STUDIES ON THE CONSTRAINTS IN EFFICIENT MICROPROPAGATION OF BAMBOO

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By

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Studies on the Constraints in Efficient Micropropagation of Bamboo

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DEDICATED
TO MY BELOVED
DAUGHTER

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on the constraints in efficient micropropagation of bamboo"** is a bonafide record of the research work carried out by **Ms. Vidya R. Sankar** under my supervision in the Department of Biotechnology, Kerala Forest Research Institute, Thrissur. The results presented in this thesis or parts of it have not been presented for the award of any other degree or diploma or any other similar titles or recognition.

This is to certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in this thesis.

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DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON THE CONSTRAINTS IN EFFICIENT MICROPROPAGATION OF BAMBOO**" for the degree of **Doctor of Philosophy** in **Biotechnology** from **Cochin University of Science And Technology** (CUSAT), Kerala, is a bonafide record of research work carried out by me under the supervision of **Dr. E.M. Muralidharan,** Senior Principal Scientist, **Kerala Forest Research Institute**, Peechi. No part of this thesis has been submitted for any other degree or diploma.

Dated: 8 November, 2018

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ABBREVIATIONS

%	:	Percentage
≈	:	Approximately
<	:	Less than
>	:	Greater than
°C	:	Degree Celsius
2, 4-D	:	2,4-Dichlorophenoxyacetic acid
2,4,5-T	:	2,4,5-Trichlorophenoxyacetic acid
AA	:	Acetic acid
ANOVA	:	Analysis of Variance
ATP	:	Adenosine Tri Phosphate
BA	:	Benzoic acid
BABA	:	β-aminobutyric acid
BAC	:	Benzalkonium chloride
BAP	:	Benzylaminopurine
BSA	:	Bovine serum albumin
CA	:	Citric acid
CAS	:	Chrome Azurol sulphonate
C_2H_5OH	:	Ethyl alcohol
cm	:	Centimeter
CFU	:	colony-forming units
C/N	:	Carbon/Nitrogen
CP	:	Calcium propionate
d	:	Day/days
dia.	:	Diameter
DMSO	:	Dimethyl sulphoxide
DMRT		Duncan's Multiple Range test
EI	:	endophyte incidence
et al.,	:	and others
EPS	:	Exopolysaccharides
FDA	:	Food and Drug Administration
Fig.	:	Figure
FYM	:	Farm Yard Manure
g	:	Grams
GRAS	:	Generally Recognized As Safe
h	:	Hours
HCl	:	Hydrochloric acid
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
$HgCl_2$	:	Mercuric chloride
HDTMA	:	Hexadecyl Trimethyl Ammonium Bromide
IBA	:	Indole-3-butyric acid
i.e.	:	that is
IAA	:	Indole-3-acetic acid
IAM	:	Indole-3- acetamide pathway
IMViC tests	:	Indole test, Methyl Red test, Voges Proskauer test and Citrate utilization test.
KIA	:	Kligler Iron Agar

Kin	:	Kinetin
l	:	Litre
LA	:	Lactic Acid
LBA	:	Luria Bertani agar
M	:	Molar
m	:	Meter
mg	:	Milligram
MR-VP test	:	Methyl Red-Voges Proskauer test
µg	:	Microgram
MH	:	Mueller Hinton
min.	:	Minutes
ml	:	Millilitre
µl	:	Microlitre
mm	:	Millimeter
mM	:	Millimolar
µm	:	Micrometre
µM	:	Micromolar
mo	:	Month
mos		Months
MP	:	Methyl paraben
MS	:	Murashige and Skoog
mT	:	metatopolin
N	:	Normality
NA	:	Nutrient Agar
NAA	:	Naphthaleneacetic
NaCl	:	Sodium chloride
NaOCl	:	Sodium hypochlorite
NaOH	:	Sodium hydroxide
NB	:	Nutrient Broth
ng	:	Nanogram
nm	:	nanometer
No.	:	Number
NOA	:	Naphthoxyacetic acid
NPN	:	1-N-phenylnaphthylamine
OD	:	Optical density
PBS	:	Phosphate buffered saline
PDA	:	Potato Dextrose Agar
PEG	:	Polyethylene glycol
PPFD	:	Photosynthetic photon flux density
PGR	:	Plant growth regulators
pH	:	Power of Hydrogen
PPM	:	Plant Preservative Mixture™
PSA	:	Phenol-Sulfuric Acid
PS	:	Potassium sorbate
ppm	:	Parts pe million
PPO	:	polyphenol oxidase
PVP	:	polyvinyl pyrollidone
ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
s	:	Seconds

SMB	:	Sodium meta bisulphite
SMM	:	Shoot Multiplication Medium
SDS	:	Sodium dodecyl sulphate
SH	:	Schenk and Hildebrandt
sp. /spp	:	Species
Sp.	:	Specific
SPSS	:	Statistical package for the social sciences
TAE	:	Tris-acetate-EDTA
TDZ	:	1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea
TE	:	Tris-EDTA
TEMED	:	N-N-N'-N'-Tetramethyl ethylene diamine
TSA	:	Tryptone Soya Agar
L-Trp	:	L-Tryptophan
UV-VIS	:	Ultraviolet-Visible
V	:	Volts
v/v	:	Volume/volume
viz.	:	Namely
wk	:	Week/weeks
WPM	:	Woody Plant Medium
w/v	:	Weight/volume

CONTENTS

ABSTRACT

ABSTRACT

There has been a tremendous increase in the global demand in recent times for fast renewable lignocellulosic biomass, which can be ideally be met only by fast growing woody plants. Bamboo the versatile woody grass that is native to the tropical and subtropical regions of the world and exhibiting great diversity, is ideal in this respect. Bamboo is finding increasing use as alternatives to conventional timber, as a feedstock for fiber and bioenergy production besides its conventional uses in the paper industry as well as in rural households as food, implements and building material.

Increased demand for planting stock for raising the bamboo plantations that are envisaged in different parts of the world has made it necessary to reevaluate the different propagation methods available for bamboo. Conventional propagation through seeds, offset, rhizomes, and culm cuttings have limitations due to the long and unpredictable flowering cycle, short seed viability, poor multiplication rates, high labor costs, which makes the methods inappropriate for large-scale application. In this regard, *in vitro* propagation or micropropagation has emerged as a promising technique for mass propagation of elite bamboos that can lead to the production of healthy, disease free plants throughout the year irrespective of season.

For the detailed study of the major constraints and bottlenecks in bamboo micropropagation including optimization of the tissue culture media, high frequency of exogenous and endogenous contamination, hyperhydricity, browning, instability of multiplication rates, the incidence of *in vitro* flowering coupled with death of cultures, low rooting response, reduced survival during hardening etc., two species of bamboo which are endemic to the western coast of India were selected. *Pseudoxytenanthera ritcheyi* (Munro) H.B. Naithani and *Pseudoxytenanthera stocksii* (Munro) T.Q. Nguyen (Syn.: *Dendrocalamus stocksii* (Munro)) are of high commercial value and improved quality and productivity of plantations is a requirement for meeting the needs of the industry. A comparison of *in vitro* culture procedure of *P. ritcheyi* was done with that of *P. stocksii,* a similar but relatively easily propagated bamboo for which micropropagation protocols are available. Major constraints faced in micropropagation include the difficulties in obtaining good multiplication and rooting rates, the incidence of latent microbial contamination and the unique issue of *in vitro* flowering that decreases the efficiency of the different steps. The standard surface sterilization procedures that are

effective in controlling microbial contamination resulting from phyllosphere (surface) flora was found to be ineffective against endophytes and the resulting latent contamination was found to cause difficulties at multiplication as well as rooting stage.

The unpredictable phenomenon of flowering in bamboos is poorly understood and is probably influenced by environmental, nutritional and physiological factors. The occurrence of *in vitro* flowering offers a very convenient model to study the phenomenon under controlled conditions. The control over *in vitro* flowering will also enable its prevention during micropropagation, where it is undesirable.

Taking into account these major problems faced during micropropagation of bamboo, the present investigation was carried out with the following objectives:

1. Standardization of an efficient and reproducible procedure for large-scale micropropagation of *Pseudoxytenanthera ritcheyi* and *P. stocksii.*
2. To study the nature of endophytes in bamboo and to develop suitable measures for control of contamination.
3. To study the phenomenon of *in vitro* flowering in bamboo and to develop measures to bring it under control during micropropagation.

A step-wise comparison of micropropagation of the two bamboo species, *P. ritcheyi* and *P. stocksii* was carried through two plant regeneration pathways viz. axillary bud proliferation and indirect somatic embryogenesis. An efficient micropropagation protocol was achieved from nodal explants from field grown culms of *P. stocksii*. Explants were used to induce multiple shoots on a modified Murashige and Skoog solid medium supplemented with 10 µM BAP and 2.5 µM NAA. Sub-culturing of shoots in MS medium with 11 µM BA and 5 µM NAA induced multiplication and rooting and rhizome induction was attained in 11 µM BA and 10 µM NAA. Axillary bud proliferation in *P. ritcheyi* was achieved by inoculating explants on MS with BAP (5 µM) + TDZ (1 µM) + NAA (2.5 µM). Solid media was found to be best for early bud break (within 4-6 d.) whereas rapid shoot proliferation (6.17 shoots/culture) was achieved in MS liquid medium with TDZ (4.5 µM) and NAA (0.5 µM). Application of meta-topolin was found to be effective for breaking the season-induced dormancy especially during monsoon for both the species. In *P. ritcheyi,* shoot necrosis was the major problem during shoot multiplication which could be overcome by adjusting the pH of the media to the acidic

range of 4.5. *P. ritcheyi* proved to be recalcitrant to all the rooting treatments attempted and severe shoot damage resulted.

Somatic embryogenesis was successfully induced in *P. stocksii* on MS+ IBA (2.5 µM) + BAP (6.66 µM) from callus derived from leaf sheath explants (85 %) and nodal segments (100 %) on MS media with 2, 4-D (13.6 µM) and kinetin (9.12 µM). In all of the explants from *P. ritcheyi,* callus induction was obtained but consisted of non-embryogenic fiber like cells.

The second objective addressed the problem of endophytic microbial contamination in the cultures, which was tackled by adopting two strategies – i. incorporating antimicrobial principles in the media and ii. through activation of the plant defense mechanism. The highest incidence of fungal contamination from endophytes in the three selected species, *B. balcooa, P. stocksii* and *P. ritcheyi* was during June-August coinciding with the monsoon season (68.19 % in *B. balcooa*) followed by September –November (51.24 % in *B. balcooa*) and the lowest during the summer months of March- May (6.02 % in *P. ritcheyi*). Among the 73 endophytic microbial strains isolated from the three bamboo species, 11 were found to cause latent contamination. Out of the endophytic population, *Bacillus subtilis* and *Fusarium oxysporum* were predominant as latent contaminants throughout the year, irrespective of bamboo species. Total eradication of the endophytes from shoot cultures of *Dendrocalamus longispathus* was done using antibiotics (gentamicin at 250 µg ml^{-1}), but the growth and survival of the shoots during later stages of culture was adversely affected and the reintroduction of the bacteria retrieved the normal growth of the host plant.

Regular surface sterilization procedures reduced the exogenous contamination of explants between 8.64 % and 9.76 % depending upon factors such as season, area of collection and the mother plant. Incidence of latent contamination was always observed in between 52.46 % and above in *B. balcooa*, *P. stocksii* and *P. ritcheyi*. On the other hand, incorporation of various preservatives that are commonly used in food such as organic acids in tissue culture media significantly reduced the levels of exogenous contamination from 6 % in control to lower than 2 % (*B. balcooa*) and latent contamination to lower than 7 % from 54 % (*B. balcooa*) in control. Methyl paraben, the preservative of choice in cosmetics, gave good control over contamination when added at 0.03 % with the contamination rate reduced to below 2 % in all the bamboo species.Thimerosal, a preservative of vaccines and anti-venom, also gave > 98.97 % aseptic cultures in the three

species with < 3.2 % latent contamination. Benzalkonium chloride, the cationic detergent used as a preservative in pharmaceutical formulations like eye/ear drops, was found to be effective for the control of contamination with < 1.6 % fungal and bacterial growths during culture initiation and < 2.81 % in various stages of bamboo tissue culture. This could be used as an alternative to $HgCl_2$ for surface sterilization of the explants.

Plant defense activation was found to be a successful strategy for the control of latent contamination in shoot cultures of the three bamboo species. Prophylactic treatment with chitosan at 1.75 % reduced the exogenous contamination less than 3 % and latent contamination lower than 2 % in all species tested. Inoculation of explants into the media with 1.75 % chitosan for 48 h successful culture establishment and growth without both exogenous and endogenous contamination in bamboo. Application of exo-polysaccharides isolated from endophytic bacteria on mother plants 24 h before explant collection reduced the latent contamination in all three bamboo species studied. Treatment of secondary branches consisting of axillary buds with 3 % H_2O_2 for 5 h reduced the initial contamination to between 0.98 % to 1.11 % and latent contamination to between 0.57 % and 1.88 % across the three species. Application of Beta-Aminobutyric acid (30 µg ml^{-1}) on the leaves of mother plant one day prior to the collection of nodes was effective against all the exogenous and endogenous contaminants in all three bamboo species and esspecially more effective against fungal contaminants. This procedure had the added advantage that it facilitated the use of sprouted buds for culture initiation especially in the case of *P. ritcheyi*. All these treatments diminished the contamination to < 3 % in all the species during culture establishment, shoot multiplication and root induction stages.

The final objective dealt with the unique phenomenon of *in vitro* flowering in multiplying shoot cultures of five species (*B. balcooa, B. tulda, B. nutans, D. longispathus* and *P. stocksii*). Photoperiod was found to be an important factor for inducing *in vitro* flowering in bamboo with the highest incidence occurring under the 16/8 h light period within 40 d. in *B. tulda*, 42 d. in *B. balcooa,* 46 d. in *B. nutans,* 52 d. in *D. longispathus* and 48 d. in *P. stocksii*. The next best photoperiod of 14/10 induced flowering in all these species, but only after 70 d. of incubation (*B. balcooa*: 71 d., *B. tulda*: 74 d., *B. nutans:* 82 d., *D. longispathus*: 95 d. and *P. stocksii*: 75 d). It was observed that incubation of shoot culture for less than 12 h did not result in floral bud development. The effect of nutrient stress on

in vitro flowering of bamboo in shoot cultures was investigated by increasing the length of each passage (through delayed subculture) of the shoot cultures and through the abrupt reduction of mineral nutrition by transfer to low salt media. Prolonged incubation of shoot cultures without sub culturing resulted in the depletion of all nutrients in the media. All the shoot cultures studied except *D. longispathus* developed flower buds in the first subculture after the prolonged incubation period of 8 wk. in 16/8 h. Shorter subculture periods did not evoke any flowering response in any shoot cultures. Their shifting into the fresh media induced only fresh vegetative growth. When shoot cultures were grown in full strength MS media for several subculture periods and then transferred to a diluted medium, the time taken for bud appearance was significantly reduced which was influenced by photoperiod and the age of the plant material. The bud break was found early in cultures established from axillary buds of adult field grown bamboo which incubated under shorter photoperiod. A combination of cytokinin and optimum photoperiod induced flowering in all species studied. All concentrations of BAP (15 µM - 90 µM) induced *in vitro* flowering in *B. balcooa* at a photoperiod of 16/8 h but the incidence of flowering increased at higher levels of BAP. BAP at 90 µM induced flowering within 30 d. and the further incubation in the same combination resulted in the tissue damage and death of the culture. Flowering was delayed by more than 70 d. at the lower levels i.e. 15 µM of BAP. Similarly, TDZ also induced flowering under 16/8 h at 1 µM and 2.5 µM within 45 d. and 30 d. respectively. Application of individual auxins at different concentrations had no influence on *in vitro* flowering in bamboo whereas, a combination of NAA and IBA (10 µM and 13 µM respectively) with the photoperiod of 10/14 h induced flowering in all shoot cultures within 45 d. All of the different carbon sources such as sucrose, glucose, maltose, fructose and lactose added at 3 %, except lactose gave *in vitro* flowering in the 16/8 h photoperiod. Sucrose was best for flower induction in bamboo cultures followed by glucose; maltose and fructose.

The major findings of this study can be summarized as follows. *P. ritcheyi* was found to be a recalcitrant species for micropropagation compared to *P. stocksii* which was more amenable for shoot multiplication as well as rooting. Further exploration of the influence of other parameters on shoot multiplication rates, rooting of shoots, induction of embryogenic calli and induction of somatic embryos and their conversion to plantlets will be required to develop a large-scale micropropagation procedure.

The study on three bamboo species revealed that the presence of endophytes could be beneficial to prevent the emergence of other microorganisms as well as for the growth of *in vitro* shoot cultures and better success during acclimatization. Rather than eliminating the endophytes totally, the use of thimerosal and lactic acid as biostatic additives in the media was found to be a relatively safe strategy for the control of microbial contamination.

All the parameters tested viz. photoperiod, growth regulators, stress factors and C/N ratio were shown to play an important role in the phenomenon of *in vitro* flowering in bamboo shoot cultures. Besides adding to our knowledge of the role of stress and photoperiod over the flowering phenomenon in bamboo, control over this hurdle to efficient micropropagation of bamboo was achieved.

INTRODUCTION

1. INTRODUCTION

Bamboo coming under Family Poaceae (Graminae) belong to the subfamily Bambusoideae, which is subsequently divided into three tribes (Clark *et al*., 2015). It has about 120 genera and 1641 species, which easily adapts to different climatic and soil conditions. Bamboo is naturally distributed in the tropical and subtropical belt between approximately 46° north and 47° south latitude, and is commonly found naturally in Africa, Asia and Central and South America (Soreng *et al*., 2015). It is widely cultivated around the world for its timber, edible shoots or on account of its ornamental value and environmental benefits. Some species may also grow successfully in mild temperate zones in Europe and North America. With large diversity of more than 1000 species belonging to 60 genera, Tropical Asia occupies the position as the central reserve for bamboo in the world (Bystriakova *et al*., 2003). Dwarf bamboo species grow to only a few centimeters, while medium-sized bamboo species may reach a few meters and giant bamboo species grow to about 30 m with a diameter of up to 30 cm.

Bamboo shoots and culms grow from the dense root rhizome system. There are two main categories of bamboo: monopodial (non-clump forming) and sympodial (clump forming) (Banik, 1995). Those from the monopodial group, most commonly found in temperate regions, have thin rhizomes that extend horizontally for long distances with stems (culms) scattered over a large area. They generate an open clump with culms distant from each other and thus can be invasive. The main genera of this group include *Phyllostachys* and *Pleioblastus*. Sympodial bamboos are naturally distributed to tropical regions and they have short and thick rhizomes with compact clump, which expands evenly around its circumference. They are non-invasive in nature and the main genera of this group are *Bambusa* and *Dendrocalamus.*

Unlike other plants, in bamboo flowering is an elusive physiological phenomena, because it is unpredictable, long periodic, gregarious, and uncontrollable. Flowering in bamboos occurs at time intervals between 10 to 120 years (known as intermast period) depending on the species. Flowering in most species is gregarious and is accompanied by seed set and death of all the plants in that population often leading to disastrous consequences for the ecosystem and the livelihoods of people in those

areas. An explanation for the peculiar flowering phenomenon of bamboo has eluded botanists for long.

Bamboos have formed an essential part of day-to-day human life in several communities around the world especially in the tropical and sub-tropical regions from ancient times. As a source of household or farm implements, construction material in the form of poles and mats, edible shoots that practice an important part of the local cuisines, bamboo has always been a significant role in rural economy and subsistence. As a resource, there is increasing interest in bamboo in recent times since it has found innumerable uses as an industrial material. Its fast renewable woody nature makes it an attractive substitute for conventional timber and biomass for energy. Bamboo edible shoots form a main ingredient in the cuisine of several countries in Asia.

The increased demand for the resources and its widening applications enforced the harvesting of bamboo from their natural habitats or from secondary forest lands. Bulk bamboo harvesting from the wild or from the rural environments leads to the over exploitation, and therefore it is necessary to make available suitable planting stocks in order to complement efforts to conserve shrinking forest habitats (Banik, 1995).

Large scale planting of bamboo requires appropriate means of mass production of planting stock. Seeds are produced in copious quantities after each gregarious flowering event, but seed viability in bamboo is unfortunately very short. Under the best of storage conditions seeds lose viability in about a year. The other great disadvantage of using seeds for propagation is that the progeny is genetically heterogeneous being predominantly outcrossing. This is an advantage in the natural conditions for maintaining long-term genetic diversity and enabling selections of desirable characteristics, but in plantations the heterogeneity is undesirable.

Vegetative propagation has been the means through which traditionally bamboo has been propagated around the world. Vegetative propagation methods make use of the vegetative parts of bamboo which include rhizomes, culm offsets, and culm cuttings. Even though, different vegetative propagation techniques are available for bamboo, these methods suffer from serious drawbacks for large scale propagation. Offsets of bamboo are expensive due to the labour intensive nature of the excavation and difficulty in transport over long distances. Only limited number of bamboo offsets

from a clump can be developed through these processes (Akinlabi *et al*., 2017) and removal of large numbers would result in degeneration of the clumps. Most of the standard practices for clonal propagation often fail to meet the demand of seedlings in large-scale plantations.

Micropropagation is accepted as a worldwide practice for large-scale propagation of bamboos. Not only are the multiplication rates several times higher than any of the conventional methods but the uniformity and small size of plantlets, the pest and disease free nature of the propagules make it an ideal means of exchange of planting stock for the global market. Being a clonal method, it highly reduces or eliminates the variation inherent in seed raised population. Indeed, the order of magnitude of the demand for bamboo planting materials indicates that micropropagation will inevitably be necessary for mass scale propagation (Subramaniam, 1994; Gielis, 2002). For mass scale propagation (> 500,000 plants per year), classical techniques are largely insufficient and inefficient, and tissue culture is the only viable method (Gielis, 2001). According to Gielis (2001), successful micropropagation can produce 0.5 million plants from a single explant.

Bamboo micropropagation is achieved through axillary bud proliferation and somatic embryogenesis. The stimulation of axillary buds with the help of plant growth regulators exploits the normal natural branch development by axillary meristems. Axillary shoot proliferation has been proven to be the most acceptable and consistent method for large scale micropropagation. Since it is highly effective for ensuring clonal fidelity and true-to-type plants of superior genotypes when explants from adult plants are used (Gielis *et al*., 2001). Somatic embryogenesis via callus is the other methods that has been successful with many species of bamboo and has the potential to be a large scale propagation method through the development of artificial seeds and its use for genetic transformation (Woods *et al*., 1995).

The major reasons limiting wider use of micropropagation of field-grown adult bamboo plants are prevalence of superficial and systemic contamination, hyperhydricity, browning, variations in multiplication rates and low *in vitro* rooting response, coupled with reduced survival during hardening and acclimatization (Gielis *et al*., 2001).

Occurrence of endophytes and latent contamination is very high in bamboo cultures and often leads to the loss of large number of cultures. One of the main reasons for the difficulty in successful micropropagation of bamboo using mature mother derived explants is systemic contamination (Oprins *et al*., 2004). Presence of endophytic bacteria in *in vitro* cultures across different plant species was reported from both in commercial laboratories and in scientific studies (Leifert *et al*., 1991), and its effect on tree micropropagation was also observed (Ulrich *et al*., 2008). To achieve sterile healthy growing plants, the endophytes were needed to be removed from the cultures (Leifert and Cassells, 2001).

Identification and eradication of these microbial contaminants is essential especially in the case of endophytes where information is not readily available. Antimicrobial agents which inhibit the growth of bacteria and fungi have been used in food preservation, cosmetics and pharmaceuticals (Corral *et al*., 1988; Herman *et al*., 2012; Xu *et al*., 2010). The inhibitory effect of preservatives varies depending on the concentration and type of preservatives, pH of the media and the species of microorganism. Because of their relative safety to human beings and environment and their efficacy, such preservatives can be used to prevent growth of stray bacteria, fungi and other pathogens in *in vitro* cultures. Hence, a proper study of the use of novel preservative chemical added to the plant tissue culture media for control of latent contaminants is useful.

Plants have revolved in an environment that consists of variety of pathogenic microorganisms and consequently their defence system can be activated by variety of external stimuli. Microbial fragments such as cell wall components, flagellin, exo-polysaccharides etc. are some of the elicitors that result in the activation of the plant defence response. Chitosan, bacterial exopolysaccharides, beta aminobutyric acid, hydrogen peroxide etc. are known to induce this response (Eschen-Lippold *et al.*, 2010; Gondim *et al*., 2012). This property could also be taken advantage of in *in vitro* culture of plants to find a way to overcome the persistent problem of microbial contamination without resorting to chemicals as has been demonstrated with chitosan in *Vitis* cultures against *Botrytis cinerea* (Barka *et al*., 2004).

In vitro flowering while offering a good model that facilitates the understanding of the physiology of flowering is mostly a hurdle to efficient micropropagation in

bamboo since it results in death of shoots at later stages. The phenomenon largely depends upon the level and interaction of exo and endogenous phytohormones, sugars, minerals and phenolics. Standard procedures such as grafting experiments and other conventional procedures do not reveal the true nature of the flowering stimulus or the mechanism in bamboo. Novel approaches involving *in vitro* flowering and molecular techniques offer unique opportunities to investigate flowering process from new perspectives especially in species which are difficult to flower or produce flowers only once in several years (Taji *et al*., 2002; Lakshmanan and Taji, 2003). The occurrence of *in vitro* flowering offers a very convenient model to study the phenomenon under controlled conditions. Such studies are expected to throw light on the primary factors involved including the molecular mechanisms and the role of growth regulators (Yuan *et al*., 2017). *In vitro* floral transition is expected to be similar to natural flowering mechanism. The scope of exploring the flowering phenomenon in various plant species is enormous since *in vitro* flowering compresses the life cycle from several years to 3-6 mon. (Ramanayake *et al*., 2001; Lin *et al*., 2007). This approach can therefore support the study of the physiological, biochemical and molecular changes that accompany floral transition. The control over *in vitro* flowering will also enable its prevention during micropropagation where it is undesirable.

Species selected for this study

For micropropagation, *Pseudoxytenanthera stocksii* (Munro) T.Q. Nguyen (Syn.: *Dendrocalamus stocksii (Munro)* M. Kumar, Remesh and Unnikrishnan) and *Pseudoxytenanthera ritcheyi* (Munro) (Syn.: *Munrochloa ritchiei* (Munro) M. Kumar & Remesh), two economically important bamboo species were selected and compared in *in vitro* culture.

Pseudoxytenanthera stocksii, commonly known as Saeme bamboo, *Managa* or *Uyi* is a thorn-less, mid-sized, erect, almost solid species endemic to the Central Western Ghats from Kasargod in Kerala to Ratnagiri in Maharashtra of India, having considerable commercial importance. Its multifarious uses in agrarian sector is manifested in the way farmers maintain it in field bunds/farm boundaries and in homesteads. Due to the solid nature of culms, it is replacing cane in the furniture industry. The potential of *P. stocksii* for exploitation as edible shoots revealed that the

macro nutritional composition was on par with three other local bamboo species. The cyanogenic glycosides responsible for the bitterness in shoots are found to be low in *P. stocksii* (Vishwanath *et al*., 2013).

The loosely spaced solid erect culms ranging from 30-50mm diameter, inter nodal length of 29 cm, height of upto 10 m, which provides flexibility in harvesting, easy management and steady income to farmers and due to its importance, the National Bamboo Mission has rightly prioritized this species for large scale cultivation in Peninsular India. Presently, it remains confined to the coastal tracts where it is cultivated in homesteads, and on farms and community lands as live fence and/or block plantation. It is a multi-utility species and recommended for variety of uses like walking sticks, umbrella handles, roofing for huts, tent poles, baskets, javelin, furniture, construction of houses etc. It is also traditionally used in pulp and paper industry. Bamboo mat-board prepared from *P. stocksii* are shown to be superior in strength compared to those from other varieties of bamboo (Kurhekar *et al*., 2015).

Natural regeneration is hampered due to over-exploitation and lack of fertile seed setting. Vegetative propagation through splitting of rhizomes (Reddy and Yekanthappa, 1989) is not dependable due to low rooting and the non-availability of propagules in required numbers.

Pseudoxytenanthera ritcheyi (Munro) H.B. Naithani locally known as erankol (Kerala), Chewa, Choomaree (Karnataka), Manga, Udhe (Maharastra), is a medium sized slender bamboo, endemic to Western Ghats, India. This also a strong bamboo with solid culm being exploited for the manufacture of umbrella handles, walking sticks, lathi and furniture. It is also used as a support for growing betel plants in northern Kerala. Due to the potential uses, this species is being utilized by the local people in large quantities, which has led to the depletion of the natural stock. Moreover, the cultivation is very limited. It has been noted that some private agencies are also exporting the valuable culm pieces of this species.

Other species used as experimental material in this study are *Bambusa balcooa* Roxb., *B. tulda* Roxb., *B. nutans* Wall. ex Munro, *Dendrocalamus longispathus* (Kurz) Kurz which are economically important bamboo species that are being

cultivated in India and micropropagation is being explored as the means of large scale propagation.

With this background, studies were taken up on some of the constraints experienced during in vitro culture of bamboo with the following objectives.

Objectives:

1. Standardization of an efficient and reproducible procedure for large-scale propagation of *Pseudoxytenanthera ritcheyi* and *P. stocksii.*

2. To study the nature of endophytes in bamboo and to develop suitable measures for the control of contamination.

3. To study the phenomenon of *in vitro* flowering in bamboo and to develop measures to bring it under control during micropropagation.

REVIEW OF LITERATURE

2. REVIEW

2.1. MICROPROPAGATION OF BAMBOO

To make available high quality planting stock for meeting the increasing global interest for commercial plantations of bamboo, it will be necessary to develop appropriate large scale propagation methods for multiplying selected adult plants. Since in most situations availability of seeds is unpredictable due to the unique flowering behavior of most species, propagation is by necessity carried out with adult clumps. The seedling populations would in any case be heterogeneous and of uncertain genetic background and in too early a stage to predict its quality for the important economic traits. Vegetative propagation has its limitations for large-scale propagation in most bamboo species. The availability of *in vitro* culture techniques for bamboo has raised high expectations as a solution to the problems faced with traditional propagation (Gielis, 2001). For mass scale propagation of many horticultural and forestry species conventional techniques are insufficient and micropropagation is alternate possible viable method. Tissue culture regeneration protocols in bamboo species help to achieve large-scale production of plants for operational planting, providing material for breeding programs and also for conservation of germplasm (Banik, 2015).

Although tissue culture studies have been extensively carried out with other grasses, especially the cereals, bamboos had received rather limited attention for a long time (Huang and Murashige, 1983). Although the first reports on regeneration of bamboo plantlets through embryo culture appeared in the late 1960's (Alexander and Rao, 1968), advances in micropropagation of adult bamboos are recent. Bamboo micropropagation broadly refers to a set of techniques that includes axillary shoot proliferation and somatic embryogenesis which has been successfully demonstrated with different types of explants (Mehta *et al.*, 1982; Saxena, 1990; Sood *et al.*, 2000; Arya *et al.*, 2002; Lin *et al*., 2012; Goyal and Sen, 2016) but many of the methods do face constraints that prevent their use as commercially viable methods.

2.1.1. Axillary Shoot Proliferation

2.1.1.1. Choice of Explant

The choice of starting material is of great significance for the initiation of cultures in bamboo (Gielis, 1999). Various explants have been in use for the culture establishment in bamboo micropropagation, but seeds and nodal explants have been preferred (Rout and Das, 1994 and Negi and Saxena, 2011). Following Alexander and Rao (1968) for his first report of micropropagation in *D. strictus* using seeds, many successful protocols were established using seeds/seedlings explants in different bamboo species like *Dendrocalamus hamiltonii* (Sood *et al*., 2002a 2002 b; Arya *et al.,* 2012); *D. strictus* (Reddy, 2006); *D. giganteus* (Devi *et al.,* 2012); *Bambusa oldhamii* (Thiruvengadam *et al.,* 2011) and in *B. bambos* (Vamil *et al.,* 2010). Unknown genetic background, limited availability, short viability of bamboo seeds, etc. restrict the application of seeds as explant.

Nodal segments, which have advantages such as year-round availability in large numbers are the other preferred type of explant. Reports using this explant are many : *B. balcooa* (Das and Pal, 2005a; Islam and Rahman, 2005; Mudoi and Borathakur, 2009; Negi and Saxena 2011a; Brar *et al.,* 2014); *B. edulis* (Lin *et al.,* 1998); *B. glaucescens* (Banik, 1987), *B. vulgaris* (Nadgir *et al.,* 1984; Gielis, 1999; Ramanayake *et al.,* 2006; Ndiaye *et al.,* 2006); *B. tulda* (Das and Pal, 2005b; Mishra *et al.,* 2007); *D. giganteus* (Ramanayake *et al.,* 1997); *D. hamiltonii* (Sood *et al.,* 2002a; Agnihotri and Nandi, 2009; Agnihotri *et al.,* 2009); *D. strictus* (Chaturvedi *et al.,* 1993 and Ravikumar *et al.,* 1998); *Pseudoxytenanthera stocksii* (Sanjaya *et al.,* 2005); *D. asper* (Singh *et al.,* 2012b; Nadha *et al.,* 2013); *Guadua angustifolia* (Rathore *et al.,* 2009; Nadha *et al.,* 2011) and many more. Some researchers have also used inflorescence explants for establishing protocols for plant regeneration through conversion to shoots as in *D. giganteus* (Ramanayake, 1998); *D. latiflorus* (Lin *et al.,* 2006); *D. asper* (Arya *et al.,* 2008 a) and *B. edulis* (Lin *et al.,* 2005).

2.1.1.2. Choice of Medium

A variety of basal media formulations have been used for bamboo species. Most of the workers preferred MS basal media for axillary bud proliferation viz. *D. hamiltonii* (Sood *et al.,* 1992, Agnihotri *et al.,* 2009), *D. giganteus* (Ramanayake and Yakandawala, 1997); *D. latiflorus* (Lin *et al.,* 2007 b); *D. asper* (Arya *et al.,* 2008 and Nadha *et al.,* 2013); *Bambusa vulgaris* (Rout and Das, 1997); *B. edulis* (Lin and Chang, 1998) and *B. balcooa* (Das and Pal, 2005 a; Negi and Saxena, 2011a and Brar *et al.,* 2014) etc. For the germination of bamboo seeds, White's medium (White, 1963) was recommended by some researchers (Alexander and Rao, 1968; Nadgir *et al.,* 1984; Nadgauda *et al.,* 1997b; Ravikumar *et al.,* 1998). In *D. asper*, Arya *et al.* (1999) used the Woody Plant Medium (LIoyd and McCown 1981) for germination of seeds. Ndiaye *et al.* (2006) tested four basal media viz. MS medium, Gamborg's B5 medium, WPM and modified MS medium for evaluating regeneration efficiency of nodal explants in *B. vulgaris* and observed that modified MS medium showed 100 % regeneration.Even with MS medium, different strengths have been employed by many researchers for inducing bud break. Sood *et al.* (1992) observed sprouting of nodal explants of *D. hamiltonii* on half strength MS medium within 10 d. Similarly, Roohi *et al.* (1991) and Yasodha *et al.* (2010) preferred half strength MS medium for better shoot proliferation in *B. balcooa* and *D. giganteus* respectively.

2.1.1.3. Role of Plant Growth Regulators (PGRs)

Cytokinins help in overcoming the apical dominance and permit growth of axillary buds from mature explants in bamboos. In general, cytokinins promote shoot formation and BAP has been the most commonly used cytokinin for effective axillary bud proliferation. Mudoi and Borthakur (2009) used 4.4 µM of BAP for continuous shoot proliferation in *B. balcooa* by sub-culturing 2-3 shoots/cluster. Arya *et al.* (2009) used 4.4 µM BAP for achieving maximum shoot multiplication rate and for better overall growth of cultures in *D. hamiltonii*. In *B. oldhamii*, Thiruvengadam *et al.* (2011) alsoreported the use of BAP at 4.4 µM for axillary shoot proliferation from mature nodal explants. Shroti *et al.* (2012) reported a multiplication rate of 15-16 folds in *D. asper* with 8.8 µM BAP. It was used at high concentration of 22 µM in *Guadua angustifolia* as reported by Jimenez *et al.* (2006) and 26.4-52.8 µM BAP was used for continuously proliferating axillary shoots in *D. giganteus* (Ramanayake and

Yakandawala, 1997). For high frequency multiple shoot induction, a combination of two cytokinins has also been used by many workers as reported in *B. wamin* (Arshad *et al.,* 2005); *B. glaucescens*, (Shirin and Rana, 2007); *B. bambos* (Anand *et al.,* 2013) and in *Ochlandra wightii* (Bejoy *et al.,* 2012). Inclusion of low concentrations of auxins in the culture medium along with the cytokinins promoted a higher rate of shoot multiplication in many cases. In *D. hamiltonii*, Agnihotri and Nandi (2009) reported enhanced shoot proliferation on medium supplemented with 8.0 μM of BAP and 1 μM of NAA. Best shoot multiplication was obtained on MS medium supplemented with 4.4 μM BAP and 0.53 μM NAA in *B. balcooa* (Brar *et al.,* 2013) while Diab and Mohamed (2008) used 2.68 μM of NAA with 4.4 μM of BAP in *Oxytenanthera abyssinica* and 20 μM BAP and 1 μM NAA was reportedly used by Sanjaya *et al.* (2005) for high frequency shoot multiplication in *Pseudoxytenanthera stocksii*. In *Thamnocalamus spathiflorus*, Bag *et al.* (2000) used of 5.0 μM of BAP in conjunction with 1.0 μM of IBA for multiple shoot proliferation.

Administration of PGRs with cytokinins like activity e.g. Thiadurazon (TDZ) and Adenine sulphate (Ads) also proved effective for shoot proliferation in many bamboo species. In *B. edulis*, Lin and Chang (1998) reported the use of 0.45 μM TDZ for induction and proliferation of shoots. Similar results were obtained by Singh *et al.* in *D. strictus* and Lin *et al.* (2007a) in *B. oldhamii*. In *Arundinaria gigantea*, a lower level of TDZ was used by Baldwin *et al.* (2009). In *D. asper*, both Singh *et al.* (2012 b) and Nadha *et al.* (2013) reported the use of adenine sulphate in conjunction with cytokinin for axillary shoot proliferation.

2.1.1.4. Role of Sucrose

Sucrose (2-3 %) is the carbohydrate of choice for the establishment and maintenance of *in vitro* cultures of most plant species. Mostly workers have reported the use of 3 % sucrose as originally used by Murashige and Skoog (1962) but some have used 2 % sucrose for the better proliferation of cultures as reported in *D. membranaceus* and *B. nutans* (Yasodha *et al.,* 1997); *D. giganteus* (Ramanayake *et al.,* 2001); *B. bambos* (Kapoor and Rao, 2006) and *B. nutans* (Yasodha *et al.,* 2008). In *B. balcooa*, Brar *et al.* (2014) reported shoot proliferation in the medium supplemented with 1 % sucrose whereas Roohi *et al.* (1991) found 6 % the best. In *D. giganteus*, a high level (4 %) of sucrose adversely affected the shoot growth, forming only 13.3 shoots compared to

34.3 and 39.2 shoots obtained on 2 and 3 % sucrose respectively (Ramanayake *et al.*, 2001). Hence, the optimum level of exogenous carbohydrate varies with every species and the same affects the physiology and differentiation of tissues.

2.1.1.5. Role of Other Additives

Inclusion of additives into the culture medium is helpful in eliminating the problems associated with browning of medium and drying of shoots including recalcitrance induced in the *in vitro* cultures. In *B. glaucescens,* Jullien and Van (1994) reported the use of 500 mg l^{-1} polyvinyl pyrrolidone (PVP) to minimize the effect of phenolic compounds during the proliferation of axillary buds. In *P. stocksii,* Sanjaya *et al.* (2005) achieved high frequency multiple shoot formation on MS medium supplemented with ascorbic acid (283.93 µM), citric acid (118.10 µM), cysteine (104.04 µM), and glutamine (342.24 µM) as additives. Banik (1987) and Dekkers and Rao (1989) reported the use of activated charcoal in the media to improve multiple shoot proliferation in *B.glaucescens* and *B. ventricosa* respectively. Addition of 10 % coconut milk (CM) for better shoot proliferation was reported in *D. longispathus* by Saxena and Bhojwani, (1993) whereas in *D. giganteus,* Ramanayake and Yakandawala, (1997) reported that the addition of casein hydrolysate (CH) to the medium has the same effect as higher levels of BAP (53.28 µM).

2.1.1.6. Rooting of Shoots

Rooting is the major problem encountered during *in vitro* multiplication of bamboos. Poor rooting frequencies form a severe bottleneck to develop micropropagation protocols. Different auxins have been used and the role of IBA and NAA in root induction is well documented. IBA was used in *B. vulgaris* (Rout and Das, 1997); *B. glaucescens* (Shirin and Rana, 2007); *B. bambos* (Anand *et al.*, 2013); *D. brandisii* (Mukunthakumar *et al.*, 1999); *D. hamiltonii*, (Agnihotri *et al.*, 2009); *D. giganteus* (Devi *et al.*, 2012); *Melocanna baccifera* (Kant *et al.*, 2009) and *Gigantochloa atroviolaceae* (Bisht *et al.*, 2010) and in many more for induction and proliferation of roots. NAA has been successfully used in *B. oldhamii*(Lin *et al.*,2007a), *B. balcooa* (Roohi *et al.*, 1991, Brar *et al.*, 2014) and *B. bambos* (Arya and Sharma, 1998). IAA has also been used for induction of roots in *D. longispathus* (Saxena and Bhojwani, 1993); *D. farinosus* (Hu *et al.*, 2011) and *B. nutans* (Negi and Saxena, 2011b). The

combination of two auxins (IBA and NAA) was also used in some species like *D. strictus* (Chaturvedi *et al.,* 1993); *D. asper* (Singh *et al.,* 2012 b) and *B. oldhamii* (Thiruvengadam *et al.,* 2011) to obtain better rooting. In *B. balcooa,* Negi and Saxena (2010) reported the use of the three auxins IBA, IAA and NAA together. Dicamba and picloram are little used PGRs with auxin like activity which were tested in *B. oldhamii* by Lin *et al.* (2007a) for induction of roots but with not more than 50 % rooting reported. Although a majority of the workers reported the use of auxins for rooting yet there a few reports of rooting on hormone free basal MS medium as in *D. strictus* (Shirgurkar *et al.,* 1996) and *Phyllostachys meyeri* (Ogita *et al.,* 2008). The role of TDZ in rooting has also been highlighted in *B. edulis* (Lin and Chang, 1998); *B. vulgaris* (Ramanayake *et al.,* 2006) and in *D. strictus* (Singh *et al.,* 2001).

Other additives have been used in bamboo tissue culture to improve the rooting efficiency. The auxin protector, coumarin and amino acids were required by certain species of bamboos to induce the *in vitro* rooting. The use of coumarin for enhanced rooting has been advocated in *B. balcooa* (Roohi *et al.,* 1991); *D. longispathus* (Saxena and Bhojwani, 1993); *D. giganteus* (Ramanayake and Yakandawala, 1997) and *D. hamiltonii* (Sood *et al.,* 2002 a). In *D. strictus* (Nadgir *et al.,* 1984), *B. edulis* (Lin *et al.,* 2004 a) and *B. vulgaris* (Ramanayake *et al.,* 2006), coconut milk (0.1 to 10 %) was used as an additive for the induction of roots. Activated charcoal is frequently added to absorb root inhibiting agents. Other phenolic compounds such as caffeic acid and phloroglucinol were also reported to regulate rooting in species that are difficult to root like *D. strictus* as reported by Chaturvedi *et al.* (1993).

Modified MS medium with major inorganic salts reduced to half or quarter strength have been used to enhance rooting percentage in bamboos. Half strength MS medium was used in *D. giganteus* (Ramanayake and Yakandawala, 1997); *D. strictus* (Saxena and Dhawan, 1999; Mishra *et al.,* 2001; Singh *et al.,* 2001 and Reddy *et al.,* 2006); *D. asper* (Singh *et al.,* 2012 b and Nadha *et al.,* 2013); *D. hamiltonii* (Bag *et al.,* 2012); *B. vulgaris* (Rout and Das, 1997); *B. bambos* (Anand *et al.,* 2013); *Melocanna baccifera* (Kant *et al.,* 2009) and many more species for rooting. Negi and Saxena (2010) used half strength MS medium for root induction in *B. balcooa* whereas full strength MS medium gave the best response as demonstrated by Brar *et al.* (2014) in

same species. Rathore *et al.* (2009) used MS medium at ¼ strength in *P. stocksii* for best rooting.

2.1.2. Somatic Embryogenesis

2.1.2.1. Choice of Explant

Because of low rooting frequency in bamboo microshoots, plant regeneration through somatic embryogenesis and conversion of embryos is a promising alternative. Plantlet regeneration through embryogenesis is influenced by many factors such as the species (including cultivars, genotypes and ecotypes), type and age of explants, type of basal medium and the type and concentration of plant growth regulators (Godbole *et al.*, 2002). Several protocols for somatic embryogenesis of bamboo species have been developed using various explants like seeds (Mehta *et al.*, 1982), shoot tip (Huang *et al.*, 1983 and Hu *et al.*, 2011); young leaf (Vasana, 1985 and Jullien and Van, 1995); inflorescence (Yeh and Chang 1986b; Gillis *et al.*, 2007); anther (Tsay *et al.*, 1990) and mature zygotic embryos (Rout and Das, 1994 and Yuan *et al.*, 2013). Regeneration through somatic embryogenesis has been reported in *D. strictus*, *D. giganteus* and *B. vulgaris* (Rout and Das, 1994); *D. strictus* (Saxena and Dhawan, 1999) and *D. hamiltonii* (Godbole *et al.*, 2002) and in many more species.

2.1.2.2. Choice of Medium

For obtaining somatic embryogenesis, callus induction from various explants were carried out by many researchers. Saxena and Dhawan (1998) successfully established somatic embryogenesis in *D. strictus* from callus initiated from seeds on MS medium containing 2, 4-D. Yuan *et al*. (2013) initiated callus from zygotic embryos after 10–20 d culture on MS media supplemented with 2, 4-D and zeatin with 15 % of the calli tending to be embryogenic in nature. Similarly, Zang *et al*. (2016) induced embryogenic calli from shoot tips explants on MS supplemented with 2, 4-D and BAP in *D. hamiltonii*. Half strength MS media was used by many workers for improvement in regeneration ability during somatic embryogenesis as reported in *B. vulgaris* by Rout and Das (1994); *B. nutans* (Mehta *et al.*, 2010); *D. strictus* (Saxena and Dhawan, 1999) and *D. hamiltonii* (Godbole *et al.*, 2002 and Bag *et al.*, 2012). In *B. bambos* and *D. longispathus*, B5 medium was demonstrated to be the best for somatic embryogenesis (Rao and Rao, 1988 and Saxena and Bhojwani, 1993).

2.1.2.3. Role of Plant Growth Regulators (PGRs)

The role of 2, 4-D in the induction of embryogenic calli was first demonstrated in *B. bambos* (Mehta *et al.,* 1982) followed by several reports of successful induction and regeneration of plantlets, as in *Sinocalamus latiflorus* (Yeh and Chang, 1987); *D. latiflorus* (Zamora *et al.,* 1988); *B. glaucescens* (Jullien and Van, 1994); *D. giganteus* (Ramanayake and Wanniarachchi, 2003) and *B. balcooa* (Gillis *et al.,* 2007 Another auxin, 2, 4, 5-T was used in the range of 8-20 µM for the induction of embryogenic calli in *D. longispathus* (Saxena and Bhojwani, 1991) and in *D. farinosus* (Hu *et al.,* 2011). Somatic embryos formed in *D. strictus* multiplied rapidly on medium containing IBA (20 µM) as reported by Saxena and Dhawan (1999) as well as in *D. giganteus* (Devi *et al.,* 2012). The use of an auxin along with cytokinin was also reported for induction of embryogenic callus in several species like *B. oldhamii* (Yeh and Chang, 1986 a); *B. beecheyana* (Chang and Lan, 1995); *D. asper* (Shroti *et al.,* 2012) and *D. hamiltonii* (Bag *et al.,* 2012). TDZ (0.45 µM) was also used effectively for embryogenic callus induction in *B. edulis* (Lin *et al.*, 2004a).

The cytokinin, BAP has been used for regeneration of plantlets from embryogenic calli in many species including *D. latiflorus* (Zamora *et al.,* 1988); *B. ventricosa* (Cheah and Chaille, 2011) while kinetin was employed in others such as *D. strictus* (Rout and Das, 1994) and *D. giganteus* (Devi *et al.,* 2012). Zeatin in the range of 20-30 µM was incorporated into MS medium for better regeneration of somatic embryos in moso bamboo as reported by Yuan *et al.* (2013).

2.1.2.4. Carbon source

As a carbon source, sucrose was the predominant sugar used for somatic embryogenesis in bamboo. Rao and Rao (1988) reported germination of mature embryos of *B. bambos* on 2% sucrose which later produced longer roots. In *D. giganteus,* Ramanayake and Wanniarachchi (2003) used 4% sucrose for conducting their experiments. High sucrose (5 to 8%) favoured somatic embryogenesis in many bamboos by reducing the frequency of albinos and providing impetus to the growing embryos leading to their germination into plantlets in *D. hamiltonii* (Sood *et al.,* 1994; Godbole *et al.,* 2002). Glucose (2%) was used for proliferation of somatic embryos in *B. nutans* (Mehta *et al.,* 2010).

2.1.2.5. Role of Other Additives

Many chemical additives including osmotica, anti-oxidants and ethylene inhibitors influence plant regeneration during somatic embryogenesis. In *B. bambos* and *D. strictus*, PVP added to the medium for the reduction of tissue browning, also improved multiplication and germination of somatic embryos (Mehta *et al.,* 1982, Saxena and Dhawan, 1999). Lin *et al.* (2004 a) incorporated 0.1 % v/v coconut milk in *B. edulis*. Ascorbic acid (1.1 μM) gave improved maturation of embryos in *B. nutans (*Mehta *et al.,* 2010).

2.1.3. Hardening and Acclimatization

Micropropagated plantlets are associated with several physiological and anatomical abnormalities that develop under *in vitro* conditions such as low photosynthesis, malfunctioning stomata, inadequate development of cuticle and a poor wax deposition on epidermis which can be attributed mainly due to high humidity inside the culture vessel (Kozai, 1991). Therefore, they need to be hardened or acclimatized before their transfer to the *ex vitro* conditions to ensure better survival. Various potting media have been used by different workers for acclimatization of bamboo plantlets. Comparison of different potting mixtures has shown a varied response in different species. Soilrite: sand (2:1) was used in *B. bambos* (Arya and Sharma, 1998) while rooted plantlets of *B. edulis* grew well when they were transferred to pots containing non-sterile vermiculite: peat: perlite 1:1:1 (Lin and Chang, 1998). In *D. brandisii*, Mukunthakumar *et al.* (1999) reported soil: farmyard manure (FYM) (1:1) as the ideal potting mixture while Singh *et al.* (2001) achieved maximum growth of *D. strictus* plants on a mixture of sand and garden soil (1:1). Negi and Saxena (2011b) tried various potting mixtures and reported hardening and survival of 98 % plantlets in *B. nutans* in the soil: agropeat (2:1) mixture. Ali *et al.* (2009) used soil: fine sand: peat moss (1:1:1) for successful acclimatization of plants in *D. asper*. A similar media (Soil: sand: FYM at 1:1:1) was used successfully in *D. hamiltonii* (Godbole *et al.,* 2002 and Arya *et al.,* 2009); *Gigantochloa atroviolaceae* (Bisht *et al.,* 2010) and *B. nutans* (Sharma and Kalia, 2012). Hence, success in hardening was seen to be dependent upon the type and proper ratio of the potting mixture for various species.

2.2. CONSTRAINTS IN MICROPROPAGATION

It is evident from the reports in literature that success with adult tissues is limited by a number of factors. Among the constraints to successful establishment of cultures from explants of field growing mother plants, exogenous microbial contamination, endophytic contamination, tissue browning and *in vitro* flowering takes precedence over other issues. Besides, hyperhydricity, instable multiplication rate, difficulty in rooting and decreased survival rate during acclimatization are the other limiting factors (Sandhu *et al.*, 2018).

Tissue browning is one of the main problems faced during *in vitro* culturing of bamboo which is imparted by the enhanced synthesis of polyphenol oxidases (PPO) in wounded tissues, either at culture initiation or during sub culturing. Factors such as species, age and position of tissue, age of mother plant and season of explant excision, nutrient medium, sterilizing agent used, etc. highly influenced the severity of browning (Compton and Preece 1986; Oprins *et al*., 2004). Browning of tissues and media associated with the increased production of polyphenol oxidases (PPO) is a problem reported in many woody plants and bamboo (Huang *et al*., 1989). Huang *et al*. (1989) found that browning intensified with increasing benzyladenine (BAP) concentration and the effect was pH dependent (Huang *et al*., 2002). Nutrient media with standard pH of 5.7 (acidic) gave a relatively low browning rate, whereas browning was higher in media having pH values of 7 or 8. Several methods have been used to overcome the tissue browning problem. Frequent subculture has been recommended to prevent browning in *D. giganteus* (Mudoi *et al*., 2014; Ramanayake and Yakandawala, 1997). Addition of polyvinyl pyrrolidone (PVP), citric or ascorbic acid and activated charcoal to the nutrient medium alleviated the problem in *D. strictus* (Saxena and Dhawan, 1999). PVP has been reported to be effective for *Sinocalamus latiflora* (Yeh and Chang, 1987) However, according to Singh *et al*. (2012b), the use of PVP and activated charcoal completely failed to suppress the browning of *D. hamiltonii* shoot cultures. Earlier reports corroborated this finding on the failure of PVP in counteracting browning in *Phyllostachys nigra* (Ogita, 2005) and of activated charcoal on *D. latiflorus* (Zamora *et al*., 1988). Huang *et al*. (2002) also found PVP, activated charcoal or PPO inhibitors, such as ascorbic acid, cysteine, ferulic acid, kojic acid, and thiourea, to be ineffective in *B. oldhamii*, *D. latiflorus* and

P. nigra. It can be concluded that although in some cases antioxidants may help reducing browning of the tissues and culture media, regular transfer to fresh medium continues to be the most effective of all the approaches.

One of the main constraints in successful micropropagation of bamboo using explants from adult mother clumps is the systemic microbial contamination (Oprins *et al*., 2004). Presence of endophytic bacteria in *in vitro* cultures was reported both in commercial laboratories and in laboratory experiments (Leifert *et al*., 1991). To obtain sterile healthy growing plants, endophytes needed to be removed from the cultures (Leifert and Cassells, 2001). Thakur and Sood (2006) reported that the large intercellular spaces and vessel cavities of bamboo shoots provided the area for microbes. Five morphologically distinct bacterial isolates were identified from tissue culture material of six *Prunus avium* L. genotypes (Quambusch *et al*., 2013).

It was reported that, the shoots derived from adult tissue in *in vitro* propagation was very difficult to root. Root induction frequency was varying with the age of the mother plant and it was fairly easier in shoot cultures derived from seedlings origin (Ramanayake *et al*., 2006).

2.2.1. Endophytic contamination

The interaction that exists between microbes and their hosts is a continuum that extends from the parasitic and commensal to the mutualistic (Malcom *et al*., 2013; Steripoulos and Gordon, 2014).The initiation and maintenance of sterile cultures from tissues collected from adult plants growing in the field is often one of the most challenging tasks in plant tissue culture of woody perennials. An extensive array of microbial communities resides on the surface of the plants and consequently only a thorough disinfection procedure using antimicrobial agents will ensure a contamination free culture. Microbial contamination can be introduced via the explants and at every stage of the tissue culture process in the laboratory (Leifert and Cassels, 2001; Reed and Tanprasert, 1995; Odutayo *et al*., 2007). It is the principal reason for losses in both scientific and commercial micropropagation systems (Leifert and Waites, 1992). In most laboratories losses averages between 3 and 15% of plants at every subculture (Leifert and Cassels, 2001). Different antimicrobial agents such as mercuric chloride, hypochlorite solutions, silver nitrite, ethanol, etc. have been used

for this purpose. As an additional step, researchers often use systemic and contact fungicides as well as broad spectrum antibiotics.

Epiphytic organisms or the phyllosphere microflora can easily be removed by the efficient surface sterilization whereas the endophytes such as those that are localized within the plant at cell junctions and intercellular spaces of cortical parenchyma (Petrini, 1991) cannot be removed through this procedure. Such endophytes can remain latent without producing any symptoms on tissues on plant growth media for long duration. These endogenous organisms consisting of bacteria, filamentous fungi and yeasts do not cause any visible symptoms during micropropagation due to the sucrose concentration, salt concentration, pH and temperature being not ideal for their growth (Cooke *et al*., 1992; Danby *et al*., 1994; Leifert *et al*., 1994a). The minor changes in media or culture conditions triggered the endophytic population and resulted in active multiplication of them and damaged the plant cultures (Leifert, 2000). These endophytic contamination resulted in reduced growth rate, retarded rooting, and even caused plant death (Leifert and Waites, 1992; Ewald *et al*., 1997; Leifert and Cassells, 2001). These microorganisms may survive in hidden form, warranting culture indexing using bacteriological media for their detection (Leifert and Woodward 1998; Thomas 2004a). Intracellular associations and a mutualistic relationship between non-cultivable endophytic bacteria residing in cytoplasmic and periplasmic spaces of host banana plants have been described by Thomas and Sekhar (2014).These microorganisms may survive in hidden form, warranting culture indexing using bacteriological media for their detection (Leifert and Woodward 1998; Thomas 2004a). A combination of cultivation and molecular identification methods revealed the great diversity that exists of uncultivable endophytes on banana (Thomas and Sekhar, 2017). These symptomless organisms cause reduced multiplication as well as low rooting rates, or death of the shoot cultures (Leifert *et al*., 1989; 1992; Ulrich *et al*., 2008). On other hand, these endophytes may induce hormone-mediated modification of *in vitro* response (Holland and Polacco, 1994), which puts into question the reproducibility of tissue culture protocols. The term ‘vitropaths’ was put forward by Herman (1989) for the endophytes that become detrimental under special growth conditions.

2.2.1.1. Common procedures for the control of microbial contamination

Amendment of tissue culture media with antibiotics is the most promising procedure for suppressing bacterial growth (Kneifel and Leonhardt 1992; Bohra *et al*., 2013). Phillips *et al*. (1981) successfully applied rifampicin in short-term cultures of *Helianthus tuberosus* to control the bacterial contaminants. In 1983, Horsch and King used the combination of streptomycin and carbenicillin for the elimination of bacterial contaminant *Hyphomicrobium* from cell cultures of *Datura innoxia* without harming the plant cells. Mathias *et al*. (1987) recommended antibiotic therapy for the regulation of microbial contamination in plant tissue culture. Cornu and Michel (1987) used different aminoglycosides, tetracyclines and rifampicin in order to eradicate the bacterial contamination that occur after several transfer *in vitro* propagation of *Prunus avium* L. Prophylactic treatments of the plants with antibiotics and their incorporation into the media have been used to suppress the bacteria in *in vitro* cultures of various plants (Fisse *et al*., 1987; Podwyzynska and Hempel, 1987; Leifert, 1990; Leifert *et al*., 1991b). Combinations of different antibiotics such as one or two β-1actams (carbenicillin and/or cephalothin), an aminoglycoside (gentamicin or streptomycin) and rifampicin or polymyxin etc. were also successfully used to eliminate bacterial contaminants in plant tissue culture. Leifert *et al*. (1991a; b) applied these combinations to eliminate the bacterial contaminants such as *Lactobacillus plantarum*, *Staphylococcus saprophyticus*, *Corynebacterium* spp., *Pseudomonas paucimobilis* etc. Nadha *et al*. (2012) identified that supplementing kanamycin (10 µg/ml) for 10 days eliminated the bacterial contaminants *Pantoea agglomerans* and *P. ananatis* during *in vitro* propagation of *Guadua angustifolia* Kunth. The incorporation of three antibiotics such as gentamicin, tetracycline and chloramphenicol into the culture medium was found to effectively reduce the incidence of bacterial contamination in *Aglaonema* cultivars tested (Fang *et al*., 2012).

In the tropics, fungal contaminants are very common due to the prevailing favourable environmental conditions (especially the high temperatures and humidity) for the proliferation of fungal pathogens on the plants. Fungal contamination is a major problem during micropropagation as fungal growth greatly reduces survival and shoot proliferation. Fungus may arrive with an explant or airborne, or enter a culture

laterally. Furthermore, the conditions in the plant culture incubation chambers are favourable for the rapid growth of fungi and other *in vitro* contaminants (Mng'omba *et al*., 2012). *Alterneria tenius, Aspergillus niger, A. fumigatus, Fusarium culmorum, Candida, Microsporum, Phialophora, Penicillium, Alternaria, Rhizopus, Cylindrocarpon* etc. were the main fungal species that cause contamination in plant tissues (Odutayo *et al*., 2007; Altan *et al*., 2010; Cobrado and Frenandez, 2016).

Fungicides are often used to reduce the pathogen loads from the stock plants to achieve culture asepsis in the culture laboratories (Mng'omba *et al*., 2012). Shields *et al.* (1984) reported that any fungicides used in plant tissue culture cannot be categorized as ideal chemical agent that kill all species of contaminating fungi without harming plant tissue.There are two classes of fungicides: contact and systemic. By preventing the germination and development of spores and mycelia, contact fungicides protected the plant tissues from the fungal attack (Yuste and Gostinear, 1999). Organic and inorganic contact fungicides have different mechanisms of action. Systemic fungicides such as benomyl, carbendazim, thiabendazole, fenbendazole, nystatin, fungizone, clotrimazole, griseofulvin, imazalil, miconazole etc. are the widely used fungicides which are relatively safe to plant tissues. Among the fungicides, benomyl is the most acceptable one and it has both systemic fungicidal property as well as the same effect as cytokinins (Yang, 1976). Shields *et al.* (1984) recommended the application of imidazoles in plant tissue culture, which was endorsed by Kowalik and Gródek (2002). Miconazole, a substituted azole, at concentrations between 5 and 20 mg 1^{-1} inhibited hyphal growth and sporulation in a wide range of fungi commonly associated with plants (Tyan *et al*., 1993). The efficiency of a fungicide in protecting *in vitro* cultures is measured by the extent of its spectrum and lack in phytotoxicity (Zenkteler, 1998). Altan *et al.* (2010) reported the effect of benomyl on fungal growth control and culture development on explants of *Lilium candidum in vitro.* Eed *et al*. (2010) reported the same in cultures of *Citrus limonia*.

Another strategy for control of bacteria and fungi in plant tissue culture is the use of iso-thiazolones. Plant Preservative Mixture™ (PPM) a technology patented by Plant Cell Technology, Washington, is a broad-spectrum biocide that consists of 5-chloro-2-methyl- 3(2H)-iso-thiazolone and 2-methyl-3(2H) - iso-thiazolone. The advantage

of PPM is that it is microbicidal against both bacteria and fungi, is heat stable and autoclavable with media. The recommended concentration of PPM to reduce or eliminate airborne contamination in media is 0.5 to 1.0 ml l^{-1} (Plant Cell Technology, 1998). The influence of PPM on plant regeneration depends on the plant species. George *et al.* (2001) tested the varying concentration of PPM with leaf explants of *Dendranthema* × *grandiflora*, *Betula pendula*, and *Rhododendron catawbiense* and effectively removed the bacterial as well as fungal contamination. Miyazaki *et al.* (2010) demonstrated that endogenous *S. paucimobilis* in contaminated tissue of cultured *Petunia* can be eradicated successfully with PPM. Rihan *et al*. (2012) evaluated the effect of PPM on the contamination during the development of cauliflower microshoots in media supplemented with 0.5 ml l^{-1} PPM and the quality of the artificial seed produced.

2.2.1.2.Control of endophytic contamination

The endogenous or endophytic microbes are often difficult to eliminate through normal surface sterilization (Herman, 1990). There are many research reports that support the beneficial interactions between endophytes and host plants in natural conditions. Helander *et al.* (1996) reported that mutualism between endophytes and the host plant depends on the prevailing plant condition. Although they are very useful outside the plant culture laboratories, endophytes become harmful to plant cultures. Mwamba (1995) studied about the symbiotic microbes (mycorrhizae) including *Uapaca kirkiana* and break-up of this mutualistic association once the host plant is stressed. Many studies point to the positive influence of these endophytes, such as biosynthesis of growth-promoting phytohormones, intensification of nutrient availability and enhanced resistance to pathogens, etc. (Vendan *et al*., 2010; Goh and Vallejos, 2013; Hassan, 2017). Adverse effect on plants due to the eradication of these endophytes is also reported. Pirttilä *et al.* (2004) observed that *in vitro* culturing of endophyte-freed buds of *Pinus sylvestris* resulted in an altered morphology, which could be restored by adding endophytic products to the plant culture medium. Root induction and active growth of root in terms of higher number and longer roots on microcuttings was observed in poplar tissue cultures when it was inoculated with the endophytic bacteria *Paenibacillus* (Ulrich *et al*., 2008a). Plant growth-promoting effects were identified during the acclimatization process in the plantlets developed

from the shoot cultures of strawberry that co-cultured with endophytic bacteria (Dias *et al.,* 2008).

Removal or eradication of the endophytes from the plants cause disturbance in equilibrium established within the microecosystem.

Antimicrobial agents used in food preservation

Selected organic and inorganic salts that are active antimicrobial agents are used extensively as food additives (Miyasaki *et al.,* 1986). Acetic, citric, succinic, malic, tartaric, benzoic, lactic and sorbic acids are the major organic acids that occur naturally in many fruits and vegetables and are used for food preservation (Foegeding and Busta, 1991). Many food additives such as lactic acid, acetic acid etc. are widely used for controlling pH, taste and texture also have a broad-spectrum antifungal activity (Corral *et al.,* 1988). The antifungal activity of many of the food additives have also been demonstrated on several plant-pathogen systems (Palou *et al.,* 2002; Arslan *et al.,* 2006; Jamar *et al.,* 2007).

In plants, animals and humans, lactic acid, or lactate, is part of regular metabolism and its salts are effective against a wide range of microorganisms. Lactate decreases water activity and undissociated lactic acid is able to pass through cell membrane and inhibit the growth of microorganisms by pH decrease (Pipek, 1998). Roth and Keenan (1971) reported that lactic acid is able to cause sublethal injury to *Escherichia coli*. Undissociated lactic acid has been reported to inhibit the growth of microorganisms (Mountney and O'Halley, 1965; Stern *et al.,* 1985a; Colberg and Izat, 1988; Marcel *et al.,* 1988; Mossel and Drake, 1990). As reviewed by Doores (1993), lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae*. Netten *et al*. (1994) categorized lactic acid as permeabilizers, which chelates divalent cations that stabilize molecular interactions in the outer membrane so that lipopolysaccharides are released, and polycations such as polyethyleneimine (Helander *et al*., 1997) or polymyxin B nonapeptide, which cause outer membrane damage without lipopolysaccharide release. Alakomi *et al*. (2000) reported that lactic acid could permeabilize Gram-negative bacteria by disrupting the outer membrane.

Phillips (1968) had reported the treatment of superficial wounds infected by *Pseudomonas aeruginosa* using acetic acid. In addition, there are many reports that support the basis for the ancient tradition of treating diseases like plague, ear, chest, and urinary tract infections with application of acetic acid (Currence, 1952; Johnston and Gaas, 2006). McManus *et al*. (1985) reported the elimination of *Bacillus pyocyaneus* (now *Pseudomonas aeruginosa*) from war wounds using acetic acid. The antibacterial action of vinegar against food-borne pathogenic bacteria, including *Escherichia coli* was reported by Entani *et al*. (1998). A recent report by Halstead *et al*. (2015) stated that acetic acid has good antibacterial activity against various biofilm forming planktonic organisms. Cartotto (2017) reviewed acetic acid as one of the topical antimicrobial agents for paediatric burns. Acetic acid at 5 % in the management of periprosthetic joint infection clinically found to eliminate 96.1 % of biofilm-associated microbes following a 20-min. treatment (Tsang *et al*., 2018).

Citric acid (CA) and its salts, sodium citrate, calcium citrate and potassium citrate have attracted much interest due to their distinctive properties as acidulates, flavoring agents and antioxidants, and are used mainly in the food and beverage industry. CA is an intermediate of the tricarboxylic acid cycle (TCA) and holds a key position in the metabolism of each microbial cell. Smith *et al*. (1966) reported that citric acid demonstrates antimicrobial property although it is not as effective an antimicrobial agent as 5.25 % NaOCl. Smith and Wayman (1986) identified the antimicrobial effectiveness of citric acid as a root canal irrigant. Minimal inhibitory concentrations of citric acid against *Shigella* were found by Ye-Won In *et al.* (2013) to be 300 ppm.

Calcium propionate is widely used as a food preservative for minimizing microbial spoilage of bread (Legan, 1993; Pateras, 1998). Schmidt-Heydt *et al*. (2007) studied the physiological relationship between food preservatives and the gene expression for ochratoxin by *Penicillium verrucosum.* Ponte and Tsen (1987) had reported on the antimicrobial effect of propionic acid on moulds and *Bacillus* spores. Ryan *et al.* (2008) tested the combined action of calcium propionate with lactic acid bacteria in bread to improve the shelf life.

Sorbic acid and its salt derivatives are the most widely used antimicrobial agents for food preservation worldwide (Al-Zaemey *et al.,* 1993; Olivier *et al.,* 1999). It is primarily used as a fungistat and as a mold and yeast inhibitor in post-harvest

handling and processing for food preservation and extension of shelf-life (Dorko *et al*., 1997). Potassium sorbate is used at 0.2 % to preserve cheeses, cakes and syrups (Sofos and Busta, 1981). Its use as a preservative in baked goods, chocolate, soda fountain syrups, fruit cocktails, cheeses, and artificially sweetened jellies has also been documented (Winters, 1989). The effectiveness of potassium sorbate against four spoilage yeasts was reported by Dai *et al.* (2009). Stanojevic *et al.* (2009) effectively applied potassium sorbate against the following species of microorganisms viz. *Bacillus subtilis, B. mycoides, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus flavus, Fusarium oxysporum, Candida albicans, Trichoderma harsianum* and *Penicillium italicum*. Guri and Patel (1998) claimed that potassium sorbate is effective as a mold inhibitor with other synthetic fungicides used in tissue culture.

Sodium benzoate is widely used in the food industry as an antifungal agent (Salkowski, 1875) to conserve margarine, fresh juices, and sweets. Hall (1992) reported that sodium benzoate when applied to citrus fruits inoculated with *Penicillium digitatum* had fungicidal activity and can control the citrus fruit decay. It was used against postharvest diseases of tomato, apple, carrots and potato as reported by Ryu and Hold (1993), Saleh and Huang (1997) and Oliver *et al.* (1998). Lope´z-Malo *et al*. (2000) found inhibition of *Saccharomyces cerevisiae* at different concentrations of potassium sorbate influenced by pH. Dai *et al.* (2009) reported the efficacy of sodium benzoate on the growth of *Saccharomyces cerevisiae, Zygosaccharomyces bailii, Brettanomyces bruxellensis*, and *B. naardenensis*. Stanojevic *et al*. (2009) tested its efficacy as an antimicrobial agent for the food industry. The fungicidal efficacy of sodium benzoate was tested in *in vitro* experiments against a soil borne pathogen *Sclerotium rolfsii* (Punja and Grogan 1982).

Salicylic acid (SA), a natural phenolic compound from *Salix alba*, which has recently been considered as a plant hormone (Davis, 2004) is involved in reducing the damage caused by various pathogens such as bacteria, fungi and viruses. It influences a variety of biochemical and molecular events associated with induction of disease resistance (Arberg, 1981).There is a very extensive use of SA as a preservative of foods, wine, beer, cider, etc., and in the form of salicylate of soda as a medicine,

especially for rheumatism. Gershon and Parmegiani (1962) screened the effect of SA against strains of five bacteria and five fungi. Hadi and Balali (2010) reported the effect of SA at 0.2 mM on the reduction of *Rhizoctonia solani* damage in tubers of potato and the further reduction in intensity of infection symptoms by an increase in the concentration of SA. Kantouch *et al*. (2013) reported that SA and three of its derivatives used to provide antibacterial properties to viscose fabrics. Exposure to a 2.5 mM SA solution for upto 120 min was found to be effective by Rocha Neto *et al*. (2015) against the blue mold (*Penicillium expansum*), the main pathogen in postharvest diseases in apples. Effectiveness of sprayed solution of SA to prevent the wilt disease of sweet green pepper (*Capsicum annuum*) caused by *Fusarium oxysporium* was studied by Yousif (2018).

Sulphites such as potassium bisulphite, sodium or potassium metabisulphite, sodium sulphite or sulphur dioxide etc. are also in use as food preservatives. They are often used as preservatives in wines (to prevent spoilage and oxidation), dried fruits and dried potato products. Sulphites also occur naturally in almost all wines. Woolford (1976) reported the antibacterial properties of sodium metabisulphite. It has been used in the preservation of fresh and frozen crustaceans up to 150 mg kg^{-1} in edible parts. Omojowo *et al.* (2009), carrying out a comparative assessment of potassium sorbate and sodium metabisulphite on the safety and shelf life of smoked catfish, reported that it can reduce the *Staphylococcus* count to zero in the fish. Noorafshan *et al*. (2014) demonstrated its effectiveness as an antioxidant agent in many pharmaceutical formulations.

The antimicrobial activity of the preservatives varies and is influenced by different factors such as concentration and nature of the preservative, pH of the medium and the species of microorganism (Restaino *et al*., 1981).

2.2.1.3.2. Antimicrobial chemicals used in cosmetics and pharmaceuticals

2.2.1.3.2.1. Methyl paraben

In order to endure the microbiological cleanliness of cosmetics during their manufacture, packing, storage and the whole period of use, some preservatives are added (Herman *et al*., 2012). Due to their well- known biological activity, the parabens – a group of alkyl esters of p- hydroxyl benzoic acid (parabens), are widely

used as antimicrobial preservatives in pharmaceuticals (Matthews *et al*., 1956; Kabara, 1984; Andersen, 2008; Seetaramaiah *et al*., 2011) cosmetics (Nes and Eklund, 1983; Grunberger *et al.*, 1988; Rastogi *et al*., 1995; Soni *et al*., 2005; Karaca, 2014)

The parabens and their salts have a broad spectrum of activity against yeasts, molds, and bacteria and are more effective against fungi than against bacteria (Elder, 1984; Rietschel and Fowler, 2001). They have been used because they are cheap, have low toxicity and kill a variety of fungi and bacteria including both the Gram positive and Gram negative (Haag and Loncrini, 1984; Charnock and Finsrud, 2007). Anthony (2009) reported that esters of 4-hydroxybenzoic acid express antimicrobial activity against a wide range of fungi and bacteria as a result of the hydroxyl group. A comparative study on inhibitory action of methyl and ethyl esters of benzoic acid was carried out against the microorganisms viz. *Escherichia coli, Bacillus cereus, Listeria monocytogenes, Fusarium culmorum* and *Saccharomyces cerevisiae* by Merkl *et al.* (2010). Carolina *et al*. (2010) reported that paraben compounds exhibited antimicrobial action against *Lactobacillus cornyformis, Listeria monocytogenes, Fusarium culmorum, Saccharomyces cervisae.* Cho *et al.* (1998) reported that 4-hydroxybenzoic acid isolated from rice hull have antibacterial activity against most Gram positive and some of Gram negative bacteria at 50 % inhibitory concentration of 160 µg/mL and concluded lipophilicity as an important factor that strongly influences the antimicrobial activity of methyl paraben. Due to their broad antimicrobial spectra with relatively low toxicity, good stability and non-volatility (Cantwell, 1976), parabens are commonly used as preservatives at very low levels ranging from 0.01 to 0.3 % (Elder *et al*., 1984, Mincea *et al*., 2009).

2.2.1.3.2.2. Thimerosal

Thimerosal, an organomercurial compound with antibacterial and antifungal properties is widely acceptable for use in topical ophthalmic preparations at concentrations ranging from 0.004 to 0.01 %. Thimerosal is a stainless and stable compound of high germicidal value, especially in serum and other protein media (Morton *et al.*, 1948). Thimerosal has generally been accepted as a safe preservative agent in eye drops, both as a preservative and the main drug for the treatment of keratomycosis (Abrams *et al*., 1965). Xu *et al.* (2010) reported that it has both

antifungal and preservative effects. Tisner *et al*. (1995) have successfully reported antifungal property of thimerosal against otomycosis. In a comparison with natamycin and amphotericin B against *Fusarium* spp., *Alternaria alternata* and *Aspergillus* spp., thimerosal was more active than other fungicides used (Xu *et al.*, 2010). Similarly, Hofling-Lima *et al*. (2005) and Xie (2003) revealed antifungal activity of thimerosal against different fungi. 0.1 % thimerosal in medium provided 100 % sterile conditions for *in vitro Chrysanthemum* culture without the need for autoclaving the media (Deein *et al*., 2013).

2.2.1.3.2.3. Benzalkonium chloride

Benzalkonium chloride (BAC) has been shown to possess antimicrobial activity against different bacteria and its therapeutic role in vulvovaginal infections has been studied (Kumar *et al*., 2011). Mosca *et al*. (2006) described the antimicrobial activity against clinical isolates of *Streptococcus agalactiae* and found out that19.2 % of which were resistant to erythromycin and clindamycin were either inhibited or killed by BAC at the low concentration of 3.12 mg L^{-1}. Fazlara and Ekhtelat (2012) studied the antibacterial influences of BAC against six important foodborne pathogens including three Gram positive (*S. aureus, Listeria monocytogenes* and *B. cereus*) and three Gram negative bacteria (*Salmonella typhimurium, E. coli* and *P. aeruginosa*). BAC is widely used for sanitation in food processing lines and surfaces in the food industry (Kuda *et al.*, 2008), as clinical disinfectant and antiseptic (topical) in health care facilities and domestic households and as antimicrobial preservative in drugs in low concentration (Mangalappalli Illathu and Korber, 2006). McComb and Bennett (1982) used 1 % benzalkonium chloride in 10 % alcohol for the disinfection of explants in *in vitro* propagation of eucalyptus. Yeh and Chang (1986) established somatic embryos from inflorescence callus of *Bambusa beecheyana* var. *beecheyana* after surface sterilization with 0.01 % Antiseptol (benzalklonium chloride 10 % and alkyl-arylpolyether alcohol 10 %). Kondo *et al*. (1996) established shoot cultures of *Spinacia oleracea* through the surface sterilization with 1/100 benzalkonium chloride solution for ten minutes followed by sodium hypochlorite solution with a drop of Tween 20 for 30 min. For developing a cost-effective explant sterilization procedure in aseptic plantlet germination from seed of *Carissa carandas,* Bhadane and Patil (2016) used BKC containing commercial bleach (Lizol).

2.2.1.3. Plant defense activation

Various stresses like microbial and insect attack, activates signalling pathways that induce different defensive protein and non-protein compounds (Denoux *et al*., 2008; War *et al.,* 2010). These proteins inhibit pathogen ingress via mechanisms such as digestion of fungal cell walls, fortification of plant cell walls, and biosynthesis of antimicrobial compounds (phytoalexins) (Lindsay *et al.,* 1993). Moscatiello *et al.* (2006) and Zipfel *et al.* (2006) reported that the defence proteins trigger the early induction of regulatory factors and genes, which encode enzymes for the synthesis of antimicrobial compounds involved in signal perception and transduction, including kinases and phosphatases. Activation of defence system occurs through the recognition of numerous pathogen-derived molecules such as cell wall fragments, flagellin etc. (Dangl and Jones, 2001; Nurnberger and Scheel, 2001; Parker, 2003; Zipfel, 2009).

There are many compounds, which activate chemical defence in plants known as elicitors (Thakur and Sohal, 2013). Defence responses induced by a primary infection can be expressed before the contact with a secondary challenging organism and a primary infection can lead to a faster activation of defence responses after challenge inoculation, a phenomenon known as potentiation (Chen and Chen, 2002). Tissue priming or conditioning and the resulting potentiation of local defence responses was demonstrated in parsley cells treated with SA, 2,6-dichloroisonicotinic acid or benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Zimmerli *et al*., 2001). Kauss and Jeblick (1995) reported the enhanced elicitation of the oxidative burst in such primed cells. Kauss *et al*. (1992, 1993) demonstrated phytoalexin production and the secretion of cell wall phenolics in such plants. Activation of biosynthetic pathways depends on the compound used and chitosan, cell wall fragments, jasmonic acid, hydrogen peroxide, salicylic acid, benzothiadiazole, benzoic acid, etc. are some of them.

2.2.1.3.1. Chitosan

Hadwiger and Beckman (1980) implicated chitosan, a polymer of β-1,4-linked glucosamine residues with a strong affinity for DNA, in the pea pod-*Fusarium solani* interaction as an elicitor of phytoalexin production, an inhibitor of fungal growth and

a chemical which can protect pea tissue from infection. Pearce and Ride (1982) demonstrated that the application of chitosan induced lignification, one of the disease resistance mechanisms in wounded wheat leaves. Addition of soluble chitosan to suspension-cultured *Glycine max* cells inhibited the rate of increase in cell fresh weight, increased their glyceollin content and altered their cell wall composition, which make the walls, became more resistant to degradation by fungal enzymes (Kohle *et al*., 1984). Conrath *et al*. (1989) reported the chitosan elicited a rapid deposition of the 1, 3-ß-glucan callose on the cell wall and formation of coumarins in suspension cultured cells of *Petroselinum crispum*. Besides, its application induced the production of reactive oxygen species (ROS) in many plants, which inhibits fungal infection (Young *et al*., 1982; Benhamou and Theriault 1992; El Ghaouth *et al.*, 1994, Lee *et al*., 1999; Amborabe *et al*., 2008). Ben-Shalom *et al*. (2003) reported that the complete inhibition of conidial germination of *Botrytis cinerea*, the causative organism of grey mould by a 50 ppm chitosan spray in cucumber plants. Barka *et al*. (2004) evaluated the effect of chitosan (1.75 %) in the culture medium for *in vitro* growth, enhancement of root and shoot biomass *Vitis vinifera* and to stimulate protection from *Botrytis cinerea* in these plantlets. According to El Ghaouth *et al*. (1994), chitosan has greatest potential as a biocontrol agent among the elicitors known to date and Povero *et al*. (2010) proved the role of chitosan on activation of plant defence system in *Arabidopsis* seedlings through the transcript profiling.

2.2.1.4.2. Exopolysaccharides

Oxalate and extracts from spinach and rhubarb leaves are known to induce protection against anthracnose caused by *Colletotrichum lagenarium* in cucumber (Doubrava *et al*., 1988). Systemic induced defence activated by *Pseudomonas aeruginosa* through exopolysaccharides **(**EPS) in bean induced protection against TMV (DeMeyer *et al*., 1999). Maurhofer *et al*. (1994) reported that Tobacco necrotic virus (TNV) in tobacco is inhibited by *P. fluorescens*. *Serratia marcescens* is one of the important bacteria known to induce resistance against various pathogens and it has been shown in betel vine to protect against foot and root rot caused by *Phytopthora nicotianae* (Lavania *et al.*, 2006). Many researchers have proven the potential of *S. marcescens* against various pathogens like *Fusarium oxysporum* f. sp. *cucumerinum* (Liu *et al*., 1995), *C. orbiculare* (Someya *et al*., 2004), bacterial pathogen, *P. syringae pv. lachrymans* (Liu

et al., 1995), and the *Cucumber mosaic virus* (Ryu *et al*., 2004). Leeman *et al*. (1995) reported that *Fusarium* wilt in carnations was systemically defended through the bacterial outer membrane lipopolysaccharide (LPS) from *P. fluorescence.* Guzzo *et al*. (1993) reported the effectiveness of crude EPS from *Xanthomonas campestris* in coffee against *Hemileia vastatrix*. The LPS from *S. marcescens* is known to possess inducer activity in tobacco against *P. solanacearum* (Graham *et al*., 1977). Ipper *et al*. (2006) reported that the culture filtrate from *Serratia* sp. controls CMV infection in tobacco. Ipper *et al*. (2008) proved the role of EPS from *Serratia* sp. strain Gsm01 in induced systemic protection in tobacco against CMV-Y and revealed its effect on plant defence related genes and compounds.

2.2.1.4.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) that plays a dual role in plant cells. H_2O_2 can act as a signalling molecule that mediates plant responses to a variety of biotic and adverse abiotic stress factors (Foyer *et al*., 1997). Pre-treatment with H_2O_2 has been shown to protect maize seedling from chilling injury by induction of peroxidases and mitochondrial catalase (Prasad *et al*., 1994a, 1994b). Karpinsk *et al*. (1999) reported the systemic signalling property of H_2O_2 and its acclimatory response to high light stress in *Arabidopsis*. Spraying with hydrogen peroxide (H_2O_2) induced various factors to develop tolerance against oxidative stress in tobacco plants (Gechev *et al*., 2002). Gondim *et al.* (2012) evaluated the effects of pre-treatment with H_2O_2 leaf sprays on plant growth in maize and found the antioxidative mechanisms involved in the response to stress, which enhanced the protective function of different enzymes against these stresses. Uchida *et al*. (2002) achieved the salinity tolerance in rice through the pre-treatment with H_2O_2. Neto *et al*. (2005) demonstrated the resistance development by exogenous application of H_2O_2 in maize. According to Wahid *et al.* (2007) antioxidant enzyme activity, lipid peroxidation and chloroplast ultrastructure was altered by the foliar application of H_2O_2 in *Cucumis sativus*.

2.2.1.4.4. Beta amino butyric acid

The non-protein amino acid, β-aminobutyric acid (BABA) has been known as an inducer of disease-resistance since 1963 and to protect about 40 plant species against

about 80 pathogens and pests, including a virus, protista, bacteria, oomycetes, fungi, nematodes and arthropods (Cohen *et al*., 2016). It has been proven that BABA is also involved in diverse physiological processes including seedling growth and stress tolerance of many plant species (Jakab *et al*., 2005). BABA-induced resistance (BABA-IR) has been frequently found in many crops against diverse bacterial and fungal species in recent decades (Hamiduzzaman *et al*., 2005; Olivieri *et al*., 2009; Walz and Simon, 2009). Chen *et al*. (2012) and Farahani *et al*. (2016) reported BABA induced resistance against *Pectobacterium carotovorum* ssp. *carotovorum* in *Arabidopsis* and tomato respectively. Resistance against late blight disease caused by *Phytophthora infestans* in potato was achieved by BABA treatment (Eschen-Lippold *et al*., 2010). Ton and Mauch-Mani (2004) revealed that the molecular genetics analysis of the BABA induced resistance in *Arabidopsis* against the necrotrophic fungi *Alternaria brassicicola* and *Plectospharella cucumerina* was independent of ABA mediated defence mechanism. Cohen *et al*. (2010) suggested that the application of BABA induced resistance in lettuce against *Bremia lactucae*, the downy mildew oomycete pathogen. Fungal resistance developed against two different classes of fungal pathogens – the necrotrophic *Alternaria brassicicola* causing black spot disease and the hemibiotroph *Colletotrichum higginsianum* causing anthracnose in kimchi cabbage by the foliar spraying of BABA (Kim *et al*., 2013). Hamiduzzaman *et al*. (2005) proved the role of BABA in protection of grapevine against downy mildew pathogen, *Plasmopara viticola* through the potentiation of some defence genes such as PR-4. BABA-induced resistance in *Brassica juncea* against *Alternaria brassicae* is mediated through an enhanced expression of some defence genes such as PR1 (Kamble and Bhargava, 2007). Similar results in potato was shown by Bengtsson *et al*. (2014) Roylawar *et al*. (2015) reported the comparative analysis of BABA against *Piriformospora indica* mediated priming of defence related genes in tomato against early blight. Induced resistance by BABA in artichoke against white mould caused by *Sclerotinia sclerotiorum* has been demonstrated by Marcucci *et al*. (2010). Amzalek and Cohen (2007) compared the efficacy of systemic acquired resistance-inducing BABA against rust infection by *Puccinia helianthi* in sunflower plants. Cohen *et al*. (2010) reported mechanisms behind the DL-b-aminobutyric acid mediated activation of resistance in *lettuce* against *Bremia lactucae*. BABA induces resistance to *Peronospora parasitica* in cauliflower without accumulation of PR proteins (Silue *et al*., 2002).

2.2.2. *In vitro* flowering

Flowering in bamboos is still a phenomenon that is largely not understood and depends probably on many factors comprising of environment, nutrition, and the physiological status of the plants (Ramanayake, 2006). The occurrence of *in vitro* flowering offers a very convenient model to study the phenomenon under controlled conditions. Such studies are expected to throw light on the primary factors involved including the molecular mechanisms and role of growth regulators (Yuan *et al*., 2017). The involvement of the genes associated with flowering in other species and comparison in bamboo will be possible. *In vitro* floral transition is expected to be similar with natural flowering mechanism. Control over *in vitro* flowering will also enable its prevention during micropropagation where it is undesirable.

2.2.2.1. *In vitro flowering* in bamboo

In vitro flowering has been reported in a dozen bamboo species mostly belonging to genera *Bambusa* and *Dendrocalamus*. The first ever report by Nadgauda *et al*. (1990) was on the species *B. arundinacea* (Syn. *B. bambos*) and *D. brandisii.* This was followed by Chambers *et al*. (1991) who reported *in vitro* flowering in shoot cultures (derived from nodal explants) of young seedlings of *D. hamiltonii* during the transfer from MS basal media with 22.2µM BA to a growth regulator-free medium after 8 wk. Sporadic flowering was reported in shoot cultures of bamboo species such as *B. nana, B. arundinacea*, *C. pergracile* etc. and it was observed that all shoots died after flowering, except those of *B. multiplex* (Prutpongse and Gavinlertvatana, 1992). *In vitro* flowering was observed by Rout and Das (1994) in shoots of *B. vulgaris, D. giganteus* and *D. strictus* developed from nodal explants taken from somatic embryo regenerated plants cultured on half-strength MS basal medium supplemented with indole-3-butyric acid (IBA), adenine sulphate and gibberellic acid (GA_3). Nadgauda *et al*. (1997) carried out a comparative study between *in vitro* and *in vivo* flowering in bamboo *B. arundinacea. In vitro* flowering with normal morphology occurred in green shoots of *B. edulis* that were maintained for 8 months on the basal medium supplemented with cytokinin (Lin and Chang, 1998). Plantlets regenerated through somatic embryogenesis in *B. oldhamii* exhibited spontaneous flowering with 75v% of viable pollen (Ho and Chang, 1998). Ramanayake *et al*. (2001) achieved *in vitro* flowering in *D. giganteus* and reported that the *in vitro* flowering was not the

expression of a species-specific mechanism believed to occur during gregarious flowering, as the mother clump did not flower. Lin *et al.* (2003) studied the *in vitro* flower formation in shoots derived from spikelet-derived, somatic embryos of *B. edulis*. Lin *et al*. (2006) described a protocol that can be used to produce large numbers of mutant inflorescences within a relatively short period in *D. latiflorus*. Green and albino regenerates of *D. latiflorus* developed from cultures that flowered after 8 mon of subculture in medium containing 4.54 µM TDZ, had normal florets but sterile pollen (Lin *et al*., 2007). Kaur *et al*. (2015) successfully transformed the vegetative meristem to a floral meristem on flower induction medium and revealed the proteomics that expressed the metabolic changes during floral transition in *D. hamiltonii.*

Factors affecting *in vitro* flowering

Flowering, the conversion of plants from vegetative phase to reproductive development, like all natural phenomena, occupies a certain position in time and space and has a certain material content (Chailakhyan, 1968). The timing of flowering is determined by endogenous genetic components as well as various environmental factors viz. day length, temperature and stress. Cho *et al*. (2017) reviewed the effect of nutrients, ambient temperature, drought, salinity, exogenously applied hormones and chemicals, and pathogenic microbes on flowering and how such stress or stimuli regulated plants either begin flowering to produce seeds for the next generation or else delay flowering by slowing their metabolism.

2.2.2.2.1. Photoperiod

Day length is an important factor for the conversion of vegetative phase of a plant into its reproductive stage (Bernier and Perilleux, 2005). Day-length threshold, typically more than 10 h, accelerated the flowering by inducing reproductive apex development in barley and wheat (Mansuri, 1969; Roberts *et al.*, 1988). Banko and Stefani (1991) reported that long photoperiod (16 h and 24 h) influenced *in vitro* flowering of *Oxydendrum arboreum* on culture media supplemented with 2, 4, or 6 µM zeatin. Jumin and Nito (1995) demonstrated the importance of photoperiod for *in vitro* flowering by inducing flowering at 16-h photoperiod but not in continuous darkness in *Murraya paniculata* plantlets that were derived from protoplasts. Vaz *et*

al. (2004) reported the interaction between photoperiod and floral spike formation in *Psygmorchis pusilla* and demonstrated that longer photoperiod i.e. 20 h or more negatively affected floral bud development, inhibiting anthesis and reducing flower longevity. Nandagopal and Kumari (2006) obtained *in vitro* flowering in *Cichorium intybus* plantlets in the MS medium with combination of cytokinin and auxin under 16/8. Influence of photoperiod was demonstrated in embryogenic callus culture developed from ovules of Kinnow mandarin and it was observed that 12-h photoperiod effectively induced highest flowering percentage (31.94 %) with maximum number (5.58) of flowers (Singh *et al.,* 2006). Kanchanapoom *et al*. (2010) reported that a photoperiod of 12/12 induced *in vitro* flowering in shoots regenerated from nodal explants of *Rosa hybrida* cv. 'Heirloom'on MS medium supplemented with BAP and Kin. Aina *et al.* (2012) reported that all the day-length (12,16 and 24 h) treatments induced *in vitro* flowering in *Arachis paraguariensis* but *in vitro* flower bud initiation was delayed in all photoperiod except 12 h and therefore this plant is considered as short-day plant. In *Arabidopsis thaliana*, long photoperiod accelerates the flowering function through the activation of *FLOWERING LOCUS T (FT)* protein, one of the main component of florigen, the systemic floral inducing substrate (Song *et al.,* 2013). Photoperiod (12/12) induced *in vitro* flowering was obtained in *Rungia pectinata* (Shekhawat *et al.,* 2016) with 30 μmol m^{-2} s^{-1} light intensity and *in vitro* induced flowers were found to be morphologically similar to those of the plants in the field.

2.2.2.2.2. Stress Factors

Stress factors of poor nutrition or low-intensity light are the main factors that can induce flowering in many plant species. In the genus *Citrus*, the stress factor, drought has an endorsing effect on flowering (Monselise, 1985). Wada and Takeno (2010) proved that the stress induced floral stimulus are transmissible through the grafting experiments in short-day plants *Pharbitis nil* and *Perilla frutescens* var. *crispa*. There are various stress factors such as high and low light intensity, exposure to ultraviolet radiation, drought, poor nutrition, poor nitrogen or oxygen etc. Besides, stress induction through pruning and suppression of root elongation can also induce flowering (Kostenyuk *et al*., 1999). Culturing of *Pharbitis nil* in nutrient-poor solution (tap water) under continuous light also exhibited faster flowering response

(Shinozaki *et al*., 1988). Adams *et al*. (1998) reported the effect of abiotic stresses by salt on accelerated flowering in *Mesembryanthemum crystallinum* and described that these effects are species-specific. Kolar and Senkova (2008) reported that the reduced mineral nutrient availability through hydroponic system had accelerated flowering in *A. thaliana*.

In tissue culture, half strength MS medium or reduced nitrogen level enhanced *in vitro* flowering in many agriculturally important as well as medicinal plants like *Orychophragmus violaceus* (Luo and Lan, 2000), tomato (Dielen *et al*., 2001) etc. Even seedlings of *O. violaceus* flowered when they were cultured on MS with a reduced amount of NH_4NO_3. In contrast, sucrose and nitrogen concentrations in the medium did not affect the rate of *in vitro* flowering in *B. edulis* (Lin *et al*., 2003). Flowering was observed when the cultures were 3 mon. age of *in vitro* shoots. The highest number of flowers per shoot was 9.4 in MS medium with reduced amount of NH_4NO_3.

2.2.2.2.3. Plant Growth Regulators

The conditions required for *in vitro* flowering is species specific and among the various factors influencing flowering, plant growth regulators (PGR) play an important role. PGRs influence many diverse developmental processes ranging from seed germination to root, shoot and flower formation (McCourt 1999). Cytokinins promote the transition in many higher plants to their reproductive stage *in vitro* (Paek *et al*., 1989; Wang *et al*., 1993). Some of the examples are ginseng (Chang and Hsing, 1980), *Phaleanopsis* (Duan and Yazawa, 1995), *Murraya paniculata* (Jumin and Ahmad, 1999), *Fortunella hindsii* (Jumin and Nito, 1996) and *Pharbitis nil* (Galoch *et al*., 2002) . Kintzios and Michaelakis (1999) reported the use of single cytokinin, such as N_6-benzyladenine (BA), zeatin and kinetin, to induce *in vitro* flowering in most studies.

Nadgauda *et al*. (1990) induced *in vitro* flowering in *B. arundinacea* and *D. brandisii* after three subcultures on MS medium containing 5 % (v/v) coconut water and 0.5 $mg1^{-1}$ BAP. Similarly, *B. vulgaris, D. strictus* and *D. giganteus* flowered *in vitro* on half-strength liquid/solid MS medium supplemented with combination of different PGRs within 12 wks of subculture (Rout and Das, 1994). Patil *et al*. (1993) indicated

that the endogenous cytokinins in ascending xylem sap can be stimulated by exogenous cytokinins that lead to the flowering. Kostenyuk *et al.* (1999) considered the role of BAP crucial in *in vitro* flowering, especially in orchids like *Cymbidium niveo-marginatum* in which it induced the flowering within 3 to 6 mon. The earlier report by Kerbauy *et al*. (1984) also supported this hypothesis and showed that BAP induce flowering *in vitro* in orchids and it significantly reduced the time taken (from years) to reach maturity (8 mon. in *Oncidium varicosum*) necessary for flowering.

Other cytokinins such as adenine sulfate, TDZ and iso pentyl adenine are also recognized to have roles in *in vitro* flowering. Flower induction in shoot cultures of *Cichorium intybus* was promoted by iso pentyl adenine and polyamines (Bais *et al.*, 2000). Flowering was induced in thin cell layers of tobacco cultures by kinetin and IAA by the enhanced production of S-adenosyl-L-homocysteine hydrolase, a cytokinin-binding protein formed during early flower bud formation (Tanaka *et al.*, 1995). Bernier *et al.* (1993) had shown the accumulation of zeatin riboside and isopentyl adenine in shoot tip shortly after floral induction in *Sinapsis alba*. Rout and Das (1994) and Singh *et al. (*2000) have shown the role of cytokinins in bamboo *in vitro* flowering. Presence of particular cytokinin or an alteration in equilibrium between the auxin-cytokinin ratios is believed to bring about flower induction *in vitro* in bamboo and this induction indicates the possibility that cytokinins trigger or contribute precursors to one or more specific compounds in the plant which trigger induction of flowering (John and Nadgauda, 1999).

Auxin induced adventitious bud formation and flowering were reported in *Torenia* stem segments cultured *in vitro* and it was demonstrated that IAA stimulated the flowering whereas the cytokinin, zeatin inhibited the conversion at all concentrations (Tanimoto and Harada 1981). According to Handro *et al*. (1983.), low concentrations of auxins induced flowering in *Streptocarpus nobilis*. Smolders *et al.* (1990) reveal that the dose of NAA determined flower bud regeneration in tobacco explants at a large range of concentrations. Combination of NAA and GA_3 induced maximum flowering in *Coriandrum* cultures (Stephen and Jayabalan, 1998), whereas the incorporation of activated charcoal to this combination enhanced the *in vitro* blooming in sunflower (Patil *et al*., 1993). Similarly, the combination of cytokinins and auxins also induced flowering in other species. In *Vitex*, flowering was obtained

with BAP in combination with NAA (Thiruvengadam *et al*., 2001). *Pisum sativum* exhibited *in vitro* flowering on medium supplemented with auxins (IBA or NAA) either alone or in combinations on the 7^{th} and 15^{th} day after their transfer (Franklin *et al*., 2000). In chicory, the maximum number of *in vitro* flowers were observed on media supplemented with BAP, IAA and ADS and this indicated that the media supplemented with adenine sulphate favored high frequency of *in vitro* flowering (Nandagopal and Kumari, 2006). In a study on the factors affecting *in vitro* flowering of *Perilla frutescens,* IBA was more efficient than NAA and IAA and IBA induced flowering in 65.6 % of the shoots (Zang 2007). Maximum flowering frequency (50%) from nodal explants of *Rauvolfia serpentina* was obtained on MS medium containing IBA, IAA and kinetin and Mondal *et al*. (2011) demonstrated the combined action of auxin and cytokinins on floral transition in this species. Shekhawat *et al.* (2016a) reported the induction of maximum numbers of *in vitro* flowers in *Aerva lanata* in the shoots culture established from nodal explants on MS medium with BAP and IAA on 12/12 photoperiod.

Tang (1999) suggested that GA_3 concentration is one of the important factors regulating *in vitro* flowering of regenerated plantlets from somatic embryogenesis and organogenesis in ginseng. Factors implicated in the promotion of floral transition of the *Ceropegia attenuata* have been identified as 4-amino-3, 5, 6-trichloropicolinic acid (picloram), 6-benzylaminopurine, sucrose and photoperiod (Chavan *et al*., 2011).

2.2.2.2.4. Sugars

Carbon sources are the most important as well as necessary factor involved in growth and development of plants in any stage. Among the different carbon sources, sucrose known to have the main role in flower induction and development (Rastogi and Sawhney, 1987). The influence of different concentrations of sucrose was evaluated among different species and it was found out that the flower induction occurred at between 30-60 g l^{-1}. The frequency and efficiency of flower bud differentiation was higher in the presence of 40 g l^{-1} sucrose in the medium; this result coincided with the earlier reports of flowering on citrus (Tisserat *et al.,* 1990) and gentian (Zhang and Leung, 2000, 2002). Lin *et al.* (2003) reported the incidence of *in vitro* flowering of *B. edulis* during the maintenance of shoot cultures on MS medium with TDZ and 30 gl^{-1} sucrose. Sucrose concentration in apical buds influenced flowering in *A. thaliana*

(Roldan *et al.,* 1999). Interaction between sucrose and cytokinins was also involved in floral induction in *Synapsis alba* by translocation between shoot and root (Havelange *et al.,* 2000). Flower bud formation on embryogenic callus from ovules of Kinnow. Mandarin orange diminished with the alteration in sucrose concentration and the optimum concentration for flower induction was found out be 40 gl^{-1} (Singh *et al.,* 2006) and the highest number of flowers (5.41) per culture with maximum percentage of cultures producing flower buds were observed at this concentration.

Garcia-Luis *et al.* (1989) reported that 170 mM glucose induced flowering in 6 % of in *vitro* cultures of *Citrus unshiu.* To test the influence of sugars on cultured buds to induce *in vitro* flowering in *Citrus*, 0, 0.1, 0.3, 1, 3, 5 and 7.5 % sucrose, fructose or glucose were substituted for the 3 % sucrose in the medium, it was found that different carbon sources and higher concentration could not induce flower bud and subsequent fruit development (Tisserat *et al.,* 1990). Sucrose was found the best for *in vitro* flowering in *Anethum graveolens* at 3 % (w/v), closely followed by fructose, glucose whereas mannose and sorbitol were totally ineffective (Jana and Shekhawat, 2011). This result coincides with earlier reports in *Pentanema indicum* (Sivanesan and Jeong, 2007), *Pisum sativum* (Franklin *et al*., 2000) and *Gentiana trifolia* (Zhang and Leung, 2002).

2.2.2.2.5. Influence of the ratio of C/N on *in vitro* flowering

Floral nutrient diversion hypothesis postulated that during flowering, C/N ratio increase in buds (Sachs, 1977) and this was supported by Tanimoto and Harada (1981). This was also proven by Konar and Nataraja (1964) and Nitsch and Nitsch (1967) who demonstrated that high C/N ratio promoted plant reproductive development while high N/C ratio encouraged plant vegetative growth. C/N ratio influenced the male flower formation in bitter melon at sucrose concentration of 150 mM which induced adverse osmolarity (Tanimoto and Harada 1981; McDaniel *et al.,* 1991), as well by decreasing the level of NH_4NO_3 to 10 mM, 5 mM or zero (Salisbury and Ross, 1985). Narasimhulu and Reddy (1984) reported in *Arachis hypogea* that the mineral formulation of the medium has an important role in the expression of flower bud primordia and they studied the role of three major inorganic salts such as ammonium, potassium and calcium nitrates and revealed the promotive influence of ammonium in the phenomenon. *In vitro* culturing of nodal explants of

Kalanchoe blossfeldiana on a low nutrient hormone-free medium resulted in flowering response and revealed the influence of nitrogen in the form of NH_4NO_3 and KNO_3 on flowering and vegetative growth (Dickens and Van-Staden, 1988). Adventitious shoot formation and *in vitro* flowering was carried out in *Phalaenopsis* Pink Leopard 'Petra' on Vacin Went medium with BAP and it was reported that the high concentration of total nitrogen inhibited the development of floral buds, and low-temperature treatments failed to induce the formation of floral buds *in vitro* (Duan and Yazawa,1995). Culturing of *Cymbidium* plantlets in MS medium containing BAP with high P and low N content than cultures grown in half-strength MS medium containing BAP without a modified P/N ratio improved the *in vitro* flowering 2.5-fold (Kostenyuk *et al*., 1999). Franklinhe *et al*. (2000) induced *in vitro* flowering and fruiting of *Pisum sativum* on transferring of elongated shoots onto MS medium containing half strength ammonium nitrate (8.25 g l^{-1}). Higher concentration of nitrogen and carbohydrate in MS media inhibited the *in vitro* flowering in tomato (Dielen *et al*., 2001). *In vitro* flowering of 52 % was achieved in *Dendrobium* on a medium containing high P and low N content whereas only 20 % of plantlets formed inflorescences in half-strength MS medium without any modification of the P/N ratio after four months (Tee *et al*., 2008). Murthy *et al*. (2010) demonstrated the effect of nutrient availability on *in vitro* flowering in *Ceropegia spiralis* derived from nodal and nodal thin cell layers. MS medium at half strength produced an average of 5.6 flower buds per microshoot with highest (90 %) flower bud induction response in *C. noorjahaniae* (Chavan *et al*., 2014).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1.GENERAL MATERIALS

3.1.1. Species

All experiments in this study were carried out with explants collected from the following bamboos species. Adult clumps of *Pseudoxytenanthera stocksii* (Munro) T.Q. Nguyen (Syn.: *Dendrocalamus stocksii (Munro)* M. Kumar, Ramesh and Unnikrishnan); *Pseudoxytenanthera ritcheyi* (Munro) (Syn.: *Pseudoxytenanthera ritcheyi* (Munro) (Syn.: *Munrochloa ritchiei* (Munro) M. Kumar & Remesh), *Bambusa balcooa* Roxb., *B. tulda* Roxb., *B. nutans* Wall. ex Munro and *Dendrocalamus longispathus* (Kurz) Kurz.

3.1.2. Location of mother clumps

Adult clumps growing in the Bambusetum of the Field Research Centre of Kerala Forest Research Institute, Velupadam, Thrissur, and Kerala were selected as mother plants and the source of explants for all the experiments that followed. In the case of *D. longispathus*, seeds were available at the time of the study and were used to initiate the cultures.

3.1.3. Glassware

Erlenmeyer conical flasks made up of borosilicate glass (Borosil Glass Ltd, Mumbai; or Scott Duran, Germany), test tubes (Borosil, India; 200 x 25 mm) and culture bottles (450 ml) capped with polypropylene film closures and petri plates (90 mm in diameter, Borosil, India) used for *in vitro* experiments. All glassware were autoclaved to disinfect from residual microorganisms from previous batch, immersed in chromic acid solution overnight, then washed well with a liquid detergent (Vitroclean, Merck, India) followed by a thorough wash in running tap water and a rinse with single distilled water and allowed to dry in an oven before being used.

3.1.4. Chemicals

All chemicals used during the course of study were of Analytical Grade procured from reputed manufacturers (Sigma Chemicals Ltd. USA; Hi - Media, India or Merck, India)

3.1.5. Culture media

For micropropagation, basal medium as per Murashige and Skoog (1962) (MS), Woody Plant Medium (WPM, Lloyd and McCown, 1980), B_5 (Gamborg *et al*., 1968) and SH (Schenk and Hildebrandt, 1972) were used. Double distilled water was used for the preparation of stocks as well as culture media. Sucrose 3 % (w/v) was used as the carbohydrate source unless otherwise specified. After adding all the constituents of medium and PGRs, the pH of the medium adjusted to 5.7 by adding required volume of 0.1N NaOH or 0.1N HCL using a digital pH meter. The medium was solidified using agar agar (0.7 %, w/v, Meron Marine Products, India) or Clerigel (0.2 %, w/v, Hi-media, India). 100 ml of medium was poured in each conical flask or to culture bottles and the vessels tightly closed. In case of test tubes, 15 ml medium was used and tightly plugged with non-absorbent cotton. The medium was autoclaved at 121ºC and pressure of 1.05 kg/cm² (15 psi) for 20 min. in a horizontal autoclave. The flasks, culture bottles and test tubes were kept in culture room after autoclaving for gelling of the medium.

3.1.6. Preparation of PGRs

Stock solution of different PGRs were prepared at 10 mg/100 ml. For preparation of 100 ml stock solution, 100 mg of desired PGR was weighed in an analytical balance and dissolved in specific solvent required for the particular PGR. All PGRs used in this study were co-autoclaved with other components in the media.

3.1.7. Preparation of sterilizing agents

Mercuric chloride solution of 0.1 % (w/v) was prepared by dissolving 10 mg in 100 ml of distilled water. Streptomycin sulphate 200 ppm was prepared by dissolving 200 mg of antibiotic in 1000 ml of distilled water. Similarly, antifungal Bavistin (1.0 %, w/v, BASF, India) was prepared by dissolving 1000 mg of powder in 100 ml of distilled water.

Toxic chemicals like $HgCl_2$, NaOCl etc. were handled carefully and kept in separate well-labelled bottles. Preliminary experiments with the use of NaOCl for surface sterilisation had given unsatisfactory results and therefore, in spite of the fact that $HgCl_2$ is highly toxic, it was used with all precautions taken in handling and disposal. Hygroscopic chemicals were stored in desiccators to avoid denaturation.

3.1.8. Aseptic transfers

All the culture inoculations and aseptic manipulations were carried out in a laminar airflow cabinet. The cabinet swabbed with rectified spirit while keeping the air- flow on. All the culture vessels and instruments such as Petri plates, forceps, scalpels, conical flasks, and test tubes sterilized were earlier by autoclaving. Prior to use, the cabinet was irradiated with UV light for 30 min. by closing the hood and airflow before inoculation. During the culture operations, forceps and scalpels were further sterilized by dipping in 70 % ethyl alcohol and flaming frequently between the transfers. In order to ensure aseptic conditions, both hands also washed properly and then swabbed with 70 % ethyl alcohol.

3.2. MICROPROPAGATION

Micropropagation of two species viz. *Pseudoxytenanthera stocksii*, and *Pseudoxytenanthera ritcheyi*, were taken up in the present investigations employing two pathways:

(a) Axillary bud proliferation

(b) Somatic embryogenesis

Various parameters were tested to standardize the protocols for micropropagation of the two species.

3.2.1. Axillary Bud Proliferation

3.2.1.1. Explants

1. Nodal segments: 2-4 cm long segments of stem collected from the secondary branches each containing a bud were used as explants.

2. Nodal buds from rhizome: Nodal buds scooped with the help of a sharp blade from the point of attachment with the swollen part of freshly emerging rhizomes were also used as explants.

Nodal segments were prepared for culture by removing the leaf sheaths to expose the tender buds which were then cleaned with a soft bristled tooth brush and liquid detergent (Tween 20; Hi-Media, India) followed by continuous rinsing with tap water. Thereafter, the explants were treated with a solution containing Bavistin (1.0 %),

Cefotaxime (200 ppm) and Tetracycline (200 ppm) for an hour with constant stirring. Finally, explants were surface sterilized in a laminar flow bench with 70 % ethanol for 1-2 min. followed by treatment with an aqueous solution of $HgCl_2$ (0.1 %) containing 1-2 drops of liquid detergent (as wetting agent) for 8 min. and again washed thoroughly 4 times with sterilized distilled water. Then, explants were dried on a sterile filter paper by gentle dabbing and were inoculated upright in test tubes containing the chosen medium.

3.2.1.2. Culture conditions

Cultures were incubated at a PPFD (photosynthetic photon flux density) of 70 ± 5 µmmol m^{-2} s^{-1} provided by cool, white, fluorescent lamps (Phillips, India) at 25 ± 2 ºC. Light and dark period were maintained at 10 /14 h light /dark cycles unless otherwise mentioned.

3.2.1.3. Culture Initiation

3.2.1.3.1. Effect of different media on initiation of cultures

Optimum bud break and growth of sprouts depend on the selection of a medium particularly suited to the species being propagated. Therefore a species specific selection of the basal media was crucial for the culture establishment and further growth. Different basal media compositions viz. MS media, WPM media, SH media and B5 media were tested for initiation of cultures and bud sprouting in two bamboo species. Basal media were evaluated by recording the number of explants (nodes) sprouted, number of sprouts formed per explant and time taken for sprouting.

3.2.1.3.2. Effects of cytokinins

It is often unnecessary to add growth regulators to the medium for inducing sprouting of axillary buds in some plants. If they are required, plant growth regulators will usually comprise a cytokinin and an auxin, at rates sufficient to support active shoot growth. To study the effect of cytokinin on axillary bud proliferation, BAP or Kin were incorporated in MS medium at concentration varying from 2.22 to 17.76 µM and 2.32 to 18.50 µM respectively. TDZ was incorporated at lower concentrations ranging from 0.04 to 4.54 µM, metatopolin (mT) 5 to 20 µM and zeatin from 0.22 to 9.12 µM.

3.2.1.3.3. Synergetic effect of auxins and cytokinins

In certain species, individually supplied cytokinins or auxins does not induce any effect on explant. Lesser concentrations of exogenous auxins together with cytokinins activated the endogenous hormones level and improve the shoot proliferation. Nodal segments collected from both the bamboo species were cultured on MS medium supplemented with combination of Kin (4.65, 9.30 and 13.95 μM) and the auxin, NAA (0.27, 0.54 and 1.34 μM), IAA (0.29, 0.57 and 1.43 μM) or IBA (0.25, 0.49 and 1.23 μM) at varying concentration so as to study the effect of cytokinin – auxin combinations on axillary shoot proliferation.

3.2.1.3.4. Addition of gelling agents

Nutritional requirements of plant cultures can be supplied by liquid media but sometimes this may adversely affect the growth and development by oxygen deprivation and hyperhydration. Control over hyperhydricity (vitrification) can be achieved by adding gelling agents to the media and by placing the tissue in contact with air. Agar and gellam gum are the main gelling agents that have been widely used in plant tissue cultures. In order to assess the effect of gelling agents on bud sprouting and culture establishment in bamboo species, these were added to the initiation media with the liquid media as control.

3.2.1.3.5. Role of carbon source on sprouting response

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is known (Koch, 1996). To study the effect of carbon sources, glucose, fructose, sucrose and lactose added individually at a concentration of 3 % in MS medium supplemented with optimal hormonal requirement.

3.2.1.3.6. Effect of size of explant on sprouting response

Shoot cultures are conventionally started from the explant up to 2.0 cm in length. Standardizing the size of the explant has an important role in culture establishment. MS medium supplemented with different cytokinins (BAP: 10 μM, Kin: 15 μM, TDZ: 1 μM, mT: 10 μM) were used for the experiment. Other variables like length, diameter and position of explant were kept constant when explants were selected.

3.2.1.3.7. Effect of Position of explants on culm on sprouting response

The correct choice of explant material can have an important effect on the success of tissue culture. Likewise, the results on proliferation also influenced by the position of the nodal segments on mother plant. In order to evaluate the effect of the position of explant on the plant, explants from a secondary branch was divided into three categories: the lower (1^{st}-3^{rd}), middle (4^{th}-7^{th}) and upper (8^{th}-10^{th}) node on the branch and the effect on sprouting was evaluated on MS medium supplemented with different cytokinins (BAP: 10 µM, Kin: 15 µM, TDZ: 1 µM, mT: 10 µM). Other variables like length, diameter and position of explant were kept constant when explants were selected.

3.2.1.3.8. Effect of season on sprouting response

To investigate the seasonal effects on *in vitro* axillary bud proliferation, the collection time in the calendar year was divided in four seasons viz. March to May, June to August, September to November and December to February. Nodal segments were collected in the first week of every season. Influence of different cytokinins on seasonal effect for sprouting response was evaluated. MS medium supplemented with different cytokinins (BAP: 10 µM, Kin: 15 µM, TDZ: 1 µM, mT: 10 µM) were used for the experiment.

3.2.1.4. Shoot Proliferation

For shoot multiplication, different combinations and concentrations of PGRs, other additives, auxin inhibitors and effect of decapitation were tested.

After initial screening, one-month-old nodal segments having 2-3 sprouted shoots were used for the experiments. Sucrose (3 %, w/v) as the carbohydrate source and agar (0.8 %, w/v) were used for shoot proliferation. Medium without PGRs served as control. Five replicates containing five shoots each were employed. Sub-cultures were performed after every 4 wks.

3.2.1.4.1. Effect of cytokinins on shoot multiplication

Application of cytokinins produce various effects in intact plants such as stimulated protein synthesis and participation in cell cycle control. They were added to the media

to overcome apical dominance and release lateral buds from dormancy. In this study, the cytokinins (BAP: 10 µM, Kin: 15 µM, TDZ: 1 µM, mT: 10 µM) were added to the media to improve the shoot proliferation of cultures of *P. ritcheyi* and *P. stocksii* at different concentrations and combinations.

3.2.1.4.2. Effect of gelling agents on shoot multiplication

Gelling agents are added to the media for ensuring the adequate contact between the tissue and the media. In plant tissue culture system, agar and gellan gum are the commonly used solidifying agents. The disadvantage of solid media is that there is minimal area in contact with the explant surfaces. As tissues grow, there may be gradient developing in nutrients, growth factors and the waste products of metabolism between medium and tissue. Gaseous diffusion is also affected by the gelling agent and gel strength. Optimization of gelling agent is therefore important for obtaining the best multiplication.

3.2.1.4.3. Effect of different media on shoot multiplication

The composition of the medium is a determining factor for growth. There are so many media in use and the amounts, particularly of nitrogen and potassium, have been found to influence maximum growth. Studies reveal that culture medium composition influences the multiplication rate as well as shoot necrosis. Therefore, optimization of strength of nutrient media was essential parameter that influenced the increased shoot development. The effect of different basal media (MS media, WPM media, SH media and B5 media) on shoot proliferation and shoot necrosis was examined especially for *P. ritcheyi*. Besides, increase in shoot numbers and shoot heights were recorded after 30 and 60 d. interval. Sub-cultures were performed after every 28 d.

3.2.1.4.4. Effect of additives on shoot multiplication

Incorporation of substances other than plant growth regulators also have certain impact on the growth and proliferation of vegetative phase in plants in *in vitro*. Coconut water has been shown to stimulate shoot proliferation in many species of plants. It was prepared from selected coconuts and processed to remove most of the protein. The product was then filter sterilized and frozen for future use. While storing, a precipitation of remaining protein became visible which should not affect the growth of the plant

tissue. The precipitate could be removed by filtering or by allowing it to settle to the bottom of the bottle and then decanting. Coconut water could be divided into smaller aliquots and used at a concentration of 5-20 % (v/v) for the growth promotion of shoots cultures in bamboo.

Myo-inositol (meso-inositol/ i-inositol) is the only one of the nine theoretical stereoisomers of inositol which has significant biological importance such as phosphate storage, cell wall biosynthesis, the production of stress related molecules, cell-to-cell communication, storage and transport of plant hormones. Myo-inositol was supplied at 20 - 1000 mg l^{-1} for proliferation of bamboo shoot culture.

For plant tissue culture, amino acids are not essential media components; but their addition as identified pure compounds, or more cheaply through casein hydrolysates (CH) can be an easy way of ensuring against medium deficiency, or of providing a source of nitrogen that is immediately available to cultured cells or tissues. For enhancing the growth, additional supplements of these compounds are frequently opted. Different concentrations of CH and glutamine individually were added to the shoot multiplication media of the two bamboo species to improve the multiplication rate.

Shoot tip browning was observed as a severe problem in shoot cultures of *P. ritcheyi*. This may be due to the osmotic properties exerted by the inorganic components of culture media. Addition of non-penetrating osmolytes such as mannitol, polyethylene glycol (PEG) etc. into the culture media can resolve this constraint. Increased levels of solidifying agents (agar) can also be used to improve the condition.

Addition of various antioxidants to the culture media was attempted to eradicate the shoot tip browning in cultures of *P. ritcheyi*. Different concentrations of ascorbic acid, citric acid and polyvinyl pyrollidone (PVP) were used to remove the phenols or reduce their accumulation in culture media. Their effects were evaluated in terms of tissue browning as well as shoot multiplication.

3.2.1.4.5. Effect of pH on shoot tip browning during shoot multiplication

Browning of explants and media is usually attributed to oxidized phenolic compounds and phenol polymers generated by elevated polyphenol oxidase (PPO) activity. In this

study attempts to prevent the activity of the enzyme through changes in the pH of the media was carried out. The pH of all media were set at 4, 4.5, 5, 5.7 (standard pH for bamboo tissue culture media), 6, 7, and 8. Cultures were visually assessed for browning of the tissues and media.

3.2.1.4.6. Effect of subculture duration on shoot multiplication

It is a common practice to shift the plant material from one media to another. Both the nature and the duration of the first and second treatments can be important and therefore the most effective duration for each species has to be determined, which is influenced by the concentration and nature of the plant growth regulator as well as the size of the shoot cultures. The subculture duration in the two species of bamboo was standardized in this study by transfers after 8, 16, 24 and 30 d. to fresh medium.

3.2.1.4.7. Role of carbon source (sucrose) on shoot multiplication

Metabolizable carbon source is an essential component of plant tissue culture media and serves to meet the energy requirements of the tissues. In addition to sucrose, which is the most widely used sugar, glucose, fructose, sorbitol and maltose have been used as a carbon source for culture. 2-3 % was the optimum concentration of sucrose used for the shoot proliferation in various plants. In this study, we examined the influence of sucrose at the range of 0 - 6.0 % in shoot cultures of bamboo *P. stocksii* and *P. ritcheyi* by assessing the increase in number of shoots at the end of the passage.

3.2.1.4.8. Effect of inoculum size on shoot multiplication

Initial inoculum density is a key factor in establishing shoot culture of any plant species because shoots cannot resume or even stop their active growth after transfer if the initial inoculum is below the critical density. Therefore the inoculum size of the shoots that gives maximum multiplication rate was standardized during the subculture period in both the species by transfer of shoot clusters consisting of 2, 3, 5 and 6 shoots each to fresh medium and evaluating the enhanced shoots number per cluster and length of shoot at the end of the passage.

3.2.1.5. *In vitro* rooting

Rooting is one of the major limiting factors in micropropagation of bamboo species. For achieving rooting in the microshoots, various parameters were used. Shoots were used either singly or in clusters of 2-3 shoots giving a fresh cut at the lower ends just below a node before transferring to auxin supplemented medium solidified with agar (0.8 %, w/v) or gellan gum (0.2 %, w/v) as gelling agent. The cultures were kept in dark as well as under 10 h illumination. Number of roots, days required for rooting, and length of roots and increase in shoot number were recorded.

3.2.1.5.1. Effect of auxins on root induction

Most of the plants responded to auxins coupled with root initiation at lower concentration and their root growth at higher concentration. Effectiveness of auxins on root formation in these two bamboo species was evaluated in terms of time taken for root initiation, length of root after incubation period and nature of the shoot portion. Well-developed shoots (≥ 5 cm long) from multiplication medium were excised and exposed to different strength of liquid MS basal media (2X, 1X, ¾ X, ½ X, ¼ X, 0) with 3 % sucrose, pH 5.8 containing 32 µM NAA and 29.5 µM IBA and maintained at 16 h photoperiod.

3.2.1.5.2. Effect of different basal media on root induction

Strength of minerals and their presence in ionized or non-ionized forms vary from medium to medium in plant tissue culture. Root induction is effectively influenced by various ions and their concentrations. In order to find out the best media for root induction, different basal media were used for root induction in these two bamboo species. Well-developed shoots (5-7 cm long) from multiplication medium were excised and exposed to different liquid basal media (MS, SH, B5, WPM with 3 % sucrose, pH 5.8) containing either 32 µM NAA or 29.5 µM IBA and maintained at 16 h photoperiod.

3.2.1.5.3. Effect of sucrose concentration on *in vitro* rooting

Root initiation and growth are processes with high energy requirements that could only occur at expense of available metabolic substrates, which were mainly

carbohydrates. The type of carbon source and its concentrations affect rooting in many plant species *in vitro.* For evaluating the effect of sucrose on root induction on *P. stocksii* and *P. ritcheyi*, sucrose at the range of 1 % - 6.0 % were tested in root induction media.

3.2.1.6. *Ex vitro* rooting

Multiple shoots (4–5 cm long) derived from axillary bud explants were excised and the cut end of the stem given a 15 min. dip in a solution of the auxins, IAA, NAA, NOA and IBA individually at concentrations of 10, 50, 100, 250, 500, 750, 1000 and 2000 ppm for root induction.

3.2.2. Somatic Embryogenesis

Induction of somatic embryogenesis as an alternate pathway for plant regeneration from explants of the two species was explored using different explants, nutrient media composition.

3.2.2.1. Explants used

i. Shoot segments: Basal part of *in vitro* shoots including the nodes were used for initiating callus cultures
ii. Leaf explants: Unopened leaf segments were taken from shoot-tips and used for initiating callus cultures
iii. Immature shoot tips: from emerging field grown adult plant

3.2.2.2. Callus Initiation

3.2.2.2.1. Effect of explant type

In order to identify the best explant for the high frequency callus induction, various explants viz. leaf sheath, leaf, internode and node segments (measuring 1-1.5 cm in length) were inoculated into MS agar medium fortified with growth hormone, 2, 4-D (13.65 µM) and Kin (9.24 µM). Cultures were maintained in dark in culture room. Observations were recorded on percentage response and intensity of callus on completion of 4 wks. of inoculation.

3.2.2.2.2. Effect of plant growth regulators

Various concentrations of auxins, 2, 4-D (4 µM – 16 µM), 2,4,5-T (4 µM-10 µM), NAA (4 µM-10 µM), IAA (4 µM – 10 µM), IBA (4 µM-10 µM), Picloram (4 µM-10 µM), coconut water (10 % v/v) and cytokinins; Kin and BAP (2.5 µM) were used either alone or in combinations using 23 treatments in the MS agar gelled medium to standardize concentration and combinations of growth regulators for callus induction from the nodal segments.

3.2.2.2.3. Effect of basal media on callus induction

The differing abilities of basal media to support callus induction were tested using MS, WPM and B5 basal media. Each medium contained 30 $g1^{-1}$ sucrose, 2, 4-D (13.65 µM) and Kin (9.24 µM) with agar. Explants were inoculated and cultures were maintained under dark condition in culture room. Observations were recorded on percentage response and intensity of callus after 4 wks. of inoculation.

3.2.2.3.Proliferation of callus

3.2.2.3.1. Effect of plant growth regulators

For proliferation of callus, MS media supplemented with different concentrations and combinations of cytokinins (BAP and Kin) with different auxins (2, 4-D, 2, 4, 5-T, NAA, IAA, IBA, Picloram and Dicamba) in factorial combinations were used. For each set of experiment, 300 mg of cream coloured, nodular and compact calli were used. Cultures were maintained in the dark and sub-culturing done at 4-5 wks. intervals.

3.2.2.3.2. Effect of basal media on callus proliferation

To check the effect of strength of basal media on embryogenic callus, basal MS, WPM and B5 were used which were supplemented with 2, 4-D (13.65 µM) and Kin (9.24 µM) or with 2, 4-D (9.1 µM) alone. For each set of experiment, 300 mg of cream coloured, nodular and compact calli were used. Cultures were maintained in the dark. Increase in biomass, change in the colour and appearance of calli were observed after 45 d. after second subculture.

3.2.2.3.3. Effect of sucrose concentration on callus growth and maintenance

To standardize the sucrose concentration for callus proliferation and maintenance, different concentrations (1.5, 3.0, 4.5, 6.0, 7.5 and 10 %) were added in combination with 2, 4-D (13.65 µM) and Kin (9.24 µM) in MS agar gelled medium. For each set of experiment, 300 mg of cream coloured, nodular and compact calli were used and cultures maintained in the dark.

3.2.2.4. Embryogenesis

For studying the feasibility of embryogenesis, the compact and nodular callus was transferred to MS fortified with different concentrations of auxins (IBA, IAA and NAA) and their combination with BAP. Effect of different basal media (MS, WP, B5 and SH) were also evaluated for the differentiation of embryos.

3.2.2.5. Maturation and germination somatic embryos

Only compact, nodular cream coloured callus lumps were able to differentiate into embryos whereas, friable callus did not show any response and turned brown after 2-3 sub-cultures.

For germination of somatic embryos, various combinations and concentrations of PGRs, different basal media were used. The experimental details were as follows:

3.2.2.5.1. Effect of PGR

To standardize the best plant growth regulators for conversion (germination) of somatic embryos different concentrations of BAP (2.22 µM - 6.66 µM) singly and in combinations with NAA, IBA and IAA were used in MS agar gelled medium.

3.2.2.5.2. Effect of basal nutrient media

In order to determine the optimum nutritional requirements of basal media for germination of somatic embryos, MS, ½ MS, ¼ MS, B5 and WPM supplemented with BAP (2.22 µM) were tested.

3.2.3. Hardening of plantlets

Rooted plants were washed thoroughly under tap water and cleaned with the help of a soft sable brush for the removal of the gel adhering to the roots of plantlets. These washed plants were then dipped in a solution of Bavistin (500 mgl^{-1}) for 5-10 min. as a protection against fungal infection and transferred to plastic pots containing moist sand. These plantlets were covered immediately with jars to maintain relative humidity of 80-85 % and maintained in a polyhouse. These plantlets were observed on daily basis to detect any causality.

After 30 d, these plants were transferred to pots containing a mixture of sand: soil: FYM in the ratio of 1:1:1. Hoagland's solution sprayed once in a month. Data after 6 mon. of transfer were recorded in terms of percent survival, number of new shoots emerging, height of plantlets and number of new leaves formed at the requisite time intervals.

33.2.4. Statistical analysis

Each treatment consisted of at least 20 explants and each experiment was repeated 5 times. Visual observation of cultivars was made every day. Percentage of culture response of explants, number of shoots per explant were recorded. Data were analyzed using one-way ANOVA. Data were analyzed by the Duncan's multiple test (p=0.05) using SPSS (ver. 18).

3.3.2. CONTROL OF ENDOPHYTIC CONTAMINATION

Isolation of frequently found contaminating microbes and effectiveness of different strategies to control endophytic contamination were evaluated in these three species.

1. *Bambusa balcooa*
2. *Pseudoxytenanthera stocksii*
3. *Pseudoxytenanthera ritcheyi*

3.3.1. Isolation and identification of endophytic contaminants

Isolation of endophytic fungi and bacteria was carried out under *in vitro* conditions. Different symptomless parts of the selected bamboo species plants such as stem cuttings and leaves were used for the isolation.

3.3.1.1. Surface sterilization of plant material

The plant material used for the isolation was first surface sterilized as follows. Plant material collected from field grown plants as small twigs with leaves were first cleaned by washing several times under running tap water and then cut into small segments of stem with single node of 2-3 cm or leaves sections of 1×1 cm. Surface sterilization was performed by sequential rinsing the plant material with 70 % ethanol (C_2H_5OH) for 30 s., then with 0.01 % $HgCl_2$ for 5 min. followed by 0.5 % NaOCl for 2-3 min. and finally rinses with sterile distilled water for 2-3 times. Plant material was then dried in between the folds of sterile filter papers.

The disinfection process was checked by pressing the disinfected plant material onto both Potato Dextrose Agar (PDA) (Himedia Ltd., Mumbai, India) and the Luria Bertani agar (LBA) (Himedia Ltd, Mumbai, India). Aliquots of water from final rinse solutions were also plated on the same media. The surface disinfected leaves and stem were used in the fungal and bacterial isolations described below.

3.3.1.2. Fungal isolation

Endophytic fungi were isolated twice from three bamboo species. A random sample from each species, consisting of 30 surface disinfected leaves or stem explants was taken, cut into 10 fragments (4–6 mm), placed onto PDA containing 100 µg ml^{-1} tetracycline. After 3–7 d. incubation at 28 °C, the number of pieces showing fungal growth was counted. The hyphal tips of each morphologically different mycelium that emerged from a leaf fragment were subcultured and transferred to PDA slants for later identification according to standard procedure by Funder (1953).

3.3.1.3. Bacterial isolation

Endophytic bacteria were also isolated twice from four bamboo species. A random sample from each species, consisting of 30 surface disinfected leaves or stem, was taken. Individual leaves were homogenized in a blender in 5 ml of sterile 0.85 % NaCl, and serial dilutions plated onto LBA. The plates were incubated at 28 °C for 1–10 d. until growth was observed, upon which the numbers of colony-forming units (CFU) were counted and the population density estimated.

In a further experiment, each leaf and stem then cut into 10 fragments (4–6 mm), which were placed onto Tryptone Soya Agar (TSA) containing 50 μgml^{-1} benomyl. After 1–10 d. incubation at 28 °C, the number of pieces showing bacterial growth counted. The endophyte incidence (EI) was calculated as the percentage of pieces showing bacterial growth.

3.3.1.3.1. Morphological and biochemical characterization of endophytes

The endophytes isolated from different plants were described and identified based on their morphological and biochemical characteristics.

The bacterial endophytes were described and identified on the basis of various morphological features like colony form, margin of the colony, its elevation, color of the colony, its surface, opacity of the colony and staining reactions while for the fungal endophytes both macroscopic and microscopic features like; growth of fungi, conidial development, size, shape of conidia, shape of conidial head, attachment of conidia were taken into account. The isolated bacterial endophytes were identified through their biochemical characteristics.

3.3.1.3.1.1. Morphological characterization of bacterial endophytes

The endophytes isolated from selected plants were described and identified on the basis of morphology of the colony. Different morphological traits were analyzed like colony form, margin of the colony, its elevation, color of the colony, its surface, opacity of colony and Gram reaction (Holt *et al*., 1994).

3.3.1.3.1.1.1. Characterization based on colony morphology:

The isolated endophytes were streaked onto nutrient agar plates and incubated at 37 °C for 48 h. At the end of the incubation period the plates were observed for colony characteristics. The colony characters were examined from 2 d. old culture of the bacteria. The colonies were examined for shape, opacity, elevation, surface, margin and color.

3.3.1.3.1.1.2. Characterization based on staining reactions:

Staining techniques are employed for differentiation and separation of bacteria in terms of morphological characteristics and cellular structures by applying specific stains:

Gram Reaction (Gram, 1884)

A thin film of culture was spread on a dry and a clean glass slide, fixed over the flame and stained with 0.5 % aqueous crystal violet solution for one minute. Excess of stain was drained off. The slide bearing the bacteria was rinsed in gram's iodine for one min. and then washed with 95 % EtOH until no more violet stain comes out. The slides were then rinsed with safranin stain for 10 s. They were then washed with water and examined under oil immersion to observe the Gram reaction of the bacterial endophytes.

Capsule stain:

A heavy smear of each organism was prepared. The slides were allowed to air dry and flooded with crystal violet for 5 min. followed by washing with 20 % copper sulphate solution and observed under oil immersion for the reaction. However, in order to further authenticate the identification of bacteria belonging to a particular genus biochemical characterization of the isolates were also performed.

3.3.1.3.1.2. Biochemical characterization of bacterial endophytes

After carrying out the preliminary morphological analysis, further biochemical analysis was carried out so as to authenticate the identification of bacteria. Bacteria were identified and classified largely on the basis of their reactions in a series of biochemical tests. The biochemical tests that were carried out under present investigation include: carbohydrate test, indole test, citrate test, catalase test, MR-VP test, hydrogen sulphide production test, gelatin test, starch and motility test. Identification of microorganisms has an important application. The characteristic biochemical activities will facilitate more specific identification of bacterial species. Control tubes where placed in each of the biochemical tests which were not inoculated with the test bacteria whereas the rest were inoculated with the test bacteria.

3.3.1.3.1.2.1. Starch hydrolysis test:

The test bacteria was streaked on starch agar medium and incubated at 37 °C for 48 h and then flooded with 1.0 % iodine solution and observed for cleared zone. Blue color indicated no hydrolysis, while a clear zone indicated hydrolysis.

3.3.1.3.1.2.2. Citrate utilization test:

The plates of citrate medium were inoculated with the test bacteria and incubated at 37 °C for 48 h and then observed for the results. A positive reaction was indicated with a Prussian blue color and growth of bacteria. A negative test had no growth of bacteria and remained green.

3.3.1.3.1.2.3. Catalase test:

A loopful of test bacteria was stirred in a drop of 10 % w/v hydrogen peroxide and then observed for the results. The appearance of gas bubbles indicated a positive test while no bubbles indicated a negative test.

3.3.1.3.1.2.4. Gelatin hydrolysis test:

The nutrient gelatin deep tubes were stab inoculated with the test bacteria and incubated at 37 °C for 48 h, then placed in a refrigerator for 30 min. and observed for the liquification. Control tubes solidified when placed on ice whereas when medium in inoculated tubes remained the same, showed positive gelatin hydrolysis test.

3.3.1.3.1.2.5. Hydrogen sulphide production test:

The production of H_2S was examined by inoculating the loopful of the culture of the test bacteria in the Kligler Iron Agar (KIA) medium. The tubes were then incubated at 37 °C for 48 h and then observed for the result. Blackening of the medium indicated a positive test.

3.3.1.3.1.2.6. Carbohydrate fermentation test:

The test bacteria were inoculated into the broth carefully so as to not disturb the Durham tube (the inverted small glass tube inserted in the broth) and incubated at 37 °C for 48 h and then observed for the results. Positive test indicates colour change from red to yellow. Negative test showed no color change. A bubble in the tube was an indication of gas production.

3.3.1.3.1.2.7. Motility test:

The agar tubes containing the media were stab inoculated with the test bacteria and incubated at 37 ° C for 48 h and then observed for the growth. Diffusion of colony indicated positive test.

3.3.1.3.1.2.8. Methyl Red-Voges Proskauer test:

The test bacteria was inoculated in the medium and incubated at 37 °C for 48 h. Five drops of Methyl Red indicator was added to the tube and change in color was observed. A positive result had a distinct red layer at the top of the broth. A negative result had a yellow layer.

The medium was inoculated with the test bacteria and incubated at 37 °C for 48 h then the VP reagents (Barritt's Reagent A and Barritt's Reagent B) were added to the tubes and observed. Pink or red indicated a positive reaction. No change in color or copper color indicated a negative reaction.

3.3.1.3.1.3. Morphological characterization of fungal endophytes

The isolated fungal endophytes were described and identified on the basis of morphological features like colony characterization, growth of fungi, color of colony (front and reverse) on PDA (Promputtha *et al*., 2005) and their microscopic appearance like conidial development, size, shape of conidia, shape of conidial head and attachment of conidia. The microscopic identification of fungal endophytes was carried out by lacto-phenol cotton blue staining method.

3.3.1.3.1.3.1. Characterization based on macroscopic features:

The isolated endophytes were inoculated onto PDA plates and incubated at 28 °C for two wks. At the end of the incubation period the plates were observed for colony characteristics. The colony characters were examined from two weeks old culture of the fungi. The colonies were examined for growth of fungi and color of colony (front and reverse).

3.3.1.3.1.3.2. Characterization based on microscopic features:

Rectangular cello tape was gently touched on the fungal colony to be identified. A small drop of lactophenol cotton blue stain was placed for 2 min. on the slide and covered with a glass cover slip. The fungus to be identified was then examined under the microscope for appearance of conidial development, size and shape of conidia, shape of conidial head and attachment of conidia.

Maintenance of endophytes:

The purified endophytic fungal isolates transferred separately to PDA slants. Bacterial species also were sub-cultured and transferred to LBA slant. All the purified endophytes were maintained at 4 °C till further use.

3.3.2. Effect of endophytes on *in vitro* growth of bamboo, *D. longispathus*

3.3.2.1. Plant material and culture conditions

Aseptic seedlings of the bamboo, *D. longispathus,* were initiated from seed collected from Tripura, India. The seeds were dehusked and given a thorough wash for 60 min. under running tap water followed by a treatment for 60 min. with a 1 % solution of Bavistin. Surface sterilization was then carried out with a treatment of 0.1 % $HgCl_2$ solution for 3 min. followed by repeated rinses with sterile distilled water to remove the traces of sterilants. The seeds were then inoculated under a laminar flow hood on a hormone free basal medium containing the basal salts of MS media solidified with 0.8 % agar-agar and supplemented with 2 % sucrose. The pH of the medium was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl prior to autoclaving (at 121 °C at 1.06 kg cm^{-2} pressure) for 20 min. All the cultures were maintained at 25 ± 2 °C under a16 h photoperiod and an average light intensity of 25 µE m^{-2} s^{-1} provided by cool white fluorescent tubes and at a RH of 50 to 60 %. The seedlings after 7 d. of germination were transferred to liquid MS media supplemented with 2 % sucrose and 2.21 µM BAP (Shoot Multiplication Medium, SMM) for induction of multiple shoots following which the shoots were maintained *in vitro* through 15-d. subcultures in the above media.

3.3.2.2. Identification of the bacterial strain

The contaminating organism was isolated by streaking a loop full of media from contaminated shoot cultures onto sterile LBA and PDA. The isolate outgrowths from LBA were collected and sub-cultured onto LBA and the cultures incubated at 28 °C for 24 h. Pure bacterial isolates were obtained by repeated sub-culturing using a serial dilution technique (Collins and Lyne, 1984) and maintained on LBA slants. The purified isolates were identified based on colony morphology, cell morphology, Gram reaction and other biochemical tests (IMViC tests) (Collins and Lyne, 1984, Krieg and Holt, 1984; Sneath *et al.*, 1986).

3.3.2.3. Antibiotic sensitivity of bacterial isolates

3.3.2.4. Effect of elimination of endophyte in shoot cultures with antibiotics

To eradicate the endophytic bacteria in shoot cultures, gentamycin, selected based on the sensitivity tests described above, was added to the liquid SMM at concentrations ranging from 10 to 500 µg ml^{-1} to determine the bactericidal dose. To confirm the efficacy of the bactericidal treatment, shoot cultures transferred to antibiotic free media after 15 d. of culture in antibiotic containing media and observed for signs of bacterial growth. Shoots were maintained over three passages through subculture to evaluate the effect of the eradication of endophyte.

3.3.2.5. Assessing the role of endophyte /culture filtrates on growth of shoot cultures of *D. longispathus*

3.3.2.5.1. Co-culturing with endophyte

Since antibiotic treatment of shoot cultures of *D. longispathus* resulted in the reduction of growth and multiplication rate of shoots, the role of the endophyte *in vitro* was tested by re-introduction of the bacteria into shoot cultures. The bamboo shoot cultures from antibiotic containing SMM was transferred into fresh SMM and inoculated with a loopful of the pure culture from agar slant and cultures maintained for three passages through subculture every 15 d.

3.3.2.5.2. Conditioning with filtrate

The media from 15 d. old shoot cultures of *D. longispathus* harboring the endophyte was centrifuged at 10000 rpm for 10 min. to remove the bacterial cells and filter

sterilized through a 0.22 µm membrane filter to obtain the sterile conditioned filtrate. This filtrate was then mixed with equal quantities of fresh, sterilized stock of SMM of 2X concentration to formulate the Spent Media. Shoot cultures of *D. longispathus* with endophytes and those free of endophytes were additionally included in the experiment.

3.3.2.6. *In vitro* assay for IAA production by bacteria

In order to check for the production of the auxin, IAA, pure culture of the bacteria was inoculated on to LBA amended with 0.5 mM L-Tryptophan (L-Trp). Petri plates (9 cm dia.) were inoculated with toothpicks into a grid pattern from agar cultures and the membrane lift assay (Bric *et al.,* 1991).The plate was overlaid with Whatman No. 1 filter paper immediately after inoculation and incubated until colonies reached 0.5 to 2 mm in diameter. Thereafter the paper was removed from the plate and treated with Salkowski's Reagent (Gordon and Webber, 1951) and saturated in a Petri dish by overlaying on a reagent-saturated filter paper until adequate color developed at room temperature. Bacteria producing IAA were identified based on the formation of a characteristic red halo within the membrane immediately surrounding the colony.

3.3.2.7. Estimation of IAA production by bacteria

The experiment was carried out in order to screen for the media for the optimal biosynthesis of auxin by the endophyte. Conical flasks (30 ml) containing 5 ml of LB broth, MS liquid media, with and without 5 mM of L-Trp, were inoculated with 1ml of the bacterial suspension and maintained at 28 °C in the dark with constant agitation at 140 rpm. After growth for 48 h, the liquid cultures were centrifuged at 7,000 g at 4 °C for 10 min. and the supernatants collected. The amount of IAA produced in culture was estimated by adding 1 ml of culture supernatant to 2 ml of Salkowski's Reagent. The mixture was kept for 120 min. in dark until red color developed, with darker red indicating a higher amount of IAA. The color intensity was measured at an absorbance of 530 nm (Asghar *et al.,* 2002) in a spectrophotometer. IAA production in shoot cultures of *D. longispathus* harboring the endophyte was assessed using the spent media after 3 d. of culture.

3.3.2.8. Assessment of growth promotion by endophyte and spent medium in shoot cultures of three non-host bamboo species

The potential for growth promotion by the endophytic bacteria isolated from *D. longispathus* on growth in other non-host bamboo species was evaluated. Shoot cultures of three species of bamboo viz. *B. balcooa*, *P. ritcheyi* and *D. strictus* derived from axillary buds of adult plants and maintained through 15 d. subcultures on SMM, were used in the experiments.

3.3.2.8.1. Co-culturing with endophyte

A single colony of the bacteria from LBA slant was inoculated into SMM and shoot cultures of each of the three species transferred to this media and maintained through subculture every 15 d. Cultures of the three species on SMM without the endophyte were maintained as control.

3.3.4.8.2. Conditioning with filtrate

The filtrate was prepared by the procedure mentioned above. This filtrate was then mixed with equal quantities of fresh, sterilized stock of SMM of 2X concentration to formulate the Spent Media. Shoot cultures of the three bamboo species were inoculated into this media and maintained by subculture to the fresh reconstituted media every 15 d. Shoot cultures of the three species on SMM were maintained as control.

3.3.2.8.3. Conditioning with auxin

In order to examine whether exogenous supply of IAA could induce the same results as that of the filtrate-conditioned media, 5.71 µM of the auxin was added to SMM into which shoot cultures of the four bamboo species were inoculated and maintained by subculture every 15 d.

3.3.2.9. Assessment of shoot multiplication rates

Shoot multiplication rates was assessed in each of the above experiments as follows. Fresh weights of shoot cultures at the beginning of experiment were calculated as the differences in the weight of the culture vessel with fresh media and that of the same vessel after inoculation, under sterile conditions, with the shoot culture. Enhancement in growth of shoots at the end of 3rd subculture was assessed by removing the shoot

cultures onto sterile blotting paper in the laminar flow chamber for 60 s to remove the liquid media before weighing. The rate of shoot multiplication was assessed as percent increase in shoot number over 3 passages, using the formula:

$$Shoot\ multiplication\ rate = \frac{No.\ of\ shoots\ after\ 3rd\ subculture - No.\ of\ shoots\ at\ 1st\ subculture}{Total\ number\ of\ explants} \times 100$$

Only shoots that had attained a length of 1 cm and above were counted. The data is reported as the mean of ten replications for each treatment. Means and the standard error of the means were calculated for the effect of different treatments. Student's t-Test was performed in order to determine the significance ($P<0.05$) of treatments in different species.

3.3.2.10. Plate assay for siderophore production by the bacterial isolate

Siderophore production by the growth promoting bacterial isolate was determined through Blue agar CAS medium containing Chrome Azurol sulphonate (CAS) and hexadecyl trimethyl ammonium bromide (HDTMA) as indicators as described by Schwan and Neilands (1987). The Blue agar medium was aseptically poured on to sterile plates and allowed to solidify. After solidifying, a paper disc was placed on the center of the CAS blue agar and the bacterial suspension cultured in LB broth was dropped onto the paper disk and incubated at 30 °C for 48 h.

3.3.2.11. Test of antagonistic effects of bacterial isolate

A 5 mm mycelial mat of *Fusarium oxysporum*, the most frequently occurring contaminating fungi in bamboo tissue culture, taken from a pure culture maintained on PDA, was placed on one side of a fresh PDA plate and the endophytic bacteria isolate was streaked on the other side of the plate and cultured at 28 ºC for 7 d. During the incubation period, antagonistic effects of the bacterial isolate against the fungal isolate was confirmed by the inhibition zone formed between the bacteria isolate and fungal isolate. The dual culture was performed in three replicates.

Only shoots that had attained a length of 1 cm and above counted. The data is reported as the mean often replications for each treatment. Means and the standard error of the

means calculated for the effect of different treatments. Student's t-Test was performed in order to determine the significance ($P < 0.05$) of treatments in different species.

3.3.3. Control of endophytes with antimicrobial food preservatives

To determine the influence of the weak organic acid food preservatives on contamination in bamboo shoot cultures, lactic, acetic, citric, benzoic, sorbic and propionic acids were used (Table:**1**) at different concentrations. The pH level is also an important consideration as preservatives have an optimum pH range for their antimicrobial activity.

Table 1: List of organic acids, their concentration and pH tested in bamboo tissue culture for controlling endogenous as well as exogenous contamination

Sl. no:	Chemical	Concentration (%)	pH
1.	Lactic acid	0.1, 0.2, 0.5	3, 4.5, 5.6, 6
2.	Acetic acid	0. 2, 0.3, 0. 4	4.5, 5.6, 6
3.	Citric acid	0.02, 0.03, 0.04	4.5, 5.6, 6
4.	Calcium propionate	0.02, 0.03, 0.04	4.5, 5.6, 6
5.	Potassium sorbate	0.05, 0.1, 0.2, 0.3, 0.5	3, 4.5, 5.6, 6
6.	Sodium benzoate	0.05, 0.10, 0.2, 0.25, 0.5	3, 4.5, 5.6, 6
7.	Sodium meta bisulphite	1, 2, 3, 4, 5	3, 4, 5.5, 5.6

All these chemicals are heat stable and added to the media prior to the autoclaving. pH range also adjusted with 0.1 N NaOH or 0.1N HCl. Disinfection and surface sterilization of explants carried out as described earlier.

To assess the antimicrobial activity of food preservatives at lower pH, shoot initiation media (MS + 12 µM BAP) were prepared with the preservative chemicals at different levels and pH adjusted by the addition of 0.1N HCl and 0.1 NaOH. Axillary buds from all the three species were inoculated into each of these media. Sprouted axillary buds without exogenous contamination were inoculate into the same media to assess the effect of the preservatives on latent contamination. Cultures were maintained upto 10 passages by 2 wk subcultures for assessing the presence of latent contamination.

3.3.4. Control of endophytes with antimicrobial preservatives used in pharmaceuticals and cosmetics

3.3.4.1. Methyl paraben

For studying the effect of methyl paraben on controlling contamination during various stages of culture, surface sterilised single nodal explants inoculated into MS supplemented with methyl paraben at the following concentrations- 0.01 %, 0.02 %, 0.03 %, 0.05 % and 0.1 %. Stock solution of paraben was prepared in acetone at concentration of 2.5 % and stored at room temperature. Aliquots from the stock solution was added to the MS media for getting the final concentrations and autoclaved. It was noticed that finely divided flakes of the solute precipitated after steam sterilization.

Effect of MP was analysed as the substitute for the $HgCl_2$ by adding to the media and inoculating the explants without treatments.

3.3.4.2. Thimerosal

Thimerosal was directly dissolved in the double distilled water and the stock solution was prepared at concentrations of 100 µg ml^{-1}.

Effect of thimerosal was analysed by two methods. In the first experiment, it added to the medium after sterilization and inoculated the surface sterilized explants.

In the second experiment, thimerosal stock directly added to the media and explants were inoculated without autoclaving the media. Effect of $HgCl_2$ treatment was also evaluated.

3.3.4.3. Benzalkonium chloride

Benzalkonium chloride, a quaternary ammonium compound is an effective chemical for the treatment in bacterial removal without affecting the structural integrity of product. This synthetic antimicrobial agent widely used as a disinfectant in processing lines and surfaces in the food industry as clinical disinfectant and antiseptic in health care facilities and domestic households and as antimicrobial preservative in drugs (Mangalappalli-Illathu and Korber, 2006). Its surfactant ability provides a great capacity to penetrate and adhere to porous surfaces of the bamboo nodal segments, which were used as explant for the establishment of shoot cultures in micropropagation.

After surface sterilization with $HgCl_2$, explants were individually placed inside sterile plastic vessels and given 50 ml washing treatment with 0.1 mg ml^{-1} BAC. The untreated explants with $HgCl_2$ after disinfection and sterile water was placed in another sterile plastic vessel. Samples were hand agitated for different period to facilitate wetting by the washing solution. After washing, explants were transferred to a new sterile vessel and subjected to a 100 ml sterile distilled water rinse for three times. Each rinse was done for 1 min. to remove chemical residues. Then, each explant was placed in fresh tissue culture media and the effect of treatment evaluated in terms of type of contamination, d. of emergence, bud breaking frequency and the time taken for bud break. Besides, observations were taken on the incidence of latent contamination, type contamination and time taken for the emergence of endophyte as contaminant.

3.3.5. Control of endophytes through the activation of the plant defense

Plant defence elicitors or boosters are a group of compounds, which act by triggering various physiological and morphological responses within the plant that help to stimulate natural defence mechanisms. The practical significance of plant defence boosters is that they can help to reduce the amount of crop protection chemicals applied to crops. Here in this study we tried to activate the plant immune system in order to suppress the emergence of its own endophytes as pathogens during various culture stages *in vitro*.

3.3.5.1. Chitosan

3.3.5.1.1. Preparation of chitosan stock

Chitosan (degree of N-deacetylation 80-85 %, prepared from crab shells) obtained from Marine Chemicals, India. Chitosan was dissolved in 100 ml 0.1 % (v/v) acetic acid with pH adjusted to 5.2 with 0.1N NaOH by stirring at 160 rpm for 24 h at room temperature (Li *et al.,* 2008) and the insoluble particles were removed by centrifugation (15 min. 10 000 × g at 24 °C). This resulting stock solution was autoclaved at 121 °C for 20 min. for further use in sterile culture. Different concentrations of chitosan were prepared in MS media.

3.3.5.1.2. Prophylactic treatment of axillary buds with Chitosan

Chitosan at different concentrations (0.5, 1, 1.5, 1.75, 2 and 2.5 %) (v/v), 0.1 %(v/v) acetic acid (as positive control) and MS medium (control) were sprayed on the prospective nodal explants on the secondary branches of *P. ritcheyi* and *B. tulda,* using a hand sprayer. The spray was continued until a uniform coating of chitosan droplets was deposited on the surface of the nodes. A set of axillary buds from a different clump was also sprayed with sterile MS media as control. The explants, one set consisting of dormant axillary buds and the other of sprouted axillary buds were collected from the plant 24 h after treatment and subjected to a surface sterilization treatment as described above and inoculated into MS media. Opened and unopened buds were cultured in polypropylene vessels (10 cm x 15 cm) and test tubes (15 mm x 25 mm, Borosil, India) respectively.

3.3.5.1.3. Control of contamination in chitosan amended media

Two sets of explants i.e. dormant axillary buds and sprouted axillary buds were collected from the plants. After surface sterilization, axillary buds were inoculated on MS medium supplemented with chitosan. Dormant buds were inoculated in test tubes of size (15 mm x 25 mm, Borosil, India) and Opened buds in polypropylene vessels (10 cm x15cm). In order to determine the optimal dose for control of contamination and allowing the best growth of *in vitro* axillary buds. Aliquots of chitosan stock were added to the medium to obtain final concentrations of 0.5, 1, 1.5, 1.75, 2 and 2.5 % before autoclaving. Media without chitosan formed the controls.

3.3.5.1.4. Evaluation of duration of exposure to chitosan on sprouting of nodal explants

For evaluating the effect of chitosan treatment on microbial contamination, explants after surface sterilization were incubated in the different concentrations of chitosan added to MS media, and incubated for different periods (12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h) after which they were transferred to MS medium without chitosan.

3.3.5.2. Exopolysaccharide

Plant endophyte fungus or bacterium can deliver various types of elicitors, which induce plant disease resistance. These elicitors can promote the production of various

secondary metabolites, such as terpenoids, alkaloids, saponins, phenols, etc. in plants (Tian et *al*., 2012). Cellular surface structures or secretions, including polysaccharides, polypeptides, glycoproteins and unsaturated fatty acids (Wang *et al*., 2015) etc. can act as the endophyte elicitors. Exopolysaccharides, the main component of cell wall often derived from digestion of the cell wall by plant hydrolases (Mohnen and Hahn, 1993). Extracellular polysaccharides secreted by endophytes can induce some complex interactions between endophytes and host plants (Hiraoka, 1995). Exopolysaccharides (EPS) are soluble or insoluble polymers secreted by microorganisms (Kumar *et al*., 2007).

3.3.5.2.1. Bacterial strain and isolation of exopolysaccharides

The endophytic bacteria *Bacillus subtilis* that commonly emerged as latent contamination was used for the experiment. In order to produce EPSs the bacteria were grown on LB media for 72 h on a rotary shaker at 28 ^{o}C. The culture supernatant was obtained by centrifugation at 12,000 rpm for 10 min. at 4 ^{o}C and concentrated to approximately 1/10th volume by freeze drying. The exopolysaccharide was precipitated by adding a double volume of ethanol. The mixture was kept at 4 ^{o}C for 12 h and centrifuged (12,000 rpm, 10 min.). The pellet was collected and air dried at room temperature and suspended in 0.1 M Tris (pH 8.0) and samples were treated with DNase (67 mg ml^{-1}) and RNase (330 mg ml^{-1}) at 37 °C for 2 h followed by proteinase K (40 mg ml^{-1}) digestion at 55 °C for 3 h. This extract was used for the experiment.

3.3.5.2.2. Estimation of EPS from *B. subtilis*

Once the EPS was extracted, the resulting EPS suspension (suspended in 10 ml distilled water) evaluated for the total amounts of sugar and protein using previously developed methods. Specifically, the sugar quantification performed with Phenol-Sulfuric Acid (PSA) method (Dubois *et al.,* 1956), and the protein was quantified using the Lowry method (Lowry *et al*., 1951).

The PSA method involved adding 50 ml 80 % (w/w) phenol solution, followed by 5 ml of highly concentrated sulfuric acid 95.5 % to 2 ml of resuspended EPS solution. The phenol, acid, and EPS suspension was incubated at room temperature (22– 25 °C) for 10 min., followed by incubation in a water bath for 20 min. at 25–30 °C. After the incubation steps, the dark yellow to brown color of the solution required an additional

4 h for stabilization, and this was done by leaving the suspension at room temperature. Final measurement of the suspension was done spectroscopically at 480 nm with Xanthan gum as a standard.

Total protein amount quantification was performed with the Lowry method using bovine serum albumin (BSA; 1 mg ml^{-1}) as the standard and measured spectroscopically at a wavelength of 500 nm. Next, 0.3 ml of suspended EPS solution (in 10 ml DI water) was put in a glass vial (20 ml in volume) and 1.5 ml alkaline copper reagent (made by combining 1 ml 2 % $Na_2C_4H_4O_6$, 1 ml 1 % $CuSO_4$. $5H_2O$, and 98 mL 2 % $NaCO_3$ in 10 µ1 M NaOH) followed by the addition of 75 ml Folin reagent (Folin and Ciocalteu's Phenol Reagent). The subsequent mixture was incubated at room temperature for 30 min. and measured in the spectrometer at 500 nm. The absorbance reading was compared to the standard curve for BSA to determine the concentration of protein in each EPS sample.

3.3.5.2.3. Prophylactic treatment of nodal explants with EPS

EPS extract was sprayed on the cut ends of the leaves of the secondary branches of three species using a hand sprayer. The spray was continued until a uniform coating of EPS droplets was deposited on the surface of the cut ends. A set of axillary buds from a different clump was also sprayed with sterile water as control. The explants consisting of dormant axillary buds were collected from the plant 24 h after treatment and subjected to a surface sterilization treatment as described above and inoculated into MS media. The sterilized explants were inoculated test tubes (15 mm x 25 mm, Borosil, India).

3.3.5.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2), the most stable among the ROS, which can rapidly diffuse across cell membranes and can act as a signalling molecule that mediates plant responses to a variety of biotic and adverse abiotic stress factors (Foyer *et al*., 1997). Pre-treatment in plants is recognized as a valuable strategy to stimulate plant defenses, leading to better plant development. Taking all these facts together, there is an intriguing possibility that prophylactic treatment with H_2O_2 can protect plants from different stress factors including contamination in *in vitro*. Foliar spray has certain advantages than the other systems like seed treatment and application in root system.

In seeds, chemical would be applied a single time before germination, whilst application to the root system requires the plants to be grown hydroponically and thus is not suitable for plants cultivated in soil.

3.3.5.3.1. Prophylactic treatment of mother plants

The secondary branches that taken as explants were sprayed with distilled water (control) or H_2O_2 solution (containing the detergent 0.025 % Tween 20 to reduce surface tension and facilitate penetration). Three preliminary experiments using different H_2O_2 concentrations (1, 10, 50 and 100 mM) were carried out in order to find the optimal H_2O_2 concentration. The plants were sprayed with 15 ml H_2O_2 or distilled water per plant at 7.30 am and again after 24 h. At 48 h after the first spray, the explants were collected and carried out normal surface sterilization procedures.

3.3.5.3.2. Pre-treatment of branch cuttings with H_2O_2

After the disinfection procedure, the long shoot segments (30 cm long) were submerged in H_2O_2 solution of different concentration for 24 h (30 cm long consists of 3-4 nodal regions) in trays before all surface sterilization.

3.3.5.4. Beta amino butyric acid

3.3.5.4.1. Prophylactic treatment of mother plants on control of contamination

The non-protein amino acid β-aminobutyric acid (BABA) solution were made up in distilled water containing 0.01 % Tween 20 and applied as foliar spray until runoff using a hand-held sprayer. Evaluation of emergence of contamination was done on the *in vitro* cultures of 1day to 80 d. old bamboo shoots. Treatments were performed 1 d. before the explant collection. All opened leaves and stem portions were sprayed with the solution. Control plants were treated with double distilled water.

3.3.5.4.2. Effect of BABA amended media on control of contamination

To assess the effects of BABA treatment on single nodal cuttings, explants were inoculated into the media with BABA. Different concentrations of filter sterilized BABA were added to the MS basal media with 10 µM BAP and 2 % sucrose. Axillary buds collected from field-grown plants were surface sterilized and trimmed into single

nodes of size 2.5 cm. Effect of BABA on explants for controlling the contamination in terms endophytes was evaluated during the later stages of culture.

3.4.2. *IN VITRO* FLOWERING

The most fundamental processes of sexual propagation in plant is flowering, fruiting and seed development and except in bamboo length of different phases in plant to reach this reproductive stage was determined (Yuan *et al*., 2017). Despite the wide application and commercial importance, very little known is about the factors that control the flowering behavior in bamboo. Inducing the flowering in shoot cultures in a sterile controlled environment without the interference of biotic and abiotic stresses lead to the revealing of important information about the various processes involved in this phase transition. In this study the phenomenon of *in vitro* flowering was examined in the light of the different factors known to influence it in various species including bamboo.

3.4.1. *In vitro* flowering induction

A flowering system *in vitro* is considered as a convenient tool for the study of the specific aspects of flowering such as initiation to senescence in bamboo. This will facilitate to identify the factors involved in switching from vegetative phase to reproductive phase of growth in this plant. This study will help to overcome problems associated with flowering in bamboo *in vivo* and *in vitro*.

3.4.2. Species selected for study:

1. *Bambusa balcooa*
2. *Bambusa tulda*
3. *Bambusa nutans*
4. *Dendrocalamus longispathus*
5. *Dendrocalamus stocksii*

In vitro proliferated shoots from axillary buds of adult clumps of five species and shoot cultures initiated from the seeds of *Dendrocalamus longispathus* were used for the experiments. All cultures were maintained on MS liquid medium with 3 % sucrose and BAP (15 µM) for 4 mon. to increase the number and uniformity of shoots. Subsequently, these multiple shoots were used to carry out experiments to evaluate the effect of photoperiods, water stress, mineral depletion, plant growth regulators, C/N ratio and additives on flower induction. Each culture bottle consisted of four shoot clumps (each

with 3-4 shoots). Subcultures were performed at an interval of 3 wks. Each treatment was replicated 10 times and all experiments were repeated at least thrice. Flower induction efficacy was determined by recording the number of flower buds per clump.

3.4.3. Physical factors

3.4.3.1. Effect of photoperiod on in vitro flowering

To examine the effect of photoperiod on *in vitro* flowering in shoot cultures of bamboo, 5 light/dark cycles (12/12, 10/14, 14/10, 16/8 and 8/16 h) were adopted. MS liquid media with BAP (15 µM) + 3 % sucrose were used for all experiment. Shoot cultures were inoculated onto the media and incubated under light (20 $\mu E/m^{-2}s^{-1}$) provided by Philips white fluorescent tubes at a temperature of 25 ± 2 ºC. Influence of light on flowering and proliferation, floral meristem formation and maturation and anthesis of flowers were evaluated.

3.4.3.2. Stress Factors

3.4.3.2.1. Effect of water stress on *in vitro* flowering

Water stress is one of the more important environmental stresses affecting the physiological mechanisms involved in cellular and whole plant system. To study the effect of water stress on flower induction in bamboo, media supplemented with agar (8, 9 & 10 %), polyethylene glycol 6000 (PEG) (5, 10 & 15 %) and mannitol (8, 9 & 10 %) and maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.3.2.2. Effect of mineral depletion induced by prolonged culture duration

Mineral depletion is one of the major hypothesis put forwarded to explain the flowering phenomenon in bamboo. To examine the effect on *in vitro* flowering, different criteria such as extended sub-culture period, incubation in low nutrient media etc. were attempted.

To examine the effect of prolonged subculture time, explants were sub-cultured to fresh liquid MS medium supplemented with 15 µM BAP with 3.0 % sucrose at intervals of 4, 6, and 8 wks and maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.3.2.3. Effect of mineral depletion on *in vitro* flowering through use of low-salt basal media

To understand the effect of lowering the nutrient levels on flowering, different dilutions (1/10, 1/100, 1/1000 or 0) of MS major salts with 3 % sucrose and 15 µM BAP were used in liquid media and cultures maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.4. Chemical factors

3.4.4.1. Effect of cytokinins on induction of *in vitro* flowering

To test the effect of plant growth regulators on *in vitro* flowering in bamboo, the shoot cultures were cultured on liquid MS basal medium with the cytokinins , BAP (15, 30, 45, 60 & 90 µM) and TDZ (1, 2.5 & 5 µM) and maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.4.2. Effect of auxin on induction of *in vitro* flowering

To test the effect of plant growth regulators on *in vitro* flowering in bamboo, the shoot cultures were cultured on liquid MS basal medium with the auxins, IBA (4.9, 24.6 &49.3 µM and NAA (5.4, 26.9 & 53.8 µM) and maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.4.3. Different carbohydrate sources on induction of *in vitro* flowering

Five carbohydrates viz. glucose, sucrose, maltose, fructose, and lactose at 3 % (w/v), were evaluated for flowering response *in vitro* on liquid MS media with BAP (15 µM) and maintained at photoperiods of 10/14, 14/10 and 16/8.

3.4.4.4. Effect of high sucrose levels in media on induction of *in vitro* flowering

Concentration of sucrose can serve as carbon and energy source and also as an osmotic agent. The effect of sucrose concentrations on *in vitro* flower induction in the different bamboo species was tested on MS liquid medium supplemented with BAP (15 µM) and different concentrations of sucrose (3, 4, 5, 6, 7, 8, 9 and 10 % w/v) tested and maintained at photoperiods of 10/14, 14/10 and16/8.

3.4.5. Statistical analysis

Each treatment consisted of at least 15 explants and each experiment was repeated 3 times. Visual observations of cultures in terms of shoot shortening, leaf browning etc. were made every day. Percentage of culture response of explants, number of flower buds per culture and time taken for flowering were recorded. Data were analyzed using one-way ANOVA. Data analyzed by the Duncan's multiple test ($p < 0.05$) using SPSS (ver. 18).

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. MICROPROPAGATION

The results of various experiments to induce high rate of axillary bud sprouting, shoot multiplication, rooting of shoots and plantlet regeneration are given below.

4.1.1. Plantlet regeneration through axillary bud proliferation

4.1.1.1. Axillary bud break

4.1.1.1.1. Effect of basal media composition on axillary bud break

The percentage bud break varied from 51–100 % in *P. stocksii* and *P. ritcheyi* on the different (MS, B5, WPM and SH) nutrient media used for shoot initiation (Fig. **1** & **2**). MS basal medium proved the best for maximum shoot induction (100 %), highest number of multiple shoots (6.98 shoots/explant) and shoot length (6.31 cm) within the 3 wk period of observation (Fig. **3** & **4**). This was followed by B5 medium with 97.84 % shoot induction, 3.88 shoots per explant and 5.92 cm shoot length. SH medium proved least effective in terms of percentage of sprout induction and shoot growth in *P. stocksii* (Table **2**).

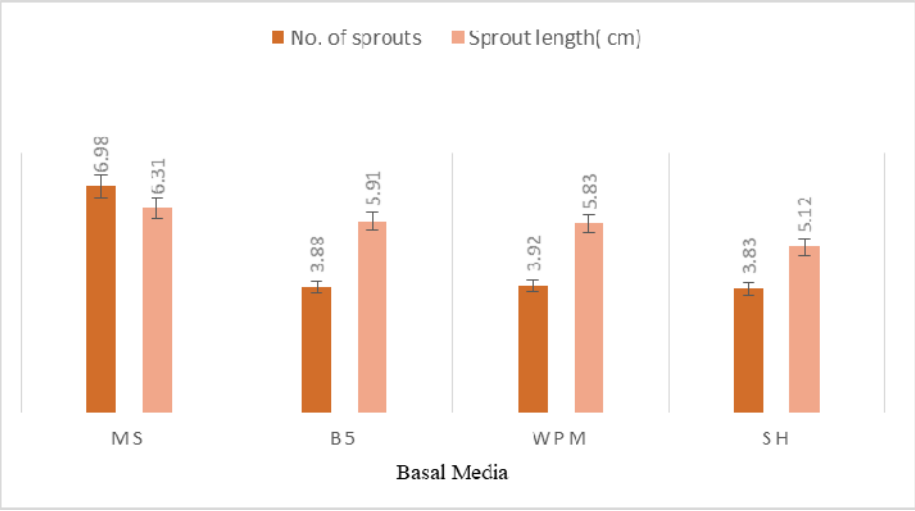

Fig.1: Effect of various basal media on shoot initiation and growth from nodal segments of *P. stocksii.*

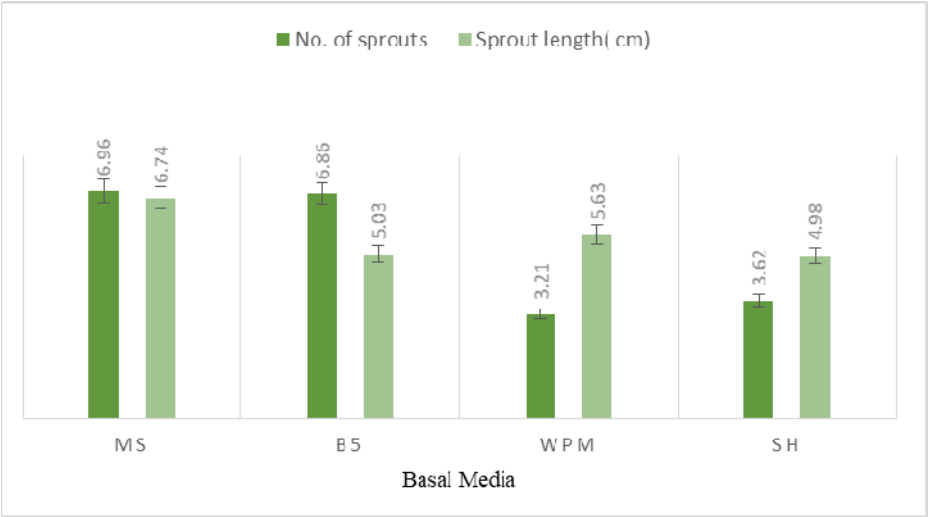

Fig.2: Effect of various basal media on shoot initiation and growth from nodal segments of *P. ritcheyi.*

Table 2: Effect of various basal media on shoot initiation and growth from nodal segments of *P. stocksii* and *P. ritcheyi.*

Nutrient media	***P. stocksii***	***P. ritcheyi***
	Response %	Response %
MS	100[a]	100[a]
B5	97.84[ab]	100[a]
WPM	49.03[e]	43.19[e]
SH	42.76[f]	41.90[f]

Media used: Basal media were supplemented with NAA (1µM) and BAP (10 µM) with Sucrose 2 %. Data were recorded at the end of 3 wk of inoculation. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

In *P. ritcheyi,* both MS and B5 solid media gave 100 % sprouting, whereas length of shoots ranged between 6.74 and 5.03 cm respectively. Many of the explants from both species inoculated on SH and WPM media exhibited a delayed sprouting response with lower number (≤ 4) of sprouted axillary shoots. The nodal explants remained mostly green but without bud break and eventually resulting in browning of the media and death of the tissues. After 3 wks. of culture, the maximum shoot number and shoot length in both the species were achieved on MS and therefore it was selected as the basal medium for all nutrient media in subsequent experiments. The results show that WPM which is considered as a medium suitable for woody plant culture and is very similar to MS in being high is salt composition, performed poorly.

The mineral salts and organic components of nutrient media play a vital role in culture initiation and differentiation from excised tissue in *in vitro* cultures. Recent reviews on micropropagation of bamboo revealed that Murashige and Skoog's media is the most commonly used media for *in vitro* cultures of bamboo (Mudoi *et al*., 2014; Sandhu *et al*., 2018). This is hardly a surprising outcome considering that MS basal media is the most widely used all formulations since its introduction. The poor response on WPM medium (which lacks ammonium ions), could be an indication of the importance of reduced nitrogen which is considered as essential for uptake of nitrate (Sathyanarayana and Blake, 1994)

Fig. 3: Axillary bud sprouting in *P. stocksii*

Fig. 4: Axillary bud sprouting in *P. ritcheyi*

Chaillou and Chaussat (1986) explained that when explants are first placed onto a nutrient medium, there may be an initial leakage of ions from damaged cells, especially metallic cations (Na^+, Ca^{2+}, K^+, Mg^{2+}) for the first 1-2 d., so that the concentration in the plant tissues actually decreases until cell commences active absorption and the internal concentration slowly rises. Phosphate and nitrogen (particularly ammonium) are absorbed more rapidly than other ions. Therefore, among the various basal media, MS medium with a higher concentration of phosphate and nitrogen could help improve the availability and encourage bud breaking in explants (George and Klerk, 2008).

4.1.1.1.2. Effect of cytokinins on axillary bud break

Nodal explants of *P. stocksii* cultured in MS basal medium without any growth regulators gave 85.80 % bud break response within 4.5 d. Addition of cytokinins improved the bud break response and reduced the time taken for sprouting. MS medium supplemented with 15 µM BAP was found to be most effective for bud break (98.56 %) with 6.83 shoots/explant after 3.83 d of inoculation (Table **3**). All other cytokinins, except Kin gave same results as that of BAP. Presence of TDZ improved the bud breaking frequency (≤ 99.90 %), but adversely effected the shoot length (≤ 3.48 cm). Meta-topolin was found to be the optimum cytokinin for inducing the bud proliferation (100 %) with minimum shoot length of 4.67 cm. Kin was observed to be the least effective cytokinin without any impact on any parameters. It adversely effected the bud breaking too (≤ 70 %).

Table 3: Effect of different cytokinins supplemented in MS solid medium on axillary bud proliferation of *P. stocksii*

Cytokinins	Concentration (µM)	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
0	0.0	4.50^{b}	85.80^{c}	5.50^{c}	4.16^{cd}
BAP	5.0	4.16^{b}	90.04^{b}	6.17^{b}	3.67^{d}
	10	4.13^{b}	92.63^{b}	6.98^{ab}	4.31^{cd}
	15	3.83^{ab}	98.56^{ab}	6.83^{ab}	4.83^{c}
	20	3.33^{a}	92.53^{b}	6.76^{a}	5.66^{b}
Kin	5.0	6.67^{fg}	60.57^{g}	1.50^{h}	3.00^{d}
	10	6.33^{f}	64.13^{g}	1.03^{hi}	3.34^{d}
	15	5.83^{d}	71.20^{f}	1.20^{h}	4.17^{cd}
	20	5.24^{cd}	70.67^{e}	2.50^{g}	5.16^{b}
TDZ	0.5	4.83^{bc}	98.53^{c}	5.75^{g}	3.48^{d}
	1.0	4.06^{b}	99.90^{c}	6.00^{b}	2.60^{e}
	2.0	3.67^{ab}	83.23^{cd}	6.90^{ab}	1.77^{f}
	4.5	3.50^{ab}	75.80^{c}	7.54^{a}	1.16^{f}
mT	5.0	5.06^{c}	100^{a}	6.22^{b}	4.67^{c}
	10	5.11^{c}	100^{a}	6.26^{b}	5.31^{b}
	15	4.33^{b}	98.84^{ab}	6.47^{ab}	5.83^{b}
	20	3.81^{ab}	94.06^{b}	6.82^{ab}	6.66^{a}

Media used: MS basal medium with Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 5 and each replicate consisted of 15 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3 wk.

A high level of bud break (97.85 %) was obtained in explants cultured on MS basal medium without any growth regulators (Table **4**) in *P. ritcheyi*. On media supplemented with cytokinin, bud break was inhibited especially on Kin and TDZ

treatment whereas on BAP and mT, there was only a marginal reduction in the number of sprouted buds, except for mT at 10 µM where it improved the response compared to the control. However, in spite of a high level of bud-break in hormone free media, shoot number and length of shoots were lower when compared to media with PGR. It was also observed that there was an inhibition in bud break with increase in concentration within each of the cytokinins. Shoot necrosis was induced in new sprouts of *P. ritcheyi* on all the cytokinins except for mT, which gave higher sprouting response (92 - 98.42 %) at different concentrations.

Table 4: Effect of different cytokinins supplemented in MS solid medium on axillary bud proliferation of *P. ritcheyi*

Cytokinins	Concentration (µM)	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
0	0.0	7.02^{c}	97.85^{ab}	1.89^{cde}	6.01^{a}
BAP	5.0	9.12^{d}	91.02^{e}	2.33^{cd}	6.0^{a}
	10	5.17^{bc}	97.00^{ab}	4.50^{b}	4.0^{bc}
	15	3.67^{a}	94.33^{c}	4.83^{ab}	3.83^{c}
	20	10.0^{de}	86.77^{fg}	2.0^{cd}	1.37^{de}
Kin	5.0	13.67^{f}	93.23^{cd}	1.67^{cde}	5.33^{ab}
	10	11.17^{e}	81.67^{h}	2.0^{cd}	6.0^{a}
	15	11.33^{ef}	81.10^{h}	3.33^{c}	4.33^{bc}
	20	18.67^{fg}	73.57^{i}	1.83^{cde}	3.67^{c}
TDZ	0.5	7.18^{c}	89.92^{f}	4.72^{ab}	1.74^{d}
	1.0	6.74^{bc}	87.02^{f}	4.81^{ab}	1.06^{de}
	2.0	5.12^{bc}	84.72^{fg}	4.92^{ab}	0.89^{f}
	4.5	5.08^{b}	82.03^{gh}	5.06^{a}	0.18^{g}
mT	5.0	5.16^{bc}	94.60^{c}	3.95^{bc}	4.10^{bc}
	10	5.02^{b}	98.42^{a}	4.39^{b}	4.32^{bc}
	15	4.97^{ab}	96.16^{b}	4.41^{b}	4.71^{b}
	20	4.82^{ab}	92.04^{d}	4.48^{b}	4.76^{b}

Media used: MS basal medium with Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 5 and each replicate consisted of 15 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3 wk.

Despite the well-documented function of cytokinins in delaying senescence, there are several lines of evidence linking cytokinins to the induction of cell death. Although BAP is currently the most common and affordable cytokinin used in micropropagation, its utilization has several drawbacks such as shoot-tip necrosis, inhibition of rooting and problematic acclimatization of plants in the greenhouse (Werbrouck *et al.,* 1996). Kunikowska *et al.* (2013) reviewed the use of cytokinins in plant tissue culture and found that the two natural cytokinins Kin and BAP at high concentrations (> 44 µM) are able to induce programmed cell death (PCD) in plants

and also that BAP is able to induce PCD in cultured plant cells whereas Kin induces this process only in living plant tissues. Among various cytokinins used in the present study, Kin gave the lowest rate of explant establishment as well as lower number of shoots.

TDZ, a phenyl urea derivative, is the other PGR, which induces maximum shoot number during sprouting. Treatment with this cytokinin however resulted in shortening of the shoots derived from the sprouted buds to as low as 0.18 cm. Lin and Chang (1998) have demonstrated that TDZ at higher concentration inhibited shoot elongation and also induced considerable vitrification in *B. edulis*. While in our studies mT improved not only the bud break, and number of shoots, but also higher shoot length, Ornellas *et al.* (2017) had showed that increasing concentrations of mT caused a reduction in culm size and an increment in branch size, with an overall increase in the proliferation rate of shoot clusters of *D. asper*. In most of the studies on bamboo micropropagation viz. (Sood *et al.,* 2002; Agnihotri and Nandi, 2009; Jimenez *et al.*, 2006), BAP is used because it is the most effective and affordable cytokinin, but in this study, application of mT in inducing axillary bud break from nodal explants was found to be more effective in ensuring establishment of culture throughout the year.

Apparently, part of the bud break and shoot proliferation response from axillary buds is an inherent response not under the influence of PGRs as seen in the controls but some of the parameters that could have a bearing on the establishment of cultures, like shoot number and length, could be influenced in a different way. In that respect, mT and BAP appear to be the PGRs that give consistently superior results in the two species.

4.1.1.1.3. Synergetic action of auxins with cytokinins on axillary bud break

Incorporation of auxin into the initiation media with cytokinin improved the bud breaking frequency along with number of sprouts and sprout length in both the species. Combination of BAP at lower levels (2.22-11 µM) with NAA (1 µM) favored culture establishment whereas the higher concentrations of the same PGR adversely effected the growth rate of the plant material in both species (Table **5**). Similarly, Kin significantly inhibited the bud break when added with NAA and reduced it to 23 % in *P. stocksii* and 36 % in *P. ritcheyi.*

Table 5: Effect of different concentrations of plant growth regulators (NAA and cytokinins) on multiple shoot initiation and growth from nodal shoot segments.

	P. stocksii			***P. ritcheyi***		
Treatments (PGRs)	Response (%)	Number of shoots/ explant	Shoot length (cm)	Response (%)	Number of shoots/ explant	Shoot length (cm)
Hormone Free	84.24^{d}	3.22^{d}	4.24^{b}	100.00^{a}	4.61^{c}	4.05^{ab}
BAP(2.22µM)	87.13^{c}	4.53^{c}	4.32^{b}	97.3^{ab}	4.66^{c}	4.31^{k}
BAP(4.44 µM)	90.72^{b}	4.62^{c}	4.54^{b}	96.12^{ab}	4.93^{c}	4.54^{j}
BAP(6.66 µM)	92.42^{b}	4.70^{c}	4.79^{b}	96.03^{ab}	6.07^{ab}	4.61^{gh}
BAP(8.88 µM)	98.93^{ab}	5.66^{b}	5.62^{a}	100.00^{a}	7.16^{a}	5.70^{cd}
BAP(11 µM)	100^{a}	6.98^{a}	5.92^{a}	98.07^{ab}	7.08^{a}	6.53^{a}
BAP(13.2 µM)	78.14^{g}	3.86^{d}	3.47^{ed}	87.42^{bc}	6.93^{ab}	6.61^{a}
BAP(22 µM)	58.71^{c}	3.34^{d}	1.5^{g}	74.10^{c}	6.16^{ab}	6.65^{a}
BAP(44 µM)	52.65^{d}	3.27^{d}	1.8^{g}	53.39^{d}	5.46^{b}	6.83^{ab}
Kin (2.3 µM)	23.20^{n}	3.16^{d}	2.86^{f}	36.17^{f}	3.31^{c}	4.43^{ij}
Kin (4.6 µM)	28.19^{m}	2.92^{e}	3.64^{cd}	38.56^{f}	4.02^{bc}	4.57^{ef}
Kin (11.5µM)	40.80^{l}	2.87^{e}	3.77^{c}	54.17^{ef}	4.46^{bc}	4.63^{e}
Kin (23 µM)	52.59^{j}	2.49^{e}	3.07^{ef}	57.16^{ef}	4.63^{bc}	4.87^{hi}
Kin (34.5 µM)	66.85^{h}	2.05^{e}	3.15^{e}	61.16^{e}	4.73^{bc}	5.15^{b}
BAP(5.5µM+Kin5.8 µM)	54.58^{j}	2.52^{e}	3.61^{d}	82.84^{b}	5.46^{b}	5.64^{bc}
TDZ (0.22 µM)	62.75i	4.54^{c}	3.59^{e}	86.16^{b}	6.63^{ab}	3.46^{b}
TDZ (0.45 µM)	78.25^{g}	4.37^{c}	4.33^{b}	98.25^{ab}	6.21^{ab}	4.23^{c}
TDZ (1.12 µM)	80.03^{d}	4.68^{c}	5.46^{a}	100.00^{a}	5.90^{b}	4.59^{e}
TDZ (2.25 µM)	81.16^{d}	5.58^{d}	4.84^{b}	100.00^{a}	5.76^{b}	3.31^{d}
TDZ (4.55 µM)	82.92^{d}	5.89^{d}	3.74^{d}	100.00^{a}	5.49^{b}	2.89^{b}
2ip (2.41 µM)	84.70^{d}	3.58^{d}	3.84^{d}	87.81^{b}	4.03^{bc}	3.05^{d}
2ip (4.82 µM)	86.12^{c}	3.89^{d}	4.74^{b}	88.03^{b}	4.16^{bc}	3.21^{b}
2ip (7.2 µM)	87.15^{c}	3.36^{d}	3.72^{d}	88.18^{b}	4.28^{bc}	3.28^{e}
2ip (9.6 µM)	91.86^{b}	3.54^{d}	3.88^{c}	90.17^{b}	4.44^{bc}	3.59^{d}
2ip (12 µM)	78.33^{g}	3.76^{e}	3.47^{c}	83.23^{b}	4.61^{bc}	4.06^{f}
2ip (24 µM)	73.14^{d}	3.81^{e}	3.93^{d}	80.02^{b}	4.14^{bc}	4.52^{d}

Media: MS+NAA (1µM) + Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 12 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3rd week.

TDZ and 2iP induced bud break over a range of 62 to 91 % with 3 to 5 shoots in *P. stocksii* whereas, it improved the bud break to 80 to 100 % with 4 to 6.6 sprouts in *P. ritcheyi* when added with NAA. Addition of NAA overcame the shoot dwarfing property of TDZ and enhanced the shoot length to 5.46 cm in *P. stocksii* and 4.59 cm in *P. ritcheyi* at 1.12 µM and 2.2 µM, respectively, but a sudden decline in shoot length was also seen in both species at higher levels. Kin in combination with IAA enhanced the axillary bud break percentage to 46 to 68 with average sprouts of 3 per explant (Table **6**). Addition of IAA into the axillary bud sprouting media stabilized the sprouting percentage within 80 in *P. stocksii* and ranged from 48 to 98 in *P. ritcheyi.* Similar in its effect to NAA, IAA also reduced the shoot dwarfing impact of

TDZ. Similarly in combination with IBA, BAP and TDZ gave a good and consistent response in *P. stocksii* with was a better response than with IAA or IBA. Kin was seen to perform the poorest with the auxins, NAA and IAA but better with IBA for both the species (Table **5, 6 & 7**) although overall, BAP together with NAA was the best combination.

Table 6: Effect of different concentrations of plant growth regulators (IAA and cytokinins) on multiple shoot initiation and growth from nodal shoot segments.

	P. stocksii			***P. ritcheyi***		
Treatments (PGRs)	Response (%)	No. of shoots/ explant	Shoot length (cm)	Response (%)	No. of shoots/ explant	Shoot length (cm)
Hormone Free	84.24^{o}	3.22^{n}	4.24^{l}	100.00^{a}	4.61^{k}	4.05^{ijk}
BAP(2.22µM)	84.32^{o}	3.48^{kl}	4.31^{k}	94.56^{o}	4.23^{kl}	4.26^{k}
BAP(4.44µM)	85.16^{n}	3.66^{ijk}	4.34^{j}	95.74^{n}	4.28^{j}	4.37^{j}
BAP(6.6µM)	87.34^{l}	3.79^{gh}	4.42^{ghi}	96.28^{l}	4.54^{gh}	4.42^{ghi}
BAP(8.8µM)	88.48^{k}	3.88^{cde}	4.65^{cd}	98.85^{a}	5.78^{cde}	4.55^{cd}
BAP(11µM)	89.21^{b}	4.26^{b}	4.87^{b}	98.62^{ab}	6.82^{b}	5.48^{b}
BAP(13.2µM)	88.64^{g}	4.58^{cd}	4.98^{fgh}	94.01^{g}	6.98^{cd}	5.63^{fgh}
BAP(22µM)	83.86^{cd}	4.72^{lmn}	3.56^{kl}	90.18^{cd}	5.72^{lmn}	6.12^{kl}
BAP(44µM)	82.01^{b}	4.98^{lm}	3.06^{k}	89.20^{b}	5.34^{lm}	6.19^{k}
Kin (2.3µM)	46.24^{n}	3.24^{ijk}	3.47^{ij}	46.01^{n}	4.06^{ijk}	4.21^{ij}
Kin (4.6µM)	48.27^{m}	3.46^{hi}	3.54^{ef}	48.22^{m}	4.22^{hi}	4.42^{ef}
Kin (11.5µM)	54.39^{l}	3.54^{i}	3.62^{ef}	50.38^{l}	4.35^{i}	4.74^{ef}
Kin (23 µM)	57.01^{j}	3.63^{hi}	3.76^{hi}	52.46^{j}	4.67^{hi}	4.81^{hi}
Kin (34.5µM)	68.29^{h}	3.68^{j}	3.82^{hi}	54.57^{h}	4.79^{j}	4.96^{hi}
BAP(5.5µM+Kin5.8 µM)	61.42^{j}	4.46^{ghi}	4.82^{def}	76.23^{j}	4.61^{ghi}	5.47^{def}
TDZ (0.22µM)	74.26^{i}	4.26^{ab}	4.27^{efg}	86.34^{i}	6.23^{a}	5.02^{a}
TDZ (0.45µM)	78.12^{g}	4.38^{b}	5.52^{bc}	98.58^{g}	6.46^{b}	4.85^{bc}
TDZ (1.12µM)	81.46^{a}	4.61^{a}	5.83^{a}	98.60^{a}	6.82^{ab}	4.02^{efg}
TDZ(2.25µM)	83.01^{a}	4.88^{de}	6.03^{def}	98.66^{a}	6.96^{de}	3.56^{def}
TDZ(4.55µM)	85.42^{a}	4.97^{c}	6.72^{b}	94.36^{a}	7.09^{c}	3.02^{b}
2ip (2.41µM)	82.38^{ef}	3.42^{de}	4.36^{def}	84.06^{ef}	4.28^{de}	4.05^{def}
2ip(4.82µM)	83.41^{def}	3.57^{c}	4.44^{b}	85.08^{def}	4.45^{c}	4.21^{b}
2ip(7.2µM)	84.63^{de}	3.55^{ef}	4.52^{ef}	87.41^{de}	4.68^{ef}	4.28^{ef}
2ip(9.6µM)	87.44^{bc}	3.59^{de}	4.61^{de}	89.48^{bc}	4.94^{de}	4.59^{de}
2ip(12µM)	78.51^{g}	3.89^{g}	4.67^{fg}	92.49^{g}	5.11^{g}	4.86^{fg}
2ip(24µM)	83.36^{f}	4.21^{f}	4.72^{de}	93.67^{f}	5.36^{f}	4.92^{de}

Media: MS+IAA (1µM) + Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 12 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3rd week.

A synergistic effect between auxin and cytokinins has often been seen to improve the development of adventitious shoot and root meristems but interactions between the two classes of regulators are often complex, and more than one combination of substances is likely to produce optimum results (George and Klerk, 2008). A low concentration of NAA in the present study promoted the growth of shoots. Bag *et al*. (2000) reported that the combined action of BAP (5.0 µM) and IBA (1.0 µM) induced

multiple shoot formation in nodal explants taken from a 2-year-old plant of *Thamnocalamus spathiflorus* and on this medium 90 % of explants proliferated with an average of 28.6 shoots per explant in 8 wk. High-frequency multiple shoot induction was achieved by Sanjaya *et al*. (2005) in *P. stocksii* from nodal shoot segments collected from superior/elite genotypes on liquid MS medium supplemented with NAA (2.68 µM) and BAP (4.44 µM).

Table 7: Effect of different concentrations of plant growth regulators (IBA and cytokinins) on multiple shoot initiation and growth from nodal shoot segments.

	P. stocksii			***P. ritcheyi***		
Treatments (PGRs)	Response (%)	No. of shoots/ explant	Shoot length (cm)	Response (%)	No. of shoots/ explant	Shoot length (cm)
Hormone Free	84.24^{o}	3.22^{n}	4.24^{l}	100.00^{a}	4.61^{de}	4.05^{ef}
BAP(2.22µM)	96.45^{a}	3.98^{c}	4.31^{bc}	94.36^{a}	6.96^{b}	6.74^{b}
BAP(4.44 µM)	96.84^{ab}	3.98^{b}	4.92^{b}	95.66^{ab}	6.86^{b}	5.94^{b}
BAP(6.6 µM)	97.09^{a}	4.92^{a}	5.83^{a}	97.19^{cd}	4.81^{de}	6.22^{ab}
BAP(8.8 µM)	97.86^{c}	5.43^{b}	6.12^{c}	97.90^{cd}	3.62^{e}	6.41^{a}
BAP(11 µM)	98.14^{a}	5.52^{a}	6.16^{a}	98.03^{b}	3.13^{e}	6.20^{ab}
BAP(13.2 µM)	98.31^{c}	5.63^{b}	6.77^{b}	98.12^{ab}	4.88^{de}	5.39^{b}
BAP(22 µM)	93.67^{fg}	5.64^{ef}	6.86^{e}	99.92^{b}	3.28^{efg}	3.46^{e}
BAP(44 µM)	86.59^{ef}	6.03^{d}	6.63^{f}	79.08^{d}	3.53^{efg}	3.78^{de}
Kin (2.3 µM)	88.19^{b}	3.28^{g}	4.44^{g}	82.15^{cd}	3.68^{de}	4.04^{cd}
Kin (4.6 µM)	92.36^{cd}	3.53^{f}	4.58^{fg}	88.72^{c}	3.46^{efg}	3.71^{de}
Kin (11.5 µM)	95.80^{c}	4.20^{ab}	5.06^{ab}	92.06^{c}	6.32^{bc}	6.74^{a}
Kin (23 µM)	97.06^{bc}	4.15^{ab}	5.24^{ab}	94.25^{bc}	6.42^{bc}	5.94^{b}
Kin (34.5 µM)	89.02^{cd}	4.42^{a}	5.96^{bc}	91.46^{cd}	6.74^{b}	6.22^{ab}
BAP(5.5µM+Kin5.8 µM)	98.12^{b}	6.06^{a}	4.61^{a}	96.76^{b}	6.76^{b}	5.64^{def}
TDZ (0.22 µM)	94.42^{c}	5.62^{b}	4.65^{a}	93.15^{c}	6.79^{b}	4.46^{a}
TDZ (0.45 µM)	94.13^{h}	6.51^{f}	3.92^{ef}	93.08^{h}	7.21^{ab}	3.93^{bc}
TDZ (1.12 µM)	95.18^{i}	7.07^{f}	3.73^{ef}	96.41^{i}	7.23^{ab}	3.59^{efg}
TDZ (2.25 µM)	97.73^{h}	8.01^{e}	3.51^{e}	98.56^{h}	7.67^{a}	3.31^{def}
TDZ (4.55 µM)	98.86^{g}	9.06^{d}	2.52^{e}	98.42^{g}	7.97^{a}	2.31^{b}
2ip (2.41 µM)	83.61^{f}	3.67^{c}	5.50^{cd}	80.29^{f}	5.53^{c}	4.05^{def}
2ip (4.82 µM)	84.04^{e}	3.94^{a}	5.61^{dc}	86.23^{e}	5.64^{c}	4.21^{b}
2ip (7.2 µM)	92.61^{cd}	4.00^{b}	6.00^{c}	91.06^{cd}	6.12^{bc}	4.28^{ef}
2ip (9.6 µM)	95.80^{c}	4.03^{ab}	7.06^{ab}	93.78^{c}	6.32^{bc}	4.59^{de}
2ip (12 µM)	97.02^{bc}	4.15^{ab}	7.24^{ab}	95.37^{ef}	6.42^{bc}	5.06^{fg}
2ip (24 µM)	97.98^{ab}	4.62^{a}	6.96^{bc}	96.75^{fg}	6.74^{b}	5.52^{de}

Media: MS+IBA (1µM).Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 12 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3^{rd} week.

4.1.1.1.4. Effect of liquid vs agar gelled media on axillary bud break

The addition of a gelling agent greatly improved the bud breaking rate in both species significantly (Table **8** & **9**). Gellan gum gave better results (100 %) than agar in all parameters except for shoot length. Bud break took longer (≥ 5.50 d.) in liquid medium. Liquid media significantly improved elongation of the new sprouts (upto 5.42 cm in hormone free media in *P. stocksii*). The number of shoots produced per explant was higher on solid media in both *P. stocksii* (Fig. **5** & **6**) and *P. ritcheyi* (Fig. **7** & **8**).

Table 8: Effect of liquid Vs agar gelled media on shoot initiation and growth from nodal shoot segments of *P. stocksii*.

Gelling agent	Cytokinin	***P. stocksii***			
		Response (%)	Time taken for bud break	No. of shoots/explant	Shoot Length (cm)
Agar	0	52.48^{f}	3.50^{b}	4.09^{ab}	2.22^{f}
	BAP (10µM)	93.26^{ab}	3.16^{a}	4.45^{a}	4.28^{c}
	TDZ (4.5µM)	88.53^{b}	3.13^{a}	4.69^{b}	3.94^{d}
Gellan gum	0	68.65^{d}	3.83^{bc}	1.08^{e}	2.17^{f}
	BAP (10µM)	**100^{a}**	3.50^{b}	**4.92^{a}**	**4.35^{c}**
	TDZ (4.5µM)	90.08^{ab}	3.16^{a}	4.97^{a}	3.44^{de}
Liquid	0	15.52^{h}	6.33^{fg}	1.28^{e}	5.42^{ab}
	BAP (10µM)	43.09^{g}	5.83^{ef}	3.46^{c}	5.64^{a}
	TDZ (4.5µM)	41.25^{g}	5.50^{e}	3.66^{c}	4.36^{c}

Media used: MS basal medium with Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 5 and each replicate consisted of 15 explants. LSD at $\alpha = 0.05$. Data were recorded upto the end of 3rd week.

Presence of PGRs influenced all the parameters significantly also exhibited the same trend for all the parameters. Sprouts from axillary buds of *P. ritcheyi* also displayed the same effect and maximum length of new sprouts (6.62 cm) was achieved in MS basal liquid media.

Inoculation of explants into liquid media with a substantial portion submerged, reduced the respiratory ability of plant tissues and resulted in a hyperhydric condition. The solutes in the culture media determine the osmotic potential and regulate the uptake of nutrients from the media.

Fig. 5: Axillary bud sprouting of *P. stocksii* in solid media.

Fig. 6: Axillary bud sprouting of *P. stocksii* in liquid media.

Table 9: Effect of liquid vs agar gelled media on shoot initiation and growth from nodal shoot segments of *P. ritcheyi*.

Gelling agent	Cytokinin	***P. ritcheyi***			
		Response (%)	Time taken for bud break	No. of shoots/ explant	Shoot length (cm)
Agar	0	98.69[b]	3.27[ab]	3.97[b]	4.02[b]
	BAP (10µM)	96.21[b]	3.14[a]	4.52[a]	4.37[b]
	TDZ (4.0µM)	94.64[b]	3.12[a]	4.63[a]	5.46[a]
Gellan gum	0	**100[a]**	3.42[b]	3.92[b]	4.19[b]
	BAP (10µM)	98.09[b]	3.23[ab]	**4.71[a]**	**4.35[b]**
	TDZ (4.0µM)	99.61[b]	3.11[a]	4.98[a]	4.44[b]
Liquid	0	05.87[f]	6.58[gh]	1.96[d]	6.62[b]
	BAP (10µM)	34.81[e]	6.46[g]	2.73[c]	5.23[b]
	TDZ (4.0µM)	39.06[e]	6.26[fg]	2.79[c]	5.14[b]

Media used: MS basal medium with Sucrose 2 %. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 5 and each replicate consisted of 15 explants. LSD at α = 0.05. Data were recorded upto the end of 3rd week.

Bornman and Vogelmann (1984) observed the effects of different degrees of rigidity of both agar and Gelrite on the uptake of radiolabeled N^6-benzyladenine (^{14}C-BA) and showed a highly significant inverse correlation between ^{14}C-BA accumulation and degree of gel stiffness in *in vitro* cultures of *Picea abies***.** Comparative studies carried out in shoot cultures of *Rosa hybrida*, *Cordyline fruticosa* and *Homalomena* revealed that whereas the gelling agent did not influence the multiplication rate of any species significantly, it influenced the shoot length and the fresh weight of shoots (Podwyszynska and Olszewski, 1995).

The type of gelling agents was shown to influence the quality of the shoot produced from *in vitro* cultures of mangosteen and abnormal morphology of shoots was seen in phytagel-solidified medium (Te-chato *et al*., 2005). Kaçar *et al*. (2010) analyzed the effect of different gelling agents (agar-agar, Agargel, Phytagel, and Plantagar) on the micropropagation of Dwarf Cavendish banana in an effort to find a combination that yields large numbers of high-quality seedlings. The type of gelling agent has an influence on the shoot number, shoot weight and shoot length.

Fig. 7: Axillary bud sprouting of *P. ritcheyi* in solid media with gellan gum.

Fig. 8: Axillary bud sprouting of *P. ritcheyi* in liquid media.

A comparative study on the effect of different gelling agents on *in vitro* shoot multiplication responses of *Amelanchier canadensis* found that the highest shoot multiplication occurred on media gelled with Guar gum, while the longest shoots developed on media with starch (Fira *et al*., 2013).

4.1.1.1.5. Role of carbon source on axillary bud break

Of the four sugars tested, sucrose and glucose gave the same good response with 100 % bud break (Table **10**) as well as number of sprouts (≥ 6.81) in both species. Shoot length was also clearly influenced by the carbon source in *P. stocksii* and *P. ritcheyi* respectively (Fig. **9** &**10**).

Table 10: Effect of various carbon sources on shoot initiation and growth from nodal shoot segments of *P. stocksii* and *P. ritcheyi.*

Nutrient media	***P. stocksii***	***P. ritcheyi***
	Response %	Response %
Sucrose	100[a]	100[a]
Fructose	97.84[ab]	83.19[c]
Glucose	100[a]	100[a]
Lactose	57.72[c]	51.90[c]

Media: MS supplemented with NAA (1µM) and BAP (10 µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

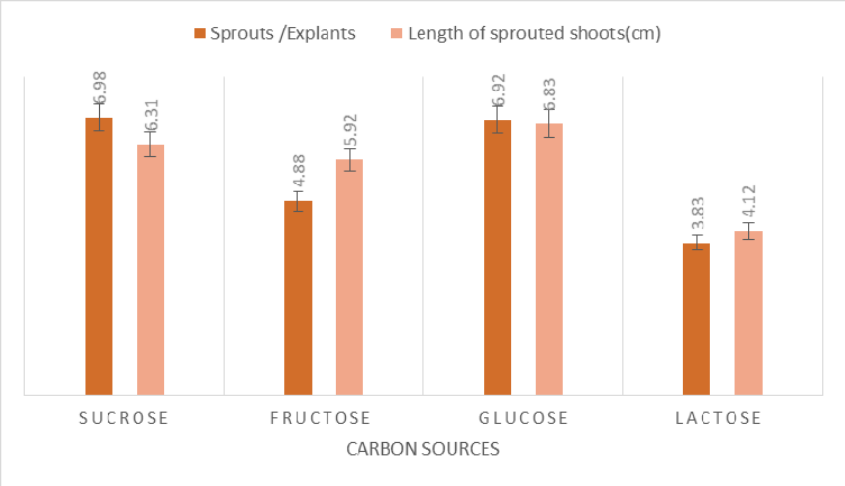

Fig. 9: Effect of various carbon sources on shoot initiation and growth from nodal shoot segments of *P. stocksii*.

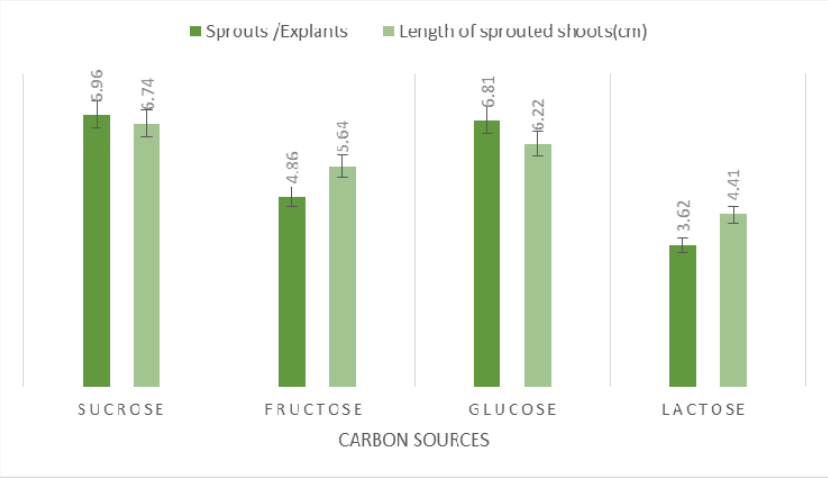

Fig. 10: Effect of various carbon sources on shoot initiation and growth from nodal shoot segments of *P. ritcheyi*.

In micropropagation, sugars serve as major carbon source to provide optimal environment for plant regeneration. Hence, it is essential to add the primary metabolite to tissue culture media for *in vitro* plant propagation (Harathi *et al*., 2016). De Pavio Neto and Otoni (2002) reported that, the regeneration frequency increases with increasing the concentration of carbon sources until an optimum is reached and decreases at higher concentration and the presence of carbon source as an energy supplement was an essential factor in *in vitro* cultures. Even though sucrose is selected as main carbon source in a vast majority *in vitro* cultures of shoots, it has been shown to be less effective in some regeneration procedures (Thomson and Thorpe, 1987). Sucrose being the easily metabolized sugar and energy source in plants gave better results compared to the others especially lactose which is not found in plants.

4.1.1.1.6. Effect of explant diameter and length on axillary bud break

Among the different diameter classes of nodes used as explants, those of 3-4 mm diameter with 2.5–3.5 cm in length proved to be the best for the multiple shoot induction in *P. stocksii* in which all nodes sprouted was high (4.92) and shoot length 6.83 cm. Smaller nodes of 1-3 mm diameter and 2-3.5 cm long worked best for *P. ritcheyi* giving 6.86 shoots per explants and a length 5.94 cm within 3 wks. (Table **11&12**). Nodal segments of higher diameter (4-5 mm) posed problems of leaching, browning and higher contamination apart from a lower sprouting response (67.72 and 51.90% for *P. stocksii* and *P. ritcheyi* respectively).

The number of multiple shoots formed too was the minimum in nodes of 1–2 mm diameter in *P. stocksii* whereas the small explants gave better response that was closer to the maximum value in *P. ritcheyi*. Explants across the different diameter classes showed bud break within 4.2 to 21.02 d. (Table **11** & **12**) in both species. Across all diameter classes, mT gave the best results in time taken for bud break, frequency of bud break, shoot number and shoot length. Generally, explants with larger diameter (> 3 mm) gave higher shoot number but with delayed response and reduced frequency of bud break. However, the length of the shoots was not influenced by this parameter. Higher shoot length was obtained on explants of size > 2 mm.

Table 11: Effect of diameter of the explant on axillary bud proliferation from nodal explants of *P. stocksii.*

Diameter of the explant (mm)	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
1-2	0	4.16^{ab}	91.04^{ab}	4.53^{c}	5.47^{bc}
	BAP	4.24^{ab}	100^{a}	4.99^{c}	5.92^{bc}
	Kin	4.79^{b}	88.61^{b}	5.81^{ab}	4.83^{c}
	TDZ	4.08^{ab}	98.27^{ab}	5.93^{ab}	3.46^{cd}
	mT	4.66^{b}	95.46^{ab}	6.05^{a}	5.43^{bc}
2-3	0	4.28^{ab}	94.31^{ab}	6.02^{a}	5.58^{bc}
	BAP	4.19^{ab}	100^{a}	6.58^{a}	5.69^{bc}
	Kin	5.37^{c}	88.94^{b}	6.16^{a}	4.87^{c}
	TDZ	3.78^{a}	98.34^{ab}	6.91^{a}	3.68^{cd}
	mT	3.96^{a}	95.02^{ab}	6.78^{a}	5.92^{bc}
3-4	0	4.12^{ab}	94.46^{ab}	5.86^{ab}	5.82^{bc}
	BAP	4.42^{ab}	100^{a}	6.87^{a}	5.95^{bc}
	Kin	8.01^{f}	86.01^{b}	5.82^{ab}	5.01^{bc}
	TDZ	4.29^{ab}	98.58^{ab}	6.95^{a}	3.79^{bc}
	mT	4.27^{ab}	98.26^{ab}	6.92^{a}	6.07^{a}
4-5	0	6.32^{d}	85.04^{c}	3.27^{d}	6.02^{ab}
	BAP	6.48^{de}	94.51^{ab}	3.86^{d}	6.49^{a}
	Kin	9.34^{fg}	74.66^{ab}	3.15^{d}	6.67^{a}
	TDZ	6.11^{d}	87.69^{b}	4.98^{b}	3.73^{cd}
	mT	6.86^{e}	91.03^{ab}	3.63^{d}	6.79^{a}

Basal media were supplemented with NAA (1μM) and BAP (10 μM). Data were recorded upto the end of 3rd week.. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

There are several reports that reveal the significance of diameter of the explant on the bud break and shoot growth in different bamboo species. Nodal segments of 1.5-2.5 mm diameter from 2-year old plants resulted in highest shoot initiation in *T. spathiflorus* (Bag *et al.,* 2000). Similarly in *P. stocksii* (Sanjaya *et al.,* 2005; Somashekar *et al.,* 2008) and *Guadua angustifolia* (Jimenez *et al*., 2006), nodal explants of 2-3 mm diameter resulted in highest shoot initiation. Kabade (2009) used different diameter classes of explants (1-2, 2-3, 3-4 and 4-5 mm), and reported that nodal shoot segments of 2-3 mm diameter favored maximum shoot initiation within 3 wks. in *B. bambos* and *D. strictus*.

Table 12: Effect of diameter of the explant on axillary bud proliferation from nodal explants of *P. ritcheyi*

Diameter of the explant (mm)	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
1-2	0	6.94^{cd}	97.02^{ab}	0.93^{ghi}	4.14^{b}
	BAP	4.26^{a}	97.40^{ab}	0.94^{gh}	4.20^{b}
	Kin	8.19^{e}	94.86^{abc}	0.95^{g}	4.46^{b}
	TDZ	6.01^{c}	97.31^{ab}	0.94^{gh}	2.52^{d}
	mT	4.56^{ab}	97.76^{ab}	0.98^{g}	4.06^{b}
2-3	0	7.02^{d}	97.85^{ab}	1.89^{efg}	6.01^{a}
	BAP	5.17^{b}	97.0^{ab}	4.50^{c}	4.00^{bc}
	Kin	11.33^{f}	81.10^{cd}	3.33^{e}	4.33^{b}
	TDZ	6.74^{cd}	87.02^{c}	4.81^{bc}	1.06^{ef}
	mT	5.02^{b}	98.42^{a}	4.39^{cd}	4.32^{b}
3-4	0	7.33^{d}	93.04^{bc}	1.99^{efg}	6.24^{a}
	BAP	5.83^{bc}	95.26^{abc}	4.87^{b}	4.42^{b}
	Kin	11.52^{f}	79.28^{e}	4.29^{cde}	4.51^{ab}
	TDZ	6.94^{cd}	84.15^{cd}	4.95^{ab}	1.32^{e}
	mT	5.27^{b}	96.03^{b}	4.46^{cd}	4.48^{b}
4-5	0	21.02^{i}	27.42^{g}	2.26^{ef}	6.26^{a}
	BAP	18.17^{g}	67.72^{f}	4.92^{ab}	4.46^{b}
	Kin	18.33^{g}	61.05^{f}	4.39^{cd}	4.53^{b}
	TDZ	19.74^{h}	67.16^{f}	4.98^{a}	1.41^{e}
	mT	8.02^{e}	72.31^{ef}	4.93^{ab}	4.62^{ab}

Basal media were supplemented with NAA (1µM) and BAP (10 µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

The size of the nodal explants was observed to influence all the studied parameters in culture establishment (Table **13 &14**). The time taken for bud break had an inverse relation to the length of the explant. In the case of frequency of bud break, the size of the explant did not have a notable effect except for the 1.5 cm class where it was significantly lower. The number of shoots formed as well as length was influenced by the size of explant and use of PGRs. Longer explants gave more number as well as longer shoots. As in the previous experiments, TDZ was found to reduce the shoot length. Across all the size classes, mT was found to reduce time taken for bud break, improve the frequency of bud break and shoot number and length of new shoots. Increase in explant length correlated with bud breaking percentage as well as sprout number in relation with all cytokinins. Time taken for bud break decreased with increase in explant length.

Table 13: Effect of length of the explant on axillary bud proliferation from nodal explants of *P. stocksii***.**

Length of explants (cm)	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
1.5	0	6.39^{c}	94.06^{ab}	3.65^{de}	4.28^{c}
	BAP	5.83^{b}	94.24^{ab}	4.05^{ab}	4.24^{de}
	Kin	8.46^{g}	83.36^{bc}	3.62^{bc}	4.06^{cd}
	TDZ	5.58^{b}	85.26^{bc}	5.78^{a}	1.97^{g}
	mT	5.72^{b}	96.34^{a}	4.53^{bc}	4.74^{a}
2	0	6.04^{d}	94.27^{ab}	3.87^{de}	4.31^{a}
	BAP	5.16^{ab}	95.39^{ab}	5.23^{a}	4.36^{a}
	Kin	8.64^{f}	83.53^{bc}	4.64^{bc}	4.33^{a}
	TDZ	5.32^{b}	85.64e	5.07^{a}	2.03^{ef}
	mT	5.61^{b}	97.09^{a}	4.69^{b}	4.85^{a}
2.5	0	5.83^{b}	95.88^{a}	3.97^{d}	4.34^{a}
	BAP	5.06^{ab}	96.08^{a}	5.51^{a}	4.12^{ab}
	Kin	7.85^{f}	82.46^{bc}	4.42^{bc}	4.38^{a}
	TDZ	5.28^{ab}	88.50^{b}	5.87^{a}	2.24^{ef}
	mT	5.54^{b}	97.26^{a}	4.98^{b}	4.47^{a}
3	0	5.92^{b}	98.26^{a}	3.99^{d}	4.25^{ab}
	BAP	5.18^{ab}	97.98^{a}	5.48^{a}	4.28^{ab}
	Kin	8.06^{f}	83.35^{bc}	4.47^{bc}	4.25^{ab}
	TDZ	6.57^{cd}	88.28^{b}	5.89^{a}	2.48^{ef}
	mT	4.98^{a}	98.67^{a}	4.99^{b}	4.52^{a}
3.5	0	5.52^{b}	71.00^{e}	3.41^{de}	4.47^{a}
	BAP	5.63^{b}	84.01^{bc}	3.96^{d}	4.69^{a}
	Kin	8.02^{g}	74.52^{de}	3.42^{d}	4.13^{ab}
	TDZ	4.86^{a}	80.68^{bc}	4.03^{b}	3.06^{bc}
	mT	4.99^{a}	86.16^{b}	3.99^{cd}	4.75^{a}
4	0	5.01^{ab}	62.78^{f}	3.00^{de}	4.78^{a}
	BAP	5.66^{b}	78.03^{d}	3.29^{d}	4.36^{a}
	Kin	8.75^{f}	71.04^{f}	2.87^{e}	4.42^{a}
	TDZ	5.02^{ab}	74.59^{e}	3.46^{c}	3.24^{bc}
	mT	5.11^{ab}	78.45^{a}	4.51^{b}	4.85^{a}

Basal media were supplemented with NAA (1µM) and BAP (10 µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

The effect of the explant size on axillary bud proliferation has been studied in *T. spathiflorus* (Bag *et al.,* 2000) and in several other plant species, in particular *Cynara cardunculus* (El Boullani *et al*., 2017), *Gerbera jamesonii* (Nhut *et al*., 2007) and cedar (*Cedrus* spp.) (Renau-Morata *et al*., 2005). Smith (2000) suggested that the explant size has an effect on the response of the tissue and this was due to the more nutrient reserves and endogenous plant growth regulators to sustain the culture.

George *et al.* (2008) reported that large explants generally survive more frequently and grow more rapidly at the outset than very small ones.

Table 14: Effect of length of the explant on axillary bud proliferation from nodal explants of *P. ritcheyi.*

Length of explants (cm)	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
1.5	0	8.43^{e}	92.34^{d}	1.72^{de}	3.83^{c}
	BAP	6.52^{cd}	94.01^{cd}	4.34^{ab}	3.28^{de}
	Kin	14.10^{g}	80.00^{fg}	3.08^{bc}	4.08^{cd}
	TDZ	7.98^{de}	84.08^{ef}	4.63^{ab}	0.92^{g}
	mT	5.96^{bc}	96.15^{bc}	3.53^{bc}	3.76^{d}
2	0	7.02^{d}	97.85^{abc}	1.89^{de}	4.01^{d}
	BAP	5.17^{b}	97.00^{abc}	4.50^{ab}	4.0^{d}
	Kin	11.33^{f}	81.10^{f}	3.33^{bc}	4.33^{c}
	TDZ	6.74^{cd}	87.02^{e}	4.81^{a}	1.06^{ef}
	mT	5.02^{ab}	98.42^{a}	3.99^{b}	4.32^{c}
2.5	0	6.96^{cd}	97.88^{abc}	1.97^{d}	4.13^{a}
	BAP	5.12^{b}	97.08^{abc}	4.58^{ab}	4.12^{cd}
	Kin	11.12^{f}	81.16^{ef}	3.42^{bc}	4.38^{c}
	TDZ	6.62^{c}	87.50^{e}	4.87^{a}	1.14^{f}
	mT	5.00^{ab}	98.44^{a}	3.99^{b}	4.41^{c}
3	0	6.92^{cd}	98.01^{ab}	1.99^{d}	4.21^{cde}
	BAP	5.08^{ab}	97.98^{abc}	4.58^{ab}	4.24^{cde}
	Kin	11.06^{f}	82.30^{ef}	3.47^{bc}	4.42^{cd}
	TDZ	6.57^{cd}	87.96^{e}	4.89^{a}	1.18^{ef}
	mT	4.98^{a}	98.62^{a}	4.69^{b}	4.52^{c}
3.5	0	6.21^{e}	70.09^{d}	2.68^{de}	3.86^{c}
	BAP	5.03^{cd}	81.36^{cd}	4.56^{ab}	4.41^{de}
	Kin	10.02^{g}	72.56^{fg}	4.27^{bc}	4.25^{cd}
	TDZ	6.72^{de}	74.21^{ef}	4.92^{ab}	1.38^{g}
	mT	4.52^{bc}	75.46^{bc}	4.42^{bc}	4.64^{d}
4	0	6.12^{e}	34.64^{c}	2.84^{de}	4.23^{d}
	BAP	4.56^{cd}	67.16^{abc}	3.59^{ab}	4.28^{d}
	Kin	10.00^{g}	54.07^{f}	3.38^{bc}	4.36^{c}
	TDZ	6.68^{de}	66.46^{e}	3.62^{a}	1.42^{ef}
	mT	4.46^{bc}	61.26^{a}	3.74^{b}	4.06^{c}

Basal media were supplemented with NAA (1µM) and BAP (10 µM Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

4.1.1.1.7. Effect of position of the node on culm on axillary bud break

Axillary buds at the middle (4-7th position) of the branch from the base gave best results in both the species, in sprouting response, number of shoots as well as shoot length. Nodes from the middle of the branch gave the best results in all the parameters (Table **15** & **16**).

The upper nodes were the next best for establishing the cultures since the lower most buds took longer to achieve bud break and had a lower frequency of bud break, number of shoots as well as the shoot length.

Physiological age of buds which means the position of buds in mother plant, is one of the important factors that determine the morphogenetic response of the explant during culture establishment. Several reports suggest that the regeneration potential of bud explants depends on the differential meristematic activity within different plant parts with respect to position (Han *et al.,* 1997).

Table 15: Effect of position of the explant on axillary bud proliferation from nodal explants of *P. stocksii*

Position	Cytokinin	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
Lower	0	10.43^{g}	67.26^{c}	2.34^{f}	5.14^{bc}
	BAP	9.64^{fg}	82.13^{b}	3.14^{c}	5.45^{bc}
	Kin	10.52^{f}	63.89^{c}	2.81^{e}	3.27^{d}
	TDZ	8.54^{f}	81.46^{ef}	3.78^{bc}	2.09^{e}
	mT	9.52^{fg}	80.34^{d}	3.49^{c}	4.37^{cd}
Middle	0	8.35^{f}	93.45^{ab}	5.59^{b}	6.21^{b}
	BAP	5.22^{a}	100.00^{a}	6.12^{ab}	6.73^{b}
	Kin	8.47^{f}	94.10^{bc}	5.64^{c}	4.56^{cd}
	TDZ	5.04^{a}	100.00^{a}	6.98^{a}	7.92^{a}
	mT	5.36^{a}	100.00^{a}	6.96^{a}	6.96^{b}
Upper	0	6.32^{b}	72.01^{cd}	2.76^{f}	4.52^{c}
	BAP	6.11^{b}	79.01^{d}	3.92^{bc}	4.81^{c}
	Kin	6.86^{bc}	66.93^{c}	2.98	4.99^{c}
	TDZ	5.16^{a}	77.51^{cd}	3.74^{bc}	2.35^{e}
	mT	5.24^{a}	70.93^{c}	3.65^{bc}	4.20^{cd}

Basal media were supplemented with NAA (1µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at α=0.05.

Explants originating from the 10 basal nodes of a shoot with at least 25 nodes gave better shoot proliferation than explants originating from the 10 distal nodes *in vitro* establishment of *Vitis rotundifolia* (Sudarsono and Goldy, 1991). The significant interactions between bud positions and shoot proliferation in nodal culture of two Guava cultivars were revealed by Shekafandeh and Khosh-Khui (2008). Devi and Sharma (2009) evaluated the effect of position of the node on lateral branches in axillary bud proliferation of *Arundinaria callosa* and they reported that mid-culm nodes are the most suitable for proliferation with high bud-break percentage and

multiplication. In the case of *B. nutans*, nodes from 5th to 7th position gave maximum shoot proliferation (Mudoi *et al*., 2014).

Table 16: Effect of position of the explant on axillary bud proliferation from nodal explants of *P. ritcheyi.*

Position	Cytokinin	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
Lower	0	13.14^{g}	51.24^{efg}	1.12^{f}	5.23^{ab}
	BAP	12.52^{f}	63.40^{de}	3.64^{bc}	3.67^{bc}
	Kin	12.52^{f}	54.11^{e}	3.26^{c}	4.01^{c}
	TDZ	11.74^{fg}	52.13^{ef}	3.05^{cd}	0.98^{f}
	mT	8.31^{e}	66.16^{d}	3.21^{c}	4.21^{bc}
Middle	0	7.02^{d}	97.85^{ab}	1.89^{e}	6.01^{a}
	BAP	5.17^{ab}	97.00^{ab}	4.50^{ab}	4.0c
	Kin	11.33^{f}	81.10^{bc}	3.33^{c}	4.33^{b}
	TDZ	6.74^{c}	87.02^{b}	4.81^{a}	1.06^{e}
	mT	5.02^{a}	98.42^{a}	3.99^{b}	4.32^{b}
Upper	0	8.47^{e}	72.01^{cd}	1.24^{f}	6.13^{a}
	BAP	6.51^{c}	74.06^{cd}	3.47^{bc}	4.21^{bc}
	Kin	11.82^{fg}	75.16^{c}	3.05^{cd}	4.35^{b}
	TDZ	7.53^{de}	73.51^{cd}	4.52^{ab}	1.14^{e}
	mT	5.67^{b}	77.93c	3.46^{bc}	4.39^{b}

Basal media were supplemented with NAA (1µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

The plant architecture is dependent on the differential behavior of the meristems in different positions on the culms as well as the nodes at different positions on the branch. Inherent dormancy of buds can be expected which can be induced to sprout due to damage to upper buds or during the process of explants collection. The interaction of PGRs in the *in vitro* conditions is a factor that further influences the response. It is therefore worthwhile to ascertain the optimum response of nodal explants from different positions not only for their initial sprouting response but also the ability to produce shoots of a quality suitable for high rate of multiplication.

4.1.1.1.8. Effect of season of explant collection on axillary bud break

Several of the parameters studied in the establishment phase such as time taken and frequency of bud break and number of shoots formed per explant were found to be influenced by the season of explant collection (Table **17** & **18**).

Bud-break frequency of the axillary buds was not uniform during the period under study. In *P. stocksii,* days required for bud break varied between 3.32 d. to 12.52 d. Explants collected during the period June- August, took the maximum duration for

the bud break ranging between 8.87-12.52 d. Explants collected during the summer months (March to May) gave maximum shoot proliferation (~ 95 %) with shoot number ≤ 8.87 and December to February the next best in *P. stocksii*. Except Kin, all other cytokinins along with the seasonal changes induced the axillary bud sprouting rate by enhancing the number of sprouted buds with > 6. Maximum length of the sprouted buds (≤ 4.9 cm) was observed during the period June - August and this found in correlation with the delayed sprouting response.

Table 17: Effect of season on axillary bud proliferation from nodal explants of *P. stocksii.* Media : MS + NAA (1µM) + Sucrose 2 %

Season	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
Mar-May	0	3.32^{a}	95.64^{ab}	2.78^{cd}	4.36^{bc}
	BAP	3.81^{a}	96.43^{ab}	6.86^{ab}	4.75^{ab}
	Kin	5.67^{b}	85.96^{e}	3.57^{cd}	4.41^{ab}
	TDZ	3.68^{a}	86.02^{d}	8.87^{a}	3.06^{g}
	mT	3.92^{a}	97.38^{a}	7.57^{a}	4.89^{ab}
Jun-Aug	0	12.52^{k}	43.35^{l}	3.34^{ef}	4.38^{bc}
	BAP	8.87^{d}	57.35^{k}	3.45^{e}	4.90^{ab}
	Kin	10.99^{d}	58.29^{j}	3.41^{cd}	4.82^{c}
	TDZ	9.86^{de}	77.13^{jk}	3.02^{cd}	3.88^{e}
	mT	8.98^{d}	86.05^{cd}	3.33^{cd}	4.98^{ab}
Sep-Nov	0	8.06^{d}	37.68^{i}	3.98^{e}	4.13^{ab}
	BAP	6.72^{f}	53.58^{g}	4.67^{bc}	4.42^{e}
	Kin	8.01^{d}	57.83^{gh}	3.78^{cd}	4.64^{ab}
	TDZ	5.26^{b}	89.68^{cd}	3.43^{cd}	3.55^{fg}
	mT	5.21^{b}	89.57^{cd}	4.89^{d}	4.67^{ab}
Dec-Feb	0	6.36^{e}	93.68^{ab}	3.46^{e}	4.41^{b}
	BAP	5.34^{b}	96.76^{ab}	5.45^{b}	4.48^{bc}
	Kin	6.28^{c}	79.46^{ef}	3.94^{d}	4.46^{ab}
	TDZ	5.19^{b}	87.47^{e}	6.07^{ab}	3.84^{fg}
	mT	5.02^{b}	96.56^{ab}	5.91^{b}	4.86^{ab}

Basal media were supplemented with NAA (1µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05.

In the case of *P. ritcheyi* too, the best season for quicker bud break was March to May and with the use of PGRs, the time taken for bud break was reduced except in the case of Kin. The frequency of bud break was lowest during the monsoon season during which new shoots are emerging from the clump and the buds collected were from the previous season. Use of PGRs did not influence the bud break significantly except in the case of media with mT where a consistency in the number of shoots produced irrespective of the season was maintained. Although bud break frequency improves during the September to November season, there no concomitant increase in

the number or length of shoot formed until the next season. As in the previous experiments, the shoot length was higher *P. ritcheyi* in the control and inhibition by TDZ was observed. The November-May period was found the best for high frequency (83-100 %) multiple shoot initiation in MS solid medium supplemented with NAA (1µM) + BAP (10 µM). Low response, delayed response and high contamination problem was encountered from the explants collected during June-September (Table **18**).

Table 18: Effect of season on axillary bud proliferation from nodal explants of *P. ritcheyi.* Media : MS + NAA (1µM) + Sucrose 2 %

Season	Cytokinins	Days required for bud break	% bud break	No. of shoots	Length of shoots (cm)
Mar-May	0	7.02^{cd}	97.85^{ab}	1.89^{e}	6.01^{ab}
	BAP	4.81^{a}	97.01^{ab}	4.50^{ab}	4.0^{cd}
	Kin	11.33^{fg}	81.10^{e}	3.33^{bc}	4.33^{c}
	TDZ	6.74^{c}	87.02^{d}	4.81^{a}	1.06^{g}
	mT	5.02^{ab}	98.42^{a}	3.99^{ab}	4.32^{c}
Jun-Aug	0	19.16^{k}	0.08^{l}	1.12^{ef}	2.00^{f}
	BAP	15.74^{hi}	7.00^{k}	3.68^{b}	2.50^{ef}
	Kin	18.61^{j}	8.09^{j}	3.01^{cd}	2.92^{e}
	TDZ	16.85^{i}	7.13^{jk}	3.24^{bc}	0.88^{h}
	mT	5.87^{b}	86.05^{de}	3.43^{bc}	4.08^{cd}
Sep-Nov	0	13.06^{gh}	31.06^{i}	1.86^{e}	2.24^{ef}
	BAP	9.72^{f}	46.08^{g}	3.82^{b}	2.76^{e}
	Kin	14.23^{h}	41.17^{gh}	3.63^{bc}	2.98^{e}
	TDZ	12.85^{g}	46.21^{g}	3.61^{bc}	1.24^{fg}
	mT	5.38^{ab}	89.14^{cd}	3.46^{c}	4.02^{cd}
Dec-Feb	0	8.11^{e}	90.21^{c}	1.73^{e}	6.32^{a}
	BAP	7.36^{cd}	91.16^{bc}	4.04^{ab}	5.22^{bc}
	Kin	12.28^{g}	78.04^{ef}	2.97^{d}	5.51^{b}
	TDZ	6.89^{c}	81.30^{e}	4.52^{ab}	1.31^{fg}
	mT	5.19^{ab}	92.23^{b}	3.76^{b}	4.47^{c}

Basal media were supplemented with NAA (1µM). Data were recorded upto the end of 3rd week. Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD: Least Significant Difference at œ=0.05

In bamboo, the seasonality of growth is pronounced and the growth phases are unique when compared to other woody plants. Annual growth does not depend on the current year's culms or the meristems present on it since a rhizome develops from the base that develops eventually to the next year's culms. Dormancy in axillary buds can be overcome under *in vitro* conditions and the variation in bud break across the different seasons can also be expected since formation of tertiary branches from the nodes of the secondary branches ceases within weeks of the growth of the new culms and buds do not show further activity after that stage.

Several studies on the appropriate time of year to collect explants for initiating cultures from nodal explants of bamboo have been carried out. In *P. stocksii* Sanjaya *et al.* (2005), reported that nodal shoot segments collected during April-September produced a significantly more number of shoots with better shoot growth. This is probably the season that coincides with the summer months in our study prior to monsoon rains. In *D. longispathus* (Saxena and Bhojwani, 1993) and *D. giganteus* (Ramanayake and Yakandawala, 1997) peak bud break were observed before the onset of monsoon rains each year as was the case in our study. February-March and September-October showed maximum bud break response in *D. falcatum* and in *B. balcooa*, respectively (Arya *et al*., 2008). February, March and December months were found to be the best for establishment of aseptic cultures in *B. nutans* (Mehta *et al.,* 2011). Other reports indicate July-August for *D. strictus* (Chaturvedi *et al.,* 1993) and October for *B. balcooa* (Das and Pal, 2005) and autumn in *B. nutans* (Mudoi *et al*., 2014) to be suitable for shoot initiation for highest bud break frequency. Low percentage of bud-break in the rainy season was reported in *A. callosa* (Devi and Sharma, 2009).

4.1.1.2. Shoot Proliferation

Nodal explants with sprouted axillary buds of *P. stocksii* and *P. ritcheyi*, were tested for a range of parameters for inducing optimum shoot multiplication after maintaining them for 4 months through subcultures. Shoots explants therefore consisted of clusters of multiplying shoots with a few shoot initials and several shoots with opened leaves. The effect of various parameters tested are as follows.

4.1.1.2.1. Effect of cytokinins on shoot multiplication

MS liquid medium with NAA (0.5 µM) + TDZ (4.55 µM) resulted in highest number of shoots (6.17 shoots/shoot cluster), but the shoots were dwarf (3.01 cm) and vitrified (hyperhydric shoots) in *P. ritcheyi*. MS liquid medium supplemented with NAA (0.5 µM) and BAP (10 µM) on the other hand proved to be the best in terms of uniformity in shoot multiplication (14.65 shoots/shoot cluster) with maximum (4.17 cm) shoot length and resulted in 4.76 fold shoot multiplication rate within 3 wks. period (Table **19**) for *P. stocksii* (Fig. **11** & **12**). Increased concentration of TDZ resulted a slight increase in the number of shoots/cluster but shoot length was decreased, in both species. Higher levels of Kin (45 µM) also was not found effective

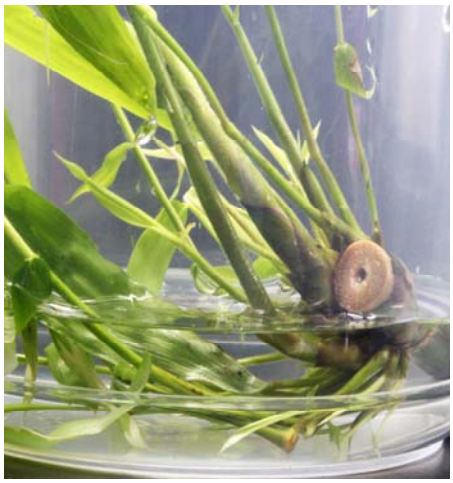

Fig. 11: Axillary shoot proliferation in *P. stocksii*

Fig. 12: Cluster of multiple shoots of *P. stocksii*

Table 19: Effect of cytokinins (BAP, Kin and TDZ) on shoot multiplication and growth.

	P. stocksii			***P. ritcheyi***		
Treatments (Cytokinins)	**Number of shoots/ clump**	**Shoot length (cm)**	**Shoot Multipli-cation rate**	**Number of shoots/ clump**	**Shoot length (cm)**	**Shoot Multipli-cation rate**
Control	4.56^{p}	3.13^{n}	1.02^{d}	3.02^{p}	6.38^{n}	0.05^{f}
Hormone Free	5.15^{n}	3.37^{jk}	1.47^{d}	3.05^{n}	6.27^{jk}	0.07^{f}
BAP(2.22µM)	8.29^{l}	3.66^{gh}	2.08^{c}	5.09^{l}	6.13^{gh}	1.48^{c}
BAP(4.44 µM)	$\mathbf{14.65^{ij}}$	$\mathbf{4.17^{nc}}$	$\mathbf{4.76^{a}}$	5.64^{ij}	6.57^{nc}	2.76^{b}
BAP(6.6 µM)	11.28^{g}	3.94^{de}	3.22^{b}	5.15^{g}	$\mathbf{6.76^{de}}$	1.53^{c}
BAP(8.8 µM)	10.19^{c}	3.46^{ij}	4.05^{a}	5.02^{e}	6.06^{ij}	1.07^{c}
BAP(11 µM)	4.52^{o}	3.28^{lm}	1.29^{d}	4.11^{o}	6.12^{lm}	0.09^{f}
BAP(13.2 µM)	5.74^{m}	3.35^{kl}	1.64^{d}	4.52^{m}	6.32^{kl}	0.46^{de}
BAP(22 µM)	7.58^{l}	3.52^{i}	2.17^{c}	4.68^{l}	6.36^{i}	0.51^{d}
BAP(44 µM)	10.58^{c}	3.86^{e}	3.02^{b}	4.72^{h}	5.96^{e}	0.53^{d}
Kin (2.3 µM)	11.37^{g}	4.14^{c}	3.25^{b}	4.78^{g}	6.03^{c}	0.56^{d}
Kin (4.6 µM)	9.18^{cd}	3.76^{f}	2.62^{c}	4.83^{k}	5.81^{f}	0.64^{d}
Kin (11.5 µM)	9.86^{cd}	4.03^{d}	2.82^{c}	4.06^{i}	4.13^{d}	0.05^{f}
Kin (23 µM)	10.13^{c}	5.12^{a}	4.24^{a}	5.01^{d}	4.01^{a}	1.03^{c}
Kin (34.5 µM)	10.96^{c}	3.87^{e}	4.50^{a}	5.52^{c}	3.45^{e}	2.01^{bc}
BAP (5.5 µM + Kin 5.8 µM)	12.24^{b}	3.62^{h}	3.93^{b}	6.04b	3.16^{h}	3.02^{ab}
TDZ (0.22 µM)	14.07^{a}	3.24^{m}	4.45^{a}	$\mathbf{6.17^{a}}$	3.01^{m}	$\mathbf{3.21^{a}}$
BAP (8.8 µM) (without auxin)	9.55^{cd}	3.72^{fg}	2.73^{c}	4.56^{j}	6.72^{fg}	0.71^{d}
Kin (5.8 µM) (without auxin)	12.14^{b}	4.31^{b}	3.47^{b}	4.14^{f}	6.31^{b}	0.08^{f}
TDZ (0.45 µM) (without auxin)	14.08^{a}	2.45^{o}	4.91^{a}	4.62^{j}	3.38^{h}	0.24^{e}

Media: MS (liquid) with and without NAA

Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (3-4 shoots/clump).LSD at $\alpha = 0.05$. Data were recorded at the end of 3 wks.

in shoot multiplication as compared to treatment with BAP (20 µM) and TDZ (4.55 µM). Combined use of BAP and Kin with NAA (0.5 µM) in the medium did not help in improving the shoots multiplication or shoot length in either species.

Addition of cytokinins to tissue culture media is seen to be necessary for shoot proliferation and is essential for plant cell division (George *et al*., 2008). Jouanneau (1970, 1975) reported that cytokinins might be required to regulate the synthesis of proteins involved in the formation and function of the mitotic spindle apparatus. In tissue culture, cytokinins regulated the cell division by arresting the cell at prophase at one stage and induced synchronized cell division during next subculture (Jouanneau, 1971). Kulaeva (1980) suggested the interaction of cytokinins with RNA and protein synthesis and this was supported by Tepfer and Fosket (1978) who found the increased level of polyribosome content in cytokinin treated soybean cell cultures. Not surprisingly, most reports in bamboo suggest that the incorporation of cytokinins in shoot cultures is beneficial (Saxena, 1990; Ling and Chang, 1998; Arya and Sharma, 1998; Ramanayake *et al*., 2001 and 2006; Chaudhary *et al*., 2004; Shirin and Rana, 2007; Jimenez *et al*., 2006, Devi and Sharma, 2009 and Kapruwan *et al*., 2014).

4.1.1.2.2. Effect of liquid vs solid media on shoot multiplication

In the comparison of liquid media with agar and gellan gum solidified media, liquid medium with BAP (10 µM) and NAA (1.25 µM) showed significantly better shoot multiplication in terms of number of shoots formed as well as in shoot length in both the species (Table **20**).

The advantage of using liquid medium for micropropagation was proven by Wimber (1965) in *Cymbidium* orchids and demonstrated that on solid medium there was only a small amount of protocorm multiplication and differentiation into shoots whereas shaken liquid medium provide continuous proliferation of protocorms. Orchard *et al*. (1979) demonstrated that cocoa shoot tip elongation in liquid medium, but on agar, growth was restricted to bud swelling and thought this was because on agar the cut surfaces of the explant became covered with a viscous exudate, which apparently restricted the uptake of nutrients.

Table 20: Effect of liquid vs solid media on shoot multiplication

Gelling agent	Cytokinin	*P. stocksii*			*P. ritcheyi*		
		No. of shoots/ clump	**Shoot length (cm)**	**Shoot Multiplication rate**	**No. of shoots/ clump**	**Shoot length (cm)**	**Shoot Multiplication rate**
Agar	0	2.036^{g}	3.05^{b}	1.02^{e}	2.92^{e}	3.27^{cd}	0.04^{e}
	BAP (10µM)	4.73^{f}	4.06^{a}	2.35^{d}	5.42^{b}	6.03^{ab}	2.76^{b}
	TDZ (4.5µM)	9.65^{a}	3.41^{b}	3.32^{c}	6.17^{a}	3.51^{c}	3.21^{a}
Gellan gum	0	4.16^{f}	3.02^{b}	0.08^{fg}	2.94^{e}	3.31^{cd}	0.04^{e}
	BAP (10µM)	4.76^{f}	4.12^{a}	1.08^{e}	4.32^{c}	3.25^{de}	1.08^{de}
	TDZ (4.5µM)	9.31^{c}	3.22^{b}	3.16^{cd}	4.49^{c}	2.04^{m}	1.17^{d}
Liquid	0	4.56^{f}	3.13^{b}	1.02^{e}	3.02^{d}	6.38^{a}	2.05^{bc}
	BAP (10µM)	**14.65^{a}**	**4.17^{a}**	**4.76^{a}**	5.58^{b}	6.57^{a}	2.98^{b}
	TDZ (4.5µM)	14.07^{b}	3.24^{b}	4.45^{b}	**6.29^{a}**	2.31^{m}	**3.32^{a}**

Media: MS + NAA (1.25 µM) + Sucrose 2 %.
Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 5 and each replicate consisted of 15 explants. LSD at α = 0.05.Data were recorded at the end of 3 wk.

Nadgir *et al*. (1984) reported the higher multiplication rates of shoot cultures in liquid media for *D. strictus.* Highest rates of multiplication and best growth were obtained in *B. tulda* on liquid media with greener and healthier shoots by Saxena (1990) who also reported that the incorporation of agar resulted in significant decline in the rate of shoot multiplication. *In vitro* clonal multiplication of bamboo, *D. longispathus* was successfully carried out in liquid media during multiplication stage of 15 passages even with the incidence of hyperhydricity which was due to the high relative humidity when the shoots are not submerged (Saxena and Bhojwani, 1993). Development of multiple shoots were carried out on semi-solid MS medium supplemented with PGR and the use of an intervening liquid medium for sub culturing has been found to enhance multiplication of shoots in *Vanilla planifolia* (George and Ravishankar,1997). During micropropagation of *D. giganteus* (Ramanayake and Yakandawala, 1997), cultures of axillary shoots were initiated in a semi-solid MS medium with PGR and continuous shoot proliferation with 1.8-fold increase in shoot number was achieved in a liquid MS medium.

The better growth obtained in liquid medium without supporting structures could be possible due to the greater contact of a significant part of entire explant with the media and avoiding the gradient for nutrients and for gases that the solid media provides. These two factors are reported to enable the efficient uptake of nutrients and

growth regulators (Debergh, 1982). In addition, toxic metabolites, which may accumulate in the vicinity of the tissue in a solidified media are effectively dispersed in liquid media (George *et al*., 2008). Additionally the very nature of the grass anatomy in which the stems are substantially hollow could result in a better tolerance of submergence and permit sufficient aeration compared to tissues of non-grass species. This effect is particularly evident when the initial inoculum consists of shoots that are partially out of the media surface rather than entirely submerged. The normal strategy of agitating shoot cultures in liquid media on an orbital shaker is therefore not necessary for bamboo shoots.

4.1.1.2.3. Effect of different basal media formulations on shoot multiplication

It was observed in both the species that shoot multiplication and shoot length varied significantly on the different basal nutrient media used. Among the various liquid nutrient media tested (MS, WPM, SH and B5) MS medium proved to be best for maximum shoot multiplication. Shoot length was also significantly higher on MS liquid medium followed by WPM and B5 medium (Table **21**). Shoot multiplication was significantly lower in SH medium.

Table 21: Effect of various liquid nutrient media on shoot multiplication and growth from the shoot clump (3-4 shoots/clump)

Nutrient media	*P. stocksii*			*P. ritcheyi*		
	No. of shoots/ clump	**Shoot length (cm)**	**Shoot Multiplication rate**	**No. of shoots/ clump**	**Shoot length (cm)**	**Shoot Multiplication rate**
MS	**14.65^{a}**	**4.17^{a}**	**4.76^{a}**	5.64^{a}	6.57^{a}	1.98^{a}
B5	9.78^{c}	3.84^{bc}	3.49^{c}	4.06^{c}	5.16^{bc}	1.12^{ab}
WPM	10.81^{b}	4.01^{b}	3.83^{b}	4.79b	5.09^{bc}	1.05^{ab}
SH	7.89^{d}	3.65^{c}	2.98^{d}	2.17^{d}	4.37^{d}	0.08^{d}

Media : MS liquid+ Sucrose 2 % + NAA (1.25 µM)+ BAP (10µM)

Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD at œ=0.05. Data were recorded at the end of 3 wks. of inoculation.

Similar, a significant factor in the success of *in vitro* culture is the choice of mineral and organic nutritional components (Gamborg *et al*., 1995). Rugmini (1984), Mehta *et al*. (2000), Lu (2005) and others who studied different nutritional media for their effect on *in vitro* shoot multiplication supported the view that different plant species

vary in their nutritional requirements and therefore they respond differently to various basal media. Shirin *et al*. (2015) evaluated *in vitro* cultures of *Saraca asoca* and reported that out of the four culture media tested, B5 medium proved to be the best culture medium for shoot proliferation and this was due to the high concentration of thiamine (vitamin B1).

Kabade (2009) analyzed different media [MS, B5, Heller's (Heller, 1953) (HE), WPM etc.] for shoot multiplication and growth in *D. brandisii, D. strictus* and *B. bambos*, HE medium was found least effective for shoot multiplication and MS the best. Similarly, in *P. stocksii*, evaluation of various media (MS, SH, B5, WPM and HE) proved that MS liquid medium was best and favoured maximum multiplication rates (21 shoots/cluster), followed by WPM (Somashekar *et al*., 2008). In a comparison between MS and B5 for shoot multiplication in *B. tulda*, MS medium was better than B5 for shoot multiplication (Saxena, 1990). Several other reports too support the view that MS media was superior for shoot proliferation as well as shoot elongation (Shirin and Arya (2003) in *B. nana*; Nadgir *et al*. (1984) and Chaturvedi *et al*. (1993) for *D. strictus ,* Das and Pal (2005a), Negi and Saxena (2010) for *B. balcooa, B. bambos* (Arya and Sharma, 1998), *G. angustifolia* (Jimenez *et al*., 2006), *A. callosa* (Devi and Sharma, 2009) and several reports on *B. nutans* (Yasodha *et al*., 2008; Mehta *et al*., 2011; Negi and Saxena, 2011).

4.1.1.2.4. Effect of adjuvants on shoot multiplication

Among the various additives (ascorbic acid, citric acid, cysteine, yeast extract and coconut water) tested alone or in combination, the maximum number of shoots (18.29) and highest shoot length (4.92 cm) was recorded in *P. stocksii* in media with ascorbic acid (50 mgl^{-1}) and citric acid (25 mgl^{-1}) (Table **22**). Addition of none of the additives however improved the shoot multiplication rate in *P. ritcheyi* whereas a marginal increase of shoot multiplication was seen in medium with ascorbic acid (50 mgl^{-1}) and citric acid (25 mgl^{-1}). However, these additives did not overcome the problem of shoot tip browning associated with the shoot cultures of P. ritcheyi as expected (Fig. **13** & **14**).

Fig. 13: Shoot culture of *P. ritcheyi* with initial stage of tissue browning

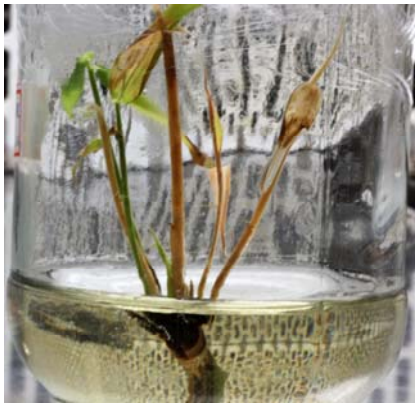

Fig. 14: Shoot culture damage due to the tissue browning in cultures of *P. ritcheyi*.

Table 22: Effect of various additives on shoot multiplication of *P. stocksii* and *P. ritcheyi*

Treatments (Additives, mgl^{-1})	*P. stocksii*			*P. ritcheyi*		
	No. of shoots/ clump	**Shoot length (cm)**	**Shoot Multiplication rate**	**No. of shoots/ clump**	**Shoot length (cm)**	**Shoot multiplication rate**
Control (without additives)	14.65^{c}	4.17^{ab}	4.76^{b}	5.64^{b}	6.57^{ab}	1.98^{ab}
Ascorbic acid, 50	15.15^{bc}	3.88^{b}	4.82^{b}	5.73^{ab}	6.20^{b}	**2.06^{a}**
Citric acid, 25	**18.24^{a}**	4.90^{a}	5.83^{a}	5.69^{b}	6.06^{bc}	1.99^{ab}
L-Cysteine, 25	17.65^{ab}	3.83^{b}	5.12^{ab}	5.63^{b}	6.18^{b}	1.96^{ab}
Ascorbic acid, 50 + Citric acid, 25	**18.29^{a}**	**4.92^{a}**	**5.83^{a}**	5.87^{a}	**6.63^{a}**	2.17^{a}
Ascorbic acid, 50 + Citric acid, 25 + L-Cysteine, 25	16.19^{b}	4.88^{a}	5.01^{ab}	5.68^{b}	6.46^{ab}	1.98^{ab}
Coconut water, 10%	15.74^{bc}	3.53^{cd}	4.78^{b}	5.67^{b}	6.51^{ab}	1.97^{ab}
Ascorbic acid, 50+ Citric acid, 25 + L-Cysteine, 25	15.58^{bc}	3.68^{cd}	4.46^{b}	5.63^{b}	6.07^{bc}	1.96^{ab}
Ascorbic acid, 50+ Citric acid, 25 + L-Cysteine, 25+Coconut water 10%	15.63^{bc}	3.46^{def}	4.51^{b}	5.71^{ab}	5.96^{d}	0.98^{d}

Media: MS (liquid) + Sucrose 2 % + NAA (1.25 µM) + BAP (10 µM)
Mean value with same letters in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 15 explants. LSD: Least Significant Difference at œ=0.05. Data were recorded at the end of 3 wks. of inoculation.

Browning of the media was observed in shoot cultures of the two species due to the phenolic exudation from the dead leaves and other tissues. In order to avoid the inhibitory effect of browning on shoot multiplication, many researchers have used various antioxidants and other chemicals. Addition of ascorbic acid, citric acid, cysteine etc. to the culture media gave positive results in shoot cultures of many plants such as banana (Mondal *et al*., 2012) and *Brachylaena huillensis* (Ndakidemi

et al., 2014). The antioxidants prevent the oxidation of phenolic compounds to quinines which are harmful to cultures. Absorption by the plant material and translocation to other parts of culture, also prevented the oxidation of phenolic compounds and browning in tissues (Singh *et al*., 1985). Besides its role as an antioxidant, ascorbic acid is also involved in cell division and elongation (de Pinto *et al*., 1999; Sujatha *et al*., 1996). The positive effect of citric acid on activity of PPO and PO enzymes activity and total phenolic production was demonstrated by Khosroushahi *et al*. (2011) in *Taxus brevifolia.* Use of such additives in the medium to overcome the problem has been reported in *D. giganteus* (Ramanayake and Yakandawala, 1997). Incorporation of additives such as ascorbic acid, citric acid, cysteine and glutamine in the shoot multiplication medium favored auxiliary effect on shoot multiplication and growth and minimized leaching in the medium in *P. stocksii* (Sanjaya *et al*., 2005 and Somashekar *et al*., 2008). Effectiveness of these additives were also shown in shoot cultures of *B. bambos* and *D. strictus* by Kabade (2009). Coconut water is itself considered as nutrient medium with high concentration of growth hormones and vitamins viz. zeatin (γ-allyl aminopurine), inositol and reduced nitrogen compounds and it increased the shoot multiplication frequency and elongation in many plants in culture (Kalpona *et al*., 2000). Coconut water was added in media in shoot cultures of bamboo viz. *B. arundinceae* and *D. brandisii* (Nadgauda *et al*., 1990) and *D. longispathus* (Saxena and Bhojwani, 1993) but apparently the effect is not significant in the species tested or due to the variation in composition of the natural product which can only be expected to vary with the variety and maturation of the coconut from which it is sourced.

4.1.1.2.4. Effect of media pH on shoot multiplication

Culture of shoots in MS liquid medium at pH 5.5 favored maximum number of shoots (14.65 shoots/clump) and shoot length (4.17 cm) in *P. stocksii* (Table **23**). In *P. ritcheyi* however, the acidic pH ranging from 4-5 gave higher multiplication rate i.e. from 6.43 -7.28. At the low pH of 2.5, reduction of multiplication (5.57 shoots for *P. stocksii* and 3.29 shoots for *P. ritcheyi*) and shoot length was observed and quality of the shoots deteriorated. In general shoots of *P. stocksii* were vigorous at pH 5.5-6.0 and shoots of *P. ritcheyi* were best in pH 4.5 (Fig. **15** & **16**). At pH higher than 7.0 shoot multiplication and growth in either species was curtailed.

Table 23: Effect of pH on shoot multiplication and growth in *P. stocksii* and *P. ritcheyi.*

Treatments (pH)	*P. stocksii*			*P. ritcheyi*		
	Number of shoots/ clump	**Shoot length (cm)**	**Shoot Multiplic ation rate**	**Number of shoots/ clump**	**Shoot length (cm)**	**Shoot Multiplic ation rate**
2.5	5.57^{fgh}	3.28^{d}	1.97^{cd}	3.29^{e}	5.08^{c}	0.47^{c}
3.0	6.42^{fg}	3.31^{cd}	2.63^{c}	4.81^{cd}	5.16^{bc}	0.92^{c}
3.5	8.78^{ef}	3.36^{cd}	2.98^{c}	5.07^{cd}	5.53^{b}	1.08^{b}
4.0	10.83^{de}	3.47^{cd}	3.29^{bc}	6.43^{b}	5.98^{b}	2.44^{b}
4.5	12.37^{c}	3.52^{b}	3.78^{b}	**7.28^{a}**	**6.02^{ab}**	**2.97^{a}**
5.0	13.22^{b}	4.53^{a}	3.92^{b}	6.89^{b}	6.49^{a}	2.74^{a}
5.5	**14.65^{a}**	**4.17^{a}**	**4.76^{a}**	5.64^{c}	6.57^{a}	1.98^{ab}
6.0	14.47^{a}	4.07^{ab}	4.34^{a}	5.26^{c}	6.64^{a}	1.72^{ab}
6.5	11.05^{d}	3.67^{b}	3.58^{b}	5.01^{cd}	6.02^{ab}	1.03^{b}
7.0	10.61^{de}	3.32^{bc}	3.24^{b}	4.95^{cd}	5.61^{b}	0.99^{c}
7.5	9.61^{e}	3.17^{cd}	3.02^{bc}	4.74^{cd}	5.27^{bc}	0.87^{c}
8.0	7.74^{f}	3.14^{cd}	2.95^{c}	4.60^{d}	5.02^{c}	0.76^{c}

MS (liquid) + Sucrose 2 % + NAA (1.25 µM)+ BAP (10 µM)
Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (3-4 shoots/clump).LSD at $\alpha = 0.05$.Data were recorded at the end of 3 wk.

Fig. 15: Shoot culture of *P. ritcheyi* rescued from shoot tip browning by culturing in media with pH 4.5

Fig. 16: Healthy shoot culture of *P. ritcheyi* in culture media with pH 4.5

The pH of a culture medium must be such that it does not disrupt the plant tissue and keeps the salts in soluble form and available for taking up. Slight variation in pH may lead to limited availability of certain mineral nutrients (Scholten and Pierik, 1998).

This pH has influence on chemical reactions especially those catalysed by enzymes. Therefore, a species specific pH had to be optimized during the course of micropropagation.The optimum pH was found to be 5.0 for the maximum shoot multiplication (4-5 fold) in *B. tulda* than pH 5.8 (3.5 fold) (Saxena, 1990). Similar results were found in shoot cultures of *Amygdalus communis* (pH 5.5) by Gurel and Gulsen (1998). Lower optimum of pH in the range of 4.0 to 6.0 have been reported in *in vitro* cultures of *Corylus avellana* (Yu and Reed, 1993) and blueberry (Wolfe *et al*., 1986). In reports of bamboo shoot cultures, pH was adjusted at 5.7 or 5.8 before autoclaving (Lin and Chang, 1998; Devi and Sharma, 2009; Arya and Sharma, 1998; Arya *et al*., 1999; Ravikumar *et al*., 1998 and Negi and Saxena, 2011). Sanjaya *et al.* (2005) and Kabade (2009) reported the optimum pH for shoot initiation and multiplication in *P. stocksii, B. bambos* and *D. strictus* as pH 6.0.

4.1.1.2.5. Effect of sub-culturing period of shoot multiplication

Out of the various treatments to optimize the sub-culture period, 24 d. proved the best in terms of number of shoots (14.65) and shoot length (4.17 cm) in *P. stocksii* (Table **24**). Similar results were obtained in *P. ritcheyi* also (7.28 shoots / cluster of maximum length 6.02 cm). There was no increase in number of shoots and length of shoot beyond the 24 d. In *P. ritcheyi*, all subculture periods except 8 d. showed browning of shoot tip and led to the decaying of the shoot tips. Furthermore, medium turned brown and shoot deteriorated) especially at 30 d. subculture interval.

Table 24: Effect of sub-culturing period on shoot multiplication in *P. stocksii* and *P. ritcheyi.*

Subculture period in Days	*P. stocksii*			*P. ritcheyi*		
	Number of shoots /clump	Shoot length (cm)	Multiplication rate	Number of shoots /clump	Shoot length (cm)	Multiplication rate
8	7.48^{d}	3.37^{c}	2.96^{c}	2.06^{e}	6.01^{a}	0.13^{c}
16	11.29^{c}	4.03^{bc}	3.17^{b}	3.72^{d}	6.01^{a}	0.64^{b}
24	**14.65^{a}**	**4.17^{a}**	**4.76^{a}**	**7.28^{a}**	**6.02^{a}**	**2.97^{a}**
30	14.67^{a}	4.19^{a}	4.76^{a}	7.27^{a}	6.04^{a}	2.99^{a}

Media: MS (liquid) + Sucrose 2 % + NAA (1.25 µM)+ BAP (10 µM)

Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (3-4 shoots/clump). LSD at α = 0.05. Data were recorded at the end of 3 wks.

Prolonged incubation of shoots for more than 3 wks. in same media caused depletion in nutrients and resulted in irregular growth of the shoots (Negi and Saxena, 2011)

and reported shoot withering in cultures of *B. nutans* after 21 d. of culture. The incidence of shoot necrosis was reported in *B. wamin* on a sub-culture cycle of more than 25 d. (Arshad *et al*., 2005). Kabade (2009) reported the decreased shoot length along with the browning of medium during the prolonged subculture periods in *B. bambos* and *D. strictus.* A species specific subculture period was demonstrated in many of the bamboo species viz. 14 d. in *D. strictus* (Ravikumar *et al*., 1998) and *P. stocksii* (Sanjaya *et al*., 2005), 10-15 d. in *B. balcooa* (Arya *et al*., 2008a) 21 d. in *B. edulis* (Lin and Chang, 1998), 28 d. in *B. bambos* (Arya and Sharma, 1998), *B. glaucescens* (Shirin and Rana, 2007) and *D. falcatum* (Arya *et al*., 2008a). Having a short subculture period would mean frequent transfers, consequently increased manpower requirement and thereby increase cost of plant production unless a proportionate benefit in terms of higher multiplication rate or quality is achieved, which is clearly not the case in the two species.

4.1.1.2.6. Effect of sucrose concentration on shoot multiplication

Sucrose in the range of 1.5- 6.0 % had a significant effect on shoot multiplication and growth in both *P. stocksii* and *P. ritcheyi*. The maximum shoot number of 15.21 for *P. stocksii* and 5.79 for *P. ritcheyi* was observed media with 4.5 % and 2.5 % of sucrose respectively (Table **25**). Deterioration of shoots within 12-15 d. of subculture was observed in the sucrose free media.

Table 25: Effect of sucrose concentrations on shoot multiplication in *P. stocksii* and *P. ritcheyi.*

Sucrose (%)	*P. stocksii*			*P. ritcheyi*		
	Number of shoots /clump	**Shoot length (cm)**	**Shoot Multiplication rate**	**Number of shoots /clump**	**Shoot length (cm)**	**Shoot Multiplication rate**
0.0	4.43^{f}	3.51^{b}	0.43^{de}	4.32^{c}	2.49^{d}	0.88^{d}
1.0	5.84^{e}	3.68^{b}	1.58^{c}	5.04^{bc}	3.77^{c}	1.24^{c}
1.5	7.36^{d}	3.74^{b}	2.72^{c}	5.21^{b}	5.97^{b}	1.36^{c}
2.0	10.74^{c}	3.83^{b}	3.06^{bc}	5.86^{a}	6.08^{a}	2.14^{ab}
2.5	12.56^{bc}	3.98^{b}	3.98^{b}	**5.79^{a}**	**6.74^{a}**	**2.38^{a}**
3.0	14.65^{ab}	4.17^{a}	4.76^{ab}	5.64^{ab}	6.67^{a}	1.98^{ab}
3.5	**14.96^{ab}**	**4.88^{a}**	4.85^{a}	5.52^{ab}	5.58^{b}	1.63^{c}
4.0	15.06^{a}	4.63^{a}	4.93^{a}	5.43^{ab}	4.92^{b}	1.47^{c}
4.5	**15.21^{a}**	4.47^{a}	4.97^{a}	5.41^{ab}	4.08^{b}	1.45^{c}
5.0	13.76^{b}	4.37^{a}	4.12^{ab}	5.25^{b}	3.67^{c}	1.39^{c}
5.5	13.04^{b}	4.34^{a}	4.06^{ab}	5.04^{bc}	3.05^{c}	1.24^{c}
6.0	12.63^{bc}	4.24^{a}	3.99^{b}	4.36^{c}	2.82^{d}	0.85^{d}

Media: MS (liquid) + NAA (1.25 µM)+ BAP (10µM)
Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (3-4 shoots/clump).LSD at $\alpha = 0.05$. Data were recorded at the end of 3 wks.

This is due to the almost totally heterotrophic nature of the shoot cultures as is true of most conventional tissues culture procedures. The higher concentration of sucrose in the medium improved shoot multiplication rates but stunting in the growth of the shoots was also observed that can be attributed to the higher osmotic effect of the sugar.

Exogenous supply of carbon sources provides energy and carbon source for growth and development of shoots in micropropagation. 3 % of sucrose was shown to be the best for shoot multiplication in many bamboo species such as *D. asper* (Arya *et al*., 1999), *P. stocksii* (Sanjaya *et al*., 2005), *G. angustifolia* (Jimenez *et al*., 2006), *B. bambos* and *D. strictus* (Kabade, 2009), *B. nutans* (Yasodha *et al*., 2008). Similarly, among the various concentrations of sucrose (1.0-6.0 %) used in MS medium, 3 % (w/v) sucrose proved the best for the maximum (18.75 shoots/clump) shoot multiplication with higher shoot length (4.05 cm) was observed in *P. stocksii* (Somashekar *et al*., 2008). However, many instances of shoot multiplication in *in vitro* culture supported by sucrose 2 % have been reported viz. *B. tulda* (Saxena, 1990), *T. spathiflorus* (Bag *et al*., 2000) *D. brandisii* and *B. arundinaceae* (Nadgauda *et al*., 1990). Sucrose is a carbohydrate of choice for most studies due to its cost effectiveness, easily translocatable nature and resistance to enzymatic degradation on account of its non-reducing nature (Pontis, 1978).

4.1.1.2.7. Effect of inoculum size on shoot multiplication

Out of the various treatments, inoculum size of 3-4 shoots/cluster proved the best in terms of number of shoots (14.65 shoots/ cluster) and shoot length (4.17 cm) for *P. stocksii* in 3 wks. And this inoculum size gave maximum multiplication rate in *P. stocksii* (Table **26**). In *P. ritcheyi*, a 3-fold increase in multiplication was observed when the sub culturing was carried out with 4-5 shoots. This also elongated the shoot length upto 6.37 cm. Higher number of shoots in the inoculum, in both species, resulted in shortening of shoots and this could be attributed to the competition among the shoots for nutrients as well as light. The dwarf clusters of shoots were also not suitable for rooting. Lowest shoot multiplication rate was observed in inoculation of 2-3 shoots/cluster.

Table 26: Effect of sub-culturing period on shoot multiplication in *P. stocksii* and *P. ritcheyi.*

Treatments (Inoculum size, shoots/ clump)	*P. stocksii*			*P. ritcheyi*		
	No. of shoots/ cluster	**Shoot length (cm)**	**Shoot Multiplication rate**	**No. of shoots/ cluster**	**Shoot length (cm)**	**Shoot Multiplication rate**
1-2	3.74^{e}	3.26^{d}	2.49^{d}	3.69^{d}	6.18^{ab}	0.92^{e}
2-3	8.37^{d}	3.81^{c}	2.35^{d}	4.35^{c}	6.42^{a}	1.01^{c}
3-4	$\mathbf{14.65^{c}}$	$\mathbf{4.17^{nc}}$	$\mathbf{4.76^{a}}$	$\mathbf{5.28^{b}}$	$\mathbf{6.02^{b}}$	$\mathbf{1.97^{c}}$
4-5	18.22^{b}	4.13^{b}	4.05^{b}	7.72^{a}	6.37^{a}	3.92^{a}
5-6	20.65^{a}	3.61^{c}	3.75^{c}	7.76^{a}	5.03^{c}	3.93^{a}

Media: MS (liquid) + Sucrose 2% + NAA (1.25 µM) + BAP (10µM).
Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. LSD at $\alpha = 0.05$. Data were recorded at the end of 3 wks.

In bamboo shoot culture, 3-5 shoots were considered as the optimum inoculum size for sub culturing in many species such as *B. tulda* (Saxena, 1990) *D. hamiltonii* (Sood *et al*., 2002), *B. wamin* (Arshad *et al*., 2005) and *G. angustifolia* (Jimenez *et al*., 2006). Ravikumar *et al*. (1998) reported that the inoculum size of 5-7 shoots was optimum for the successful multiplication of the bamboo *D. strictus.* Arya *et al*. (1999) and Sanjaya *et al*. (2005) suggested the inoculum size as 3-4 shoots in *D. asper* and in *P. stocksii* respectively. Inoculum size with 3 shoots was widely accepted in some other bamboo such as *B. nutans* (Negi and Saxena, 2011), *B. bambos* (Arya and Sharma, 1998), *B. glaucescens* (Shirin and Rana, 2007) and in *A. callosa* (Devi and Sharma, 2009).

Larger inoculum size will facilitate the survival of culture in fresh media but will also result in a competition for the nutrients and the proportion of older shoots that are close to senescence is higher than in the case of smaller inoculum where they get dispersed into different vessels during subculture. Smaller inoculum would mean more effort and time goes into excising the shoots from the shoot cluster. Larger shoot explants are also expected to have a larger number of nodes that contributes to the proliferation of multiple shoots but in the present case no such clear advantage is seen since the medium sized shoot cluster of 3-4 shoots gave the best response.

4.1.1.2.8. Effect of auxins on *in vitro* rooting of microshoots

Shoots rooted within 10 d. of inoculation in the medium consisting of IBA (9.84 µM) in *P. stocksii*. Improved *in vitro* rooting (100 %) was also observed in the medium with high levels of auxins (NAA, IAA and IBA). Among the various treatments, IBA (9.84 µM) produced the maximum root number (6.22) with a root length of 5.54 cm

(Table **27**). Interestingly micro-rhizomes were also induced along with the rooting in several treatments of NAA and IBA (Fig. **17**). Least effective root induction response (<50 %) was obtained at lower levels of NAA, IAA and NOA. Thick swollen unelongated roots (1.0-2.0 cm in length) was observed in the medium with 2, 4-D (0.45 and 1.13 µM) and picloram (4.14 µM). Basal brown callus without any sign of rooting was formed in all treatments supplemented with higher concentrations of auxins in *P. ritcheyi*. All the shoot clumps of *P. ritcheyi* completely dried out without root induction in auxin free medium (control) and at lower (2 µM) concentration of auxins. In general, root induction experiments *in vitro* were without any success in this species.

In many species of bamboo, root induction was reported as a severe constraint even while 100 % root induction was reported in many species such as *D. strictus* (Nadgir *et al*., 1984*), D. brandisii* and *B. arundinacea* (Nadgauda *et al.*, 1990), *D. strictus* and *D. giganteus* (Das and Rout, 1991) and *B. nana* (Shirin and Arya, 2003).

Table 27: Effect of various auxins on *in vitro* rooting from shoot clumps (2-3shoots/clump)

P. stocksii			
Treatments (µM)	**Response (%)**	**Rooting**	
		No. of roots / clump	**Root length (cm)**
Control	0.0	0.0	0.0
NAA 2.68	43.75^{i}	2.64^{gh}	2.76^{ef}
NAA 5.37	81.25^{c}	3.09^{f}	3.68^{d}
NAA 10.74	94.37^{b}	3.64^{d}	4.08^{c}
NAA 26.87	100^{a}	4.33^{b}	4.35^{b}
IAA 2.82	21.87^{l}	1.46^{lm}	2.63^{f}
IAA 5.71	40.62^{k}	1.79^{k}	2.76^{ef}
IAA 11.42	65.26^{f}	2.44^{hi}	3.65^{d}
IAA 28.55	100^{a}	3.26^{ef}	4.05^{c}
IBA 2.46	56.25^{h}	2.07^{j}	2.88^{e}
IBA 4.92	62.66^{g}	2.73^{g}	3.62^{d}
IBA 9.84	**100^{a}**	**6.22^{a}**	**5.54^{a}**
IBA 24.6	100^{a}	6.14^{a}	5.39^{a}
NOA 2.47	18.75^{m}	1.33^{m}	2.03^{g}
NOA 4.95	43.33^{ij}	2.28^{ij}	2.76^{ef}
NOA 9.9	62.5^{g}	2.49^{ghi}	3.62^{d}
NOA 24.75	68.75^{e}	3.17^{f}	4.26^{b}
2,4-D 0.22	0.0	0.0	0.0
2,4-D 0.45	41.66^{jk}	1.65^{kl}	1.67^{h}
2,4-D 1.13	72.24^{d}	3.52^{de}	1.03^{j}
Picloram 4.14	61.25^{g}	4.06^{c}	1.27^{i}
Picloram 8.28	0.0	0.0	0.0

Media: MS 1/4 agar gelled medium. Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (2-3 shoots/clump).LSD at $\alpha = 0.05$.Data were recorded at the end of 4 wks.

Fig. 17: Root and rhizome induction in *P. stocksii*

Mukunthakumar *et al*. (1999) reported 65 % rooting in 60 d. from shoots pulse treated with IBA (492 µM) for 24 h, followed by transfer to MS agar gelled medium with IBA (4.92 µM) in *D. brandisii*. Similarly, in *T. spathiflorus*, transferring of the shoot cultures from half strength MS medium with IBA (300 µM) after 14 d. to half strength MS hormone free medium favored 100 % rooting (Bag *et al*., 2000). Shoot culture in media supplemented with NAA (16.11µM) or the high level of IBA (49.2 µM) induced 95-98 % rooting in 30 d. in seedling cultures of *D. asper* (Arya *et al*., 1999). Four wks. incubation in MS medium supplemented with IBA (37.12 µM) resulted 95 % rooting in *B. wamin* (Arshad *et al*., 2005). Prolonged exposure to IBA enhanced the root formation upto 100 % in certain species like *B. vulgaris* '*striata'* (Ramanayake *et al*., 2006) and *B. glaucescens* (Shirin and Rana, 2007).

Some species required combined action of various PGR for root induction. Sanjaya *et al*. (2005) induced rooting in *P. stocksii* within 2 wks. of culture in half strength MS liquid medium with BAP (0.44 µM) and IBA (4.90 µM). Similarly, Devi and Sharma (2009) reported that root formation was improved to 75 % in *A. callosa* through the incubation of shoots for 8 wks. in half strength MS liquid medium with IBA (25 µM) and BAP (0.05 µM). 100 % rooting in *B. nutans* was obtained similarly in half strength MS liquid medium supplemented with a combination of IBA (9.8 µM), IAA (2.85 µM) and NAA (2.68 µM) within 3 wks. (Negi and Saxena, 2011).

It is evident therefore that the optimum requirement of specific auxins, its concentration and combinations varied widely with the bamboo species. In the present study, IBA alone yielded high rate of rooting when compared to the other

auxins (IAA, IBA and NOA) which may partly be due to photo stability and thereby the availability of IBA activity in culture for longer period.

4.1.1.2.9. Effect of basal nutrient media on *in vitro* rooting

Rooting percentage and number of roots formed were significantly influenced by different media used in *P. stocksii*. No rooting was obtained in any of the treatments on *P. ritcheyi* as in the previous experiments. Among the various media (MS, ½ MS, 1/3 MS , ¼ MS, 1/6 MS, B5, WPM and White's Media) tested with IBA (9.84 µM), ¼ MS medium was found most effective for rooting (100 %), produced more number of roots (6.58) and better (5.22 cm) root length in 4 wks. (Table **28**). This was followed by 1/3 MS, ½ MS, 1/6 MS, B5, WPM and White's medium in the order of rooting response. MS full strength medium was not found suitable for rooting. Shoot clusters remained green and healthy but without root induction in MS hormone free medium whereas, on the other media without auxins, shoots did not survive for long.

Table 28 : Effect of different basal media salt concentration and IBA on *in vitro* rooting in *P. stocksii*

Treatments (With IBA9.84 µM)	**Response (%)**	**Roots**	
		Number of roots/clump	**Root length (cm)**
MS	0.0	0.0	0.0
MS + IBA	27.13^{i}	3.53^{bc}	3.25^{e}
MS	34.24^{h}	2.07^{f}	2.55^{g}
½ MS + IBA	68.75^{c}	3.22^{cd}	3.72^{d}
1/3 MS	38.44^{g}	2.86^{de}	3.06^{f}
1/3 MS + IBA	77.36^{b}	3.62^{bc}	4.17^{c}
¼ MS	0.0	0.0	0.0
¼ MS + IBA	**100^{a}**	**6.58^{a}**	**5.22^{a}**
1/6 MS	0.0	0.0	0.0
1/6 MS + IBA	66.81^{c}	3.57^{bc}	4.33^{c}
WPM	0.0	0.0	0.0
WPM + IBA	52.26^{e}	3.25^{cd}	4.25^{c}
White's	0.0	0.0	0.0
White's IBA	46.81^{f}	2.55^{ef}	3.57^{d}
B5	0.0	0.0	0.0
B5 + IBA	56.25^{d}	3.83^{b}	4.58^{b}

Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (2-3 shoots/clump).LSD at $\alpha = 0.05$.Data were recorded at the end of 4 wks.

Nutritional requirements at rooting stage is considered to be different from that during the shoot multiplication stage. Low nutrient media resulted in root formation and this nutrient requirement is species specific. Quarter strength of MS basal medium was found to be the most effective in highest (100 % response) rate of rooting and root

initials appeared within 10-12 d. after incubation. Shoots produced 6.58 roots (per shoot clump) with 5.22 cm root length at the end of 4 wks. Schiefelbein and Benfey (1991) reported that the structure of root system is determined by the interaction between external factors and an internal developmental program. Hutchings and de Kroon (1994) supported this view and demonstrated that one important factor among these external factors was mineral nutrients. Zhang and Forde (1998) revealed the contrasting effects of nitrate and phosphate availability on root formation. Lower concentration of ammonium nitrate was found as important factor for root induction in many bamboo species such as *B. tulda* (Saxena, 1990). Shirin and Arya (2003) reported the higher root induction (90 %) in *B. nana* in MS full strength medium supplemented with IBA (25 µM) than other different (B5, WP and White's) media evaluated. Induction of higher number of shoots (17.27) and maximum rooting (90 %) were observed in *B. vulgaris* on MS medium supplemented with NAA (25 µM) among the different (MS, B5, WP and White's) media (Shirin *et al*., 2003). Whereas, ½ MS and ¼ MS media induced the maximum (100 %) rooting in *P. stocksii* out of the various media each supplemented with NAA (5.37µM) (Somashekar *et al*., 2008). Similar results for the low salt basal media was obtained in D. *giganteus* (Ramanayake and Yakandawala, 1997), *T. spathiflorus* (Bag *et al*., 2000), *D. strictus* (Singh *et al*., 2001), *P. stocksii* (Sanjaya *et al*., 2005) and *B. wamin* (Arshad *et al*., 2007)

4.1.1.2.10. Effect of sucrose concentration on *in vitro* rooting

Rooting percentage and root growth varied significantly in *P. stocksii* with concentration of sucrose (1.0-6.0 %) used in ¼ MS agar gelled medium supplemented with IBA (9.84 µM). 3.0 % sucrose in the medium was found the best for high (100 %) rate of rooting with maximum number of roots (5.84) and root length (4.58 cm). Treatments of 3.0 % and 4.0 % sucrose were on par in terms of percentage rooting, number of roots and root length (Table **29**). Higher (5-6 %) and lower concentration (1-2 %) of sucrose in the rooting medium decreased rooting percentage as well as a reduction in number of roots and length. Sugar free medium was not suitable for rooting.

Table 29: Effect of sucrose concentrations on *in vitro* rooting of shoot of *P. stocksii*

Treatments (Sucrose, %)	Response (%)	Roots	
		Number of roots/ clump	Root length (cm)
0	0.0	0.0	0.0
1	29.17[e]	2.08[d]	1.37[d]
2	50.36[d]	3.16[c]	2.55[b]
3	**100[a]**	**5.84[a]**	**4.58[a]**
4	100[a]	5.78[a]	4.42[a]
5	83.24[b]	4.65[b]	3.32[b]
6	71.31[c]	3.43[c]	2.36[c]

Media: ¼ MS (solid) +IBA (9.84 µM)
Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 shoot clumps (2-3 shoots/clump).LSD at $\alpha = 0.05$.Data were recorded at the end of 4 wks.

Carbon source in the culture medium is an essential component as a source of energy and for maintaining osmoticum (Sul and Korban, 1998 and Cuenca and Vietiez, 2000). Carbohydrate requirement for rooting in shoots depends upon availability of auxins, nitrogen and light (Bonga and Von Aderkas, 1992). Sucrose is often used as the carbohydrate in cell and tissue culture media, because, it is the main sugar translocated in the phloem of many plants (Giaquinta, 1980; Strickland *et al*., 1987). Out of the various concentrations of sucrose (1.0-6.0 %) tested, maximum rooting (100 %) was obtained with 3 % sucrose and IBA (9.84 µM) in 4 wks. 1.0 and 2.0 % sucrose exhibited lowest response (29.17 and 50.36 % respectively) on rooting. Incorporation of 3 % sucrose had favored maximum (5.84) number of roots and root length (4.58 cm) as compared to lower and higher concentration of sucrose. Higher concentration (5-6 %) of sucrose not only lowered the rooting response but also yielded fewer and shorter roots. It is also evident from the experiment that except for control (sucrose free medium), rooting was observed in all sucrose supplemented medium. This indicates sucrose has a vital role rooting. Reports on the effect of different concentrations of sucrose on *in vitro* rooting are scanty. In *Dalbergia latifolia*, 80 % of shoots gave root induction with IBA (9.84 µM) and 2 % sucrose (Swamy *et al*., 1992). Sanjaya *et al*. (2005) also reported rapid rooting obtained on ½ MS liquid medium with BA (0.44 µM) + IBA (4.90 µM) supplemented with 2 % sucrose in *P. stocksii*. Yasodha *et al*. (2008) however reported that addition of glucose (88 mM) to MS medium along with 49.0 µM, IBA during the root induction phase gave 85 % rooting success as compared to sucrose (88 mM) supplemented medium in

B. nutans. Whereas in another report, maximum (100 %) rooting was observed on half strength MS liquid medium supplemented with 3 % sucrose in *B. nutans* (Negi and Saxena, 2011). In most of the *in vitro* studies, sucrose is the carbohydrate of choice mainly due to its resistance to enzymatic degradation on account of its non-reducing nature (Pontis, 1978). Poor growth of root and shoots at low levels of sucrose may be attributed to the fact of inadequate availability of the energy source.

4.1.1.2.11. Effect of auxins on *ex vitro* rooting

Significant differences were observed with respect to different auxins tested on root induction frequency, number of roots and its length. All the auxin treatments induced root induction, which were significantly higher than control. Among the various auxins tested at 2000 ppm, shoot pulse treated (15 min.) with IBA and planted in sand and vermiculite (3:1, ratio) medium showed highest *ex vitro* rooting response (78.96 %) with 4.36 roots and root length (5.62 cm). This was followed by the NAA, IAA and NOA (Table **30).** The lowest rooting (12.57 %) was in the treatment without any auxin (control).

Table 30: Effect of various auxins (15 min pulse treated) on *ex vitro* root induction in sand: vermiculite (3:1, v/v) rooting mediumin *P. stocksii*

Treatments (2000 ppm)	Response (%)	Roots	
		Number of roots/clump	Root length (cm)
Control (HF)	12.57^{e}	1.43^{e}	2.26^{d}
IAA	58.01^{c}	2.81^{c}	3.25^{b}
NAA	63.68^{b}	3.52^{b}	3.43^{b}
IBA	**78.96^{a}**	**4.36^{a}**	**5.62^{a}**
NOA	31.22^{d}	2.15^{d}	2.86^{c}

Mean values with same letter(s) in columns do not differ significantly according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 plastic cups. Each cup contains 4 shoot clumps (2-3 shoots/clump). LSD: at $\alpha = 0.05$. Data were recorded at the end of 4 wks.

Among the auxins tested, shoots treated with NOA was least (31.22 %) effective for *ex vitro* rooting but this treatment promoted the activated shoot growth during hardening.

High frequency (92.29 % and 99 %) *ex vitro* rooting was obtained on sand by pulse treatment of shoot with NAA (2000 ppm) and NAA (1000 ppm) in *B. bambos* (Kabade, 2009) and *P. stocksii* (Somashekar *et al*., 2008) respectively in a 4 wk period under greenhouse conditions. In *Embelia ribes*, shoots treated with IBA (4.93

μM) solution for 30 min. favored the highest (95.2 %) *ex vitro* rooting with maximum shoot length (4.5 cm) observed within 4 wks. (Annapurna and Rathore, 2010). In many horticultural crops *ex vitro* rooting is standard practice to improve the efficiency of micropropagation and reduce the costs. *Ex vitro* rooting besides bypassing the step of *in vitro* rooting, reduces mortality during hardening and cost of production and also improves the physical quality of plants (Augustine and D'Souza, 1997). *Ex vitro* rooting overcomes the problem of accumulation of ethylene during *in vitro* rooting (de Klerk, 2002). To induce *ex vitro* rooting efficiently, desired light intensity, temperature and relative humidity is a crucial requirement. Choice of auxin, its concentration and mode of treatment may vary from species to species. In order to increase endogenous level of auxin, exogenous application by pulse treatment is useful.

4.1.2. Somatic Embryogenesis

Tender shoot tips from field grown plants were used as source of explants for callus induction, further induction of somatic embryogenesis and regeneration of plantlets. The results of experiments that tested the effect of explant type, basal media and PGRs are as follows.

4.1.2.1. Callus initiation

4.1.2.1.1. Effect of explant type on callus induction

Of the different type of explants used in *P. stocksii*, early callus initiation response (within 2 wks. of inoculation) was noticed in leaf sheath segment in the form of swelling at the cut ends. An unorganized mass of callus formed around the wounded region. When the callus response of individual explant type was assessed, the percentage of callus initiation from the leaf sheath was highest (100 %) (Fig. **18** & **19**), followed by nodal segment (85.75 %) within 4 wks. in *P. stocksii* (Fig. **20**). There was a distinct difference in color and texture between the calli obtained from different explants. The callus obtained from nodal explants was creamish-white but that from root segment was brownish in color. Explants like leaf segment and inter-nodes did not respond to the treatments (Table **31)** and therefore the calli obtained from leaf sheath and nodal shoot segment were used for further multiplication in *P. stocksii*.

Among the different explants of *P. ritcheyi* used for callus induction, early callus formation was found in nodal segments collected from the axillary shoots within 2 wks. of inoculation (Fig. **21**). At both the cut ends of the nodal segments, an un-organized mass of callus was first induced. Callus initiation on the nodal segments was highest (93.78 %), followed by leaf sheath (64.11 %) (Fig. **22**). All the calli developed from different explants exhibited same texture and color. The calli could be described as soft and fibrous in nature. Explants with or without response exhibited severe deterioration and tissue browning at the end of 4th wk. (Table **31).** Callus obtained from nodal segments and leaf sheaths were used for further experiments.

Dudits *et al*. (1995) opines that various stress factors including PGRs reprogrammes the gene expression which lead to a series of cell divisions and resulted the formation of unorganized callus growth. Instead of all cells developing by an embryogenic pathway, only a few cells in the primary explant are competent for this process and only their genes involved in the generation of embryogenic cells have been activated by these factors. In this study different explants from immature leaf sheath and leaf segment from field grown plants, inter-node and nodal shoot segment from *in vitro* shoots were assessed for their ability on callus induction.

Table 31: Effect of explant type on callus initiation

Type of Explant	Response (%)	Callusing intensity	Callus type	Remarks
P. stocksii				
Leaf Sheath	100	++++	Compact & nodular	Embryogenic Callus
Leaf segment	51.93	++	”	”
Inter-node	28.72	+	”	”
Nodal shoot segment	85.74	++++	Mucilaginous, Compact translucent	”
P. ritcheyi				
Leaf Sheath	64.11	++	Soft & fibrous	Non-embryogenic
Leaf segment	05.57	+	”	”
Inter-node	06.09	+	”	”
Nodal shoot segment	93.78	+++	”	”

MS solid medium supplemented with 2, 4-D (13.65 µM) kin (9.24 µM).
Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 Petri plates. Each Petri plate consists of 4 callus clumps. LSD at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data were recorded at the end of 4 wks.

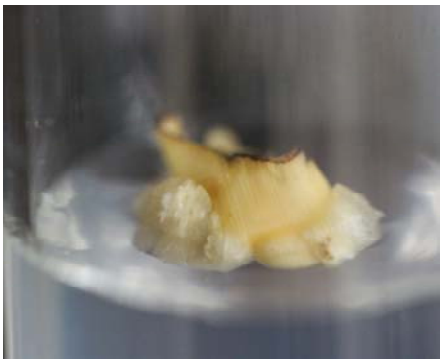

Fig. 18: Callus induction in *P. stocksii* (leaf sheath segments)

Fig. 19: Callus induction in *P. stocksii* (leaf sheath segments)

Fig. 20: Callus induction in *P. stocksii* (intermodal segments)

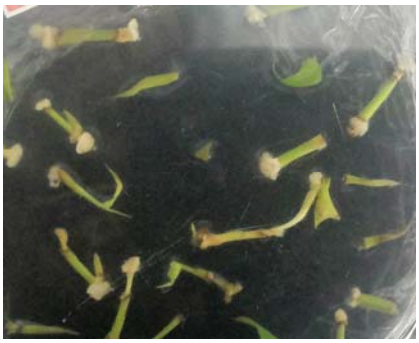

Fig. 21: Callus induction in *P. ritcheyi* (intermodal segments)

Fig. 22: Callus induction in *P. ritcheyi* (leaf sheath)

Among various explants tested in both species in this study, maximum response on callus induction was observed from leaf sheaths and nodal segments of *in vitro* shoots in *P. stocksii* and *P. ritcheyi* respectively within 4 wks. period. Wounded portions exhibited the swelling within 10 d. of inoculation and resulted in the creamish –white compact callus mass within 4 wks.in *P. stocksii.* In *P. ritcheyi,* the calli formed developed into a loose mass of cells without any of the compactness typically seen in

embryogenic calli. Absence of mucilaginous, viscous and compact embryogenic callus was noticeable in both species.

Nodal explants from mature clumps were used as explants in many bamboo species for culture initiation such as *B. nutans* (Mehta *et al*., 2011), *D. asper* (Arya *et al*., 2008b), *P. stocksii* (Somashekar, 2007), *B. edulis* (Lin *et al*., 2004), *D. giganteus* (Ramanayake and Wanniarachchi, 2003), *D. hamiltonii* (Godbole *et al*., 2002) and *B. vulgaris* (Rout and Das, 1997). Somatic embryogenesis and plant regeneration from leaf sheath of immature shoots of adult clumps, as in the present studies is not very common but was reported to in *Phyllostachys bambusoides* (Komatsu *et al*., 2011) where only albino shoots and rhizogenesis was seen. In *D. farinosus,* Hu *et al*. (2011), reported plantlet regeneration in leaf sheath callus induced on 2, 4, 5 T.

4.1.2.1.2. Effect of plant growth regulators (PGRs) on callus induction

Among the 23 different treatments of various PGRs viz. (auxins: 2, 4-D, 2, 4, 5-T, NAA, IAA, IBA and picloram and cytokinins: Kin and BAP) and coconut water, the medium with 2, 4-D (13.65 µM) and kin (9.24 µM) resulted in the best response (100 %) with maximum intensity of whitish compact and nodular callus obtained from nodal shoot explants in *P. stocksii* (Table **32 a** & **b).** Higher concentrations of 2, 4-D (18.2 µM) did not improve callus intensity. MS medium with 2, 4-D in combination with Kin or BAP (1.1 µM), found less effective as compared to 2, 4-D (9.1 µM) alone with respect to percentage response (78.24 and 57.46 %, respectively) and callus growth.

In the case of P. *ritcheyi* the response to all the combinations of PGR was not very encouraging. All the explants gave callus induction as well as proliferation in 2, 4-D alone but the callus formed were found to be of a soft wet type which revealed a mass of elongated fiber like cells. Explants inoculated into media with 2, 4-D and Kin gave callus with compact nature. Callus induction was absent in the medium without growth regulators. Incorporation of other auxins (NAA, IAA and IBA) at a concentration of 4.92 µM and 9.84 µM alone did not induce any callus. Treatments 2,4-D (9.1 µM), 2,4-D (18.2 µM), 2,4-D (9.1 µM) and CW 10 %, 2,4,5-T (7.82 µM), 2,4,5-T(15.64 µM)

Table 32a: Effect of plant growth regulators on callus initiation from leaf sheath as explants in *P. stocksii*

Treatments (PGRs @ µM)	Response (%)	Callus intensity	Remarks
Control	0.0	-	Explants turned brown
2,4-D (4.55)	76.54[e]	++	White, compact, nodular callus
2,4-D (9.1)	78.24[e]	++	Pale yellow compact callus
2,4-D (13.65)	78.24[e]	++	High frequency compact nodular callus
2,4-D (18.2)	57.46[i]	++	White soft callus
2,4-D (4.55)1.0 + CW 10%	92.66[b]	+++	Soft, mucilaginous callus
2,4-D (9.1)+ CW 10%	57.46[i]	++	Compact nodular callus
2,4-D(13.65)+ CW 10%	67.00[a]	++	High frequency compact nodular callus
2,4-D (18.2) + CW 10%	88.72[c]	+++	”
2,4-D (9.1) + Kin (4.65)	100.00[a]	+++	”
24-D (13.65)+ Kin (9.3)	100.00[a]	++++	”
2,4-D(4.55)+BAP(1.11)	42.90[d]	++	”
2,4-D(9.1) + BAP(1.11)	57.46[i]	++	White soft callus
2,4,5-T(7.82)+ CW 10%	78.32[e]	++	High frequency compact nodular callus
2,4,5-T (3.91)	78.32[e]	++	Soft, mucilaginous, callus
2,4,5-T (7.82)	100.00[a]	++++	”
2,4,5-T(15.64)	65.35[g]	++	”
2,4,5-T(3.91)+ CW 10%	61.82[h]	++	”
2,4,5-T(7.82)+ CW 10%	72.21[f]	++	Compact callus
2,4,5-T(15.64)+CW10%	78.32[e]	++	”
NAA (5.37)	0.0		No callusing
NAA (10.74)	0.0		”
IAA (5.71)	0.0		”
IAA (11.42)	0.0		”
IBA (4.92)	0.0		”
IBA (9.84)	0.0		”
Picloram(4.14)	65.35[g]	++	Brown Friable callus
Picloram (8.28)	72.21[f]	++	”
Picloram (16.56)	82.37[d]	+++	”

Medium: MS (solid) with PGRs
Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 Petri plates. Each Petri plate consists of 4 callus clumps. LSD at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data were recorded at the end of 4 wks.

and CW 10 % and picloram (8.28 µM) were more or less similar with 100 % of callus induction. However, medium fortified with 2, 4-D and Kin favored early (within 2 wks.) induction of a compact embryogenic callus in both species.

The frequency of callus induction from nodal shoot segments of *P. ritcheyi* varied significantly with different combinations and concentrations of PGRs used. Among the PGRs (2, 4-D, 2,4,5-T, IAA, IBA, NAA, Picloram, BAP, Kin and coconut water) tested either alone or in combinations in MS

medium, high frequency (100 %) callus initiation was observed on medium with 2,4-D (9.1 µM).

Table 32b: Effect of plant growth regulators on callus initiation *in vitro* nodal segments in *P. ritcheyi.*

Treatments (PGRs @ µM)	Response %	Callus intensity	Remarks
Control	0.0	-	Explants turned brown
2,4-D (4.55)	34.72[e]	++	Soft, fibrous, callus
2,4-D (9.1)	100.00[a]	++++	”
2,4-D (13.65)	100.00[a]	++++	”
2,4-D (18.2)	57.46[i]	++	”
2,4-D (4.55) + CW 10%	92.66[b]	+++	”
2,4-D (9.1) + CW 10%	57.46i	++	”
2,4-D(13.65)+ CW 10%	100.00[a]	++++	”
2,4-D (18.2)+ CW 10%	24.19[c]	+++	”
2,4-D (9.1) + Kin (4.65)	34.26[e]	++	Compact callus
24-D (13.65)+ Kin (9.3)	89.44[d]	+	”
2,4-D(4.55)+BAP(1.11)	67.34[d]	+	Soft, fibrous, callus
2,4-D(9.1)+ BAP (1.11)	78.36[i]	++	”
2,4,5-T(7.82)+ CW 10%	65.23[e]	++	”
2,4,5-T (3.91)	0.0		No Callus
2,4,5-T (7.82)	0.0		”
2,4,5-T (15.64)	0.0		”
2,4,5-T(3.91)+ CW 10%	0.0		”
2,4,5-T(7.82)+ CW 10%	0.0		”
2,4,5-T(15.64)+CW10%	0.0		”
NAA (5.37)	0.0		”
NAA (10.74)	0.0		”
IAA (5.71)	0.0		”
IAA (11.42)	0.0		”
IBA (4.92)	0.0		”
IBA (9.84)	0.0		”
Picloram (4.14)	65.35[g]	++	Brown Friable callus
Picloram (8.28)	72.21[f]	++	”
Picloram (16.56)	82.37[d]	+++	”

Medium: MS (solid) with PGRs
Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 Petri plates. Each Petri plate consists of 4 callus clumps. LSD at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data were recorded at the end of 4 wks.

Early callus induction (within 10 d.) was also observed on the same medium as compared to other PGRs tested. But the callus produced was of a “soft and wet” texture and consisting of fibre like thin cells. The callus developed from explants inoculated on 2, 4 –D and kinetin was found to be compact in nature. Incorporation of cytokinins (Kin and BAP) at lower concentration in combination with 2, 4-D was found to enhance the callus response and the callus was compact in *P. stocksii*. Whereas, at a lower concentration, induction was low and callus remained slow-growing. Media with 2, 4, 5-T and picloram at concentrations of 9.1 µM were

equivalent in terms of callus induction with that of 2, 4-D (9.1 μM). But by taking into consideration of early callus response, 2, 4-D (9.1 μM) was better than the other PGRs evaluated in both the species. Treatments consisting of the auxins IAA, IBA and NAA, did not favour callus induction and eventually explants deteriorated. Callus was not initiated in controls with media without any auxin.

Various reports suggest 2, 4-D as the most potent auxin for embryogenic callus induction and more frequently 9.1 μM were used in the medium. Many researchers such as Mehta *et al*., (1982); Rout and Das (1994); Sood *et al*. (2002) reported the potential of this auxin in inducing somatic embryogenesis in bamboo. Auxin concentration and combinations for embryogenesis is species specific and certain species required very high levels for the induction of embryogenic calli. For *D. giganteus*, nodular callus was induced and proliferated at a high levels of 2, 4-D and NAA (Ramanayake and Wanniarachi, 2003). Similarly, 30-40 % of inoculated shoot segments responded for callus induction medium supplemented with 2, 4-D (22.75 μM), but no response was recorded at lower or higher concentrations of 2, 4-D in *B. nutans* (Mehta *et al*., 2011). Arya *et al*. (2008b) reported that 91.6 % callus induction was obtained from nodal shoot segments of *D. asper* on MS medium supplemented with still higher (30 μM) levels of 2,4-D.

Combination of 2, 4-D with cytokinins enhanced the prospects of this auxin as was shown in *B. oldhamii* (Yeh and Chang, 1986a); *D. hamiltonii* (Godbole *et al*., 2002) and *D. hamiltonii* (Sood *et al*., 2002). Ogita (2005) reported that combination of BAP with auxin negatively affected the callus induction from *P. nigra.* But similar to results in *P. stocksii* in the present study*,* Lin *et al*. (2004) reported the induction of embryogenic calli from nodal shoot segments from *in vitro* plantlets of *B. edulis* on medium supplemented with 2,4-D and Kin.

On all auxins other than 2, 4- D, explants either did not respond or produced non-embryogenic calli. The use of other auxins has been reported for embryogenic callus induction, such as 2, 4, 5-T for young shoot explants of *D. farinosus* (Hu *et al*., 2011) and picloram for leaf sheath explants in *P. bambusoides* (Komatsu *et al*., 2011).

The use of *in vitro* shoots as explants for inducing embryogenesis has the advantage that sterile tissues are available on a continuing basis and loss due to contamination is

considerably reduced. Seasonal variation in response is also avoided by using *in vitro* shoots as source of explants.

4.1.2.1.3. Effect of basal nutrient media on callus induction

The treatment consisting of 2, 4-D (13.65 µM) and Kin (9.24 µM) in MS media was found to be the best for callus initiation (100 %) followed by in WPM (72.53 %) in *P. stocksii*. Whitish compact callus and good growth was observed in the MS medium as compared with other media tested (Table **33**). Lower frequency of callus induction was apparent in B5 (65.17 %) medium with 2, 4-D (9.1 µM).

The same pattern was followed by *P. ritcheyi* also. Nodal segments from *in vitro* grown axillary buds gave the maximum callus formation on 2, 4-D (13.65 µM) and kin (9.24 µM) followed by WPM, but the texture of the callus varied among the combinations.

Table 33: Effect of various nutrient media compositions with 2, 4-D and Kin on callus initiation from leaf sheath in *P. stocksii* and nodal segments in *P. ritcheyi.*

Treatments (Nutrient media +PGRs @ µM)	***P. stocksii***			***P. ritcheyi***		
	Response (%)	**Callus intensity**	**Callus texture and color**	**Response (%)**	**Callus intensity**	**Callus texture and color**
MS Control	0.0	-	Explants turn brown	0.0	-	Explants turned brown
MS+2,4-D (9.1)	82.38[b]	++	Brown yellow compact callus	91.25[b]	+++	Whitish fibrous callus
MS+2,4-D (13.65) + Kin (9.24 µM)	**100[a]**	+++	Friable vigorous callus	**98.09[a]**	+++	White fibrous vigorous callus
B5+ 2,4-D (9.1)	38.81[f]	+	Creamish gel like callus	21.98[f]	+	Creamish gel like callus
B5+2,4-D(13.65) + Kin (9.24 µM)	65.17[d]	++	Friable callus	42.48[d]	++	Soft callus
WPM+2,4-D(9.1)	54.11[e]	++	Compact callus	46.62[e]	++	Less frequency nodular callus
WPM+2,4-D (13.65) + Kin (9.24 µM)	72.53[c]	++	Compact callus	62.18[c]	++	Compact callus

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Each treatment with 3 replicates and each replicate consisted with 3 Petri plates. Each Petri plate consists of 4 callus clumps.LSD: Least significant difference at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data evaluation were carried out at end of 4th wks.

MS media has been widely accepted for inducing somatic embryogenesis in bamboo by many researchers. Lin *et al*. (2004) reported that nodal segments from *in vitro*

grown plants of *B. edulis* gave embryogenic callus induction on MS media. Nodal segments of *D. asper* gave best results on MS medium (Arya *et al*., 2008b). Zhang *et al*. (2010) found MS medium the best for vigorous, granular and compact callus induction from mature zygotic embryos of *D. hamiltonii* among different basal media (MS, ½ MS, NB etc.) evaluated.

Young shoots of *D. farinosus* gave 29.7 % callus induction frequency on MS medium as compared to 22.3 % on WPM medium (Hu *et al*., 2011). Other reports of role of MS media on callus formation was from *D. strictus* (Saxena and Dhawan, 1999) *B. vulgaris, D. strictus* and *D. giganteus* (Rout and Das, 1994) and *B. glaucescens* (Jullien and Tran Thanh Van, 1994) among others. However, successful reports on bamboo somatic embryogenesis in other basal media have also been reported such as *D. strictus* on B5 medium (Rao *et al*., 1985) and B5 medium in *D. strictus* and *B. bambos* (Rao *et al*., 1990).

The studies revealed that the mineral concentrations and combination of auxins have significant role on callus induction. Cellular differentiation in plant cell was critically influenced by mineral nutrition in combination with growth hormones (Beasley *et al*., 1974). The cellular uptake of the mineral nutrients is generally proportional to the medium concentration up to a concentration of twice that of MS (Williams, 1993). Among the three basal media tested, MS media provided the maximum nutrients which lead to the active proliferation of cells to produce callus in short time.

4.1.2.2. Callus multiplication

Compact nodular embryogenic callus along with nodal segments sub-cultured on new medium promoted multiplication of callus. Callus in growth phase maintained through sub-cultures on MS medium with 2, 4-D (4.55 µM) and Kin (4.65 µM) at regular interval of 2 wks. for 3-4 mon. was used to study the effect of auxins, nutrient media and sucrose concentrations on embryogenic callus multiplication. Results were given below. According to Arya *et al*. (2008b), somatic embryogenesis in bamboo involved three different types of calli viz. yellow friable non-embryogenic, compact, white, nodular embryogenic and translucent mucilaginous partially embryogenic type.

4.1.2.2.1. Effect of auxins on callus growth and maintenance

Table 34: Effect of different concentrations of auxins on callus multiplication in MS medium in *P. stocksii* and *P. ritcheyi.*

Auxins (µM)	*P. stocksii*			*P. ritcheyi*		
	Fresh weight (gm)	**Callus intensity**	**Texture and color of the callus**	**Fresh weight (gm)**	**Callus intensity**	**Texture and color of the callus**
Control	0.32^{f}	+	Mucilaginous	1.06^{b}	++	Brown black
2,4-D(4.55)	2.07^{cd}	++	Callus deteriorated	2.17^{a}	+++	Creamish gel
2,4-D(9.1)	1.35^{ef}	++	Callus turned brown	2.46^{a}	++++	Soft callus
2,4-D(13.65)	1.08^{f}	+++	Slightly mucilaginous	1.92^{ab}	+++	Less frequency nodular callus
2,4-D(18.2)	0.88^{ef}	++	Callus turned brown	1.99^{ab}	++++	Compact callus
2,4-D(9.1) + Kin (4.65)	4.35^{b}	++++	Callus was embryogenic	1.93^{ab}	+++	White fibrous vigorous callus
2,4–D (13.65) + Kin (4.65)	2.57^{ef}	+++++	Slightly compact	1.36^{b}	++	Creamish gel like
2,4–D(18.2)+ Kin (4.65)	1.38^{ef}	++	Callus soft creamish yellow	1.82^{ab}	+++	Soft callus
2,4 -D(13.65) + Kin (9.3)	5.02^{a}	++++++	Slightly compact & granular	1.67^{ab}	+++	Less frequency nodular callus
2,4-D(18.2) + kin (9.3)	3.93^{c}	++++	Callus turned brown black	1.06^{bc}	++++	Compact callus
2,4-D(9.1) +BAP 4.44	1.38^{ef}	++	Callus turned brown black	1.96^{ab}	++	White fibrous vigorous callus
2,4-D(13.65) + BAP (4.44)	1.41^{ef}	++	Slightly compact	2.07^{a}	+++	Creamish jelly callus
2,4-D(18.2) + BAP (4.44)	1.52^{ef}	++	Callus soft creamish yellow	1.38^{b}	++	Soft callus
2,4-D(13.65) + BAP (8.88)	2.08^{cd}	+++	Compact	1.52^{b}	++	Slightly compact
2,4-D(18.2) + BAP (8.88)	2.13^{c}	+++	Nodular embryogenic white callus	1.57^{b}	++	Compact callus
2,4,5-T (3.91)	1.12^{ef}	++	Slight mucilage yellow creamish	0.07^{fg}	+	White fibrous callus
2,4,5-T (7.82)	0.67^{f}	+	Slightly mucilaginous	0.22^{ef}	+	Creamish jelly callus
2,4,5-T (11.73)	0.44^{ef}	+	Nodular embryogenic white callus	0.35^{e}	+	Soft callus
2,4,5-T (15.64)	0.12^{h}	+	Callus white healthy and nodular	0.41^{e}	+	Nodular callus
Picloram (4.14)	0.95^{f}	+	Compact creamish	0.64^{d}	+	Soft callus
Picloram (8.28)	0.88^{f}	+	White brownish mucilage callus	0.27^{ef}	+	Fibrous vigorous callus
Picloram (12.42)	0.35^{g}	+	Callus soft creamish yellow	0.35^{e}	+	Creamish jelly callus
Picloram (16.56)	0.57^{g}	+	White brownish mucilage callus	1.41^{b}	++	Slightly mucilaginous
Dicamba (4.52)	0.35^{gh}	+	Slight mucilage yellow creamish	1.64^{ab}	++	Soft callus
Dicamba (9.04)	0.13^{h}	+	White and compact	1.07^{bc}	++	Soft callus
Dicamba (13.56)	0.02^{hi}	-	mucilaginous	0.94^{c}	+	White fibrous vigorous callus

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Each treatment with 3 replicates and each replicate consisted with 3 Petri plates. Each Petri plate consists of 4 callus clumps. LSD at $\alpha = 0.05$ by Duncan test.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data evaluation were carried out at end of 4^{th} wk.

It was observed that, callus fresh weight and frequency of callus induction varied significantly with different concentrations of auxins (2, 4-D, 2, 4, 5-T, NAA, IAA, IBA, Picloram and Dicamba) in *P. stocksii* and *P. ritcheyi*. Medium fortified with 2, 4-D (4.55 µM) was found suitable for embryogenic callus multiplication as related to other auxins tested (Table **34**).

Callus remained compact, nodular and creamish-white in colour with appreciable callus growth in 2, 4-D containing medium. Callus fresh weight was relatively lower (1.27 g and 1.07 g) in the 2, 4, 5-T (7.82 µM) and picloram (8.28 µM) containing media respectively. There was no significant differences in the callus fresh weight on IAA, IBA and NAA and furthermore callus turned brown and deteriorated without any further growth. Addition of Kin and BAP enhanced the callus growth to almost double than that obtained in the media containing auxin alone and the combination of 2,4-D 9.1 µM with Kin 4.65 µM gave maximum proliferation (4.35 gm) in *P. stocksii* (Fig. **23**).

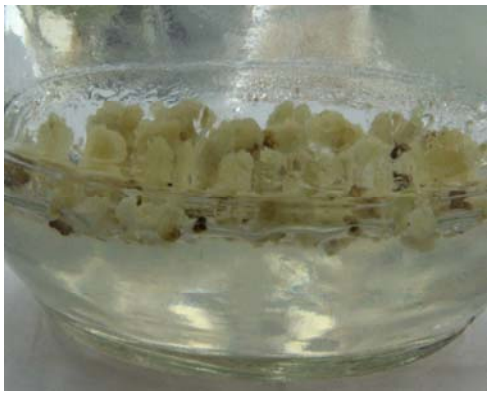

Fig. 23 Callus multiplication in *P. stocksii*

Fig. 24: Callus multiplication in *P. ritcheyi*

In *P. ritcheyi,* callus proliferation was highest on the media with 2, 4-D at 9.1 µM with 2.46 gm of weight. Callus proliferation was found to be relatively lower in the species than of *P. stocksii* in all media tested. Over all, callus growth was the best in 2, 4-D supplemented media among the auxins tested. The morphology of the callus varied greatly across the combinations and the compactness was found only in three combinations. Moreover, callus turns black-brown and deteriorate over a period.

Most of the calli that developed in *P. ritcheyi* was found to be non- embryogenic (Fig. **24**).

Callus proliferation was favored by the combination of 2, 4-D and Kin in MS media in *S. latifolia* (Yeh and Chang, 1987). Saxena and Dhawan (1999) reported that the combination of 2, 4-D, Kin and IBA was substantially better in multiplication of embryogenic callus cultures of *D. strictus* over a 5 wk. period. The combination of 2, 4-D with BAP improved the callus proliferation in *D. hamiltonii* (Godbole *et al.*, 2002). Lin *et al*. (2004) observed maximum (526 mg) fresh weight of callus and better callus multiplication on MS medium supplemented with 2,4-D (13.6 µM) and TDZ (0.046 µM) in *B. edulis*. In *D. asper*, the combined use of 2,4-D (9.0 µM), BAP (0.88 µM) and IAA (2.85 µM) favored better callus multiplication (3.5 fold) with maximum callus fresh weight (175 g) as compared to other phytohormone treatments (Arya *et al*., 2008b).There were no reports of callus proliferation in other auxins such as IAA and NAA.

4.1.2.2.2. Effect of basal nutrient media on callus growth and maintenance

Among the various nutrient media (MS, B5 and WPM) used, MS medium with 2, 4-D (9.1 µM) and Kin (4.65 µM) was found best and giving the highest (2.17 g) fresh weight of embryogenic callus. MS media supplemented with 2, 4-D (4.55 µM) and 2, 4-D (9.1 µM) with Kin (4.65 µM), shared statistically similar trait in fresh weight of callus (Table **35**) while a lower rate of callus multiplication was observed in B5 medium as compared to WPM. These data suggest that MS medium was superior for callus multiplication and growth.

Most of the researchers have used MS media for multiplication of embryogenic callus viz. *B. beecheyana* (Yeh and Chang, 1986b), *P. viridis* (Hassan and Debergh, 1987), *B. glaucescens* (Jullien and Tran Thanh Van, 1994), *B. vulgaris*, *D. strictus* and *D. giganteus* (Rout and Das, 1994), etc. Lin *et al*. (2004) used MS medium with 2, 4-D and TDZ for successful callus proliferation in *B. edulis*. Superiority of MS medium over the B5 and WPM on callus multiplication of *P. stocksii* and *P. ritcheyi* may be attributed to the high nutrient level (macro and micro salts) in MS medium, which supported better embryogenic callus multiplication.

Table 35: Effect of various nutrient media on callus multiplication in *P. stocksii* and *P. ritcheyi.* *Media: MS + Sucrose 2% + Kin (4.65 µM)

Treatments (Nutrient media)	*P. stocksii*		
	Fresh wt. (gm)	**Callus intensity**	**Callus texture and color**
MS Control	0.36[e]	-	Brown black deteriorated Callus
MS+2,4-D (9.1)	2.17[a]	++	Brown yellow compact callus
MS+2,4-D (13.65)	2.43[a]	+++	White compact vigorous callus
B_5+2,4-D (9.1)	0.74[d]	+	Compact nodular embryogenic callus
B_5+2,4-D (13.65)	1.16[c]	++	Compact and nodular
WPM+2,4-D (9.1)	1.25[bc]	++	Healthy callus
WPM+2,4-D (13.65)	72.53[c]	++	Compact callus
	P. ritcheyi		
MS Control	0.0	-	Callus turned brown
MS+2,4-D (9.1)	4.04[a]	++++	Compact nodular embryogenic callus
MS+2,4-D (13.65)	2.75[b]	+++	Compact callus
B_5+2,4-D (9.1)	1.96[b]	++	Creamish jelly callus
B_5+2,4-D (13.65)	0.89[c]	+	Soft callus
WPM+2,4-D (9.1)	0.11[e]	+	Fibrous non embryogenic callus
WPM+2,4-D (13.65)	1.03[c]	++	Compact and granular

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Each treatment with 3 replicates and each replicate consisted with 3 Petri plates. Each Petri plate consists of 4 callus clumps. LSD at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data evaluation were carried out at end of 4th wk. * Hormone concentrations are described in µM.

4.1.2.2.3. Effect of sucrose concentration on callus growth and maintenance

During prolonged callus multiplication on MS medium supplemented with 2, 4-D (9.1 µM) and kinetin (4.65 µM), the prevalence of mucilaginous/non-embryogenic type of callus was observed which hampered further somatic embryogenesis in *P. ritcheyi* and *P. stocksii.* To overcome proliferation of mucilaginous callus, various concentrations of sucrose was used in MS medium supplemented with additives, 2, 4-D (9.1 µM) and kinetin (4.65 µM). Among the different sucrose levels (1.5, 3.0, 4.5, 6.0, 7.5 and 10 %) used, 7.5 % sucrose proved best to minimize the problem of mucilage and improve callus fresh weight (3.11 g).

Sucrose at 10 % also improved the callus fresh weight (3.36 g) but was statistically on par to the medium supplemented with 10 % sucrose in 4 wks. (Table **36**). Sucrose concentration below 6 % was not found suitable for obtaining competent callus. Sucrose is known to act not only as a carbohydrate source, but also as osmoregulator. The osmotic potential of a medium can regulate the early development of somatic embryogenesis (Thorpe *et al*., 2008). In *D. hamiltonii,* only compact, nodular and creamish-white callus was capable to differentiate into somatic embryos rather than

the fast growing and friable callus (Godbole *et al*., 2002). In the present study also the friable callus did not show any response in both species. In *P. ritcheyi*, when callus was multiplied for long periods, friable and mucilaginous callus growth was observed along with nodular callus. To overcome the problem of mucilaginous cells in callus during prolonged callus multiplication in *P. ritcheyi*, different sucrose concentrations (1.5, 3.0, 4.5, 6.0, 7.5 and 10 % w/v) were used in MS medium supplemented with 2, 4-D (4.55 µM). Media with 7.5 % sucrose proved to be the best in reducing mucilaginous callus and favored maximum (3.11g) callus fresh weight. High concentration of sucrose (7.5 %. and 10 %) also increased callus fresh weight. The quality of callus improved from mucilaginous (non-embryogenic) to white compact homogenous (embryogenic) callus on the medium with 7.5 % sucrose.

Table 36: Effect of sucrose concentrations in MS medium supplemented with 2, 4-D (9.1 µM) and kinetin (4.65 µM) on callus multiplication in *P. stocksii* and *P. ritcheyi.*

Sucrose (%)	*P. stocksii*			*P. ritcheyi*		
	Fresh weight (gm)	Callus intensity	Callus texture and color	Fresh weight (gm)	Callus intensity	Callus texture and color
0	0.05^{g}	-	Deteriorated callus	0.37^{f}	+	Brown callus deteriorated
1.5	1.66^{e}	++	Compact and nodular callus	1.08^{e}	++	Creamish white nodular mucilage callus
3	3.93^{b}	++	Slightly compact granular and embryogenic callus	1.78^{d}	++	Creamish white nodular mucilage callus
4.5	2.17^{b}	++	Slightly compact and friable callus	2.53^{c}	+++	White compact nodular and less mucilage callus
6	2.08^{a}	+++	Compact and friable callus	2.92^{b}	+++	White compact and nodular callus
7.5	1.26^{a}	++++	Creamish white nodular mucilage callus	3.11^{ab}	+++ +	White granular compact homogenous callus
10	1.02^{e}	++++	Creamish white compact nodular callus	3.36^{a}	+++ +	Creamish white compact nodular callus

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT.Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 callus clumps. SD: Least significant difference at α = 0.05.Callus intensity: ─: no response; +: slow callus growth; ++: Moderate growth; +++: Good growth. Data were recorded at the end of 4 wks.

Osmotic effect of sucrose in culture medium for callus multiplication is well documented by the earlier workers in different species. In *P. stocksii,* 3 % sucrose was found to be effective for conversion of the callus into embryogenic compact callus. In *B. oldhamii* and *B. beecheyana* high levels of sucrose (6 % w/v) was used in the callus multiplication medium (Yeh and Chang, 1986a and b). In contrast, Woods *et al*. (1992) reported that medium with 2 % sucrose was more conducive than 5 % sucrose for maintenance of embryogenic callus in Mexican Weeping Bamboo. Comparison with another protocol (Lin *et al*., 2004) indicated among the sucrose concentrations (1.5, 3.0, 4.5 and 6.0 %) tested, lower percentage of callus browning and maximum callus fresh weight was observed on MS medium supplemented with 4.5 % sucrose in callus proliferation of *B. edulis*. Litz (1988) revealed that 6 % sucrose in B5 medium favored multiplication of embryogenic callus of *Euphorbia longan*. In *Zea mays* high concentration of sucrose (12 %) in MS medium proved the best for induction of embryogenic callus from immature seed embryos (Lu *et al*., 1982). Similarly, Ho and Vasil (1983) used 6-10 % sucrose in MS medium to promote the formation of proembryoids from young leaves of *Saccharum officinarum*. However, Ahloowalia and Maretski (1983) showed that somatic embryo formation from callus of *S. officinarum* was best in MS medium with 3 % sucrose, but growth of the embryo into complete plantlets require 6 % sucrose in MS medium.

4.1.2.3. Somatic embryo induction and maturation

To carry out further studies on somatic embryo induction and maturation, healthy compact white embryogenic callus clumps were used. Although, the process of somatic embryo induction from cells in callus culture is not completely understood, it is believed that the presence of auxin can trigger the proembryogenic cells in the callus cultures to synthesize all the gene products necessary to complete the globular stage of embryogenesis (Steward *et al*., 1958; Gahan and George, 2008). Effect of various factors on induction of somatic embryos gave the following outcome.

4.1.2.3.1. Effect of plant growth regulators (PGRs) on somatic embryogenesis

Among the different PGRs (NAA, BAP, IBA and IAA) used in MS medium, treatment with IBA (2.46 µM) and BAP (6.66 µM) favored the highest (71.63 %) (Fig. **25** & **26**) response in induction of somatic embryos from embryogenic callus

followed by a combination of NAA (8.05 µM) and BAP (6.66 µM) (64.28 %) in *P. stocksii* (Table **37**).

Treatments with PGR alone induced a compact calli which were failed to the regenerate embryos. Combined action of BAP along with auxins enhanced the induction of nodular compact callus but the combination of BAP and IBA only induced homogenous nodular embryogenic nodular callus.

Fig. 25: Embryogenic callus of *P. stocksii*

Fig. 26: Somatic embryogenesis in *P. stocksii*

Table 37: Effect of plant growth regulators in MS agar medium on somatic embryo induction and maturation in *P. stocksii* and *P. ritcheyi*.

Treatments (PGRs, µM)	*P. stocksii*		*P. ritcheyi*	
	Response %	**Callus texture and color**	**Response %**	**Callus texture and color**
Control	0.00	White compact callus	00.44^{g}	Callus deteriorated
IAA (5.71)	11.22^{e}	Slight compact callus	03.44^{g}	Callus deteriorated
IBA (4.92)	14.22^{e}	Slight compact callus	04.71^{e}	Callus deteriorated
NAA (5.37)	17.66^{e}	Slight compact callus	08.33^{f}	Slight compact callus
BAP (4.44)	34.12^{e}	Slight compact callus	10.36^{c}	Slight compact callus
BAP (6.66)	19.78^{e}	Nodular compact callus	11.19^{bc}	Slight compact callus
IBA(2.46)+BAP (6.66)	95.71^{c}	Homogenous nodular compact greenish callus	19.34^{a}	Nodular compact callus
NAA (2.68) + BAP (6.66)	66.08^{d}	Nodular compact callus	04.26^{e}	Callus deteriorated
IAA (2.8)+ BAP (4.44)	59.67^{d}	Nodular compact callus	13.81^{b}	Slight compact callus

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 clumps. LSD at α = 0.05.Data were recorded at the end of 4 wks.

Callus from *P. ritcheyi* found to be brown and senescent in media with auxins alone. In this species also, combination of IBA and BAP induced a compact nature to the friable explant callus (Fig. **28** & **29)**. None of the other PGRs was capable of inducing embryogenesis in *P. ritcheyi.*

PGRs profoundly influence the growth and differentiation of plant cells, tissues and organs. They function as chemical messenger for intercellular communication and are known to play an important role in somatic embryogenesis in bamboo, but their mode of action is not known (Zhang *et al*., 2010).

Fig. 27: Soft and fibrous callus of *P. ritcheyi*

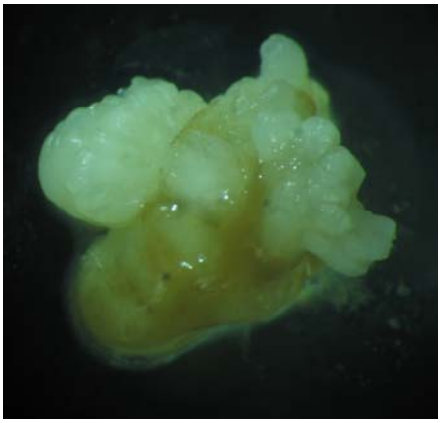

Fig. 28: Compact callus of *P. ritcheyi*

In the present study, various PGRs were used for somatic embryo induction and maturation from the homogenous compact nodular callus clumps obtained from the callus multiplication phase. Among different PGRs used in MS medium, treatment supplemented with IBA (2.46 µM) and BAP (26.4 µM) resulted in the highest (71.63 %) response with nodular and compact white colored embryos. It was also revealed that use of NAA or BAP individually in the medium gave a lower frequency of somatic embryos induction whereas a combination of NAA and BAP in the medium was most effective in induction and maturation of somatic embryos. Woods *et al*. (1992) also reported importance of BAP in production of somatic embryos in Mexican Weeping bamboo.

Godbole *et al*. (2002) found, incorporation of BA (10 µM) in ½ MS medium to be the best for somatic embryo induction and differentiation in *D. hamiltonii*. Mehta *et al*. (2011) revealed that compact slightly green callus in *B. nutans* obtained on MS medium supplemented with BAP and 2, 4-D resulted in maturation of somatic embryo.

4.1.2.3.2. Effect of basal nutrient media compositions on somatic embryogenesis

Significant effect of basal nutrient media on percentage embryo induction, synchronization and maturation of embryos. Among different nutrient media (MS, WP, B5and SH) tested supplemented with IBA (2.46 µM) + BAP (6.66 µM), maximum (98.82 %) percentage of callus of *P. stocksii* responded with somatic

Table 38: Effect of various nutrient media supplemented on somatic embryo induction and maturation in *P. stocksii and P. ritcheyi.*

P. stocksii		
Treatment	**Response %**	**Callus texture and color**
MS	98.82[a]	Homogenous compact nodular greenish callus
WPM	43.73[c]	Compact white callus
B5	58.22[b]	Compact white callus
SH	63.45[c]	Compact white callus
P. ritcheyi		
MS	63.29[a]	Compact callus with green patches
WPM	10.71[b]	Compact white callus
B5	13.70[b]	Compact white callus
SH	19.21[b]	Compact white callus

Media: Basal media + Sucrose 3% + IBA (2.46 µM) + BAP (6.66 µM)
Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 clumps. LSD at α = 0.05.Data were recorded at the end of 4 wks.

embryo induction in MS medium as compared to other three media (Table **38**). Frequency of embryo induction varied from 10.71 % to 63.29 % in *P. ritcheyi*. The embryogenic callus transformed to embryo like structures in the form of compact nodular and granular structures. Godbole *et al*. (2002) used ½ MS medium with BAP (10 µM) alone for somatic embryo maturation in *D. hamiltonii*. In contrast, Rao *et al*. (1985) obtained embryogenic callus on B5 medium with 2, 4-D and secondary embryo developed in about 67 % of callus cultures. Similarly, Patenat *et al*. (2002) used B5 basal medium with varying levels of 2, 4-D (2 µM - 20 µM), coconut milk (10 %) and sucrose (6 %) for somatic embryo induction in *Mangifera indica*. It was revealed from the present findings and reports in other species that the nutrient medium required for embryo induction and maturation in different species is attributed to different nutritional requirements particularly of macronutrients and different levels of nitrogen and the ratio between nitrate and ammonium.

4.1.2.4. Somatic embryo germination

Compact nodular embryo like structures were used as an inoculum to obtain germination of somatic embryo into plantlets. The results of testing the effect of PGR types, their concentration and nutrient media composition which have influence on somatic embryo germination into complete plantlet are as follows.

4.1.2.4.1. Effect of plant growth regulators (PGRs) on somatic embryo germination

Significant differences in conversion of somatic embryo was observed when different PGRs were tested in embryogenic cultures of *P. ritcheyi* and *P. stocksii*. Addition of PGR considerably enhanced the regeneration of somatic embryos and improved embryo survival. BAP alone induced the embryo germination upto 93.11 % (Fig. **31, 32, 33, 34** & **35**) at 2.22 µM but the regeneration potential reduced with increasing levels of the cytokinin. Combination of auxins with BAP also adversely effected the embryo germination, whereas it increased the tissue damage (Table **39**).

Table 39: Effect of plant growth regulators in MS medium on somatic embryo germination in *P. stocksii* and *P. ritcheyi*

Treatments (PGRs, µM)	*P. stocksii*		*P. ritcheyi*	
	Per cent regeneration	Remarks	Per cent regeneration	Remarks
Control	38.74[g]	53.96 %	Nil	Poor germination
BAP (2.22)	93.11[c]	00.77 %	Nil	”
BAP (4.44)	72.63[f]	38.44 %	Nil	”
BAP (6.66)	61.38[e]	39.70 %	Nil	”
IBA (2.46) + BAP (4.44)	56.89[b]	43.11 %	Nil	”
IBA (4.92) + BAP (4.44)	57.44[d]	42.56 %	Nil	”
IBA (2.46) + BAP (6.66)	52.56[a]	47.44 %	Nil	”
IBA (4.92) + BAP (6.66)	52.32[a]	47.68 %	Nil	”
IAA (2.85) + BAP (4.44)	27.01[b]	59.70 %	Nil	”
IAA (5.71) + BAP (4.44)	27.44[d]	72.56 %	Nil	”
IAA (2.85) + BAP (6.66)	22.56[a]	76.34 %	Nil	”
IAA (5.71) + BAP (6.66)	12.32[a]	87.68 %	Nil	”
NAA (2.68) + BAP (4.44)	20.80[b]	79.20 %	Nil	”
NAA (5.37) + BAP (4.44)	22.16[d]	77.84 %	Nil	”
NAA (2.68) + BAP (6.66)	21.51[a]	78.49 %	Nil	”
NAA (5.37) + BAP (6.66)	10.06[a]	89.94	01.86[a]	9% germination

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT.Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 clumps. LSD at $\alpha = 0.05$. #: Normal somatic embryogenesis (with intact shoot and root) and only shoot. Data were recorded at the end of 4 wk.

Fig. 31: Germination of somatic embryos in *P. stocksii*

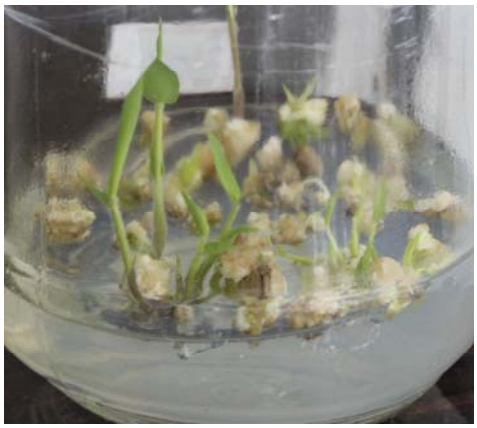

Fig.32: Germinated embryos of *P. stocksii*

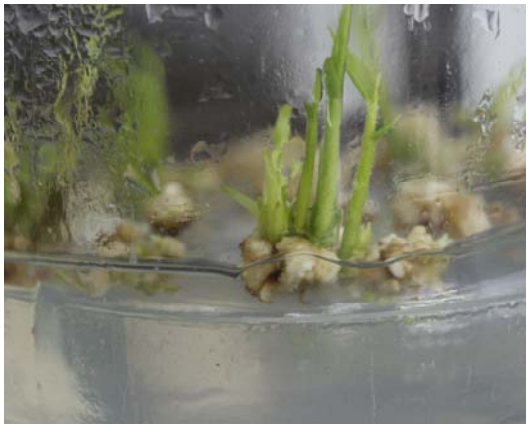

Fig. 33: Elongated shoots of *P. stocksii*

Fig. 34: Regenerated plantlets of *P. stocksii*

Fig. 35: Regenerated plantlets of *P. stocksii*

Fig. 36: Regenerated callus of *P. ritcheyi*

In *P. ritcheyi,* none of the PGR combination gave any indication of embryo germination except NAA (5.37 µM) and BAP (6.66 µM) (Fig. **36**). This suggests that the auxin has no significant effect on embryo germination on BAP containing

medium in *P. stocksii*. Earlier reports also confirmed role of cytokinins in somatic embryo germination in many bamboo species. Godbole *et al*. (2002) found that BAP (11.1 µM) alone in ½ MS medium was the best for embryo differentiation into plantlets in *D. hamiltonii*. Lin *et al*. (2004) reported that somatic embryo germination had declined (28%) in the medium supplemented with NAA (5.37 M) and TDZ (0.046 M), whereas, maximum (84 %) germination was observed on MS medium with only TDZ (0.455 M) in *B. edulis*.

Godbole *et al*. (2002) reported that MS medium without phytohormones favored germination of somatic embryo in *D. hamiltonii*. Hassan and Debergh (1987) showed that hormone free MS medium favored germination of somatic embryos into plantlets in *P. vividis*. Similarly, Yeh and Chang (1986a and b) also observed that hormone-free medium proved best for embryo conversion in *B. oldhamii* and *B. beecheyana* var *beecheyana* respectively. The present study also concurs with the use of BAP for the germination of somatic embryos of *P. stocksii*.

Success of somatic embryogenesis relies on the efficient germination of the matured embryos. For *P. ritcheyi*, the hurdles were abnormal development, poor or abnormal germination and low frequency of normal conversion into plantlets. Improper embryo maturation and immobilization of the reserves that were accumulated during the maturation process were reported to be some of the reasons for this poor performance (Marion-Poll, 1997).

4.1.2.4.2. Effect of basal nutrient media on somatic embryo germination

Among different media (MS, ½ MS, ¼ MS, B5 and WPM) supplemented with BAP (2.22 µM), MS medium proved to be best in obtaining maximum germination of somatic embryos into plantlets (64.24 %). This was followed in ½ MS (42.88 %), WPM (38.54 %) and B5 (32.66 %) medium in that order. Least plantlet recovery was obtained in ¼ MS (15.31 %) medium in 4 wks. (Table **40**).

In *P. ritcheyi*, no regeneration was found, but only green patches on the surface of the calli. Some callus on MS media gave some shoot regeneration without roots and but these shoots too exhibited abnormal morphology.

Table 40: Effect of nutrient media supplemented with BAP (2.2µM) on somatic embryo germination in *P. stocksii* and *P. ritcheyi*

Nutrient media	*P. stocksii*			*P. ritcheyi*		
	Per cent regeneration	Only Shoot	Remarks	Per cent regeneration	Only shoot	Remarks
MS	97.84[a]	0.48[b]	1.68 % deterioration	0.07[b]	3.11[b]	Poor germination
MS/2	42.88[b]	3.56[a]	53.56 % deterioration	0.26[a]	0.27[a]	Poor germination
MS/4	15.31[e]	4.75[c]	79.94 % deterioration	0.53e	2.01[c]	Poor germination
B5	32.66[d]	0.93[b]	66.41 % deterioration	0.75[c]	3.61[b]	Poor germination
WPM	38.54[c]	6.23[c]	55.23 % deterioration	0.89[d]	1.27[c]	Poor germination

Mean values with same similar alphabet in columns are statistically par with each other according to DMRT. Numbers of replicates for each treatment were 3 and each replicate consisted of 5 culture bottles. Each culture bottles contains 4 callus inoculum. LSD at $\alpha = 0.05$.Data were recorded at the end of 4 wks.

MS medium was found to be best suited for germination of somatic embryos in many bamboo species. Somatic embryos developed from mature zygotic embryo exhibited 89.5 % of germination in *D. hamiltonii* on full MS medium than on ½ MS and ¾ MS (Zhang *et al*., 2010). Similar results were reported by Arya *et al*. (2008b) with 70 % germination of the somatic embryo into plantlets in *D. asper* under a 16 h photoperiod within 4 wk. period. Spontaneous germination of somatic embryos on MS media were reported in many other bamboo species such as *B. oldhamii* (Yeh and Chang, 1986a), *D. farinous* (Hu *et al*., 2011), *B. nutans* (Mehta *et al*., 2011), *B. balcooa* (Gillis *et al*., 2007), *P. nigra* (Ogita, 2005) and *D. giganteus* (Ramanayake and Wanniarachchi, 2003). On the contrary, Godbole *et al*. (2002) reported ½ MS medium was better than MS medium for embryo maturation and germination in *D. hamiltonii*. Similarly, Rout and Das (1994) also observed that ½ MS medium with Kin (2.32 µM) favored germination of somatic embryos which subsequently develop into plantlets in three bamboo species (*B. vulgaris*, *D. giganteus* and *D. strictus*). On the other hand, Woods *et al*. (1992) observed more than 95 % conversion of somatic embryos into shoots and roots on MS medium from zygotic embryo of *Otatea acuminate aztecorum.*

From the above discussion it can be concluded that nutrient requirement for embryo germination is also species dependent. During somatic embryogenesis in *P. stocksii*, most of somatic embryos converted into normal plantlets (shoots with intact roots), and a very few gave shoots alone. In *P. ritcheyi,* this abnormal somatic embryo

conversion may be due to unsynchronized maturation and development of somatic embryos. Shoots regenerated from somatic embryos did not elongate beyond 1 cm and did not develop any root at all. Other than the confirmation that shoot meristems are formed in the culture it offers little to suggest that a viable propagation protocol can be developed. The future course of action to improve upon these results would be to improve the embryogenic competence of the callus like visual characterization of the embryogenic regions, synchronous development of embryos, proper maturation under a precise regime of PGRs and other well known factors that control maturation and conversion to plantlets.

4.1.3. Hardening and acclimatization

The regenerated plantlets, raised through axillary shoot proliferation and somatic embryogenesis were hardened in polybags with sand and soil (1:1 v/v for 4 wks. before keeping in open nursery (Fig. **37, 38, 39** & **40**). New root and shoot growth was noticed within 3 wks. during hardening in green house. Within 6 m. these *in vitro* regenerated plants developed miniature rhizomes.

Table 41: Growth performance of *in vitro* regenerated plants of ***P. stocksii*** through axillary shoot proliferation and somatic embryogenesis

***In vitro* regenerated plants**	**Survival (%)**	**Number of tillers**	**Plant height (cm)**
Plantlets raised through axillary shoot proliferation	96.56 ± 0.09^{a}	3.19^{b}	7.73^{a}
Plantlets raised through somatic embryogenesis	97.91 ± 1.12^{a}	3.62^{a}	7.64^{a}

Assessment after primary hardening (one mon.) in green house.
Mean values with same similar alphabet in columns are statistically par with each other according to DMRT.

No significant differences in survival percentage between axillary shoot proliferated and plantlets derived through somatic embryogenesis during primary hardening was seen (Table **41**) but a higher number of tillers were produced in somatic embryo derived plantlets.

Success of micropropagation is evaluated based on the survival rate of hardened plants in nursery and field and cost effectiveness of the process. The tissue cultured plants are often characterized by abnormal leaf morphology, anatomy, poor photosynthetic efficiency (Donnelly and Vidaver, 1948), stomatal dysfunction and

Fig. 37: Hardened plants of *P. stocksii* derived through indirect somatic embryogenesis

Fig. 38: Hardened plants of *P. stocksii* (from axillary bud proliferation)

Fig.39: Hardened plants of *P. stocksii* derived through somatic embryogenesis in open nursery

Fig.40: One year old plant of *P. stocksii* (derived through somatic embryogenesis) in the field

scanty epicuticular layer (Ziv and Chen, 2008). The heterotrophic mode of nutrition and poor mechanism to control water loss, render micropropagated plants vulnerable to transplantation shocks. Acclimatization conditions have to be standardized for each species and the procedure continues to be a major bottle neck in the micropropagation of many plants (Conner and Thomas, 1981; Ziv, 1986; Chandra *et al*., 2010). Among the *in vitro* regenerated plants, high percentage of survival was observed in axillary shoot proliferated plants as well as in somatic embryogenesis developed plants in *P. stocksii*. The high survival rate of *in vitro* regenerated plants through somatic embryogenesis of *P. stocksii* can be attributed to the higher number of tillers, higher number of roots and higher root length per shoot clump.

Negi and Saxena (2011) reported the high rate (96 %) of survival of *in vitro* raised plantlets through axillary shoot proliferation in *B. nutans* during hardening and acclimatization stage in nursery. The same species in a different study gave 90 % survival in somatic embryo-derived plantlets (Mehta *et al*., 2011). Only 60-70 % of survival was observed during acclimatization phase of *A. callosa* plantlets propagated through axillary shoot proliferation from mature plants (Devi and Sharma, 2009). Sanjaya *et al*. (2005) reported 92 % success rate during hardening and acclimatization stage of the plants raised through axillary shoot proliferation in *P. stocksii*. Plants of *D. hamiltonii* regenerated through axillary shoot proliferation showed 85 % survival during hardening under greenhouse condition (Agnihotri *et al*., 2009). Godbole *et al*. (2002) obtained survival in field of 78 % of plantlets derived through somatic embryogenesis in *D. hamiltonii*.

Based on protocol developed, about 2000 plants were produced through somatic embryogenesis and 500 plants through axillary shoot proliferation in *P. stocksii*. At six months, these micropropagated plants did not show any apparent morphological or phenotypic variations, which suggested suitability of the protocol for large-scale production of this species. However, it is prudent to carry out a genetic fidelity test with a suitable molecular marker to assess the risk of variability before the plants derived through somatic embryogenesis via callus is adopted for a large-scale propagation programme.

4.2. CONTROL OF ENDOPHYTIC CONTAMINATION

4.2.1. Isolation and identification of endophytic contaminants

A total of 11 isolates were identified as frequently appearing latent contaminants in shoot cultures of bamboo. These were confirmed to be endophytes and include six bacteria and five fungi (Table **42**). Through biochemical tests of these 11 endophytes, two were confirmed as species of *Bacillus* and two of *Aspergillus* genus.

The highest incidence of fungal contamination was in the monsoon season (68.19 %) followed by winter (51.24 %) and summer (14.32 %). The isolation rates of endophytic fungi that caused contamination in different stages of *in vitro* cultures of three species of bamboo were also higher in monsoon season (84.56 %) than in summer (24.12 %) (Table **42**). Similarly, contamination induced by endophytic bacteria was highest in monsoon and the minimum in summer. Grass endophytes usually colonize internal tissues of aerial parts of host plants by forming a systemic and perennial association with their hosts (Leuchtmann, 1992).

Table 42: List of different endophytic microorganisms causing contamination in bamboo shoot tissue cultures in different season

		Season		
		February-May	**June-September**	**October- January**
B. balcooa	**Bacteria**	*Bacillus subtilis* *Staphylococcus aureus*	*B. cereus* *B. subtilis* *Corynebacterium sp* *Pseudomonas flourescens*	*B. subtilis* *P. flourescens*
	Fungi	*Fusarium oxysporum* *Cladosporium acalyphae* *Saccharomyces sp*	*Aspergillus fumigates* *A. niger* *F. oxysporum* *F. proliferatum* *Alternaria tenius*	*F. oxysporum* *Rhizopus nigricans* *Cladosporium cladosporioides*
D. stocksii	**Bacteria**	*B. subtilis* *S. aureus*	*B. subtilis* *P. flourescens* *S. aureus*	*B. subtilis* *P. flourescens*
	Fungi	*F. oxysporum* *C. acalyphae*	*F. oxysporum* *Alternaria alternata* *Penillium sp*	*F. oxysporum* *Alternaria alternata* *Penillium sp.*
P. ritcheyi	**Bacteria**	*B. subtilis* *Alcaligenes faecalis*	*B. subtilis* *Alcaligenes faecalis* *P. flourescens* *S. aureus*	*B. subtilis* *S. aureus* *Moraxella sp.* *Brachybacterium sp.*
	Fungi	*F. oxysporum*	*F. oxysporum* *Alternaria alternata*	*F. oxysporum* *Colletotrichum tropicale*

Among the 73 endophytic bacterial strains isolated from three bamboo species, only six species were found to result in latent contamination. Out of these, *Bacillus subtilis* and *Fusarium oxysporum* played the major role throughout the year irrespective of bamboo species.The diversity of endophytic population depends on various factors such as host, climatic factors, geographical regions, etc. Mother plants selected for the two bamboo species *P. ritcheyi* and *P. stocksii* were growing in the same location but the endophytes isolated from these two species exhibited great variation. Zhou and Hyde (2001) defined the distribution of endophytes as having host specificity, host selectivity or host preferences. Host specificity was exemplified through the mangrove saprobes, *Hypophloeda rhizophorae* and *Rhizophila marina*, which have been found only in *Rhizophora* species and none of the other mangrove species (Hyde and Lee, 1995).

But some endophytes exhibit a wide host range among taxonomically unrelated plants and Kandel *et al*. (2017) provided an example of broad host range and cross-species colonization of endophytes from the woody dicots, poplar and willow poplar (Salicaceae) with the monocot grass, maize. Here in all the three bamboo species studies, the bacterial species *B. subtilis* was distributed and found throughout the year. Distribution of endophytes found throughout the organs and tissues of plants and its diversity and abundance varying among the plant organs such as leaf and root (Porras -Alfaro and Bayman, 2011; Fernandes *et al*., 2015). Chareprasert *et al*. (2006) studied the relative frequency of total endophytes in mature leaves of teak and rain tree (*Samanea saman*) and found out that the number of genera and species with colonization frequency was higher than in tender leaves and their incidence in leaves increased during rainy season. This result was supported by the study in *Gingko biloba* of Thongsandee *et al.* (2012) who suggest that the distribution of endophytes was organ-specific and differed within seasons.

Photita *et al*. (2004) isolated seven endophytic fungi from wild banana (*Musa acuminata*) and tested their pathogenicity and reported that only *Deightoniella torulosa* was able to cause leaf spots on banana leaves *in vitro*. And they confirmed that these fungal pathogens may be latent in their host as endophyte long before the outbreak of disease symptoms. Muller and Krauss *et al*. (2005) reported that the

symbiosis between vertically transmitted asexual endophytic fungi and grasses is common and generally considered to be mutualistic, but the interacting environmental factors can modify the nature of the symbiosis into mutualism–parasitism transition. Schulz and Boyle (2005) reported that far from being neutral, endophyte-host interactions involved a balance of antagonisms, irrespective of the plant organ infected. Hyde and Soytong (2008) hypothesized that the endophytes become primary saprobic decomposers and this was supported by the study of Promputtha *et al*. (2007).

4.2.2. Growth promotion by endophytes in shoot cultures of *D. longispathus*

4.2.2.1. Identification, characterization and elimination of endophyte in *D. longispathus*

In all the shoot cultures of *D. longispathus*, presence of bacteria was indicated by the turbidity in the media, as early as in the third passage (25 d.). Since the contamination from the phyllosphere microflora because of inadequate surface sterilization usually appears in the first passage itself and commonly consists of bacterial and fungal contaminants, the presence of latent microbes in the cultures was suspected.

Table 43: Identification of bacteria isolated from plant tissue cultures of the bamboo, *D. longispathus*

Criteria	**Isolate**
Gram reaction	+
Acid fastness	-
Morphology of bacterial cells	**Rods**
Endospores	+
Motility	+
Urease	+
Nitrate reduction	+
Carbon source	
Citrate	+
Starch	-
Indole	-
Methyl red	-
Voges paskuer	+
Methyl red	-

Moreover, the shoot cultures showed no signs of damage and growth continued unabated. Based on cultural characteristics, the IMViC test and the comparison with the Bergey's Manual of Determinative Bacteriology, the circular, creamy and smooth colonies were identified as Gram-positive *Sporosarcina pasteruii* (*Bacillus pasteruii*) (Table **43**). Molecular methods of identification of the strain were not deemed necessary for this study since the emphasis was for developing a routine simple and rapid method of identifying the potential for *in vitro* growth promoting activity.

4.2.2.2. Antibiotic sensitivity assay for endophytes

Out of 20 antibiotics tested, *S. pasteurii* was found to be sensitive only to tobramycin, kanamycin, gentamicin and streptomycin (Table **44**) and the level of susceptibility was highest for kanamycin followed by gentamicin.

Table 44: Antibiotic sensitivity of the endophytic bacterial species *S. pasteurii* of *D. longispathus*

Antimicrobial Agent	Disc content (µg)	Zone of inhibition (Diameter in cm)
Imipenem	10	0
Ciprofloraxin	5	0
Tobramycin	10	1.4066±0.075
Moxifloraxin	5	0
Ofloxacin	5	0
Sparfloxacin	5	0
Levofloxacin	5	0
Norfloxacin	10	0
Co-Trimoxazole	25	0
Colistin	10	0
Nalidixic acid	30	0
Augmentin	30	0
Kanamycin	30	2.590±0.05
Gatifloxacin	5	0
Gentamicin	10	2.563±0.076
Amikacin	30	0
Streptomycin	25	1.5533±0.1043
Ceftriaxone	30	0
Cefopodoxime	10	0
Ticarillin	75	0

The sensitivity assay revealed that at 250µg ml^{-1}, both kanamycin and gentamicin were bactericidal when added to shoot cultures of *D. longispathus* (Table **44**). Both are broad-spectrum bactericides for Gram positive and Gram-negative bacteria (Reed

et al., 1995). Although kanamycin treatment of shoot cultures for 15 d. was found to be effective against bacterial contamination, it also caused severe damage to the expanded leaves and newly emerging shoots. Gentamicin at the safe bactericidal level of 250 µg $ml^{-1}$was therefore used in this study.

4.2.2.3. Effect of endophyte on *in vitro* growth of *D. longispathus* shoots

The beneficial role of the endophyte in maintaining steady growth and multiplication of shoot cultures was demonstrated when elimination of the endophyte with gentamicin resulted in a significant reduction in these parameters when compared to cultures with the endophyte (Table **45**). Reintroduction of the pure isolate and Spent media, improved the multiplication rate as well as the fresh weight above that of the antibiotic treated cultures but did not quite achieve that of the controls in the 21 d. of culture since multiplication rates and growth will take longer to pick up.

Table 45: Assessment of the influence of endophyte *S. pasteurii* on shoot cultures of *D. longispathus*

Treatment	Fresh weight*	Multiplication rate*
Control	3.174±0.087^{c}	5.40±0.16^{a}
Antibiotic treated	0.089±0.027^{a}	2.43±0.39^{d}
Co-culture with isolate	2.716±0.190^{b}	4.87±0.21^{c}
Addition of Spent Medium	0.935±0.001^{a}	2.69±0.06^{a}

*Consolidated increase in shoot fresh weight in gm and percentage increase in shoot number rate after 3 passages. Values in a column followed by different letters are significantly different at p>0.05 level based on mean comparison range using t -Test.

Pirtilla *et al.* (2004) reported that the mitigation of browning in callus cultures of *Pinus sylvestris* through endophytic conditioning was due to the consumption of nutrients from the media by endophytes and production of metabolites. According to Thomas (2004a and b), these results indicate that the reproducibility of tissue culture protocols rely on the presence or absence of the endophytes which are involved in various pathways in the normal growth and development of the plants. Routine use of endophytes in micropropagation can of course be recommended only after the risks it poses in a facilty is evaluated and significant benefits are clearly demonstrated.

4.2.2.4. *In vitro* screening of endophyte for IAA production

The membrane lift assay confirmed the production of detectable levels of IAA by endophyte *S. pasteruii* when grown on media containing the precursor L-Trp. Color development was visible within minutes of overlaying and increase in intensity continued for a period of 30 min. (Fig. **41**). The minimum detectable level of IAA in the test is approximately 50 pmol in a $2mm^2$ spot (Bric *et al*., 1991). Many of the microorganisms associated with plants are involved in phytohormone biosynthesis and the amount of hormone produced may vary greatly between the strains within a species, and some strains may not produce any phytohormone (Antoun *et al*., 1998). This method is therefore suitable for rapid screening of endophytes associated with plants to take advantage of their growth promoting ability for improving the efficiency of *in vitro* cultures.

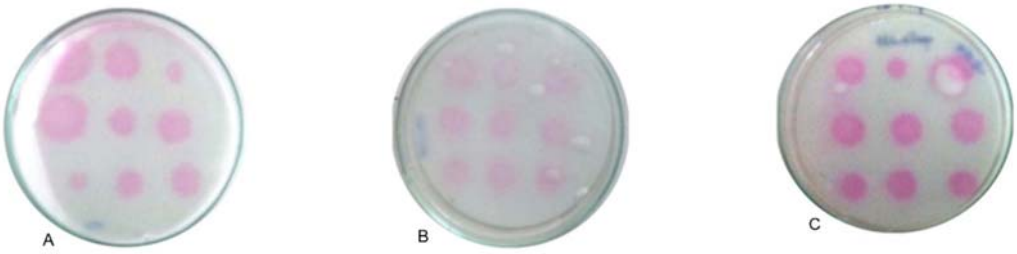

Fig 41: Color development due to the IAA production by *S. pasteruii* in different media. A: LB +Trp; B: MS+ Trp; C: Media from *D. longispathus* shoot culture

IAA quantified from filtrates of bacterial cultures in LB broth, LB Broth + L-Trp, MS media, MS + L- Trp and from shoot cultures of *D. longispathus* after 48 h incubation with bacteria. The results (Fig. **42**) demonstrated that IAA production in shoot cultures harbouring the endophyte (98.76 µg ml^{-1}) is 8 times more than that of the isolate grown in the basal tissue culture media (MS) (12.00 µg ml^{-1}) indicating the importance of the plant-microbe interaction for the growth promotion. Several reports suggest that IAA biosynthesis via the Indole-3- acetamide pathway (IAM) is widespread in the plant kingdom and that L-Trp can serve as the precursor for the

auxin (Pollmann *et al*., 2006; Mano *et al*., 2010). Higher production of IAA in the media containing L- Trp revealed that the bacterium relies on this Trp-dependent pathway for auxin production (Woodward and Bartel, 2005; Pollmann *et al*., 2006; Zhao, 2010). Due to the simplicity of the colorimetric assay, this test can be recommended as a routine method of screening of auxin-producing endophytic bacteria in plant species for which micropropagation was attempted without the need for more sophisticated quantitative methods.

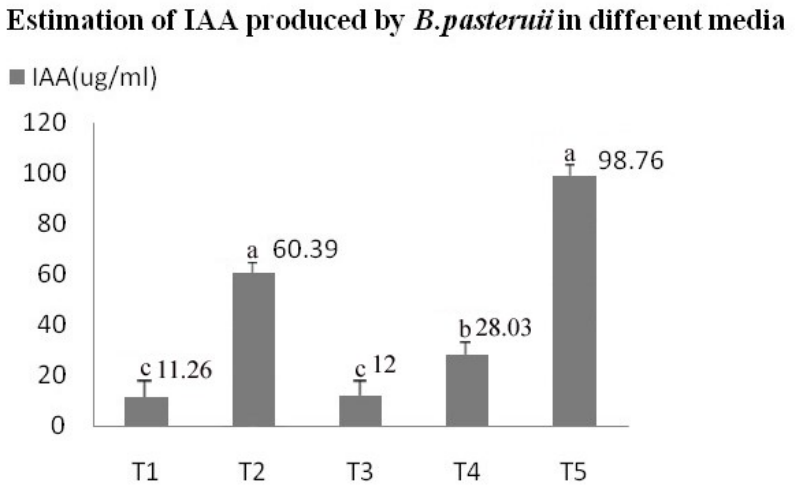

Fig. 42: Spectrophotometric analysis of IAA production by endophytic *S. pasteruii* in different media T1: LB + Endophyte; T2: LB + L-Trp + Endophyte; T3: MS + Endophyte; T4: MS + L-Trp + Endophyte; T5: Filtrate from shoot culture with endophyte

Accurate identification and quantification of the metabolites of the endophyte would require more sophisticated, cost and time-consuming methods of detection like HPLC and mass spectrometry. However, as a simple routine screening procedure for detecting IAA producing endophytes with growth promoting activity for plants in general, the lift membrane assay can be advantageous for those attempting to develop protocols for micropropagation.

4.2.2.5. Assessment of growth promotion by endophyte and Spent Medium in shoot cultures of three non- host bamboo species

The pure culture of the endophyte when inoculated into shoot cultures of the three non-host bamboo species did not show the expected effect of growth enhancement as

was seen in host species (Table **46** & **47**). The bacteria instead caused a contaminated culture where the multiplication of the shoot culture was suppressed and damage to the shoots, typical of contaminated cultures, was seen within 3 d of inoculation. This confirmed the host specificity of the endophyte and ruled out its use as a beneficial organism for *in vitro* culture of other species.

Addition of Spent Media as a supplement did not produce a significant increase in growth or multiplication rate in any of the three bamboo shoot cultures (Table **46** & **47**). Although the presence of IAA in the filtrate has been established in this case, the benefit is limited since no further auxin production is possible in the absence of the endophyte and the auxin present is soon used up. The possibility of the filtrate containing other metabolites those are not conducive to growth and multiplication of bamboo cultures cannot be ruled out.

Table 46: Assessment of growth enhancement in shoot cultures of three non-host bamboo species treated with Spent Medium and bacterial isolate

Treatment	***B. balcooa****	***D. strictus****	***P. ritcheyi****
Control	0.964 ± 0.049^{b}	0.353 ± 0.086^{b}	0.367 ± 0.237^{a}
Co-culture with isolate	0	0	0
Addition of Spent Medium	0.716 ± 0.273^{a}	0.456 ± 0.261^{b}	0.381 ± 0.331^{a}

*Increase in fresh weight of shoots in gm measured after 3 passages. Values in a column followed by different letters are significantly different at $p>0.05$ level based on mean comparison range using t-test

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Table 47: Assessment of multiplication rate in shoot cultures of three non-host bamboo species treated with Spent Medium and bacterial isolate

Treatment	***B. balcooa****	***D. strictus****	***P. ritcheyi****
Control	5.23 ± 0.12^{a}	1.21 ± 0.41^{a}	2.38 ± 0.21^{b}
Co-culture with isolate	0	0	0
Addition of Spent Medium	5.54 ± 0.06^{b}	2.16 ± 0.11^{b}	2.53 ± 0.19^{a}

*Percentage increase in shoot number after 3 passages. Values in a column followed by different letters are significantly different at $p>0.05$ level based on mean comparison range using t-test.

The effect of exogenous supply of IAA on growth of shoots and multiplication is clearly seen in all three bamboo species (Table **47**). In the host species, no significant improvement in growth was seen even when the multiplication rate showed some

improvement. Although the other non-host bamboo species do show significant improvement either in shoot growth or in multiplication rates, in the presence of added IAA, the levels of auxin required for optimum growth would need to be ascertained separately as is the case when media composition is being standardized.

It is thus established that the beneficial effect of the endophyte in shoot cultures of *D. longispathus* is not surpassed by the supplements of Spent Medium or exogenous auxin (Table **48**), indicating the uniqueness and importance of host–endophyte interaction in producing the effect. The effectiveness of endophyte as growth promoting agents, besides host specificity, depends on factors like population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance to biotic stresses (Kandel *et al*., 2017). It is to be assumed that the endophyte in *D. longispathus* meets one or more of these requirements.

Table 48: Assessment of growth in shoot cultures treated with exogenous IAA in four bamboo species

Species	Fresh weight		Multiplication rate	
	Control*	IAA(5.71 µM)*	Control*	IAA(5.71 µM)*
D. longispathus	0.8314±0.0028[a]	0.8569±0.1239[b]	2.61±0.22[c]	2.65±0.18[b]
B. balcooa	0.964±0.049[b]	1.1911±0.09[a]	5.23±0.12[a]	6.14±0.01[b]
D. strictus	0.353±0.086[b]	0.6458± .0014[b]	1.21±0.41[a]	2.11±0.08[b]
P. ritcheyi	0.367±0.237[a]	0.6782± 0.0224[b]	2.38±0.21[b]	3.09±0.16[a]

*Consolidated increase in shoot fresh weight in gm and percentage increase in shoot number after 3 passages. Values followed by different letters in a row for the same parameter are significantly different at p>0.05 level based on mean comparison range using t -Test.

CAS blue agar assay (Fig. **43**) confirmed that the endophytic bacteria produce siderophores. Schwyn and Neilands (1987) reported that siderophores could remove the iron molecules to develop a typical colored zone of yellow to orange in the media. This biochemical property is important as a plant growth-promoting trait as well as for microbial antagonism shown by the isolate. In addition to inhibiting the growth of pathogenic microorganisms, siderophores indirectly stimulate the growth

of plant (Glick, 2012). Gururani *et al*. (2012) reported that siderophore producing bacteria significantly influence the uptake of various metals, including Fe, Zn, and Cu by plants.

The formation of inhibition zones after 7 d. incubation (Fig. **44**) confirmed the biocontrol potential of *S. pasteruii*. The dual culture *in vitro* test conducted with *F. oxysporum* (an ascomycete with a chitin–glucan containing cell wall and a common fungal contaminant of plant tissue cultures), demonstrated that the siderophore producing endophyte from *D. longispathus* can control the emergence of other contaminating organisms. Antagonistic effects of *Bacillus* isolates against various soil borne fungi viz. *F. oxysporum, P. capsici, R. solani, S. sclerotiorum* analysed through the dual culture tests by Kim *et al*. (2008) showed that the degree of inhibition varied depending on strains and their siderophore producing trait. Wahyudi *et al*. (2011) reported that endophytes can activate biosynthetic pathways of various antimicrobial compounds in host plants which inhibit different pathogenic organisms as well as induced host resistance against various stress factors. The isolate from *D. longispathus* showing high siderophore producing activity can thus be further studied for its ability to confer disease resistance in host plants.

Fig 43: Siderophore production on CAS blue agar by the endophyte, *S. pasteruii* antagonistic effect of endophytic bacteria *in vitro*

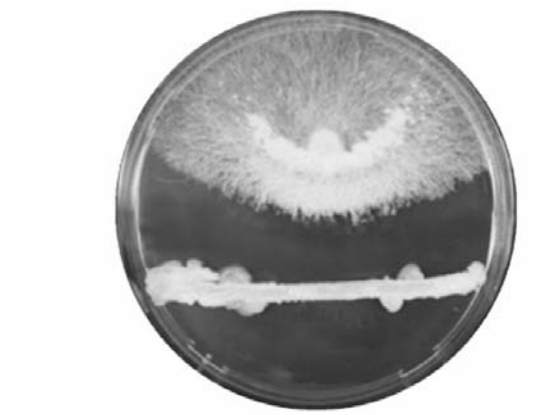

Fig 44: Siderophore production on CAS blue agar by the endophyte, *S. pasteruii* antagonistic effect of endophytic bacteria *in vitro*

4.2.3. Control of endophytes with antimicrobial food preservatives

4.2.3.1. Lactic Acid

The axillary buds excised from sterilized secondary branches of *B. balcooa*, *P. stocksii* and *P. ritcheyi* were inoculated on MS medium supplemented with 12 µM BAP and different concentrations of lactic acid (LA) at different pH levels for 3 min. to study the effect of LA on controlling the contamination at various stages of tissue culture. The explants derived from three species exhibited variation in incidence of contamination. Inoculation of explants with normal surface sterilization exhibited contamination of range 8.64 to 9.76 % at initial stage (Fig. **45**, **46** & **47**). 0.1 % reduced microbial growth during culture initiation between 3.54-3.94 % at pH 5.6. The effectiveness of LA was influenced by concentration. Maximum control over exogenous contamination (*P. stocksii*: 0.79 %) and endophytic contamination (*P. ritcheyi*: 1.5 %) occurred in 0.5 % of LA. Exposure of explants and shoot cultures to the higher concentration (0.5 %) adversely effected the bud break of axillary buds and caused tissue damage to the new sprouts in shoot cultures and hence could not be recommended.

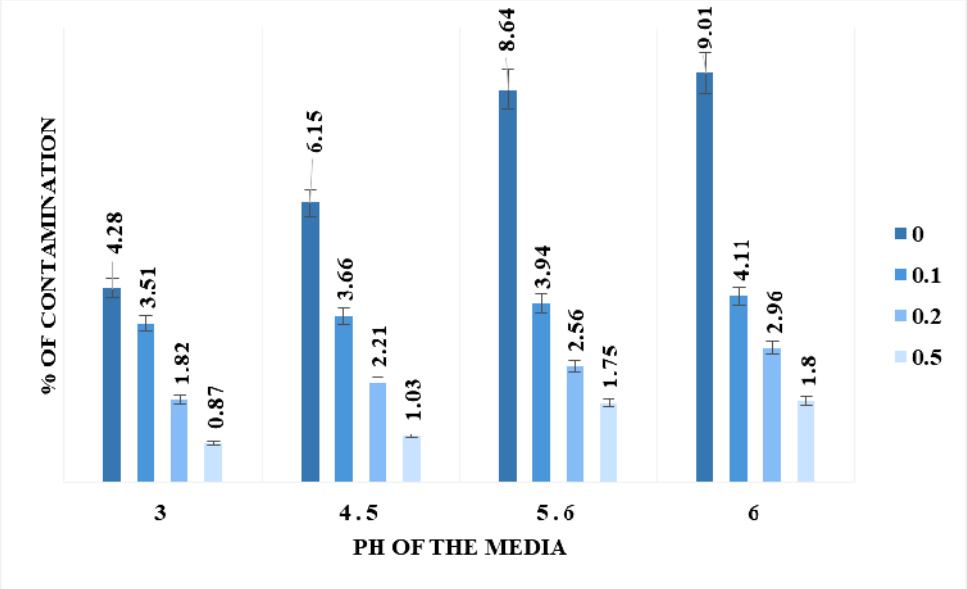

Fig 45: Effect of Lactic Acid on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) in 10 d. of inoculation.

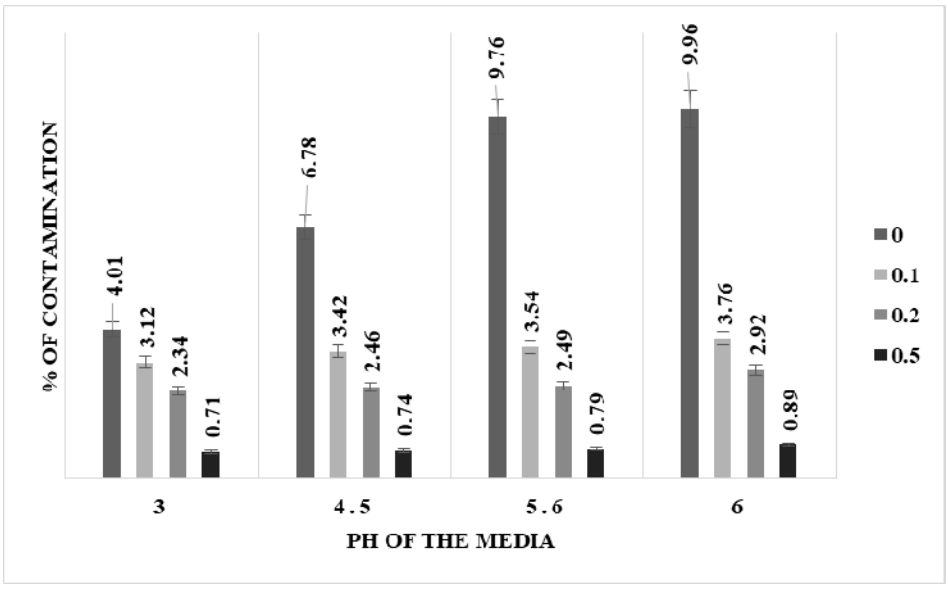

Fig 46: Effect of Lactic Acid on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi) within 10 d. of inoculation

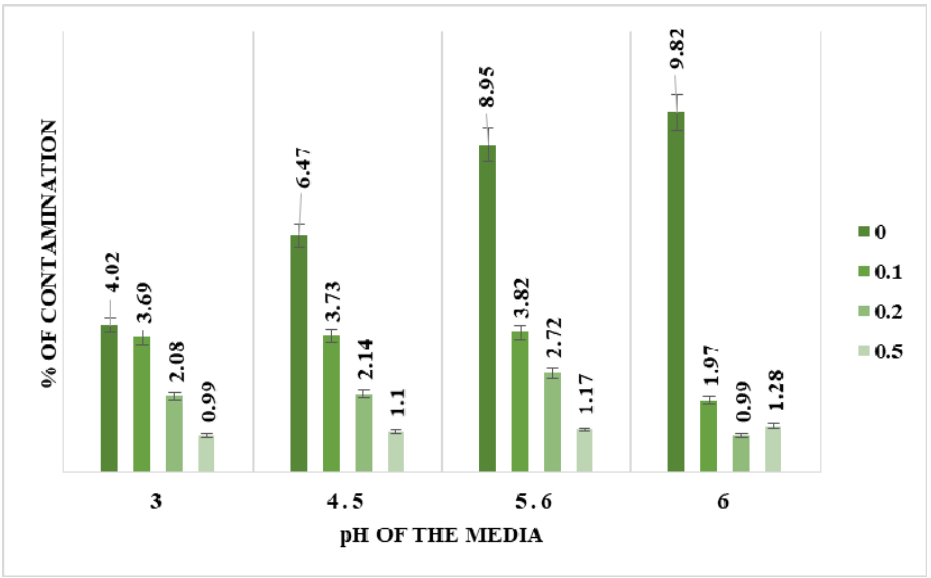

Fig 47: Effect of Lactic Acid on exogenous contamination in *P. ritcheyi*. Data represent the percentage of contamination (both bacteria and fungi) within 10 d. of inoculation

The antibacterial action of LA is assigned to the membrane disintegrating property and it was proven by the lipopolysaccharide release from treated organisms and their uptaking of 1-N-phenylnaphthylamine (NPN) hydrophobic probe (Alakomi *et al.,*

2000). Ray and Sandine (1992) reported that the undissociated form of LA could penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force. This study showed that the antimicrobial property of LA is not only due to its acidification, but also through the membrane disintegration as well as disruption of proton motive force and inhibition of metabolic activities. The same mechanism affected the fungal growth also (Hassan *et al.,* 2015). Higgins and Brinkhaus (1999) reported that the growth and morphology of fungi are influenced by the pH of media.

LA at 0.1 % was sufficient to inhibit latent contamination in all three (Table **49**).

Table 49: Effect of Lactic acid on controlling latent contamination bamboo shoot cultures.

LA %	pH	***B. balcooa***	***P. stocksii***	***P. ritcheyi***
0.0	3	12.4± 0.30 ^{c}	19.8± 0.15^{c}	19.4± 1.16^{c}
	4.5	16.2 ± 2.28 ^{c}	14.5 ± 1.62^{c}	12.5 ± 0.12^{c}
	5.6	56.21± 1.64^{f}	52.46± 0.01^{f}	53.5± 1.94^{f}
	6	54.6 ± 1.21^{f}	57.1 ± 0.41^{f}	57.1 ± 2.01^{f}
0.1	3	11.2± 1.87 ^{c}	10.7± 1.13^{c}	10.9± 0.86^{c}
	4.5	11.4 ± 0.70 ^{c}	11.1 ± 0.72^{c}	11.3 ± 1.05^{c}
	5.6	11.8 ± 1.31 ^{c}	11.8 ± 0.40^{c}	12.2 ± 1.04^{c}
	6	13.1 ± 1.56 ^{c}	12.2 ± 0.71^{c}	12.9± 0.02^{c}
0.2	3	8.7± 1.12 ^{b}	7.9± 0.42 ^{b}	7.2± 1.27^{b}
	4.5	8.7 ± 0.76 ^{b}	7.6 ± 1.81 ^{b}	7.2± 1.08 ^{b}
	5.6	8.6 ± 1.07 ^{b}	7.2± 0.45 ^{b}	7.1 ± 0.91 ^{b}
	6	8.2 ± 0.96 ^{b}	7.1 ± 1.02 ^{b}	7.1 ± 0.06 ^{b}
0.5	3	2.9± 1.16 ^{a}	1.9± 1.03^{a}	1.9± 1.86 ^{a}
	4.5	2.8 ± 1.79 ^{a}	1.8 ± 0.70^{a}	1.8 ± 0.10 ^{a}
	5.6	2.8 ± 0.81 ^{a}	1.7 ± 1.01^{ab}	1.5 ± 0.31 ^{a}
	6	2.2 ± 1.02 ^{a}	1.7 ± 0.06 ^{a}	1.1 ± 1.02 ^{a}

Data represent the percentage of contamination (both bacteria and fungi). Data taken after 100 d of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

Incorporation of LA into culture media reduced the pH to 2.5-2.9. In food products, lactic acid prevents microbial growth through the pH regulation. Acidification of culture media at pH 3 reduced the emergence of contamination in three tested species (between 4.01 % and 4.28 % at the intial stage and 12.4 % and 19.4 % in later stages) (Fig. **45, 46 & 47** and Table **49**). MS media amended with LA at different

concentrations effectively reduced the exogenous contamination to as low as 3.12 % (at 0.1 % LA in *P. stocksii*) to 0.71 % (at 0.5 % LA in *P. stocksii*). Incorporation of LA (0.1% at pH 3 and 4.5) into tissue culture media throughout the culture period greatly reduced the incidence of endophytic contamination.This result is supported by the studies of Parischa *et al.* (1979) who reported that lactic acid retained its antimicrobial property even after the pH of the peptone water was brought back to 7.3.

4.2.3.2. Acetic Acid

Addition of acetic acid (AA) into plant tissue culture media reduced the incidence of contamination depending on its concentration and pH. Routine surface sterilization (control) cause initial contamination of ~10 % in 3 species and ~56 % of latent contamination, which occurred within 80 d. of culture. Addition of AA at lower concentration (0.2 %) at three pH levels significantly reduced the incidence of latent contamination (≤ 18.02 %) (Table **50**) indicating that the effectiveness of AA depended on the pH. At 0.4 % and pH 4.5, AA reduced the contamination effectively (Fig. **48, 49** & **50**).

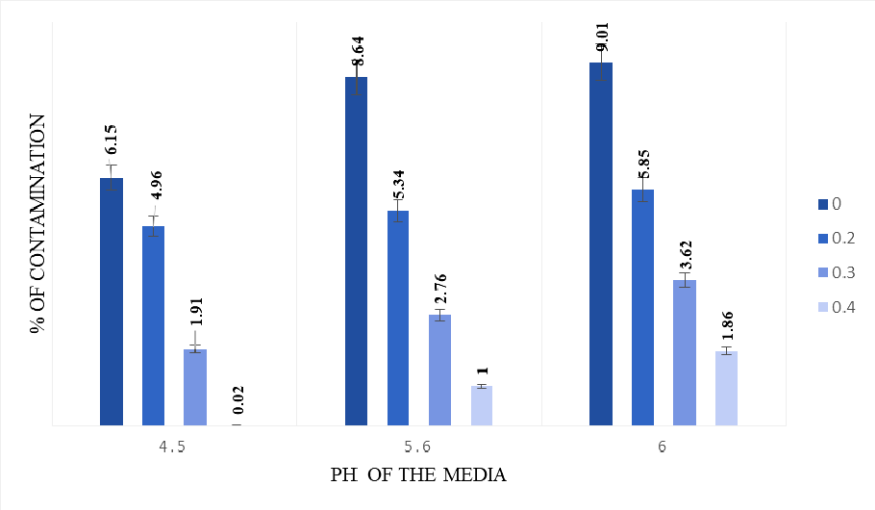

Fig 48: Effect of Acetic Acid on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) in ten d. of inoculation

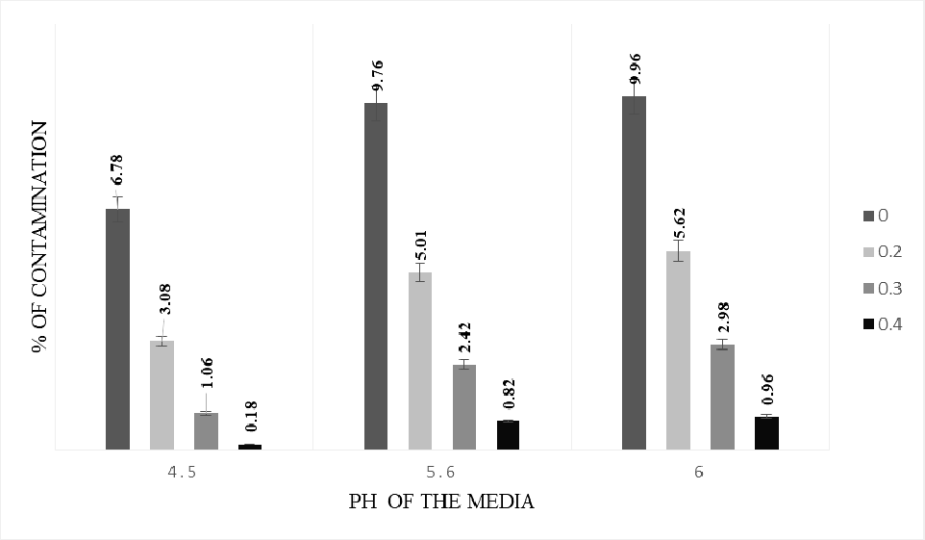

Fig 49: Effect of Acetic Acid on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi) and taken within ten days of inoculation

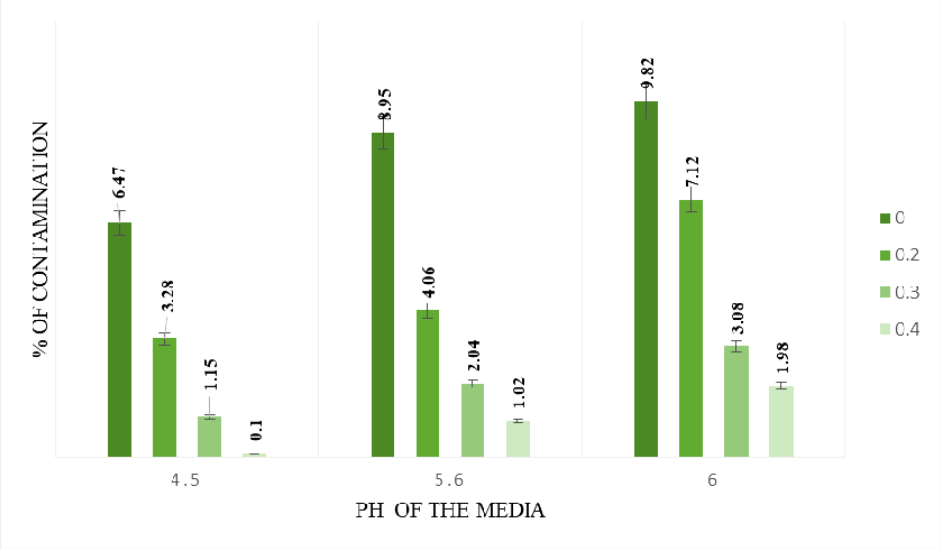

Fig 50: Effect of Acetic Acid on exogenous contamination in *P. ritcheyi*. Data represent the percentage of contamination (both bacteria and fungi) and taken within ten days of inoculation

McDonald *et al.* (1990) had reported that undissociated AA could alter the internal pH of the microorganisms. In addition, it was found that growth of *Leuconostoc mesenteroides* ceased when the internal pH of the cells reached from 5.4 to 5.7, whereas growth stopped in *Lactobacillus plantarum* at pH 4.6 to 4.8 by the addition of 160 mM sodium salt of AA, which reduces the external pH to 3.0. The inhibitory

concentration and effective pH of AA varies with the microbial species. Species-specific antimicrobial activity of AA at particular pH was proven by Kirby *et al.* (1937) against black bread molds, *A.niger* and *R.nigrificans* at pH 5.8 (0.4 %) and pH 5.0 (0.2 %) respectively. This supported by Levine and Fellers (1939) and proven that the lower concentrations of AA than 160 mM needed to inhibit *S. cerevisiae* (pH 3.9) and *A. niger* (pH 4.1) and *B. cereus* (pH 4.9).

Table 50: Effect of Acetic acid on controlling the latent contamination* in bamboo shoot cultures.

A.A. Con. (%).	pH	Species		
		B. balcooa	***P. stocksii***	***P. ritcheyi***
0.0	4.5	16.2 ± 2.28 ^{gh}	14.5±1.62 ^{bc}	12.5 ± 0.12 ^{g}
	5.6	56.21± 1.64 ^{J}	52.46±0.01 ^{bc}	53.5± 1.94 ^{J}
	6	54.6 ± 1.21 ^{J}	57.1±0.41 ^{bc}	57.1 ± 2.01 ^{J}
0.2	4.5	10.01 ± 0.00 ^{g}	6.71±0.02 ^{bc}	7.66 ±0.02 ^{bc}
	5.6	14.28 ± 1.01 ^{g}	11.28 ±0.01 ^{bc}	10.42±0.01 ^{g}
	6	18.01 ± 0.16 ^{gh}	17.02±0.26 ^{g}	18.02±0.00 ^{g}
0.3	4.5	2.02 ± 0.06 ^{bc}	2.0±2.00 ^{bc}	2.28±0.01 ^{bc}
	5.6	4.01 ± 1.09 ^{bc}	3.29±0.04 ^{bc}	4.16± 0.62 ^{bc}
	6	4.27 ± 0.12 ^{bc}	4.16±0.01 ^{bc}	6.51 ± 0.01 ^{g}
0.4	4.5	0.04± 0.09 ^{a}	0.08 ±0.10 ^{a}	0.03 ± 0.06 ^{a}
	5.6	0.82 ± 0.26 ^{a}	0.78±1.46 ^{a}	0.98 ± 2.71 ^{a}
	6	1.21 ±0.02 ^{ab}	1.29±0.04 ^{bc}	1.19± 0.02 ^{ab}

Data represent the percentage of contamination (both bacteria and fungi).*Data taken after 100 d of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

However, addition of AA, while controlling the microorganisms, negatively affected axillary bud proliferation. Amendment of AA at 0.2 % prevented bud breaking (≤ 58 %) and delayed it to 8th day in *P. ritcheyi*. Time taken for bud breaking in *B. balcooa* and *P. stocksii* were 8.5 and 10 d. AA at 0.4 % decreased the bud-sprouting rate to ≤ 22 % (when it was > 98 % in control) in three species tested. There are reports that AA at higher concentrations adversely affect plant and animal tissues. Maikinen (1958) reported that presence of lower concentration of AA (0.0001 and 0.001 M) regulated the spindle activity and retarded anaphase in the mitotic cycle of the root tips of the onion, *Allium cepa.* Manna and Mukherjee (1966) provided evidence that damage from AA resembled that of ionizing radiation or radiometric chemical

damage. Therefore, even though AA is natural product that finds use in pickles, sauerkraut and vinegar, application of AA cannot therefore be recommended in plant tissue culture as an antimicrobial agent.

4.2.3.3. Citric Acid

Citric acid (CA), one of the tricarboxylic acids with highly water-soluble property can control the pH. Gardner (1972) reported that it could act along with antioxindants to prevent the rancidity by chelating metal ions. Application of CA in plant tissue culture scenario controlled contamination in different stages of cultures irrespective of plant species. The lowest concentration added to the media (0.02 %) reduced the exogenous contamination to ≤ 2.68 % at pH 4.5 (Fig. **51, 52** & **53**). Addition of CA at the lower concentration (0.2 %) effectively reduced latent contamination at pH 4.5 (10.02 % in *P. ritcheyi*) (Table **51**). Effectiveness of CA decreased by increasing pH and the incidence of contamination raised to ≤ 4.96 % at pH 6. Similarly, pH 6 prevented the antimicrobial activity of CA on latent contamination and resulted the contamination rate at ~16 % in all three species.

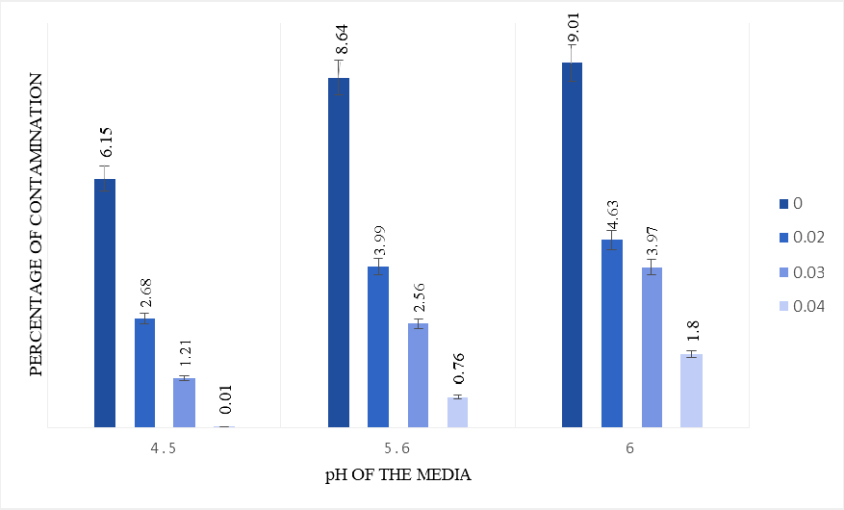

Fig 51: Effect of Citric Acid on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) and taken within ten days of inoculation.

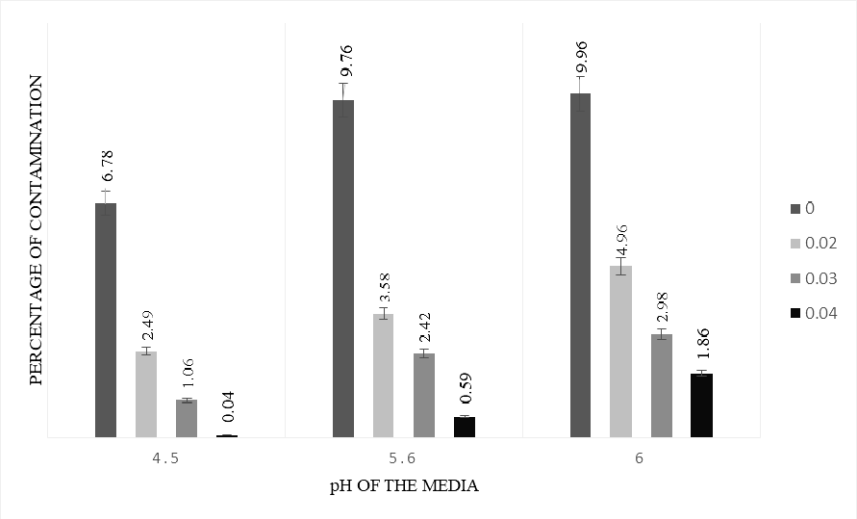

Fig 52: Effect of Citric Acid on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi).*Data taken within ten days of inoculation.

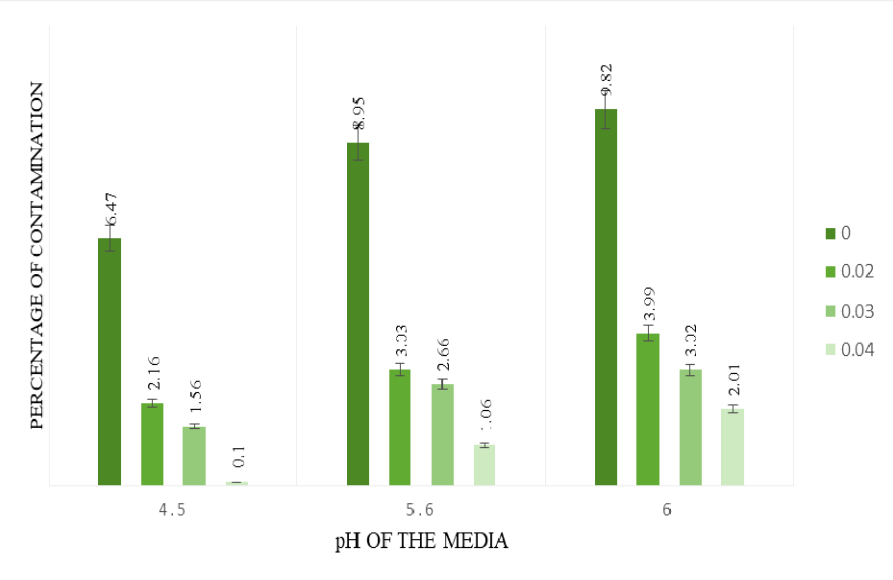

Fig 53: Effect of Citric Acid on exogenous contamination in *P. ritcheyi*. Data represent the percentage of contamination (both bacteria and fungi).*Data taken within ten days of inoculation.

Addition of CA at 0.04 % at pH 4.5 found to be most effective treatment where it reduced the contamination incidence very close to zero in all three-bamboo species.

This concentration completely eradicated the latent contamination (~0.02) at pH 4.5 from all shoot cultures tested.

Table 51: Effect of Citric acid on controlling *the latent contamination in different stages of bamboo shoot cultutures.

C.A. Con. (%).	pH	Species		
		B. balcooa	***P. stocksii***	***P. ritcheyi***
0.00	4.5	16.2 ± 2.28 [d]	14.5 ± 1.62 [d]	12.5 ± 0.12 [e]
	5.6	56.21± 1.64 [f]	52.46± 0.01 [f]	53.5± 1.94 [fg]
	6	54.6 ± 1.21 [f]	57.1 ± 0.41 [f]	57.1 ± 2.01 [fg]
0.02	4.5	10.02 ± 0.10 [e]	10.06 ± 0.02 [d]	11.01±1.02 [e]
	5.6	12.8 ± 0.24 [ab]	11.24 ± 0.10 [d]	12.61±0.09 [e]
	6	16.1±1.02 [ab]	16.28 ± 0.01 [d]	16.32±0.02 [e]
0.03	4.5	3.47 ± 0.02 [ab]	2.64 ± 0.07 [ab]	1.29± 0.06 [a]
	5.6	4.26 ± 0.07 [ab]	4.28± 0.28 [ab]	4.18 ± 0.01 [c]
	6	5.72 ± 0.06 [ab]	8.61 ± 1.16 [ab]	7.02 ± 0.03 [d]
0.04	4.5	0.02 ±0.00 [a]	0.03 ± 0.00 [a]	0.02 ± 0.10 [a]
	5.6	1.01 ± 0.01 [a]	0.26 ±0.09 [a]	0.5 ± 0.91 [a]
	6	2.05 ± 0.02 [ab]	1.67 ± 0.27 [a]	1.12 ± 0.02 [ab]

Data represent the percentage of contamination (both bacteria and fungi). *Data taken after 100 d of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

Larger dissociation constant was one of mechanisms of antimicrobial activity of citric acid. Due to the lipophilic property, CA induce hydrogen ion outflow across the membrane, resulting in decreased interior pH and inhibition of nutrient uptake. Dissociation of CA to citrate anion, an easily transpotable ion disrupt the energy yielding metabolism of cell. Addition of 0.04 % CA at pH 4.5 found to be effective for control over initial and latent contamination. At same time this concentration was did not cause any harmful effect during the course of shoot culture. Sprouting percentage of the axillary buds in all three species was same as that of control. Transferring of the shoot cultures from CA containing media to CA free shoot culture media resulted in the emergence of contamination. This expressed the biostatic properties of CA, which helped to bring down the emergence of endophytes as contaminants without causing damage shoot cultures. Therefore CA can be selected as good media additive for ensure 100 % aseptic shoot cultures in micropropagation.

4.2.3.4. Calcium Propionate

Addition of calcium propionate (CP) also showed promise for the suppression of exogenous as well as endophytic contamination. The optimum pH for use of CP found to be 6 at which, it worked efficiently in all concentrations tested. It effectively reduced the rate of contamination during explant stage below 2.15 % (Fig. **54, 55** & **56**).

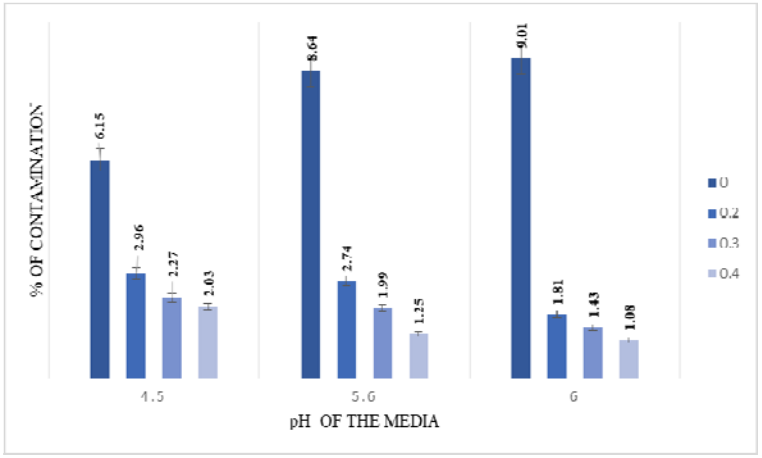

Fig 54: Effect of CP on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) and taken within 10 d after inoculation.

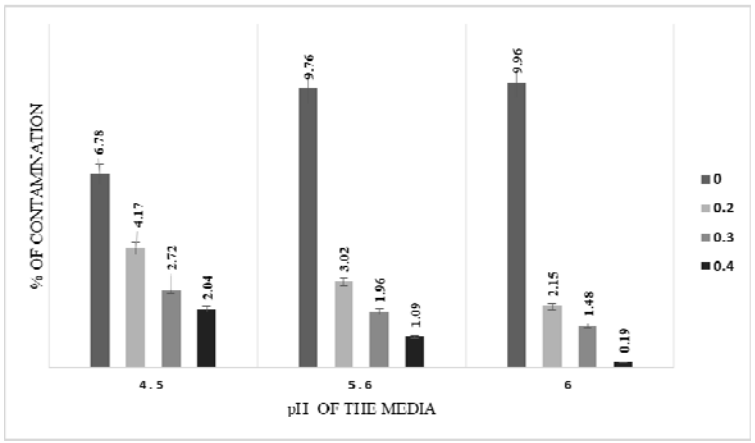

Fig 55: Effect of CP on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi) and taken within 10 d. after inoculation.

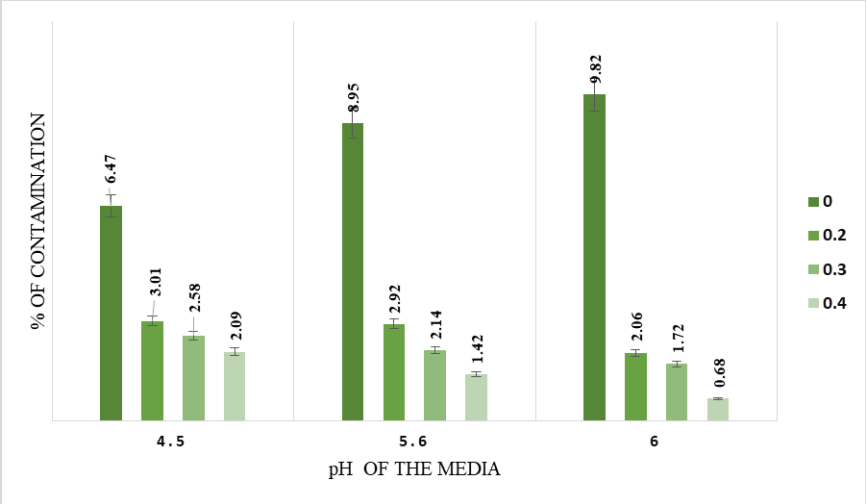

Fig 56: Effect of CP on exogenous contamination in *P. ritcheyi*. Data represent the percentage of contamination (both bacteria and fungi) and taken within 10 d after inoculation.

The higher concentration (0.04 %) of CP at pH 4.5 was not effective on exogenous contamination in *B. balcooa* as much as 0.02 % at pH 6. Similar trends observed in control of endogenous contamination also (Table **52**).

Table 52: Effect of Calcium propionate on controlling the *latent contamination in different stages of bamboo shoot cultutures.

C. P. Con. (%).	pH	Species		
		B. balcooa	***P. stocksii***	***P. ritcheyi***
0.00	4.5	16.2 ± 2.28 ^{f}	14.5 ± 1.62 ^{f}	12.5 ± 0.12 ^{f}
	5.6	56.21± 1.64 ^{gh}	52.46± 0.01 ^{gh}	53.5± 1.94 ^{gh}
	6	54.6 ± 1.21 ^{gh}	57.1 ± 0.41 ^{gh}	57.1 ± 2.01 ^{gh}
0.02	4.5	10.16± 0.02 ^{e}	12.02 ± 1.12 ^{f}	11.74 ± 0.02 ^{f}
	5.6	9.12 ± 1.01 ^{e}	8.06 ± 0.07 ^{cd}	7.02 ± 0.84 ^{f}
	6	1.01±0.01 ^{ab}	1.23±0.01 ^{ab}	1.00± 0.02 ^{a}
0.03	4.5	2.06±0.21 ^{a}	2.56 ± 1.06 ^{a}	3.28± 1.08 ^{bc}
	5.6	1.82±1.04 ^{a}	2.28± 0.42 ^{b}	2.16 ± 0.91 ^{b}
	6	1.42±0.06 ^{a}	1.73 ±1.24 ^{a}	1.38 ± 0.06 ^{ab}
0.04	4.5	1.62 ± 1.01 ^{ab}	2.00 ± 0.10 ^{b}	1.71 ± 0.19 ^{ab}
	5.6	0.2 ± 0.00 ^{ab}	1.02 ± 0.24 ^{ab}	1.04 ± 0.06 ^{ab}
	6	0.1 ± 0.02 ^{a}	0.05 ± 0.06 ^{a}	0.21 ± 0.03 ^{a}

Data represent the percentage of contamination (both bacteria and fungi).*Data taken after 100 d of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

At pH 4.5, CP 0.04 % brought down the latent contamination to 1.62 %, 2.00 % and 1.71 % in *B. balcooa, P. stocksii* and *P. ritcheyi,* respectively, but at the same time, CP at a lower level (0.02 %) could reduce the latent contamination to 1.01 %, 1.23 % and 1.00 % in *B. balcooa, P. stocksii* and *P. ritcheyi* respectively when used at pH 6. The pH of the media clearly very important to improve the efficacy of CP treatments.

However, the prolonged exposure (> 50 d.) of the plant material to the lower concentration of CP (0.02 %) itself cause undesirable effects on the new shoots. It induced shoot tip decaying in newly emerged shoots and reduced the multiplication rate of the shoot culture in three bamboo species tested. Therefore, the continuous exposure of plant material to CP is not recommended in plant tissue culture.

4.2.3.5. Potassium Sorbate

The most effective concentration of potassium sorbate (PS) was 0.2 %. At this concentration, it reduced the exogenous contamination in all three species to <1 % (Fig. **57**, **58** & **59**). It also suppressed the emergence of endophytic contamination between 0.01 to 1.24 %. Altering the pH of the media to a highly acidic range of 3 and 4.5, an increased antimicrobial potential of the compound was observed in terms of control over contamination (Table **53**).

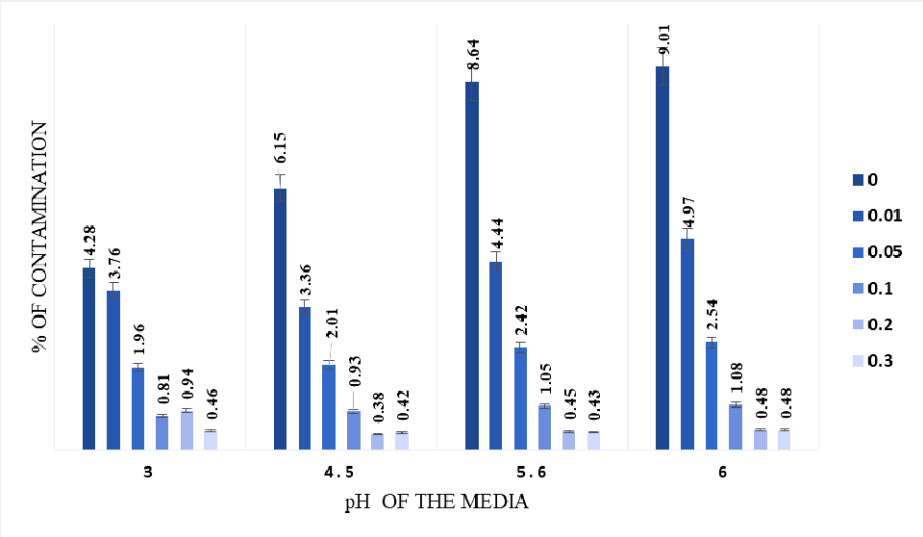

Fig 57: Effect of Potassium Sorbate (PS) on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation.

Sorbic acid (2, 4-hexadienoic acid), one of the short-chain organic acids together with their salts, have been shown to exhibit antimicrobial properties. Sorbic acid has low solubility in aqueous plant tissue culture media.

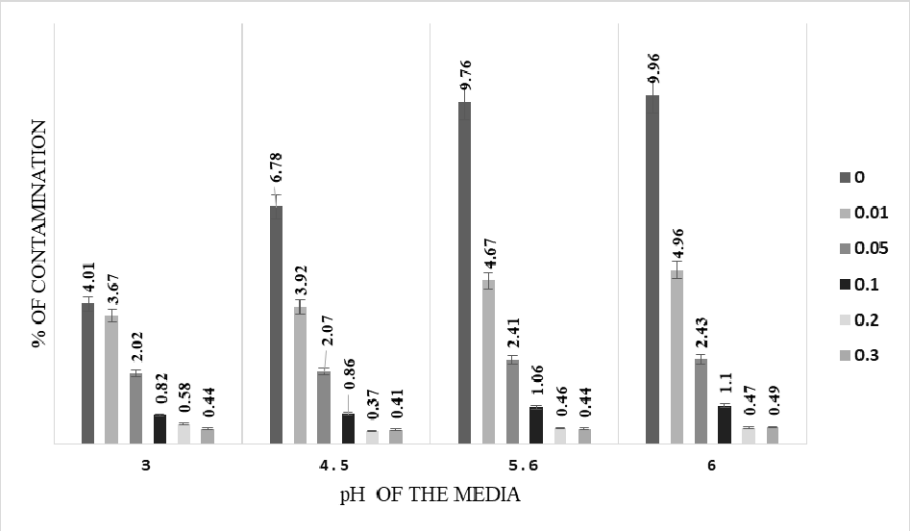

Fig 58: Effect of Potassium Sorbate on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation.

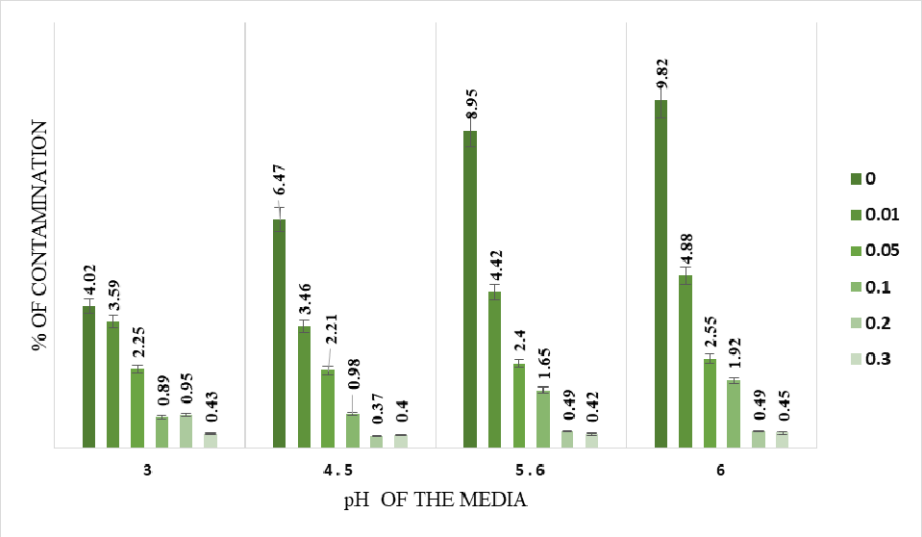

Fig 59: Effect of Potassium Sorbate on exogenous contamination in *P. ritcheyi.* Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation.

Addition of sorbic acid in MS media resulted in precipitation and was rectified with the use of potassium sorbate. There are many reports that show the influence of sorbic acid concentration and pH of the media on inhibition of growth of microbes

(Bell *et al*., 1959; Sofos *et al*., 1980). Cherrington *et al*. (1991) report that the acidic range of 3 and 4.5 retain 50 % of the acid in its effective undissoicated form.

Table 53: Effect of Potassium sorbate on controlling the latent contamination* in bamboo shoot cultutures.

P.S Con. (%)	pH	Species		
		B. balcooa	***P. stocksii***	***P. ritcheyi***
0.00	3	12.4± 0.30 ^{e}	19.8± 0.15 ^{a}	19.4± 1.16 ^{a}
	4.5	16.2 ± 2.28 ^{e}	14.5 ± 1.62 ^{e}	12.5 ± 0.12 ^{e}
	5.6	56.21± 1.64 ^{g}	52.46± 0.01 ^{g}	53.5± 1.94 ^{g}
	6	54.6 ± 1.21 ^{g}	57.1 ± 0.41 ^{cd}	57.1 ± 2.01 ^{g}
0.01	3	4.01 ± 0.01 ^{c}	4.12 ± 0.11 ^{c}	3.99 ± 0.04 ^{bc}
	4.5	3.90 ± 0.02 ^{bc}	3.86 ± 0.02 ^{bc}	3.92 ± 0.01 ^{bc}
	5.6	4.81 ± 0.02 ^{c}	4.80± 0.03 ^{a}	4.80 ± 0.01 ^{c}
	6	4.89 ± 0.04 ^{c}	4.84 ± 0.20 ^{a}	4.86 ± 0.02 ^{c}
0.05	3	1.31 ± 0.16 ^{ab}	1.61 ± 0.09 ^{ab}	1.28 ± 0.10 ^{ab}
	4.5	1.08 ± 0.22 ^{ab}	1.62 ± 0.02 ^{ab}	1.13 ± 0.02 ^{ab}
	5.6	1.91 ± 0.14 ^{ab}	2.01 ± 0.05 ^{b}	1.89 ± 0.02 ^{ab}
	6	2.01 ± 0.11 ^{b}	2.18 ± 0.01 ^{b}	2.00± 0.01 ^{ab}
0.10	3	1.12 ± 0.10 ^{ab}	1.23 ± 0.04 ^{ab}	1.21 ± 0.01 ^{ab}
	4.5	1.10 ± 0.12 ^{ab}	1.09 ± 0.01 ^{ab}	1.09± 0.02 ^{ab}
	5.6	1.22 ± 0.02 ^{ab}	1.36 ± 0.02 ^{ab}	1.20 ± 0.12 ^{ab}
	6	1.47 ± 0.12 ^{ab}	1.41 ± 0.02 ^{ab}	1.46 ± 0.01 ^{ab}
0.20	3	0.21 ± 0.09 ^{a}	1.02± 0.01 ^{ab}	0.81 ± 0.10 ^{a}
	4.5	0.10 ± 0.05 ^{a}	0.12 ± 0.03 ^{a}	0.72 ± 0.02 ^{a}
	5.6	1.01 ± 0.01 ^{a}	1.01 ± 0.11 ^{a}	0.91 ± 0.11 ^{a}
	6	1.01 ± 0.01 ^{a}	1.24 ± 0.02 ^{a}	0.99 ± 0.02 ^{a}
0.30	3	0.90 ± 0.07 ^{a}	0.85 ± 0.02 ^{a}	0.79 ± 0.40 ^{a}
	4.5	0.09 ± 0.01 ^{a}	0.04 ± 0.11 ^{a}	0.72 ± 0.01 ^{a}
	5.6	0.88 ± 0.01 ^{a}	0.86 ± 0.02 ^{a}	0.78 ± 0.01 ^{a}
	6	0.84 ± 0.09 ^{a}	0.87 ± 0.01 ^{a}	0.81 ± 0.02 ^{a}

Data represent the percentage of contamination (both bacteria and fungi).*Data taken after 100 d of culture. Means in the column with same letter were not significantly different at p> 0.05 level based on mean comparison range using t -Test.

Analysis of rate constants (k) explained the influence of pH on the dissociation and release of sorbates (Flores *et al.,* 2007) and reported that the k values were significantly higher at pH 4.5. Generally, fatty acid preservatives cause cell stasis or arrest the cells in lag phase of the cell cycle rather than killing the microbe (Stratford and Anslow, 1996). Undissociated form of this fatty acid penetrate the cell wall of the microbe, get into dissociated form under the higher cytoplasmic pH and released free H^{+}, which inhibit glycolysis pathway (Stratford and Anslow, 1996; Piper *et al*., 2001). Antimicrobial activity of these chemicals are pH linked (Chichester and Tanner, 1972).

Sorbates exerts antimicrobial activity against many bacteria and fungi (Russell, 1991). This was specifically effectiveagainst catalase positive organisms through the inactivation of fumerase resulting in the inhibition of oxidative metabolism. Implication of ATP depletion and inhibition on several sulfhydryl-containing enzymes such as aspartase, succinic dehydrogenase, yeast alcohol dehydrogenase etc. induced the antimicrobial action of this chemical, PS inactivate the dehydrogenase enzymes involved in fatty acid oxidation of fungi. ATP depletion has been reported as one of the possible reasons for the germination instability of the conidia of *A. parasiticus* in the presence of sorbate (Przybylski and Bullerman, 1980). York and Vaughn (1964) postulated that sorbic acid inhibited various enzymes involved in oxidative phosphorylation and its higher concentration prevented the amination of α-ketoglutarate. Whitaker (1959) reported that sorbic acid forms stable compounds with sulfhydryl containing enzymes through a thiohexenoic acid derivative.

4.2.3.6. Benzoic Acid

Benzoic acid (BA), one of the oldest food preservatives was successfully used as an antimicrobial agent in bamboo shoot cultures. Different concentrations tested (0.05 % - 0.5 %) effectively reduced the contamination in different stages of shoot cultures in all three the species tested. BA at pH 5.6, incidence of contamination decreased with increasing concentrations in three species tested (Fig. **60, 61** & **62**). On adjusting the pH to the highly acidic range (pH 3 and 4), BA more effectively controlled the microbial growth in shoot culture in initial as well as later stages of shoot culture. At this pH, lower concentration (0.05 %) of BA reduced the exogenous contamination to 3.23 % (*P. stocksii*) and latent contamination to 4.02 % (*P. ritcheyi*). Among the various concentration tested, 0.25 % was selected as the optimum and this concentration at pH 3 reduced the contamination level less than 0.5 % in all stages of culture (Table **54)**.

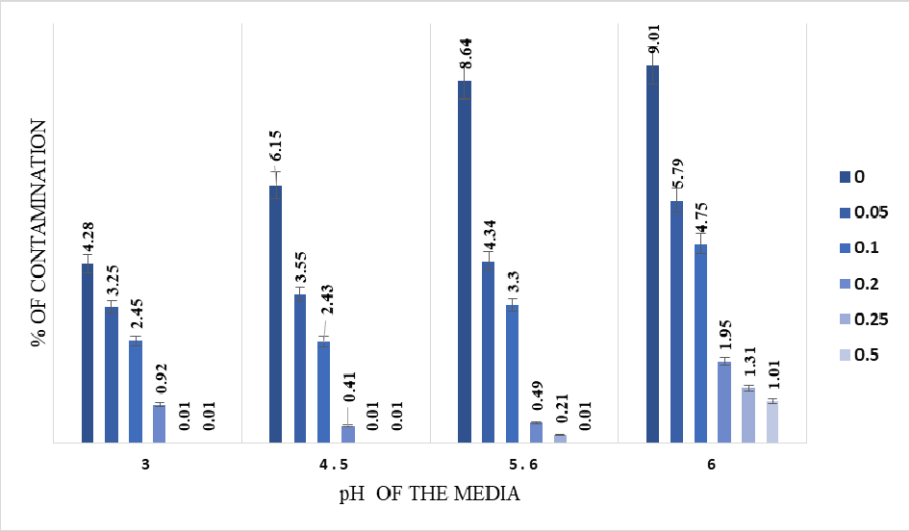

Fig 60: Effect of Benzoic Acid on exogenous contamination in *B. balcooa*. Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation

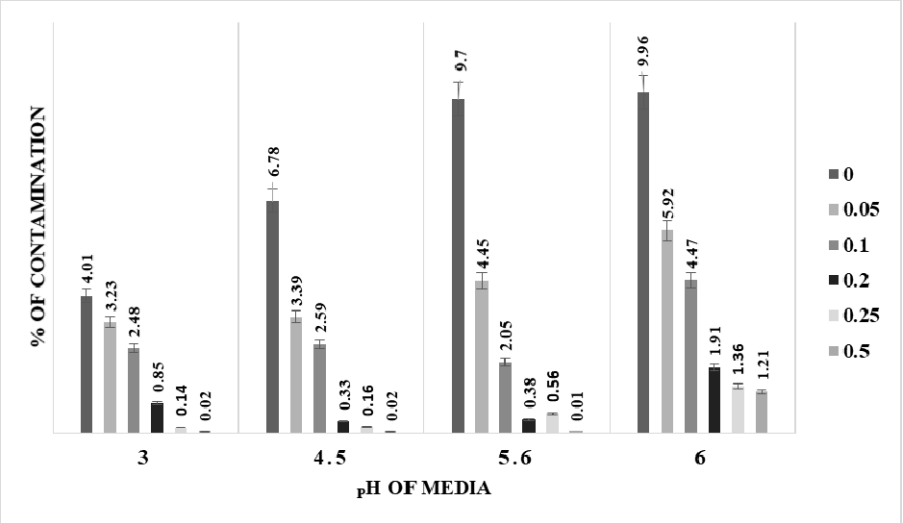

Fig 61: Effect of Benzoic Acid on exogenous contamination in *P. stocksii*. Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation.

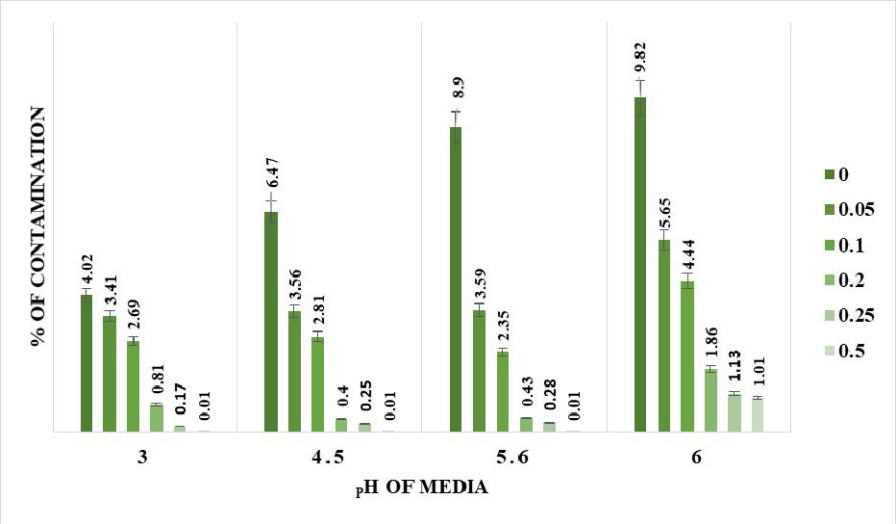

Fig 62: Effect of Benzoic Acid on exogenous contamination in *P. ritcheyi*. Data represent the percentage of contamination (both bacteria and fungi) and taken after 10 d. of inoculation.

Table 54: Effect of Benzoic acid on controlling the latent contamination* in different stages of bamboo shoot cultures.

BA Conc.%	pH	*B. balcooa*	*P. stocksii*	*P. ritcheyi*
0.00	3	12.4± 0.30 [f]	19.8± 0.15 [f]	19.4± 1.16[f]
	4.5	16.2 ± 2.28 [a]	14.5 ± 1.62 [f]	12.5 ± 0.12[f]
	5.6	56.21± 1.64 [g]	52.46± 0.01 [g]	53.5± 1.94 [g]
	6	54.6 ± 1.21 [g]	57.1 ± 0.41 [cd]	57.1 ± 2.01 [g]
0.05	3	6.32±0.05 [cd]	4.11±0.01 [cd]	4.02±0.06 [c]
	4.5	4.39±0.0 [cd]	4.26±0.02 [cd]	4.27±0.09 [c]
	5.6	5.48±0.02 [cd]	5.21±0.01 [cd]	5.35±0.12 [cd]
	6	6.76±0.03 [cd]	6.14±0.01 [cd]	6.63±0.02 [a]
0.10	3	4.32±0.04 [cd]	2.21±0.02 [b]	3.42±0.01 [bc]
	4.5	2.81±0.01 [b]	2.74±0.01 [b]	2.47±0.02 [b]
	5.6	2.86 ±0.41 [b]	2.76 ±0.2 [b]	2.53 ±0.01 [b]
	6	3.89 ± 0.01 [a]	3.71± 0.01 [a]	3.64 ± 0.12 [bc]
0.20	3	1.07 ± 0.04 [ab]	0.87 ± 0.12 [a]	1.06± 0.12 [ab]
	4.5	0.77 ± 0.27 [a]	1.30 ± 0.11[ab]	1.42 ± 0.11 [ab]
	5.6	0.49 ± 0.16 [a]	1.26 ± 0.12 [ab]	1.25 ± 0.12 [ab]
	6	1.01 ± 0.02 [ab]	1.70 ± 0.02 [ab]	2.84 ± 0.02[b]
0.25	3	0.61±0.00 [a]	0.33 ± 0.28 [a a]	0.68 ± 0.05 [a]
	4.5	0.44 ± 0.06 [a]	0.36±0.23 [a]	0.75±0.23 [a]
	5.6	0.50±0.19 [a]	0.54 ±0.47 [a]	0.81 0.26 [a]
	6	0.79 ±0.17 [a]	0.91±0.72 [a]	0.93±0.42 [a]
0.50	3	0.16±0.01 [a]	0.19 ± 0.21 [a]	0.22 ± 0.21 [a]
	4.5	0.28 ± 0.52 [a]	0.28±0.09 [a]	0.32±0.09 [a]
	5.6	0.42±0.74 [a]	0.48 ±0.11 [a]	0.69 0.14 [a]
	6	0.68 ±0.04 [a]	0.86±0.41 [a]	0.85±0.13 [a]

Data represent the percentage of contamination (both bacteria and fungi).*Data taken after 100 d. of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

According to Gabel (1921) (cited by Chipley, 2005), antimicrobial activity of BA is directly correlated with pH and it is bactericidal at acidic pH at lower concentration (0.1 %) but inactive at alkaline pH even when the concentration is high (0.2 %). Observations of Cruess and Richert (1929) support this result against fungi and yeast. The factor responsible for the antimicrobial property of BA is the undissociated form. The acidic pH stabilises the structural properties of BA and this is the reason for elevated antimicrobial action of BA at lower pH (Rahn and Conn, 1944). Cellular uptake of undissociated BA was maximum at lower pH and this enhanced the saturation level BA in microbial cytoplasm. Saturated level of BA causes a general energy loss through ATP depletion (Warth, 1991) resulted with metabolic effects of reduced glucose consumption and decline of glucose-elicited reactions within cells, followed by the prevention of glycolysis, which significantly reduced the growth rate of microbes.

No noticeable phytotoxicity was found during different shoot culture stages, instead a delayed sprouting response (~ 9 d.) was found at the higher concentration of BA in all three species at all pH range tested.

4.2.3.7. Sodium Meta bisulphite

Sodium meta bisulphite (SMB) effectively reduced the rate of contamination to ~0 % at a concentration of 5 % in all three-bamboo species. This was found as the effective concentration for reducing the latent contamination in all pH ranges tested in these three species (<0. 05 % in all species after 90 d. of culture) (Table **55**). Among these, pH 4 gave the maximum reduction of contamination in initiation as well as multiplication stages of shoot culture (0 % in all species). Davidson and Taylor (2007) reported that the optimal pH for antimicrobial activity of sulphites was below pH 4. pH 7.5 and 10 gave the same results as pH 4 on the antimicrobial action of SMB. In the present study, it was found that SMB exhibited maximum antimicrobial activity at pH 4 than at pH 3 without any harmful effect on shoot cultures. For plant tissue, culturing shoots in neutral and alkaline pH (pH 10) was not advisable (Kaiser and Hartung, 1981). Therefore, pH 4 was selected as the optimal for the control of contamination using SMB in plant tissue culture media.

Table 55: Effect of Sodium Meta Bisulphite on controlling the contamination in different stages of bamboo shoot cultutures.

S.M.B. Con. (%).	pH	Species					
		B. balcooa		***P. stocksii***		***P. ritcheyi***	
		Initial	Latent	Initial	Latent	Initial	Latent
0.00	3	4.28±2.11 ^{d}	12.4±0.30 ^{g}	4.01±1.09 ^{a}	19.8±0.15 ^{g}	4.02±1.06 ^{a}	19.4±1.16 ^{g}
	4	6.03±1.03 ^{ef}	14.8 ±2.01 ^{g}	6.32±0.12 ^{f}	12.3±0.02 ^{g}	6.32±0.01 ^{ef}	11.7±1.02 ^{g}
	5	7.12±0.19 ^{f}	27.8±0.06^{h}	7.18±0.06 ^{f}	36.4±1.01 ^{i}	7.52±0.91 ^{a}	38.6±2.03 ^{i}
	5.6	8.64±2.15 ^{f}	56.21±1.64 ^{i}	9.76±0.12 ^{f}	52.46±0.01 ^{i}	8.95±1.27 ^{a}	53.5± 1.94 ^{i}
1.0	3	3.96±0.21 ^{bc}	3.32±0.05 ^{a}	3.93±0.09^{c}	3.04±0.01 ^{bc}	3.41±0.06^{c}	3.12±0.06 ^{bc}
	4	2.48±0.13 ^{b}	2.39±0.02 ^{b}	2.46±0.18^{b}	2.21±0.02 ^{b}	2.42 ±0.24^{b}	2.27±0.09 ^{b}
	5	3.34±0.03 ^{bc}	2.48±0.02 ^{b}	2.45±0.13^{b}	2.26±0.01 ^{b}	2.59±0.10^{b}	2.35±0.12 ^{a}
	5.6	4.79±0.04 ^{c}	3.76±0.03 ^{bc}	3.99±0.22^{bc}	3.14±0.01 ^{bc}	3.65±0.16^{bc}	3.63±0.02 ^{bc}
2.0	3	3.61±0.07 ^{bc}	2.60±0.18 ^{b}	3.84±0.74 ^{bc}	2.60±0.18 ^{a}	3.08±0.14 ^{bc}	2.17±0.15 ^{b}
	4	1.81±0.10 ^{ab}	1.06±0.07 ^{ab}	2.29±0.18 ^{b}	1.20±0.10 ^{ab}	2.09±0.74 ^{a}	1.32±0.12 ^{ab}
	5	5.56±0.11 ^{d}	1.81±0.13 ^{ab}	2.31±0.12 ^{a}	1.76±0.35 ^{ab}	2.46±0.07 ^{b}	1.73±0.10 ^{ab}
	5.6	4.45±0.12 ^{cd}	2.33±0.17 ^{a}	3.87±0.60 ^{a}	2.06±0.07 ^{a}	3.79±0.18 ^{bc}	2.60±0.18 ^{a}
3.0	3	3.05±0.08 ^{bc}	1.32±0.04 ^{a}	3.08±0.09^{bc}	1.71±0.02 ^{a}	2.49±0.14^{b}	1.42±0.01 ^{ab}
	4	1.03±0.06 ^{ab}	0.81±0.01 ^{a}	1.59±0.10^{ab}	1.04±0.01 ^{ab}	1.18±0.20^{ab}	0.47±0.02 ^{a}
	5	1.30±0.04 ^{ab}	0.86 ±0.41 ^{a}	1.86±0.48^{a}	1.06 ±0.2 ^{a}	1.35±0.03^{ab}	0.53±0.01 ^{a}
	5.6	3.75±0.02 ^{bc}	1.89 ± 0.01 ^{ab}	3.04±0.18^{bc}	1.20± 0.01 ^{a}	2.64±0.07^{b}	1.64 ± 0.12 ^{ab}
4.0	3	1.92±0.11 ^{ab}	0.77 ± 0.04^{a}	1.85±0.01 ^{ab}	1.08 ± 0.12 ^{ab}	1.81±0.02 ^{ab}	0.06± 0.12 ^{a}
	4	0.01±0.03 ^{a}	0.07 ± 0.27 ^{a}	0.26±0.01 ^{a}	0.26 ± 0.11 ^{a}	0.40±0.01 ^{a}	0.25 ± 0.11 ^{a}
	5	0.09±0.04 ^{a}	0.09 ± 0.16 ^{a}	0.34±0.02 ^{a}	0.30 ± 0.12 ^{a}	0.43±0.12 ^{a}	0.42 ± 0.12 ^{a}
	5.6	1.95±0.02 ^{ab}	0.81 ± 0.02 ^{a}	1.91±0.12 ^{ab}	1.70 ± 0.02 ^{ab}	1.86±0.01 ^{ab}	0.84 ± 0.02 ^{a}
5.0	3	0.01±0.02 ^{a}	0.06±0.0 ^{a}	0.18±0.01 ^{a}	0.09 ± 0.21 ^{a}	0.06±0.10 ^{a}	0.06± 0.21 ^{a}
	4	0.00±0.01 ^{a}	0.00 ± 0.06 ^{a}	0.00±0.02 ^{a}	0.01±0.09 ^{a}	0.00±0.01 ^{a}	0.02±0.09 ^{a}
	5	0.01±0.00 ^{a}	0.01±0.19 ^{a}	0.00±0.02 ^{a}	0.04 ±0.11 ^{a}	0.01±0.01 ^{a}	0.02 ±0.14 ^{a}
	5.6	0.01±0.01 ^{a}	0.09 ±0.17 ^{a}	0.04±0.11 ^{a}	0.06±0.41 ^{a}	0.04±0.01 ^{a}	0.05±0.03 ^{a}

Data represent the percentage of contamination (both bacteria and fungi).*Data taken within 10 d of inoculation and ** after 100 d. of culture. Means in the column with same letter were not significantly different at p >0.05 level based on mean comparison range using t -Test.

SMB hydrolyses in tissue culture media to bisulfite ions and reacts with membrane protein S-S groups by interchange, freeing a SH group on one protein but leaving a thiolsulfate ester on the other protein. This ester is hydrolysed by water to give a free SH group on the protein and a sulfate ion (SO_4^{2-}) and disrupt the membrane integrity. Teresa *et al*. (2003) reported the lethal effect of SMB on germination, stomatal development, stomatal index, chlorophyll content, yield and biomass of *Vigna sinensis*. Continuous exposure of bamboo shoot cultures on to SMB also resulted in reduced multiplication of shoots as well as in biomass.

4.2.4. Control of endophytes with antimicrobial preservatives used in pharmaceuticals and cosmetics

4.2.4.1. Methyl paraben

Addition of methyl paraben (MP) into tissue culture media was found to effective in inhibiting both bacterial and fungal contamination in the three species tested. Bacterial contamination in initiation stage was reduced to 0.03 %, 0.22 % and 0.36 % in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively at 0.05 % of MP (Table **56**) from 8.59 %, 8.67 % and 9.51 %.The lowest concentration (0.01 %) tested did not influence the exogenous contamination or the latent contamination. Exogenous contamination was brought down to < 0.83 % by inoculating $HgCl_2$ treated explants in media with 0.03 % MP. This concentration efficiently reduced fungal contamination also (≤ 0.20 %). Higher concentration (0.05 %) of MP reduced the bud break (~67 %) and delayed culture initiation to 7 d. Addition of higher concentration of MP (0.03 % and 0.05 %) significantly influenced the incidence latent contamination upto 87th d. of culture period and effectively reduced the rate < 2 %.

Table 56: Effect of methyl paraben on control of contamination in *in vitro* growth response of bamboo species($HgCl_2$ treated)

	M.P %	Contamination*				Latent contamination**	
		Bacteria %	Fungi %	Sprouting rate (%)	Days taken for bud break	Contamination (%)	Day
P. ritcheyi	0	8.59± 1.16^{e}	9.17± 0.08^{f}	100±0.00^{a}	3.97±0.15^{b}	53.5± 1.94^{e}	17.67±0.33^{g}
	0.01	8.43±1.01^{d}	9.0±0.57^{f}	98.52±2.41^{ab}	4.27±1.00^{c}	50.46± 1.26^{e}	26.48±0.27^{f}
	0.02	4.24±0.60^{c}	5.76±0.66^{cd}	94.54±0.02^{b}	4.30±0.46^{c}	21.46± 0.52^{c}	32.57±0.82^{e}
	0.03	0.25±0.16^{a}	0.20±0.76^{a}	94.12±1.51^{b}	5.54±0.97^{d}	1.38± 0.18^{a}	88.02±0.35^{a}
	0.05	0.03±5.02^{a}	0.07±0.35^{a}	71.06±5.32^{d}	6.58±0.67^{e}	1.31± 1.08^{a}	88.46±0.12^{a}
B. balcooa	0	8.64±2.08^{d}	8.67±0.31^{f}	99.92±0.10^{ab}	3.98±0.22^{b}	56.21± 1.64^{e}	12.33±0.33^{gh}
	0.01	8.27±0.68^{d}	7.92±2.57^{ef}	94.51±6.31^{b}	4.18±0.42^{c}	54.62± 3.61^{e}	39.01±1.05^{e}
	0.02	4.68±5.09^{c}	3.89±0.57^{b}	93.26±0.57^{b}	4.38±0.22^{c}	27.84± 0.26^{c}	50.53±0.06^{d}
	0.03	0.83±0.14^{d}	0.04±0.02^{a}	92.01±2.52^{b}	5.58±0.11^{d}	1.09 ± 0.46^{a}	87.19±0.17^{a}
	0.05	0.22±0.41^{a}	0.02±0.53^{a}	55.46±0.87^{e}	6.94±0.17^{e}	0.07± 0.87^{a}	89.01±9.32^{a}
P. stocksii	0	9.51± 0.43^{f}	9.52± 1.90^{f}	99.84±0.16^{a}	3.28±0.82^{a}	52.46± 0.01^{e}	15.80±0.32^{gh}
	0.01	9.02±0.27^{f}	8.88±0.24^{d}	98.14±0.37^{ab}	3.46±0.79^{ab}	51.58± 0.48^{e}	33.42±2.17^{e}
	0.02	4.04±3.52^{c}	3.56±0.32^{b}	93.01±1.34^{b}	4.29±3.71^{c}	24.17± 0.98^{c}	64.45±7.91^{c}
	0.03	0.67 ± 0.81^{a}	0.62±0.73^{a}	92.58±0.07^{b}	5.64±0.24^{d}	1.92 ± 0.14^{a}	88.03±0.26^{a}
	0.05	0.36±0.09^{a}	0.52±0.02^{a}	67.93±0.27^{de}	6.98±0.77^{e}	1.48± 1.18^{a}	88.21±0.67^{a}

Data represent the percentage of contamination (both bacteria and fungi).*Data taken within ten days of inoculation and ** after 100 d. of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

Although $HgCl_2$ is the most widely used surface sterilant in micropropagation, it is an extremely toxic chemical. In attempting to find a replacement for $HgCl_2$, a comparision of the different concentrations of MP for their effectiveness in reducing exogenous contamination during culture establishment was done. Short-term treatment (10 min.) prior to inoculation with different MP concentrations had no significant outcome on eradication in contamination. By adding four different concentrations of MP to the *in vitro* culture medium, various levels of contamination control were observed in three bamboo species. These results indicated the positive effect of $HgCl_2$ treatment on explant sterilization (Table **57**). Addition of MP in culture medium (0.01 %) resulted in establishment of aseptic cultures in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively whereas 0.03 % of MP facilitated the establishment of sterile cultures 80.03 %, 81.41 % and 78.65 % in these species with bud breaking frequency 87.31 %, 84.09 % and 86.52 % respectively (Fig. **63, 64** & **65**).

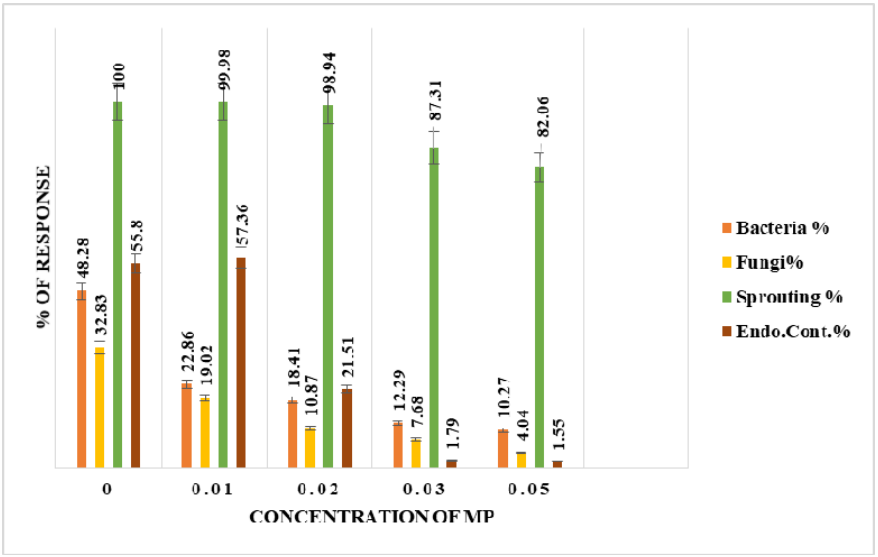

Fig 63: Effect of Methyl Paraben on control of contamination in shoot cultures of *P. ritcheyi*.

Although the treatment with $HgCl_2$ was ineffective in inhibiting endogenous contamination in all shoot cultures, use of MP made a difference. While the lower concentrations of MP (0.01 % and 0.02 %) were ineffective in controlling latent contamination, at 0.03 % it was found very effective, as contamination was brought

down to ≤ 1.92 % and delayed the incidence upto ~ 83 d. MP therefore emerges as an effective alternative for $HgCl_2$.

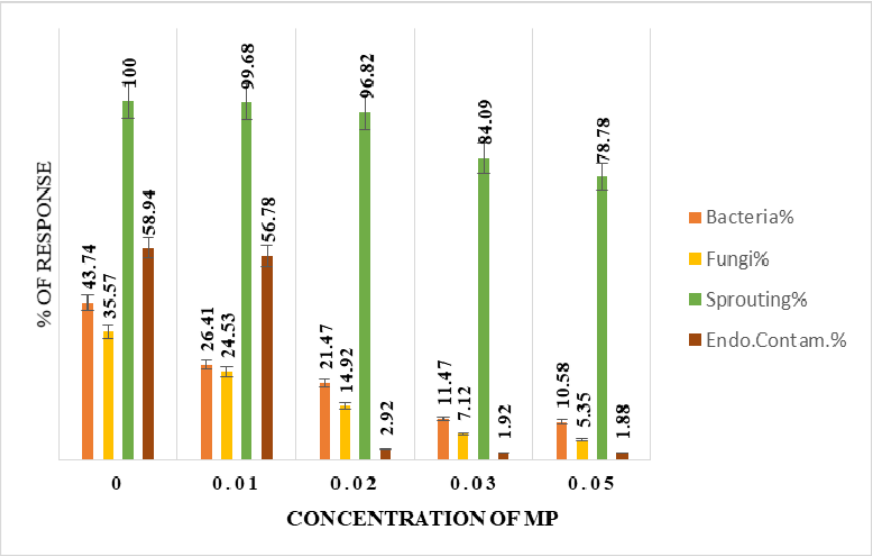

Fig 64: Effect of Methyl Paraben on control of contamination in shoot cultures of *B. balcooa*

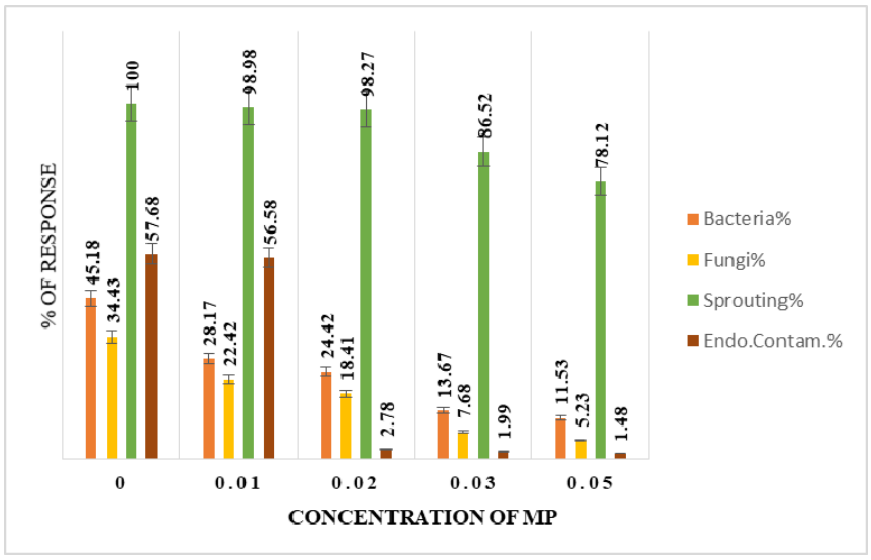

Fig 65: Effect of Methyl Paraben on control of contamination in shoot cultures of *P. stocksii*

Table 57: Effect of methyl paraben on control of contamination in *in vitro* growth response of bamboo species (without $HgCl_2$ sterilization treatment)

Species	MP %	Days taken for bud break	Latent contamination*(Day)
P. ritcheyi	0	3.68±0.46^{ab}	15.68 ±0.38^{f}
	0.01	3.69±2.01^{ab}	26.43±6.29^{e}
	0.02	3.92±0.53^{b}	32.78±0.79^{e}
	0.03	4.02±0.47^{bc}	87.73±3.02^{a}
	0.05	4.56±2.46^{c}	89.65±0.07^{a}
B. balcooa	0	3.32±1.58^{a}	12.84±0.68^{f}
	0.01	3.72±0.01^{ab}	46.90±0.06^{e}
	0.02	3.96±0.02^{b}	80.14±0.18a^{e}
	0.03	4.12±3.04^{bc}	82.67±0.83^{a}
	0.05	4.59±0.94^{c}	84.07±0.28^{a}
P. stocksii	0	3.28±0.82^{a}	14.80±0.32^{f}
	0.01	3.56±4.74^{ab}	48.18±9.05^{e}
	0.02	3.89±0.97^{ab}	84.59±2.69^{a}
	0.03	4.06±2.16^{bc}	83.48±0.85^{a}
	0.05	4.31±1.89^{c}	87.02±1.67^{a}

Data represent the percentage of contamination (both bacteria and fungi).*Time taken for emergence of latent contamination. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

MP is an ester of p-hydroxybenzoic acid and widely used as antimicrobial agents in a large variety of food, pharmaceutical, and cosmetic products at very low level of 0.01 to 0.3 %. They are stable, effective over a wide pH range (4–8), relatively low in toxicity (Cantwell, 1976), and active against a broad spectrum of microorganisms. The inhibitory effect of MP is through its effect on membrane transport and mitochondrial function. It works by disrupting the integrity and increasing the fluidity and permeability of membrane, which results in transmembrane transport disruption and leakage, and uncoupling of metabolic processes (Denyer and Stewart, 1998 and Mrozik *et al*., 2002). Rietschel and Fowler (2001) reported that MPs are more effective against fungi than against bacteria; their antibacterial activity is greatest against Gram-positive organisms and poorest against *Pseudomonas* species. This finding supported the present results. The latent contamination observed in bamboo shoot cultures grown in MP amended media was only of bacteria. This compound completely suppresses the fungal contaminants during culture period. Continuous exposure of shoot cultures to MP amended media showed some growth

retardation in new sprouts. Alternating the subculture of shoots between MP amended media and MP free media facilitates recovery of the young sprouts from toxicity.

4.2.4.2. Thimerosal

Thimerosal, a preservative used widely in vaccines and anti-venom, showed success in tackling contamination in bamboo tissue culture (Table **58**) The lowest rate of bacterial contamination (0.13 % in *P. stocksii*) was obtained in explants inoculated into the media containing 0.02 % thimerosal after 10 d. of inoculation. Similarly, fungal contamination was lowest in *P. ritcheyi* (1.64 %) obtained in same concentration (0.02 %) of thimerosal after 12 d. of inoculation.

Table 58: Effect of thimerosal with different concentrations on *in vitro* growth response and contamination of bamboo species ($HgCl_2$ treated)

	Thimerosal %	Bacteria %	Day *	Fungi %	Day*	% of bud break	Days to bud break
P. ritcheyi	Control	8.65±2.06^{c}	2.78±0.00^{a}	9.76±1.01^{c}	3.97±0.02^{a}	99.00±2.08^{j}	2.33±0.17^{a}
	0.005	5.85±0.64^{b}	7.32±0.17^{b}	6.86±0.28^{b}	8.85±0.76^{c}	62.09±1.86^{g}	6.04±0.50^{b}
	0.01	2.84±0.71^{a}	9.89±0.03^{c}	3.33±0.17^{a}	9.32±0.07^{c}	54.75±0.64^{f}	13.67±0.67^{d}
	0.02	0.69±0.17^{a}	10.89±0.20^{c}	1.64±0.29^{a}	10.07±0.96^{c}	49.12±0.79^{ef}	23.03±0.04^{c}
B. balcooa	Control	8.71±2.01^{c}	2.08±0.95^{a}	8.97±1.01^{c}	3.99±0.06^{a}	98.04±0.85^{j}	3.01±0.29^{a}
	0.005	5.28±0.38^{b}	8.45±1.08^{c}	6.06±0.76^{b}	8.06±0.02^{c}	66.77±1.62^{g}	6.17±0.44^{a}
	0.01	2.65±0.17^{a}	9.88±2.68^{c}	3.82±0.82^{a}	9.62±0.16^{c}	54.31±0.95^{f}	12.98±0.86^{b}
	0.02	0.35±0.13^{a}	10.94±0.19^{c}	2.07±0.29^{a}	12.04±0.02^{d}	46.98±0.07^{e}	23.01±0.09^{c}
P. stocksii	Control	8.92±1.74^{c}	2.72±0.48^{a}	9.67±0.78^{c}	3.98±0.04^{a}	94.33±0.84^{j}	2.09±0.37^{a}
	0.005	4.81±0.07^{b}	7.97±0.18^{b}	8.67±0.88^{c}	8.97±2.09^{c}	61.10±0.90^{g}	6.50±0.86^{a}
	0.01	2.44±0.25^{a}	9.01±0.74^{c}	4.33±0.60^{b}	10.89±0.06^{c}	56.97±0.02^{f}	14.98±0.10^{b}
	0.02	0.13±0.40^{a}	10.36±0.07^{c}	1.67±1.45^{a}	12.05±0.46^{d}	47.05±0.29^{e}	24.04±0.08^{c}

***** Time taken for first incidence of contamination.Thimerosal added to the media after sterilization. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

Contamination emerged only after the shifting of explants into thimerosal free media. Media with thimerosal however resulted in delay of bud break (≥ 6.04 d. in *P. ritcheyi* at 0.005 %) and also reduced the bud breaking rate (≥ 47.05 % at 0.02 % in *P. stocksii*). Sprouting of axillary buds was obtained only after the first subculture into thimerosal free media. Thimerosal in the media helped to circumvent the routine

practice of surface sterilization with $HgCl_2$ (Table **59**). It reduced the contamination in all three species, at all the concentrations tested more effectively than 0.1 % $HgCl_2$.

Table 59 : Effect of thimerosal with different concentrations on *in vitro* growth response and contamination of bamboo species (without $HgCl_2$ sterilization step)

	Thimerosal %	Bacteria%	Day*	Fungi %	Day*	% of bud break	Days to bud break
P. ritcheyi	Control	42.80±2.72^{i}	2.17±0.33^{a}	38.56±0.29^{h}	3.10±0.49^{a}	98.99±0.74^{j}	3.6±0.98^{a}
	0.005	8.58±1.00^{b}	7.02±0.17^{b}	9.10±0.64^{b}	12.44±1.05^{c}	58.48±0.18^{f}	8.26±1.38^{c}
	0.01	4.89±0.02^{a}	12.41±1.02^{c}	7.88±0.52^{b}	23.46±1.68^{e}	52.60±0.06^{f}	11.42±0.43^{c}
	0.02	1.78±0.41^{a}	16.21±0.26^{d}	3.75±0.16^{a}	31.68±0.09^{g}	46.87±0.01^{e}	14.16±0.04^{d}
B. balcooa	Control	38.09±1.66^{h}	3.43±0.17^{a}	38.67±0.69^{h}	3.50±0.68^{a}	99.73±0.03^{j}	3.65±0.02^{a}
	0.005	8.78±0.02^{b}	7.25±0.26^{b}	9.81±0.02^{b}	11.82±0.03^{c}	61.50±0.27^{g}	7.84±0.53^{b}
	0.01	4.83±0.18^{a}	10.08±0.15^{b}	5.62±0.27^{b}	21.57±0.16^{e}	53.05±1.48^{f}	12.01±0.26^{d}
	0.02	1.42±0.10^{a}	13.46±0.89^{c}	3.65±0.02^{a}	30.38±0.67^{f}	45.24±2.09^{e}	13.44±0.68^{d}
P. stocksii	Control	39.33±1.33^{h}	2.83±0.32^{a}	33.67±1.85^{g}	2.95±0.38^{a}	99.82±1.06^{j}	3.48±0.09^{a}
	0.005	7.41±0.07^{b}	8.08±0.22^{b}	9.24±0.18^{b}	11.98±0.16^{c}	71.28±1.29^{h}	7.89±0.04^{b}
	0.01	3.72±0.08^{a}	12.57±0.28^{c}	5.27±0.25^{b}	24.27±0.08^{e}	51.45±0.58^{f}	12.44±0.16^{d}
	0.02	1.81±0.35^{a}	17.47±0.38^{d}	3.47±0.38^{a}	30.18±0.46^{f}	49.86±1.47^{e}	14.46±0.08^{d}

* Time taken for first incidence of contamination. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test. Thimerosal added to the media after sterilization.

Explants in the control treatment had high rate of contamination (≥ 42.80 % bacteria in *P. ritcheyi* and ≥ 38.67 % fungi in *B. balcooa*) within 4 d. of inoculation. This was reduced to 1.42 % of bacteria and 3.65 % of fungi in *B. balcooa* in the presence of 0.02% thimerosal. Inhibition of axillary bud break by thimerosal was observed even with the lowest level (0.005 %) tested delaying the bud breaking to ≥ 7.84 d. This was overcome by transferring the explants into thimerosal free media. Thimerosal is thus a promising alternative for the highly toxic $HgCl_2$ and needs to be explored further for routine applications.

In the experiment where the potential use of thimerosal to replace the sterilization of media by autoclaving (Table **60** and Table **61**) along with impact of $HgCl_2$ treatment. Media supplemented with low concentration of thimerosal can control the contamination only upto 8 d but the ontamination was significantly reduced from the

Table 60 : Effect of different concentrations of thimerosal on *in vitro* growth response and contamination of bamboo species ($HgCl_2$ treated)

	Thimerosal %	Bacteria%	Day*	Fungi%	Day*	% of bud break	Days for bud break
P. ritcheyi	Control	8.92±2.06^{e}	2.78±0.00^{a}	9.76±1.01^{e}	3.98±1.02^{a}	98.99±0.74^{j}	2.33±0.17^{a}
	0.005	5.33±0.87^{c}	7.36±0.18^{b}	6.86±0.28^{d}	5.67±0.39^{b}	58.53±2.12^{f}	6.04±0.50^{b}
	0.01	2.82±0.01^{b}	14.89±0.24^{d}	3.33±0.17^{b}	29.08±0.18^{f}	52.10±0.92^{f}	13.67±0.67^{d}
	0.02	0.67±0.17^{a}	18.69±0.23^{e}	1.64±0.29^{a}	38.01±0.48^{h}	46.83±1.01^{e}	23.03±0.04^{f}
B. balcooa	Control	9.06±2.01^{e}	2.06±0.78^{a}	8.89±1.01^{e}	3.99±0.06^{a}	99.73±0.03^{j}	3.01±0.29^{a}
	0.005	5.33±0.44^{c}	8.65±0.09^{c}	6.06±0.76^{d}	5.06±0.02^{b}	60.67±1.45^{g}	6.17±0.44^{b}
	0.01	2.67±0.24^{b}	14.67±0.37^{d}	3.82±0.82^{b}	38.62±0.16^{h}	53.0±1.71^{f}	12.98±0.86^{d}
	0.02	0.37±0.07^{a}	18.03±0.06^{e}	2.07±0.29^{b}	41.54±0.02^{i}	45.33±1.48^{e}	23.01±0.09^{f}
P. stocksii	Control	8.76±1.74^{e}	2.42±0.56^{a}	9.61±0.78^{e}	3.98±0.04^{a}	99.82±1.06^{j}	2.09±4.07^{a}
	0.005	4.83±0.67^{c}	9.78±0.18^{c}	8.68±0.28^{e}	4.82±1.09^{a}	71.20±2.60^{h}	4.80±1.76^{b}
	0.01	2.66±0.05^{b}	15.25±0.34^{d}	4.18±4.76^{c}	36.82±0.12^{h}	51.13±2.09^{f}	15.38±0.16^{d}
	0.02	0.17±0.32^{a}	18.24±0.17^{e}	1.68±0.79^{a}	42.17±1.84^{i}	49.83±2.09^{e}	23.34±7.08^{f}

***** Time taken for first incidence of contamination. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test. Thimerosal added to the media without autoclaving.

Table 61: Effect of different concentrations of thimerosal on *in vitro* growth response and contamination of bamboo species (without $HgCl_2$ sterilization step)

	Thimerosal %	Bacteria %	Day*	Fungi %	Day*	% of bud break	Days for bud break
P. ritcheyi	Control	35.80±2.72^{d}	2.17±0.33^{a}	38.56±0.29^{g}	2.10±0.49^{a}	99.00±2.08^{p}	3.6±0.98^{a}
	0.005	28.50±0.50^{c}	5.00±0.28^{a}	19.04±1.16^{d}	4.42±0.60^{a}	62.09±1.86^{i}	8.33±2.34^{b}
	0.01	6.83±0.33^{a}	12.33±1.45^{c}	6.83±0.60^{b}	19.83±0.60^{d}	54.75±0.64^{h}	11.20±1.50^{c}
	0.02	4.50±0.00^{a}	16.67±0.33^{d}	4.34±0.10^{a}	28.23±0.62^{f}	49.12±0.79^{d}	14.13±1.84^{c}
B. balcooa	Control	35.02±1.66^{d}	3.43±0.17^{a}	36.67±0.69^{h}	1.50±0.68^{a}	98.04±0.85^{p}	3.65±0.02^{a}
	0.005	27.17±0.34^{c}	5.16±0.33^{a}	19.72±0.14^{d}	4.68±1.02^{a}	66.77±1.62^{j}	7.67±1.86^{b}
	0.01	8.66±0.73^{a}	8.16±1.20^{b}	7.50±0.86^{b}	21.10±1.11^{e}	54.31±0.95^{h}	11.82±2.20^{c}
	0.02	4.56±1.07^{a}	14.0±0.41^{c}	3.67±0.16^{a}	30.64±1.99^{f}	46.98±0.07^{d}	13.57±1.10^{c}
P. stocksii	Control	34.33±1.33^{d}	2.83±0.32^{a}	32.67±1.85^{g}	1.95±0.38^{a}	94.33±0.84^{o}	3.48±0.09^{a}
	0.005	27.34±0.44^{c}	5.16±0.16^{a}	16.33±1.46^{d}	4.04±0.28^{a}	60.65±0.46^{i}	7.83±1.36^{b}
	0.01	6.77±0.14^{a}	7.83±0.73^{b}	6.33±0.44^{b}	24.53±0.62^{e}	55.38±0.12^{f}	12.47±1.13^{c}
	0.02	3.83±0.34^{a}	17.90±0.42^{d}	4.50±0.76^{a}	30.66±0.88^{f}	46.95±0.28^{d}	14.53±1.30^{c}

* Time taken for first incidence of contamination. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test. Thimerosal added to the media without autoclaving.

control (≤ 9.06 % of bacteria and ≤ 9.76 % fungi). Incidence of contamination occured within 3-4 d. in control. However, the addition of thimerosal significantly

influenced the bud breaking percentage as well as delayed the sprouting response to as much as 23 d. (*P. ritcheyi*).

Explant inoculation without $HgCl_2$ treatment enhanced the contamination rate as well as bud breaking percentage. On media with 0.005 % thimerosal, 28.50 %, 27.17 % and 27.34 % of bacterial contamination was found in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively. Among the three concentrations of thimerosal tested, 0.01 % was found to be optimum for controlling contamination in explants without $HgCl_2$ treatment, autoclaving free media etc. At this concentration, reduction in the contamination (≤ 8.66 % of bacteria and ≤ 7.50 % of fungi) was same as that of the contamination rate of $HgCl_2$ treated explants inoculated in sterile media (≤ 8.92 % of bacteria and ≤ 9.76 % of fungi).

Table 62 : Effect of different exposure period of explants in 0.1 % thimerosal on *in vitro* growth response and contamination of bamboo species ($HgCl_2$ treated)

	Duration (day)	Bacteria%	Day*	Fungi%	Day*	% of bud break	Days for bud break
P. ritcheyi	Control	8.92±2.06^{f}	2.78±0.00^{a}	9.76±1.01^{g}	3.98±1.02^{a}	98.99±0.74^{e}	2.33±0.17^{a}
	1	6.01±0.27^{e}	3.04±0.46^{b}	4.82±0.51^{b}	4.08±0.89^{a}	98.45±0.21^{e}	3.74±0.62^{b}
	2	5.48±0.05^{d}	4.56±0.68^{c}	4.51±0.77^{b}	5.73±1.28^{a}	95.27±2.63^{e}	5.28±1.19^{d}
	3	5.01±0.44^{d}	4.79±0.52^{c}	4.26±1.96^{b}	8.18±1.04^{b}	84.01±0.31^{d}	6.62±1.14^{e}
	4	3.99±0.42^{b}	6.08±3.23^{e}	4.02±0.35^{b}	9.02±0.01^{c}	76.53±0.02^{c}	7.36±0.09^{f}
	5	3.58±2.08^{b}	8.63±0.16^{g}	3.66±0.92^{a}	12.08±0.02^{d}	69.48±0.36^{b}	8.15±1.62^{g}
	6	3.07±1.57^{b}	10.46±0.03^{i}	3.46±0.08^{a}	21.02±0.31^{f}	58.68±1.49^{a}	9.57±0.26^{h}
B. balcooa	Control	9.06±2.01^{g}	2.06±0.78^{a}	8.89±1.01^{f}	3.99±0.06^{a}	99.73±0.03^{e}	3.01±0.29^{b}
	1	6.24±0.06^{e}	3.38±0.39^{b}	6.52±0.98^{d}	4.48±0.23^{a}	98.66±1.54^{e}	3.78±0.90^{b}
	2	5.72±0.16^{d}	4.74±0.04^{c}	5.73±0.01^{c}	8.82±0.56^{b}	96.87±2.17^{e}	5.32±1.82^{d}
	3	5.26±0.28^{d}	5.93±0.07^{d}	4.83±1.02^{b}	9.25±0.74^{c}	85.71±0.29^{d}	6.44±1.40^{e}
	4	4.09±0.07^{c}	7.36±0.65f	4.57±1.06^{b}	11.49±1.24^{c}	74.59±0.47^{c}	7.65±3.01^{f}
	5	3.67±0.05^{b}	8.89±0.03^{g}	4.36±0.08^{b}	18.34±1.78^{e}	63.42±0.85^{b}	8.29±0.02^{g}
	6	2.78±0.03^{a}	10.00±0.57^{i}	4.02±0.57^{b}	28.89±0.57^{i}	58.46±1.06^{a}	9.64±0.32^{h}
P. stocksii	Control	8.76±1.74^{f}	2.42±0.56^{a}	9.61±0.78^{g}	3.98±0.04^{a}	99.82±1.06^{e}	2.09±4.07^{a}
	1	6.81±0.38^{e}	3.58±0.03^{b}	6.71±1.06^{d}	4.39±0.72^{a}	98.06±0.29^{e}	3.28±0.78^{b}
	2	5.73±0.96^{d}	4.89±0.71^{c}	5.03±0.03^{c}	5.86±0.62^{a}	95.01±0.09^{e}	4.87±1.09^{c}
	3	5.22±0.03^{d}	5.05±0.86^{d}	4.88±1.03^{b}	8.64±0.04^{b}	85.38±0.07^{d}	6.25±0.80^{e}
	4	4.06±0.78^{c}	7.63±0.91^{f}	4.62±1.^{80b}	9.71±0.91^{c}	77.89±0.12^{c}	7.28±0.26^{f}
	5	4.58±1.65^{c}	8.98±0.83^{g}	4.39±0.07^{b}	12.65±0.05^{d}	68.09±0.12^{b}	8.04±0.52^{g}
	6	2.96±0.35^{a}	12.78±0.06^{k}	4.18±4.76^{b}	29.17±0.05^{i}	55.08±0.13^{a}	15.38±0.16^{i}

* Time taken for contamination. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test. Thimerosal added to the media without autoclaving.

Continuous exposure of the explant to the thimerosal in the media reduced the sprouting rate as well as delayed bud sprouting. In order to optimize the exposure period of the explant of thimerosal, 0.1 % $HgCl_2$ treated explants inoculated into MS media containing 0.01 % thimerosal. These explants were shifted into thimerosal free sterilized media and evaluated for the effect on explant contamination as well as the sprouting response (Table **62**). It was found that the incubation of explants for 3 d in thimerosal containing media effectively reduce the contamination of both bacteria (5.01 %, 5.26 %, and 5.22 % in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively) and fungi (4.26 %, 4.83 %, and 4.88 % in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively). Improved bud breaking frequency in all three species tested (≥ 84.01 %) and time taken for bud breaking (within ~4 d. after shifting to the thimerosal free media) was also observed with this step.

Thimerosal is an organo-mercurial compound, which inhibits sulfhydryl-containing active site of various enzymes and binds to sulfhydryl compounds such as glutathione, cysteine, and sulfhydryl groups of proteins. In addition, thimerosal activates the calcium channel on the endoplasmic reticular membrane, thereby triggering the release of intracellular calcium resulting in a calcium-induced calcium-influx of extracellular calcium. Therefore, thimerosal may induce or inhibit various cellular functions that are dependent on the signalling of calcium. Thimerosal was found as an alternative for $HgCl_2$ since its addition to media ensured prevention of contamination, but its application as a surface sterilizing agent was not successful in bamboo tissue culture (data not shown). The option of amendment of thimerosal in culture media for the taking advantage of the antimicrobial property was however sucessful. Prolonged exposure of the plant material to this chemical adversely effected bud breaking as well shoot elongation. Exposure of tender shoots or sprouts to thimerosal resulted in tissue damage. Short span exposure of explant (mature tissue for 3-4 d. and transferring the material to the normal media is feasible. The addition of 0.1% thimerosal in medium in *in vitro* cultures of *Chrysanthemum* provided 100% sterile conditions without even the need for autoclaving the media (Deein *et al*., 2013). One of the advantage of this chemical is that long term storage (upto 6 mos.) and re-usability (8 to 10 times) of the thimerosal containing media. No effect of

thimerosal on latent contamination was seen and therefore its use is limited to the control of exogenous contamination alone.

4.2.4.3. Benzalkonium chloride

In all three species, Benzalkonium chloride (BAC) caused significant antimicrobial effects. $HgCl_2$ of 0.1 % was used in these experiments as a positive control and reduced the contamination rate ≤ 9.06 % of bacteria and ≤ 9.92 % of fungi (Fig. **65**, **66** & **67**). Even with 3 min. exposure BAC, significant reduction of contamination (~7 % of bacteria and ~ 8 % of fungi) seen after 3 d. of inoculation without affecting the sprouting rate. An exposure time dependent linear decline in control of contamination observed. Deterioration in bud sprouting was however observed in all three species tested (59 %, 55 % and 53 % in *P. ritcheyi, B. balcooa* and *P. stocksii* respectively). Based on the various parameters for successful culture establishment, 7 min. of exposure of nodal segments to BAC after surface sterilization was found as the optimum treatment (Table **63**).

In order to find out the role of BAC as an alternative to $HgCl_2$, an additional experiment was carried out in which nodal segments (explants) after treatment with antibiotics and fungicides treated with the BAC.

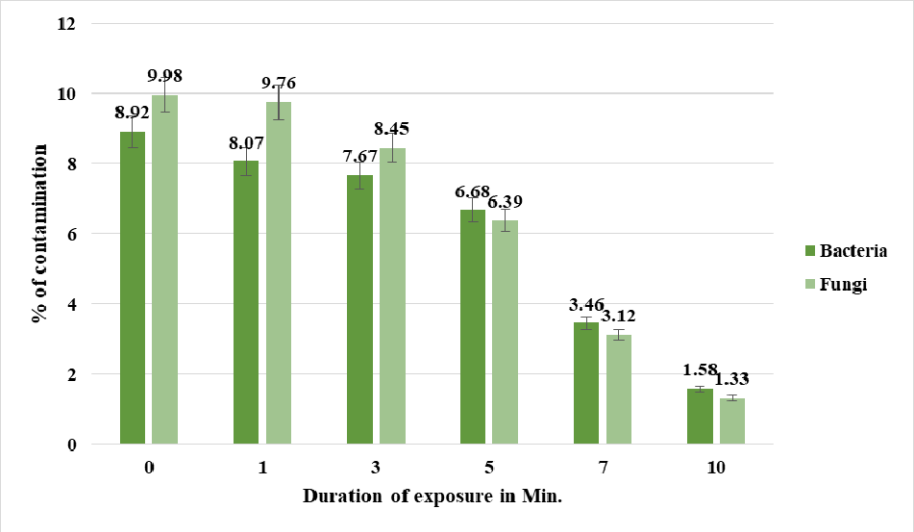

Fig. 65: Effect of Benzalkonium chloride (0.1mg ml^{-1}) on contamination *of P. ritcheyi* ($HgCl_2$ treated)

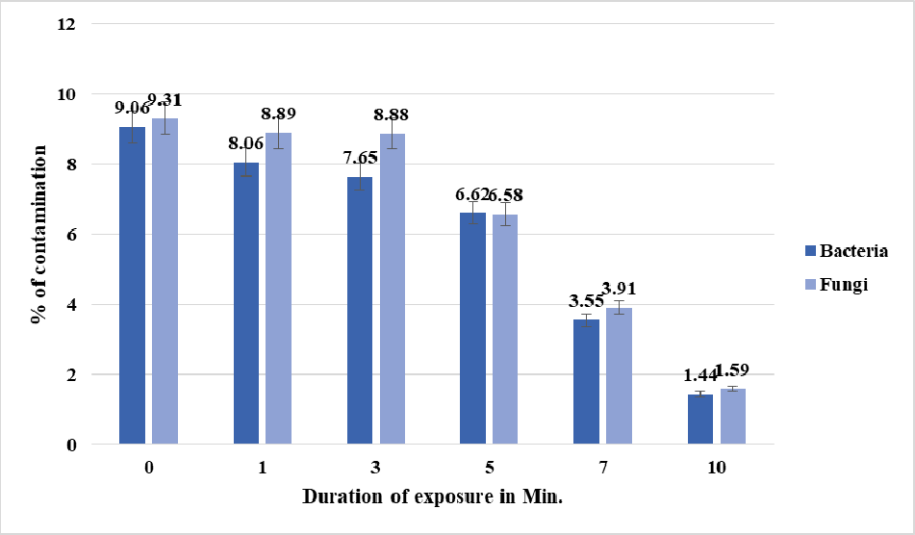

Fig. 66: Effect of Benzalkonium chloride (0.1mg ml^{-1}) on contamination *of B. balcooa* ($HgCl_2$ treated)

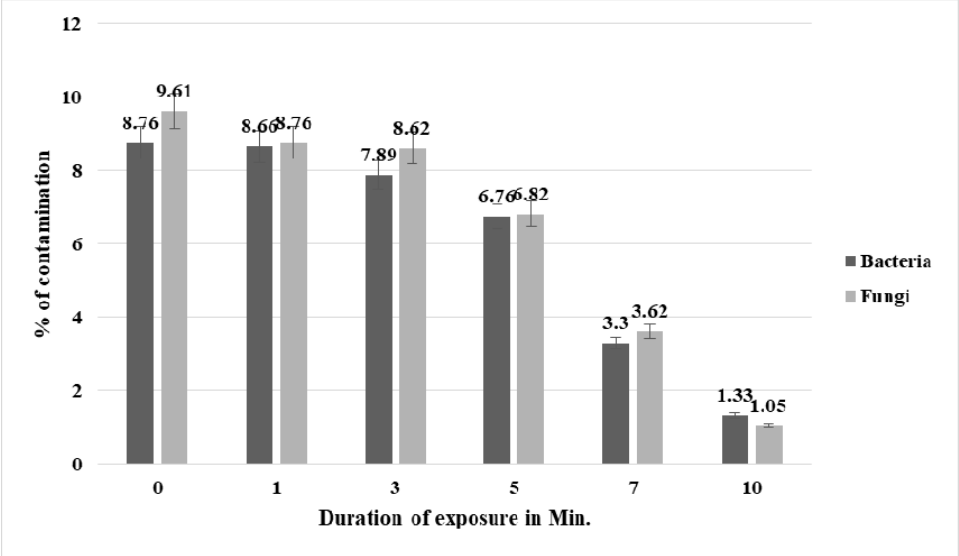

Fig. 67: Effect of Benzalkonium chloride (0.1mg ml^{-1}) on contamination *of P. stocksii* ($HgCl_2$ treated)

Table 63 : Effect of Benzalkonium chloride (0.1mg ml^{-1}) on *in vitro* growth response and contamination of bamboo species ($HgCl_2$ treated)

	Duration (min)	Day*	Day*	% of bud break	Day required for bud break
P. ritcheyi	0	2.78 ± 0.00^{a}	3.98 ± 1.02^{a}	98.99 ± 0.74^{c}	2.33 ± 0.17^{a}
	1	3.45 ± 0.21^{b}	4.34 ± 0.01^{b}	96.84 ± 1.73^{c}	3.87 ± 0.16^{b}
	3	3.90 ± 0.14^{b}	5.28 ± 0.12^{c}	95.87 ± 1.72^{c}	3.90 ± 0.53^{b}
	5	6.02 ± 0.10^{d}	8.38 ± 0.28^{e}	89.98 ± 0.50^{b}	3.97 ± 0.17^{b}
	7	9.36 ± 0.92^{e}	9.31 ± 0.60^{f}	88.04 ± 0.13^{b}	4.78 ± 0.67^{c}
	10	10.21 ± 0.42^{f}	9.71 ± 0.28^{f}	59.24 ± 0.14^{a}	6.73 ± 0.50^{d}
B. balcooa	0	2.06 ± 0.78^{a}	3.99 ± 0.06^{a}	99.73 ± 0.03^{c}	3.01 ± 0.29^{b}
	1	3.90 ± 0.45^{b}	4.48 ± 0.17^{b}	98.11 ± 0.40^{c}	3.41 ± 0.50^{b}
	3	4.00 ± 0.24^{c}	5.17 ± 0.20^{c}	94.52 ± 0.76^{c}	3.70 ± 0.58^{b}
	5	6.53 ± 0.85^{d}	6.89 ± 0.09^{d}	87.33 ± 0.91^{b}	3.80 ± 0.14^{b}
	7	8.67 ± 0.15^{e}	9.77 ± 0.70^{f}	84.50 ± 0.30^{b}	4.73 ± 0.50^{c}
	10	9.73 ± 0.49^{f}	9.84 ± 0.50^{f}	55.06 ± 0.16^{a}	7.67 ± 0.77^{e}
P. stocksii	0	2.42 ± 0.56^{a}	3.98 ± 0.04^{a}	99.82 ± 1.06^{c}	2.09 ± 4.07^{a}
	1	3.10 ± 0.09^{b}	4.61 ± 0.56^{b}	98.10 ± 0.28^{c}	3.65 ± 0.65^{b}
	3	3.62 ± 0.35^{b}	6.76 ± 0.20^{d}	95.98 ± 0.50^{c}	3.33 ± 0.77^{b}
	5	6.66 ± 0.53^{d}	8.10 ± 0.3^{e}	88.28 ± 0.61^{b}	3.95 ± 0.70^{b}
	7	8.92 ± 0.10^{e}	9.69 ± 0.32^{f}	83.22 ± 0.66^{b}	4.35 ± 0.20^{c}
	10	9.33 ± 0.11^{f}	10.37 ± 1.20^{g}	53.91 ± 0.17^{a}	6.77 ± 0.90^{d}

* Time taken for first incindence of contamination. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test.

The results show that the shorter exposure periods (1min, 3min, and 5 min) had no significant effect on control of contamination. BAC treatment for 7 min. and 10 min. reduced the contamination effectively (≤ 7.87 % of bacteria and ≤ 9.66 % of fungi). Bud breaking significantly affected by the long exposure period (~82 % of bud break) (Fig. **68**, **69** & **70**). From this, it was clear that the reduced rate of axillary bud sprouting in above experiment by the combined action of BAC and $HgCl_2$ (Table **64**).

Benzalkonium chloride (BAC), a cationic detergent with tetra-substituted ammonium salt was shown in the experiments to be working well in control of contamination on the nodal explants of bamboo as same as that of $HgCl_2$. In addition, it effectively reduced the initial contamination in all species tested. Reduction in contamination rate was linearly depending on the concentration of BAC.

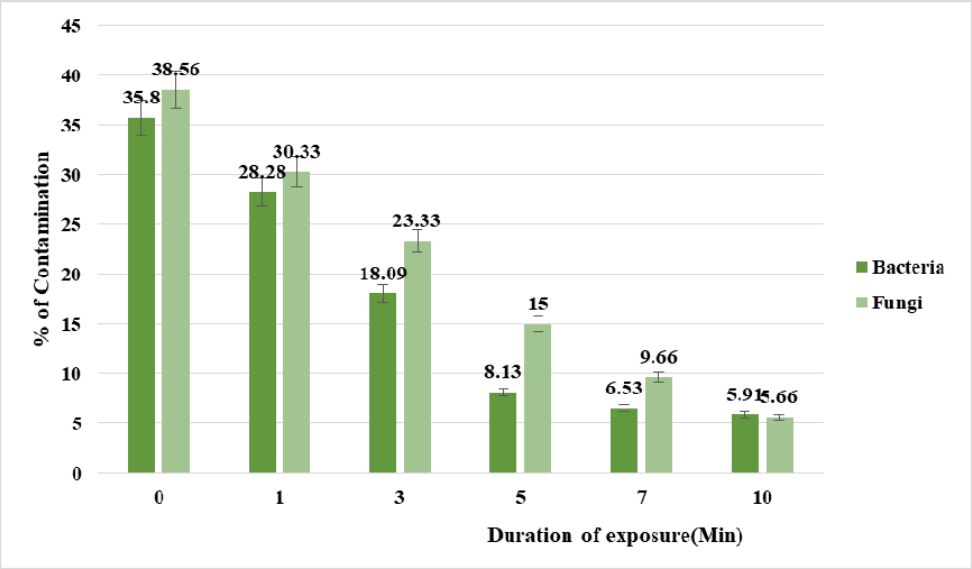

Fig. 68: Effect of Benzalkonium chloride (0.1mg/ml) on contamination *of P. ritcheyi* ($HgCl_2$ non-treated)

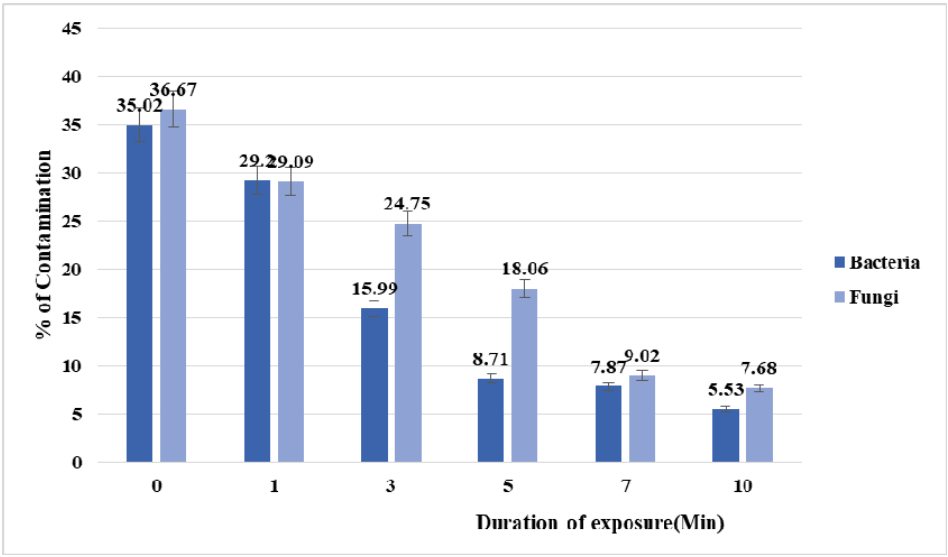

Fig. 69: Effect of Benzalkonium chloride (0.1mg/ml) on contamination *of B. balcooa* ($HgCl_2$ non-treated)

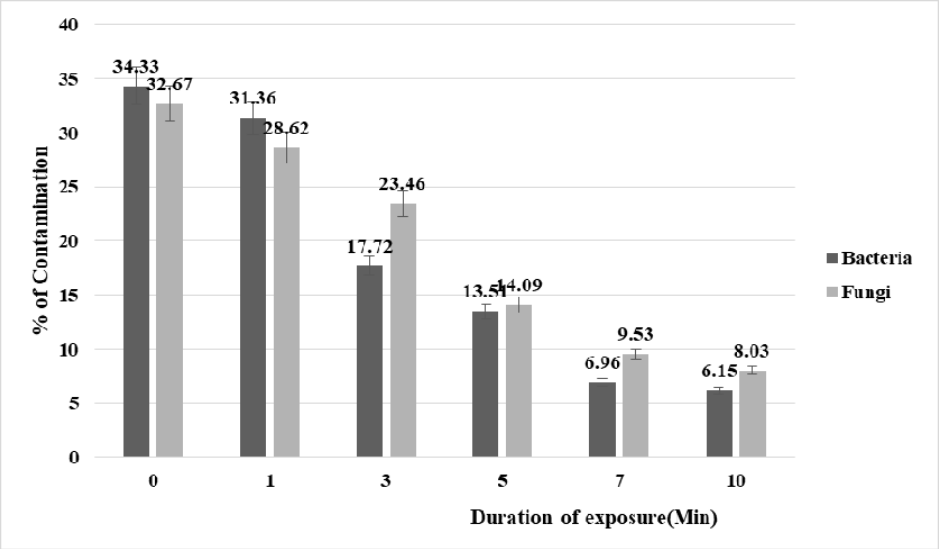

Fig. 70: Effect of Benzalkonium chloride (0.1mg/ml) on contamination *of P. stocksii* ($HgCl_2$ non-treated)

Table 64: Effect of Benzalkonium chloride (0.1mg ml^{-1}) on *in vitro* growth response and contamination of bamboo species ($HgCl_2$ non-treated).

	Duration (min)	Day *	Day*	% of bud break	Day required for bud break
P. ritcheyi	0	2.17±0.33^{c}	2.10±0.49^{d}	99.00±2.08^{a}	3.62±0.98^{a}
	1	2.65 ± 0.21^{c}	3.00 ± 2.88^{c}	98.43 ± 7.6^{a}	3.94 ± 1.54^{a}
	3	3.13 ± 0.20^{b}	3.66 ± 1.66^{c}	97.66 ± 1.66^{b}	4.00 ± 1.44^{b}
	5	3.80 ± 0.14^{b}	3.89 ± 1.03^{c}	95.66 ± 1.28^{d}	4.18 ± 1.11^{b}
	7	4.71 ± 0.14^{a}	4.00 ± 5.66^{b}	94.00 ± 5.76^{d}	4.34 ± 1.62^{b}
	10	4.89 ± 0.29^{a}	4.10 ± 5.54^{b}	81.33 ± 5.62^{e}	4.58 ± 1.11^{b}
B. balcooa	0	3.43±0.17^{b}	2.50±0.68^{d}	98.64±0.85^{a}	3.65±0.02^{a}
	1	3.60 ± 0.39^{b}	3.66 ± 5.36^{c}	98.06 ± 5.21^{a}	3.61 ± 2.17^{a}
	3	3.76 ± 0.40^{b}	3.71 ± 1.11^{c}	97.55 ± 0.55^{b}	4.00 ± 2.89^{b}
	5	3.94 ± 0.80^{b}	3.95 ± 1.00^{c}	94.89 ± 1.82^{d}	4.11 ± 1.38^{b}
	7	4.23 ± 0.12^{a}	4.66 ± 0.83^{b}	93.22 ± 1.21^{d}	4.22 ± 1.47^{b}
	10	4.84 ± 0.20^{a}	6.60 ± 1.33^{a}	81.08 ± 7.75^{e}	5.00 ± 13.8^{c}
P. stocksii	0	2.83±0.32^{c}	1.95±0.38^{e}	98.33±0.84^{a}	3.48±0.09^{a}
	1	3.22 ± 0.89^{b}	2.09 ± 3.92^{d}	97.03 ± 6.26^{b}	3.70 ± 9.01^{a}
	3	3.32 ± 0.60^{b}	3.56 ± 5.52^{c}	96.98 ± 7.43^{c}	3.87 ± 1.12^{a}
	5	3.95 ± 0.30^{b}	3.86 ± 1.31^{c}	96.47 ± 6.69^{c}	3.92 ± 1.31^{a}
	7	4.07 ± 0.95^{a}	4.06 ± 3.98^{b}	93.04 ± 3.19^{d}	4.08 ± 6.16^{b}
	10	4.46 ± 1.18^{a}	4.57 ± 8.42^{b}	82.06 ± 12.3^{e}	4.86± 9.72^{b}

* Time taken for first incindence of contamination. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test.

Incubation of explants in higher concentration (≥ 0.15mg ml^{-1}) significantly reduced the sprouting rate as well as delayed the bud breaking response. This was because of their positive charge and their interaction with cell surface of the emerging bud region. Simultaneously, it was proven that the treatment with BAC did not influence emergence of latent contamination. The mode of action of this compound against bacterial cells involves a general perturbation of lipid bilayer membranes. At low concentrations, they are bacteriostatic and at high concentrations, they are bactericidal (Houari and Di Martino, 2007). It is also well documented that they bind to the fatty acids of cell membranes of organisms, which makes them useful as biocides and treatment of shoot primordia with BAC helped to established sterile cultures in *Spinacia oleracea* (Kondo *et al*., 1991) proved it efficiency in plant tissue culture also.

4.2.5. Control of endophytes through the activation of the plant defense

4.2.5.1. Chitosan

4.2.5.1.1. Prophylactic treatment of axillary buds with chitosan

Spraying chitosan on bamboo plants 24 h before collection of explants caused a decrease in the incidence of contamination in both cases of fungi and bacteria (Table **65**). Reduction in contamination was directly proportional to the chitosan concentration; concentrations of 1.75 to 2.5 % resulted in contamination percentages of < 2 % in the case of exogenous contamination and of latent contamination in all three species. This strategy opened up the possibility of using opened or sprouted buds for culture establishment, which is particularly useful in establishing cultures in a recalcitrant species such as *P. ritcheyi.*

Axillary buds with sprouts collected from the field grown clumps exhibited high percentage of establishment with reduced contamination rate (Table. **65** and **66**). Control of microbial contamination during culture establishment phase and in the later stages of shoot culture in these experiments could be attributed to either the biocidal property of chitosan or its ability to induce defense in plants, or a combination of these factors.

Table 65: Effect of prophylactic treatment (*opened buds*) with different concentrations of chitosan on *in vitro* growth response of bamboo species.

	Chitosan (%)	Exogenous Contamination %*		Latent contamination% **
P. ritcheyi		**Bacteria**	**Fungi**	
	Control	24±2.01^{d}	30±1.01^{e}	53.5± 1.94^{d}
	AA (0.1)	22±1.27^{d}	28±0.29^{e}	49.42 ± 0.23^{d}
	0.5	21± 1.16^{d}	24±0.25^{d}	6.21 ± 1.14^{c}
	1	18±1.31^{c}	23±0.27^{d}	2.73 ± 1.69^{b}
	1.5	6±1.19^{b}	11±0.11^{c}	2.38 ± 1.57^{b}
	1.75	1.17±2.11^{a}	1.03±0.31^{b}	1.76 ± 0.07^{a}
	2	1.08±1.09^{a}	0.98±0.02^{a}	1.38 ± 0.28^{a}
	2.5	0.52±0.03^{a}	0.21±0.01^{a}	1.07± 1.21^{a}
B. balcooa	Control	23±2.98^{d}	39±1.03^{e}	56.21± 1.64^{de}
	AA (0.1)	21±3.04^{d}	36±0.39^{e}	50.36 ± 1.12^{d}
	0.5	21±1.16^{d}	24±0.27^{d}	6.19 ± 0.14^{c}
	1	11±1.13^{c}	13±0.23^{c}	2.36 ± 1.07^{b}
	1.5	8±0.23^{b}	9±0.31^{b}	2.98 ± 1.27^{b}
	1.75	1.26±0.97^{a}	3±0.16^{a}	2.76 ± 0.69^{b}
	2	1.12±1.18^{a}	2±0.02^{a}	1.89 ± 1.78^{a}
	2.5	0.82±1.03^{a}	1±0.81^{a}	1.16 ± 0.27^{a}
P. stocksii	Control	22±2.14^{d}	34±1.26^{e}	52.46± 0.01^{d}
	AA (0.1)	20±0.22^{d}	26±0.12^{d}	6.02 ± 0.12^{c}
	0.5	10±0.01^{c}	12.1±0.17^{c}	2.16 ± 0.09^{b}
	1	7±1.09^{b}	8.22±0.01^{b}	2.05 ± 1.18^{b}
	1.5	1.76±0.02^{a}	3.65±0.04^{a}	1.92 ± 0.12^{b}
	1.75	1.01±0.01^{a}	0.32±1.00^{a}	1.04 ± 1.01^{a}
	2	0.98±0.27^{a}	0.11±1.91^{a}	0.16 ± 0.01^{a}
	2.5	0.01±0.03^{d}	0.2±0.01^{d}	0.01 ± 0.02^{c}

Values represent the means of 15replicates ± the standard error of the mean ($P < 0.05$).). *Data collected after 10 d. of culture. **Data taken after 100 d. (10 passages) of culture

Although the severity of contamination was decreased significantly with increase in chitosan concentration, a concomitant inhibition of sprouting of axillary buds was also observed. The lowered efficacy of chitosan of 0.5 % against contamination in various stages of shoot culture necessitates the selection of 1.75 % as the best concentration of chitosan.

Spraying of chitosan in unopened buds resulted in much less contamination percentages (Table **66**); concentrations increased resulted in decreased percentages of bud sprouts. Prophylactic pre-harvest chitosan sprays applied at intervals on strawberry fruit was reported by Bhaskara Reddy *et al*. (2000) to enhance the resistance against post-harvest infection by the fungi, *Botrytis cinerea* and improved quality of fruit by enhancing shelf life.

Table 66: Effect of prophylactic treatment (unopened buds) with different concentrations of chitosan on *in vitro* growth response of bamboo species.

Species	Chitosan (%)	Response		
		Dormant buds		
		Sprouting %*	Exogenous Contamination %*	Latent contamination%**
P. ritcheyi	Control	96.6±1.63[a]	7.31±0.29[d]	62.11 ± 1.43[e]
	AA. (0.1)	63.94±1.28[d]	3.90±0.19[b]	11.42 ± 0.16[e]
	0.5	96.21±1.01[a]	6.01±1.84[c]	7.47 ± 1.28[d]
	1	95.71±1.40[a]	5.09±0.15[c]	5.46 ± 1.37[c]
	1.5	89.63±2.11[a]	4.29±0.31[b]	4.76 ± 1.23[c]
	1.75	87.59±0.61[a]	3.01±0.03[b]	2.99 ± 0.35[a]
	2	74.01±0.92[b]	2.05±0.90[a]	2.81 ± 0.23[a]
	2.5	61.14±1.73[c]	2.01±0.15[a]	2.63± 1.13[a]
P. stocksii	Control	97.92±1.04[a]	8.16±0.05[d]	57.89 ± 0.08[e]
	AA (0.1)	61.52±0.02[d]	5.06±0.34[b]	17.02 ± 0.02[e]
	0.5	96.21±1.01[a]	5.13±0.01[c]	6.15 ± 0.14[d]
	1	96.16±0.09[a]	5.02±0.06[c]	5.02 ± 1.01[c]
	1.5	91.42±0.13[a]	4.12±0.08[b]	4.11 ± 0.03[c]
	1.75	90.12±0.37[a]	3.04±1.00[b]	2.42 ± 0.01[a]
	2	83.99±1.02[b]	1.87±0.20[a]	1.89 ± 0.14[a]
	2.5	63.20±1.08[c]	1.01±0.13[a]	0.42± 0.01[a]
B. balcooa	Control	98.9±0.10[a]	7.25±0.09[d]	63.12 ± 1.24[e]
	AA (0.1)	78.3±1.63[d]	6.18±0.37[c]	12.18 ± 0.05[e]
	0.5	97.5±1.63[a]	6.46±0.80[c]	8.33 ± 1.07[d]
	1	89.17±1.63[a]	6.03±0.49[c]	4.62 ± 0.23[c]
	1.5	83.52±1.63[a]	6.01±0.44[c]	3.92 ± 1.13[b]
	1.75	80.09±1.63[a]	3. 35±0.01[b]	2.76 ± 0.69[a]
	2	62.5±1.63[b]	3.08±0.16[b]	2.69 ± 1.78[a]
	2.5	48.04±1.63[c]	1.05±0.29[a]	2.16 ± 1.17[a]

Values represent the means of 15 replicates ± the standard error of the mean ($P < 0.05$).). *Data collected after 10 d. of culture. **Data taken after 100 d. (10 passages) of culture

4.2.5.1.2. Control of contamination in chitosan amended media

When added to culture media, a relatively low concentration of chitosan (0.5%) caused upto 98 % inhibition of contamination at the initiation stage and as well as later stages of culture. A higher concentration (2.5 %) microbial growth was almost completely controlled. Chitosan however also reduced the bud sprouting in the experiments. The bud break percentage was approximately 65 in 0.5 % of chitosan amended with media (Table **67**), whereas 2.5 % chitosan, it was about 30 %. Among the various concentration of chitosan applied, 1.75 % in all three species tested gave better response in terms of control over initial contamination (< 0.29 %), latent

contamination (< 0.18 %) and gave an acceptable sprouting response of more than 50 %.

Prolonged exposure of explants to high concentration of chitosan induced tissue damage and death of explants. This could be due to the formation of thick chitosan film on the explants that is inserted into the media, which can act as a barrier for nutrient and gases uptake and slow the metabolic activity. The coating of fruits with chitosan to form semi-permeable film to retard ripening is practiced in the industry. Lowings and Cutts (1982) reported that the coating can retard ripening by modifying the internal CO_2, O_2 and ethylene levels. According to Bai *et al.* (1988), chitosan coating is likely to modify the internal atmosphere without causing anaerobic respiration. Similar phenomenon could be acting on the explants placed on the chitosan containing media.

Table 67: Culture Initiation with Chitosan amended media

Species	Chitosan (%)	Sprouting %*	Exogenous Contamination % *	Latent contamination %**
P. ritcheyi	Control	95.68±1.63^{a}	7.22±0.00^{f}	51.06 ± 0.83^{e}
	A. A (0.1)	24.44± 2.07^{d}	3.30±0.44^{b}	10.11 ± 1.16^{e}
	0.5	64.44±0.19^{b}	1.97±0.95^{d}	1.81 ± 0.61^{d}
	1	58.67±0.03^{b}	0.92±0.10^{c}	1.51 ± 1.27^{c}
	1.5	56.92±0.81^{b}	0.47±0.43^{b}	0.81 ± 1.83^{b}
	1.75	54.90±0.31^{b}	0.27±0.15^{b}	0.18 ± 0.91^{a}
	2	34.44±0.73^{c}	0.05±0.75^{b}	0.11 ± 0.63^{a}
	2.5	21.12±0.06^{c}	0.01±0.06^{b}	0.07 ± 1.37^{a}
P. stocksii	A. A (0.1)	96.33±4.01^{a}	8.16±1.02^{f}	56.41 ± 0.16^{e}
	0.5	32.13± 0.21^{d}	3.57±0.21^{b}	10.42 ± 0.12^{e}
	1	65.18±0.01^{b}	2.01±0.14^{d}	3.02 ± 0.14^{d}
	1.5	59.35±0.12^{b}	1.88±0.02^{c}	1.48 ± 1.01^{c}
	1.75	54.02±0.01^{b}	1.07±0.01^{b}	0.32 ± 0.22^{b}
	2	53.00±1.08^{b}	0.63±1.04^{b}	0.15 ± 0.01^{a}
	2.5	39.32±0.01^{c}	0.25±0.21^{b}	0.09 ± 1.07^{a}
	A. A (0.1)	28.98±0.01^{c}	0.11±0.12^{b}	0.03 ± 0.01^{a}
B. balcooa	0.5	98.9 ± 0.38^{a}	7.25±0.09^{f}	53.05 ± 1.48^{e}
	1	23.3±1.13^{d}	3.13±0.50^{b}	11.05 ± 1.63^{e}
	1.5	63.3±0.51^{a}	2.16±0.80^{e}	2.19 ± 0.72^{d}
	1.75	59.6±0.62^{b}	1.03±0.07^{d}	1.78 ± 1.59^{c}
	2	54.1±1.81^{b}	0.50±0.44^{b}	0.31 ± 1.07^{a}
	2.5	52.7±1.10^{b}	0.29±0.11^{b}	0.19 ± 0.52^{a}
	A. A (0.1)	35.5±0.97^{b}	0.20±0.71^{b}	0.16 ± 1.16^{a}
	0.5	28.0±1.91^{c}	0.05±0.44^{a}	0.01 ± 0.09^{a}

Values represent the means of 15replicates ± the standard error of the mean (P < 0.05).). *Data collected after 10 d. of culture. **Data taken after 100 d. (10 passages) of culture.

4.2.5.1.3. Evaluation of exposure time to chitosan for control of contamination

Initial exposure to chitosan and subsequent withdrawal of chitosan from the medium was found to be very effective for overall culture establishment from explants. This two-step procedure (inoculation in chitosan containing medium followed by transfer to chitosan-free medium) resulted in good sprouting rate within 4–6 d. after the subculture. Among the various exposure durations tested with chitosan (Table **68**), sprouting declined (96-52 %) when exposure time increased from 12h to 168h; At 48h the maximum sprouting (88 % and 89 %) was obtained in all three species along with reduced exogenous (4 % and 3.7 %) and latent contamination (1.62 % and 0.83 %). Sprouting percentage gradually reduced by increasing the incubation period.

Table 68: Effect of different exposure time of chitosan (1.75%) on dormant buds *in vitro*

Species	Duration (hrs)	Sprouting (%)*	Exogenous Contamination %*	Latent contamination%**
P. ritcheyi	Control	95.68±1.63^{a}	7.22±0.00^{e}	58.11±0.13^{d}
	12	95.06 ±1.03^{a}	7.13±0.17^{e}	2.36 ± 1.07^{c}
	24	94.44±0.69^{a}	5.70±1.00^{c}	1.32 ± 0.18^{c}
	48	88.44±0.52^{b}	1.19±0.00^{b}	0.72 ± 0.01^{c}
	72	74.90±0.28^{c}	1.06±1.02^{a}	0.63 ± 0.10^{c}
	96	71.40±0.06^{c}	0.97±0.09^{a}	0.60 ± 1.04^{a}
	120	69.20±0.14^{d}	0.49±1.02^{a}	0.59 ± 0.08^{a}
	144	59.10±0.22^{e}	0.13±0.01^{a}	0.53 ± 1.15^{a}
	168	54.90±0.31^{e}	0.07±2.00^{a}	0.48 ± 0.91^{a}
P. stocksii	Control	95.68±0.21^{a}	8.64±0.90^{e}	57.09± 0.06^{d}
	12	96.06 ±1.18^{a}	7.08 ± 0.17^{e}	2.89 ± 0.82^{c}
	24	93.42±1.65^{a}	6.82±1.00^{c}	1.09 ± 1.13^{c}
	48	88.04±0.27^{b}	1.48±1.27^{b}	0.63 ± 0.01^{c}
	72	76.90±0.02^{c}	1.24±0.70^{a}	0.48± 2.02^{a}
	96	72.40±1.04^{c}	1.07±0.18^{a}	0.46± 1.04^{a}
	120	69.09±0.11^{d}	0.96±1.26^{a}	0.41 ± 1.03^{a}
	144	57.19±0.26^{e}	0.39±0.60^{a}	0.28 ± 0.24^{a}
	168	55.07±0.01^{e}	0.06±0.15^{a}	0.02 ± 0.01^{a}
B. balcooa	Control	98.9±0.38^{a}	7.25±0.09^{e}	57.06 ± 1.56^{d}
	12	96.20±1.18^{a}	6.50±0.11^{d}	2.30 ± 0.12^{c}
	24	94.30±1.01^{a}	4.23±0.38^{b}	1.28 ± 0.07^{b}
	48	89.10±1.21^{b}	1.64±0.53^{a}	0.83 ± 0.03^{a}
	72	73.40±1.93^{b}	0.92±0.93^{b}	0.61 ± 0.46^{a}
	96	65.02±1.48^{b}	0.57±0.27^{b}	0.24 ± 0.09^{a}
	120	61.05±0.16^{b}	0.39±1.53^{b}	0.20± 1.79^{a}
	144	54.61±1.10^{b}	0.26±0.81^{b}	0.19 ± 1.08^{a}
	168	52.70±1.10^{b}	0.05±0.11^{b}	0.19 ± 0.52^{a}

Values represent the means of 15 replicates ± the standard error of the mean ($P < 0.05$). *Data collected after 10 d. of culture. **Data taken after 100 d. (10 passages) of culture

This may because of the interaction of chitosan with the cell membrane altering cell permeability or due to the chelating property of chitosan towards some trace metals that would inhibit their absorption by the explants from culture media (Kong *et al*., 2010).

It is hypothesied that chitosan interferes with the negatively charged residues of macromolecules exposed on the fungal cell surface and thereby changes the permeability of the plasma membrane (Benhamou, 1996). The role of chitosan in the elicitation of several defence-related enzymes was studied by various investigators (Abeles and Forrence, 1979; Inui *et al*., 1997; Vander *et al*., 1998). These enzymes are known to participate in early defence mechanisms and to prevent pathogen infections (Smith, 1996). Chitosan has also been reported to stimulate other systems involved in resistance, such as lipoxygenase and phenylalanine ammonia lyase activities, and lignin formation in wheat leaves (Bohland *et al*., 1997; Vander *et al*., 1998). El-Ghaouth *et al*. (1994) had previously showed elicitation of chitosanase by chitosan in bell pepper fruit and highly acetylated oligomers and polymers of chitosan strongly elicited peroxidase activity in wheat leaves (Vander *et al*., 1998). In our preliminary study, we had found that mixing chitosan with conidia of *B. cinerea* just before applying them to the leaves was much more effective at controlling the disease than spraying chitosan on the leaves (data not shown). Our results in these experiments suggest that the main activity of chitosan is antifungal, acting through a fungistatic effect, a phenomenon previously mentioned in the literature (El-Ghaouth *et al*., 1992). There is evidence that the enzymes b-1, 3-glucanase and chitinase may be responsible for limiting fungal development in cucumbers by inducing systemic resistance (Ji and Kuc, 1996). However, Schneider and Ullrich (1994) did not find good relationships between increases in specific enzyme activities such as those of chitinase and b-1, 3-glucanase, and the induction of resistance in cucumber leaves.

4.2.5.2. Exopolysaccharides (EPS)

4.2.5.2. 1. Estimation of EPS from *B. subtilis*

The EPS in the complex separated by dissociation of high ionic medium. The isolated EPS from *B. subtilis* cultured in LB Broth estimated at 2.82 ± 0.36 mg/100ml of fresh weight and 1.64 ± 0.18 mg/100ml of dry weight.

Carbohydrate and protein estimation for *B. subtilis* indicated an optical density of 0.96 ± 0.07 mg/100ml and 0.14 ± 0.12 mg/100ml respectively. Compared to both, protein estimation in bacteria gave higher optical density.

4.2.5.2.2. Effect of prophylactic treatment of nodal explants with EPS

In order to verify that EPS is truly an effective stimulator of plant defence system, extracted EPS from *B. subtilis* was applied to three bamboo species. The results showed that application of EPS increased resistance against incidence of contamination during initial phase as well as later stages of culture (Table **69**). This could mean that the crude polysaccharide mixture is able to serve as elicitors, so the lower concentration itself can effectively activate the defense system inplants. This experiment confirmed that the EPS at 4 mg ml^{-1} was the optimum concentration for the elicitation and the higher levels exhibited the same effect. Treatment with EPS reduced the endogenous contamination to < 3 % in all three species and it.

Table 69: Effect of exopolysaccharides with different concentrations on *in vitro* growth response and contamination of bamboo species.

Sp.	EPS (mg ml^{-1})	Exogenous Contamination*				Latent contamination**	
		Bacteria%	Days for emergence of contamination	Fungi%	Days for emergence of contamination	Contamination%	Days for emergence of contamination
P. ritcheyi	0	24.02±2.01^{h}	2.00±0.00^{a}	30.19±1.01^{g}	3.97±0.15^{a}	53.5± 1.94^{e}	17.67±0.33^{f}
	2	4.89±0.01^{a}	3.27±0.19^{b}	13.25±0.16^{d}	3.14±0.14^{a}	15.02± 0.72^{d}	58.68±0.02^{b}
	4	4.26±0.00^{a}	6.60±1.27^{f}	2.66±0.03^{a}	5.91±0.02^{c}	2.36± 0.05^{a}	82.42±1.01^{a}
	6	4.14±0.63^{a}	6.67±0.09^{f}	2.51±0.02^{a}	6.01±0.04^{d}	2.22± 0.02^{a}	88.18±1.06^{a}
B. balcooa	0	22.31±0.01^{h}	2.16±0.27^{a}	28.13±0.28^{g}	3.46±0.92^{a}	52.6± 1.02^{e}	15.42±0.28^{f}
	2	4.83±0.06^{a}	3.38±0.46^{b}	12.23±0.11^{d}	3.25±0.17^{a}	16.02± 0.01^{d}	52.64±0.12^{b}
	4	4.61±0.09^{a}	5.78±0.28^{d}	2.56±0.92^{a}	5.27±1.08^{c}	2.78± 0.15^{a}	86.01±0.19^{a}
	6	4.52±0.31^{a}	6.02±0.06^{f}	2.41±0.02^{a}	5.46±0.07^{a}	2.64± 0.02^{a}	86.24±0.02^{a}
P. stocksii	0	23.16±0.48^{h}	2.21±0.63^{a}	31.04±0.25^{g}	3.25±0.08^{a}	56.1± 1.06^{e}	16.24±0.41^{f}
	2	4.62±0.78^{a}	3.52±0.51^{b}	14.04±0.81^{d}	3.58±0.02^{a}	14.65± 0.25^{d}	57.02±0.86^{b}
	4	4.31±0.60^{a}	6.18±0.21^{f}	2.42±0.36^{a}	5.92±0.18^{c}	2.13± 0.06^{a}	88.02±0.01^{a}
	6	4.28±0.01^{a}	6.20±0.20^{f}	2.39±0.14^{a}	5.97±0.62^{c}	2.05± 0.34^{a}	88.67±0.02^{a}

Data represent the percentage of contamination.*Data taken after 10 d of inoculation and ** after 100 d of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

It is known that the induction of plant defence responses depend on the specific combination of the strain and the host plant genotype (Parniske *et al*., 1994). The

EPS identified from the endophyte could significantly stimulate the biosynthetic as well as metabolic pathways. Here the endophytic strain *B. subtilis* was used for the production of EPS. This is because of the wide distribution of the organism in all three-bamboo species and its ability to produce the higher quantity of EPS. The majority of elicitors are polysaccharides, glycoproteins, proteins (or polypeptides), and unsaturated fatty acids (Zhao *et al*., 2005). EPS and flagellin are mainly used as a structural component of the cell recognition during pathogenicity in bacteria. The extracted EPS act only as signaling molecules and play a role in plant defense response of the host without developing disease.

De Pinto *et al.* (2003) reported the effect of exopolysaccharides produced by plant pathogenic bacteria on the ascorbate metabolism in *Nicotiana tabacum.* For endophytes, polysaccharide elicitors would act as "antagonism balancers" between the host plant and the endophyte, promoting a relationship of coexistence (Chen *et al*., 2016). The cell wall component from *Leptosphaeria maculans* can induce defence response of the host, *Brassica napus* (Kim *et al*., 2013).The endophytic fungus *Fusarium oxysporum* stimulated the accumulation of the secondary metabolite, diosgenin in the host *Dioscorea zingiberensis* through the hydrolytic cell wall polysaccharides (Li*et al.,* 2011).

4.2.5.3.. Hydrogen peroxide (H_2O_2)

4.2.3.3.1. Effect of prophylatic treatment of mother plants

Spraying of leaves with H_2O_2 influenced the exogenous contamination as well as the latent contamination form (Table **70**) during axillary bud proliferation of bamboo. Delayed expression and reduced percentage of latent contamination was the significant effect of H_2O_2 leaf spray (Fig.**71, 72** & **73**). While 1 mM has no substantial effect on the contamination in any of the bamboo species both bacterial and fungal contamination was effectively reduced with 10 mM and above of H_2O_2. Spraying of hydrogen peroxide successfully reduced bacterial contamination to < 10 % and fungal contamination to < 14 %.

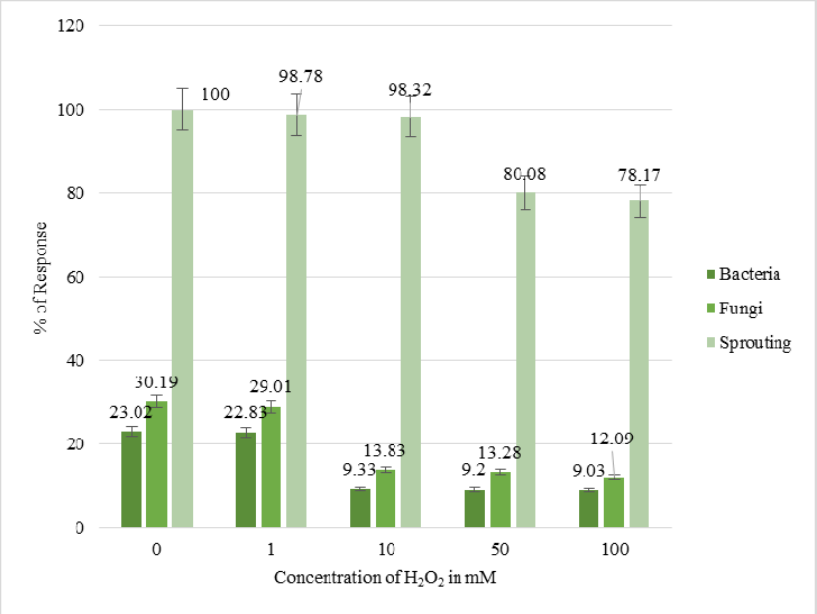

Fig. 71: Effect of prophylactic treatment with different concentrations of H_2O_2 on control of contamination in bamboo species *P. ritcheyi*.

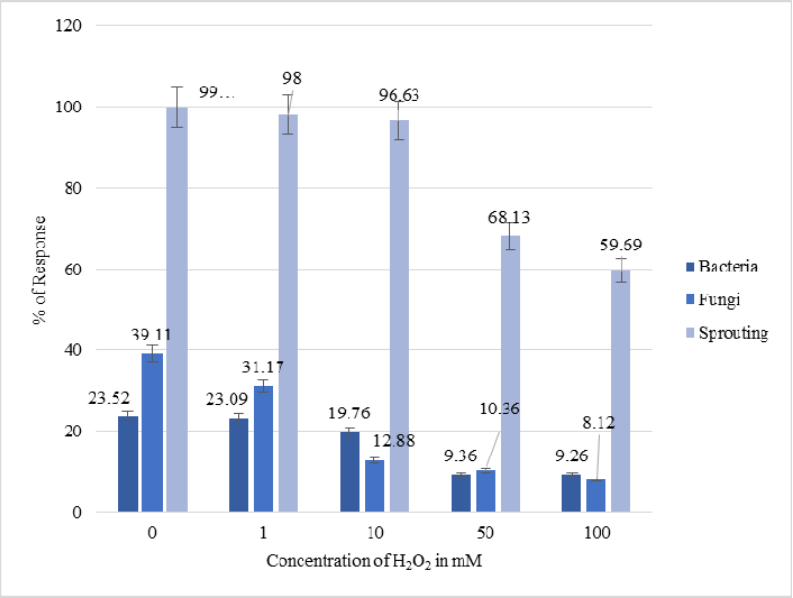

Fig. 72: Effect of prophylactic treatment with different concentrations of H_2O_2 on control of contamination in *in vitro* growth response of bamboo species *B. balcooa*.

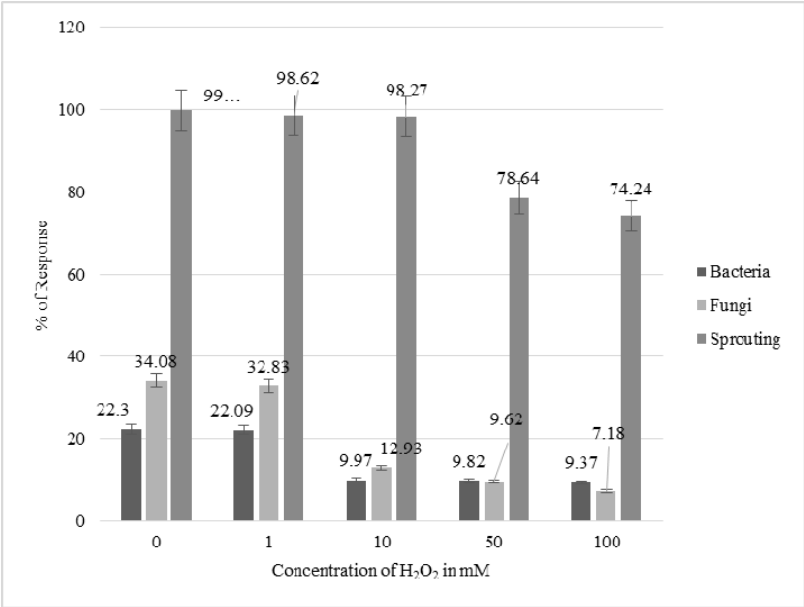

Fig. 73: Effect of prophylactic treatment with different concentrations of H_2O_2 on control of contamination in *in vitro* growth response of bamboo species *P. stocksii*.

Table 70: Effect of prophylactic treatment with different concentrations of H_2O_2 on control of contamination in *in vitro* growth response of bamboo species

Species	H_2O_2 mM	Days taken for bud break	Latent contamination**	
			Contamination %	Days for emergence
P. ritcheyi	Control	3.97±0.15^{h}	53.5± 1.94^{e}	17.67±0.33^{e}
	1	4.06±0.09^{h}	50.6± 0.06^{e}	46.32±2.05^{e}
	10	4.31±1.08^{h}	1.99± 0.28^{e}	82.18±0.26^{e}
	50	4.65±0.28^{h}	1.62± 1.01^{e}	83.64±0.02^{e}
	100	4.99±0.01^{h}	1.48± 0.04^{e}	88.06±0.18^{e}
B. balcooa	Control	3.98±0.22^{h}	56.21± 1.64^{e}	12.33±0.33^{e}
	1	3.98±0.56^{h}	54.44± 0.17^{e}	49.82±1.16^{e}
	10	4.36±0.64^{h}	2.18± 0.09^{e}	80.14±2.13^{e}
	50	4.49±0.17^{h}	1.93± 1.37^{e}	82.06±0.28^{e}
	100	4.76±0.39^{h}	1.79± 0.28^{e}	86.43±1.02^{e}
P. stocksii	Control	3.28±0.82^{h}	52.46± 0.01e	15.80±0.32^{e}
	1	3.78±0.11^{h}	50.22± 1.51^{e}	48.18±9.05^{e}
	10	4.27±0.01^{h}	2.08± 0.16^{e}	84.02±0.17^{e}
	50	4.45±0.06^{h}	1.86± 0.68^{e}	85.52±0.48^{e}
	100	4.96±0.28^{h}	1.41± 0.19^{e}	88.34±0.02^{e}

Data represent the percentage of contamination.*Data taken within 10 d of inoculation and ** after 100 d of culture. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test.

Spraying with10 mM H_2O_2 and higher concentrations effectively reduced the endophytic contamination ≤ 2.18 and delayed the time of emergence up to 80 d. Higher concentrations had no substantial difference from that of the 10 mM H_2O_2

treatment. The frequency of bud break was inversely proportional to the H_2O_2 concentration. Increased concentrations of H_2O_2 reduced the bud breaking frequency to ≤ 68 %. Prophylactic treatments with H_2O_2 had no effect on the time taken for bud sprouting.

4.2.5.3.2. Effect of pretreatment of branch cuttings with H_2O_2

Submerged treatment with H_2O_2 on long shoots gave more effect in controlling the initial contamination (Table **71**). Application of 1 mM H_2O_2 reduced bacterial contamination less than 10 % and fungal contamination to 17 %. Treatment with 10 mM H_2O_2 decreased the bacterial and fungal contamination ≤ 2.16 % and ≤ 6.84 % respectively in all three species (Fig**.74, 75& 76**). Occurrence of latent contamination was delayed upto ~88 d. with rate of contamination ≤ 1.97 %. Exposure of shoots to the higher concentrations of hydrogen peroxide reduced the bud-breaking rate and it overdue to ~7 d. From these two experiments, 10 mM H_2O_2 was selected as the optimum concentration for the activation of plant defence system.

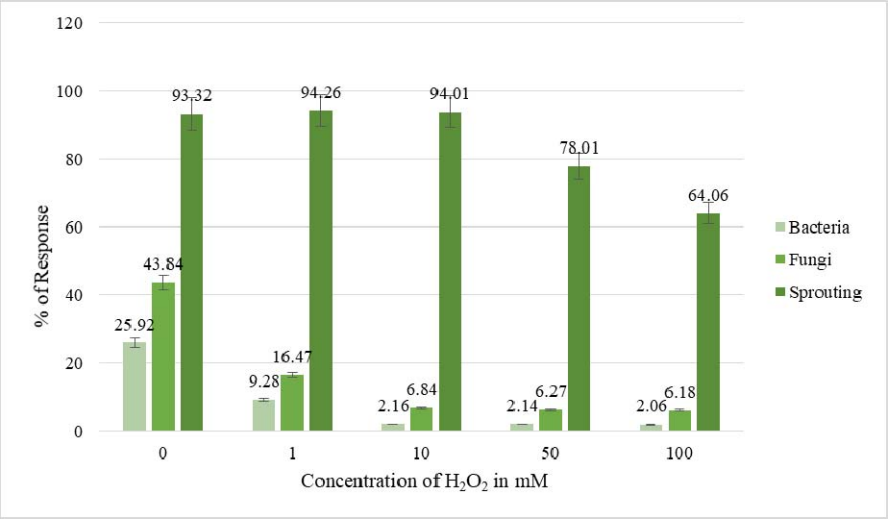

Fig. 74: Effect of pretreatment of branch cuttings with H_2O_2 on contamination and sprouting of explants of *P. ritcheyi* ($HgCl_2$ treated).

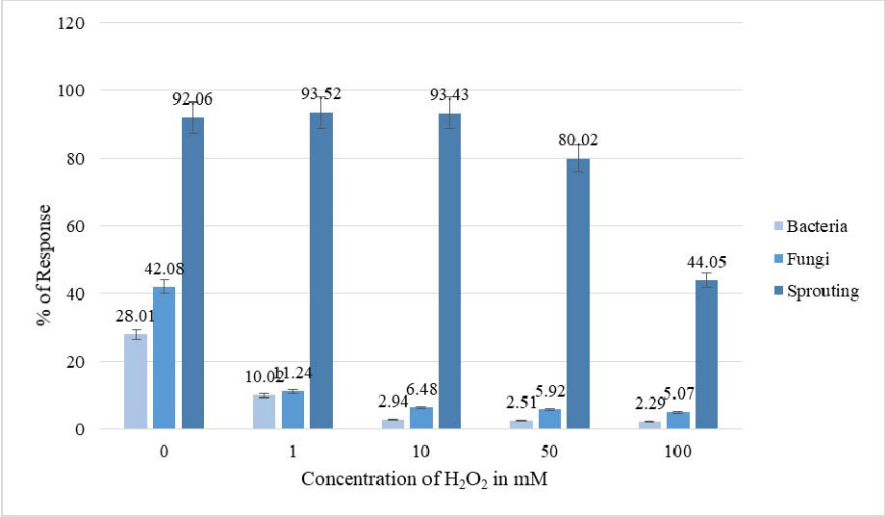

Fig. 75: Effect of pretreatment of branch cuttings with H_2O_2 on contamination and sprouting of explants of *B.balcooa* ($HgCl_2$ treated).

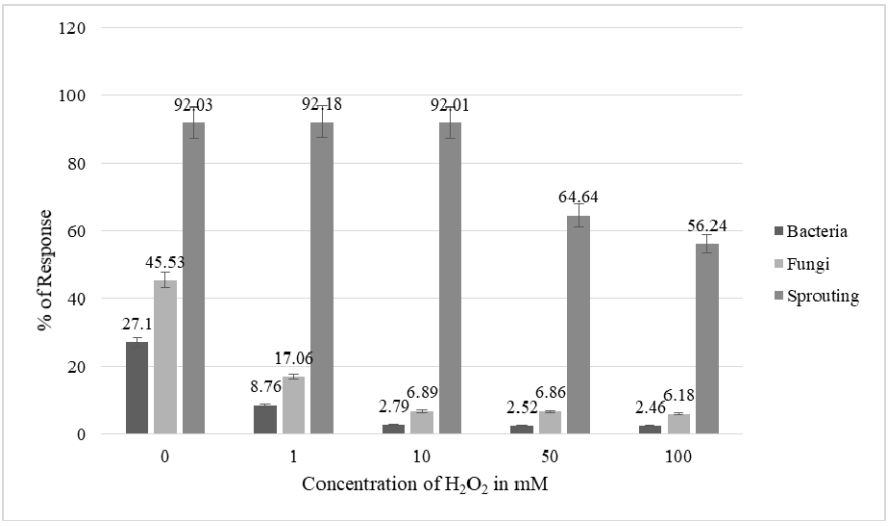

Fig. 76: Effect of pretreatment of branch cuttings with H_2O_2 on contamination and sprouting of explants of *P. stocksii* ($HgCl_2$ treated).

Table 71: Effect of pretreatment of branch cuttings with H_2O_2 on contamination and sprouting of explants ($HgCl_2$ treated).

	H_2O_2 mM		Latent contamination**	
		Days taken for bud break	Contamination %	Days for emergence
P. ritcheyi	Control	3.94±1.79[a]	56.02± 1.08[h]	18.27±0.08[g]
	1	5.47±1.43[b]	10.28±1.28[b]	66.05±2.28[c]
	10	5.72±0.86[b]	1.78± 0.45[a]	88.73±0.97[a]
	50	6.98±2.57[c]	1.43± 1.89[a]	88.74±1.98[a]
	100	7.06±1.84[d]	1.27± 2.08[a]	89.28±0.02[a]
B. balcooa	Control	5.06±0.91[b]	62.09± 0.37[h]	15.04±0.06[g]
	1	5.24±1.93[b]	36.03± 0.64[e]	58.02±1.07[e]
	10	5.36±0.01[b]	1.97± 3.28[a]	83.64±0.84[a]
	50	6.17±4.03[c]	1.72± 2.41[a]	83.85±1.05[a]
	100	6.68±0.05[c]	1.58± 0.22[a]	86.97±0.62[a]
P. stocksii	Control	5.06±0.06[b]	54.95± 1.73g	17.28±0.49[g]
	1	6.01±1.78[c]	20.98± 0.30[d]	54.06±0.43[d]
	10	6.26±0.64[c]	1.88± 1.34[a]	86.41±1.08[a]
	50	6.44±5.91[c]	1.68± 0.05[a]	86.74±1.26[a]
	100	7.06±1.43[d]	1.47± 0.83[a]	88.52±0.12[a]

Data represent the percentage of contamination.*Data taken after 10 d. of inoculation and ** after 100 d. of culture. Means in the column with same letter were not significantly different at p>0.05 level based on mean comparison range using t -Test.

In explants derived from branch cuttings pretreated with H_2O_2 incidence of contamination was reduced to <10 % in case of bacteria and < 20 % fungi. Application of 1 mM H_2O_2 reduced bacterial contamination to < 10 % and fungal contamination to 17 %. Treatment with10 mM H_2O_2 decreased the bacterial and fungal contamination ≤ 2.16 % and ≤ 6.84 % respectively in all three species. Occurrence of latent contamination was delayed upto ~88 d. and only ≤ 1.97 % explants were effected.

Exposure of shoots to the higher concentrations reduced the bud break and it overdue to ~7 d. From these two experiments, 10 mM H_2O_2 selected as the optimum concentration for the activation of plant defence system. Exogenous application of H_2O_2 imparts a stress condition and the plant system responds accordingly. In this study, reduction in the incidence of contamination especially of latent contamination was in explants derived from plants treated with H_2O_2 leaf sprays. The beneficial effect of H_2O_2 treatment was most evident at 10 mM. This method could be useful in explant collection for tissue culture and provides another option to reduce the toxic chemicals for the disinfection purposes.

4.2.5.4. Beta-Aminobutyric acid (BABA)

4.2.5.4.1. Effect of prophylactic treatment of mother plants on control of contamination

Twenty-four hours after BABA treatment, the explants were inoculated into the culture medium. To determine the level of priming and/or direct defence, type of contamination, site of emergence and time taken for emergence etc. were observed. In secondary branches treated with 100 and 200 µg ml^{-1} BABA did not activate the immediate immune system, whereas treatment with 200 µg ml^{-1} triggered plant systemic resistance and it reduced thee latent contamination to < 6 %. However, treatment with 200 µg ml^{-1} and higher concentrations of BABA conferred enhanced levels of systemic defence response and it was indicated by the reduced level of contamination after 10 d. of inoculation both in the case opened and dormant buds of bamboo (Table **72** and Table **73**). Treatment with 100 µg ml^{-1} BABA resulted in 50 % culture loss due to the latent contamination within 10 wks of culture period.

Table 72: Effect of prophylactic treatment (*opened buds*) with different concentrations of BABA on *in vitro*

Sps	BABA (µg/ mL)	Exogenous Contamination*				Latent contamination**	
		Bacteria%	Days for emergence of contamination	Fungi%	Days for emergence of contamination	Contamination%	Days for emergence of contamination
P. ritcheyi	Control	44.52±0.21^{h}	2.00±0.00^{f}	36.68±1.01^{g}	3.97±0.15^{h}	51.24± 0.09^{e}	17.67±0.33^{e}
	100	20.27±1.02^{e}	3.33±0.33^{d}	0.22±0.19^{c}	8.12±0.62^{g}	49.42 ± 0.23^{d}	23.00±1.00^{f}
	200	2.17±0.16^{d}	5.67±0.33^{c}	0.16±0.25^{b}	9.08±2.01^{f}	6.21 ± 1.14^{c}	82.33±0.89^{c}
	300	2.01±1.01^{c}	6.43±0.18^{b}	0.03±0.02^{a}	11.46±0.02^{d}	2.73 ± 1.69^{b}	85.00±2.89^{c}
	500	1.03±0.09^{a}	7.33±0.33^{a}	0.01±0.01^{a}	14.02±0.03^{a}	2.38 ± 1.57^{b}	86.33±0.89^{a}
B. balcooa	Control	43.87±0.81^{h}	1.40±0.25^{g}	45.06±0.21^{g}	3.69±0.02^{h}	57.07± 0.23^{e}	12.33±0.33^{e}
	100	21.9±0.03^{g}	3.98±0.11^{d}	3.96±0.39^{e}	9.05±0.73^{f}	48.36 ± 1.12^{d}	26.67±0.89^{f}
	200	2.31±1.01^{c}	5.04±0.32^{c}	1.96±0.76^{d}	10.98±0.02^{e}	2.89 ± 0.14^{b}	53.33±4.41^{d}
	300	1.81±0.03^{b}	6.23±0.30^{b}	0.43±0.23^{b}	12.04±1.18^{c}	2.06 ± 1.07^{b}	88.33±3.33^{c}
	500	1.24±0.01^{a}	7.82±0.01^{a}	0.32±0.31^{b}	12.97±0.75^{c}	1.98 ± 1.27^{a}	84.33±1.67^{b}
P. stocksii	Control	42.82±0.67^{g}	1.63±0.18^{g}	54.08±1.07^{g}	3.84±0.29^{h}	72.31± 9.01e	15.80±0.32^{e}
	100	20.21±0.56^{f}	4.02±0.15^{e}	6.93±0.12^{e}	8.96±0.63^{g}	46.02 ± 0.12^{c}	28.00±1.00^{f}
	200	2.54±0.01^{c}	5.27±0.60^{c}	2.01±0.17^{d}	10.01±0.74^{e}	2.16 ± 0.09^{b}	46.67±1.67^{e}
	300	1.89±0.02^{b}	6.48±0.02^{b}	0.62±0.01^{b}	12.54±0.98^{c}	2.05 ± 1.18^{b}	88.33±6.01^{c}
	500	1.01±0.06^{a}	7.93±0.01^{a}	0.55±0.04^{b}	13.67±0.67^{b}	1.92 ± 0.12^{a}	89.67±4.41^{b}

Data represent the percentage of contamination.*Data taken after 10 d. of inoculation and ** after 100 d. of culture. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test.

Treatment with BABA effectively decreased the emergence of fungal contamination in all stages. In *P. ritcheyi*, the spraying with 100 µg ml^{-1} controlled the incidence of fungi below 0.22 % whereas the bacteria caused 20 % contamination. In other two species also, the effectiveness of BABA is higher against fungi. However, this concentration was insufficient to induce defence against the latent contamination in all three species (49.42 %, 48.36 % and 46.02 % in *P. ritcheyi*, *B. balcooa* and *P. stocksii* respectively). Traits activated by BABA were not significantly varied from 300 µg ml^{-1} on and therefore it was concluded that 300 µg ml^{-1} was the optimum concentration for activation of defense system in bamboo.

Table73 : Effect of prophylactic treatment (*dormant buds*) with different concentrations of BABA on *in vitro*

Species	BABA (µg/ mL)	Exogenous Contamination*				Latent contamination**	
		Bacteria%	Days for emergence of contamination	Fungi%	Days for emergence of contamination	Contamination%	Days for emergence of contamination
P. ritcheyi	Control	24.02±2.01^{h}	2.00±0.00^{f}	30.19±1.01^{g}	3.97±0.15^{h}	53.5± 1.94^{e}	17.67±0.33^{e}
	100	21.06±8.13^{e}	3.52±0.26^{d}	1.88±0.04^{c}	3.81±0.16^{g}	5.46 ± 0.29^{d}	23.92±0.73^{f}
	200	3.34±1.06^{d}	3.66±1.04^{c}	2.80±0.24^{b}	4.03±0.31^{f}	4.27± 1.02^{c}	81.92±0.14^{b}
	300	3.24±1.29^{c}	3.78±1.17^{b}	1.72±0.14^{a}	4.15±0.02^{d}	1.42 ± 0.38^{b}	84.02±2.07^{b}
	500	3.26±0.01^{a}	3.97±0.24^{a}	1.97±1.06^{a}	4.72±0.18^{a}	1.39 ± 0.02^{b}	86.13±0.02^{b}
B. balcooa	Control	23.52±0.12^{h}	1.40±0.25^{g}	39.11±1.03^{g}	3.69±0.02^{h}	56.21± 1.64^{e}	12.33±0.33^{e}
	100	21.02±0.01^{g}	3.04±0.85^{d}	3.96±0.02^{e}	6.01±1.25^{f}	6.02 ± 0.12^{d}	22.04±0.24^{f}
	200	3.72±0.26^{c}	3.14±0.94^{c}	1.42±1.51^{d}	4.42±1.02^{e}	2.37 ± 0.19^{b}	62.41±0.02^{d}
	300	3.58±1.28^{b}	3.29±0.06^{b}	2.71±0.04^{b}	3.25±1.05^{c}	1.71 ± 0.04^{b}	85.62±0.02^{b}
	500	3.29±1.01^{a}	4.04±2.31^{a}	2.05±0.05^{b}	4.62±0.56^{c}	0.98 ± 1.02^{a}	92.05±0.12^{a}
P. stocksii	Control	22.3±0.11^{g}	1.63±0.18^{g}	34.08±1.26^{g}	3.84±0.29^{h}	52.46± 0.01e	15.80±0.32^{e}
	100	19.43±2.06^{f}	4.02±0.01^{e}	3.79±0.02^{e}	4.18±0.03^{g}	5.68 ± 3.02^{c}	26.05±0.06^{f}
	200	3.54±1.65^{c}	3.38±0.09^{c}	2.82±0.18^{d}	3.46±0.14^{e}	2.74 ± 0.19^{b}	44.02±0.74^{e}
	300	2.84±0.31^{b}	4.01±0.12^{b}	1.69±1.01^{b}	4.51±0.68^{c}	1.96 ± 1.02^{b}	85.21±0.06^{b}
	500	3.64±0.12^{a}	3.93±1.91^{a}	3.62±0.04^{b}	4.60±0.42^{b}	1.91 ± 0.03^{a}	89.08±0.18^{b}

Data represent the percentage of contamination.*Data taken after 10 d of inoculation and ** after 100 d of culture. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t -Test.

Spraying of BABA into plant activates the defence mechanism and reduced the contamination in later stages in all three-bamboo species even in explant stage. When explants were inoculated on BABA containing media, the sprouting response of buds delayed with increasing concentration of BABA without effecting the sprouting percentage. The results clearly show that BABA did not have any antimicrobial action on microorganisms. Incorporation of BABA into the media did not supress the

exogenous contamination, which was effectively suppressed by the prophylactic treatment.

4.2.5.4.2. Effect of BABA amended media on control of contamination

Combination of BABA in culture media led to enhanced resistance of bamboo shoots against contamination. Significant difference was observed in the rate of fungal contamination in initial stage. Whereas the rate of bacterial contamination was reduced to half of that of control treatment. In other words, the initial contamination by both bacteria and fungi was due to the exogenous organisms that escaped from the surface sterilization treatment. These organisms were escaped from the effect of induced systemic resistance. Continuous exposure of the explants in BABA prevent the emergence of endophytes that got into stressed conditions of *in vitro* as contaminants.

Table 74: Effect of amendment of MS media with different concentrations of BABA on *in vitro* growth response of bamboo species

Species	BABA (µg/ mL)	Exogenous Contamination*				Latent contamination**	
		Bacteria%	Days for emergence of contamination	Fungi%	Days for emergence of contamination	Contamination%	Days for emergence of contamination
P. ritcheyi	0	24.31±0.01^{e}	2.00±0.00^{f}	30.42±0.01^{f}	3.97±0.15^{b}	53.5± 1.94^{f}	17.67±0.33^{g}
	10	11.81±0.01^{d}	2.71±0.02^{d}	3.84±1.21^{b}	4.18±0.79^{a}	0.61 ± 9.07^{b}	81.92±1.76^{d}
	20	10.78±0.32^{c}	2.82±0.06^{c}	3.69±0.76^{b}	4.26±1.91^{a}	0.47 ± 0.12^{b}	89.91±0.01^{d}
	30	10.72±2.11^{c}	3.02±0.29^{b}	2.92±1.63^{a}	4.41±0.72^{a}	0.03 ± 1.01^{a}	100.00±2.06^{a}
	50	10.24±1.19^{b}	3.12±0.34^{a}	3.28±6.01^{b}	4.32±1.03^{a}	0.01 ± 0.01^{a}	100.00±0.42^{a}
B. balcooa	0	23.45±2.98^{e}	1.40±0.25^{g}	39.03±1.03^{e}	3.69±0.02^{b}	56.21± 1.64^{f}	12.33±0.33^{g}
	10	10.98±1.25^{c}	1.99±0.73^{d}	2.89±6.05^{a}	3.73±0.21^{b}	0.85 ± 0.98^{b}	86.21±0.04^{d}
	20	10.02±1.06^{b}	2.01±0.16^{c}	3.62±0.01^{b}	4.06±3.02^{a}	0.36 ± 1.37^{b}	87.94±0.36^{d}
	30	10.34±1.13^{b}	2.26±0.42^{b}	2.94±1.74^{c}	4.44±1.07^{a}	0.04 ±0.07^{a}	100.00±0.36^{a}
	50	09.22±0.23^{a}	2.81±0.61^{a}	2.78±0.28^{a}	3.99±0.03^{b}	0.02 ± 0.01^{a}	100.00±0.01^{a}
P. stocksii	0	22.02±0.14^{e}	1.63±0.18^{g}	34.16±1.26^{e}	3.84±0.29^{b}	52.46± 0.01^{f}	15.80±0.32^{g}
	10	10.12±0.22^{c}	2.01±0.01^{e}	2.84±0.08^{a}	3.99±0.23^{b}	0.14 ± 3.04^{b}	78.94±1.27^{f}
	20	11.01±0.01^{d}	2.24±0.03^{c}	3.75±1.63^{b}	4.06±0.21^{a}	0.11 ± 1.06^{b}	89.18±1.03^{e}
	30	09.82±1.09^{a}	2.62±0.12^{b}	2.29±0.91^{a}	3.52±1.42^{b}	0.06 ± 0.81^{a}	100.06±0.01^{c}
	50	09.27±0.02^{a}	2.96±0.16^{a}	3.05±1.65^{b}	3.68±0.72^{b}	0.03 ± 0.02^{a}	100.00±0.01^{b}

Data represent the percentage of contamination.*Data taken after 10 d. of inoculation and ** after 100 d. of culture. Means in the column with same letter were not significantly different at $p > 0.05$ level based on mean comparison range using t - Test.

This resulted in the drop of contamination to half in the case of bacteria and fungi in explant stage. Seven d incubation in BABA containing media induced the plant systemic resistance and reduced the latent contamination < 1 % in all species tested. In addition, it delayed the occurrence of latent contamination only after 80 d of culture. The results (Table **74**) indicated that increased concentration has no particular role on activation, while the minimum quantity of BABA required for the plant defence activation *in vitro* was 30 µg ml^{-1}.

BABA induces defence responses in plants by both physical and biochemical mode of action. BABA has been shown to protect about 40 plant species against about 80 pathogens and pests, including a virus, Protista, bacteria, oomycetes, fungi, nematodes and arthropods (Cohen *et al*., 2016). It is not clear how BABA interacts with plant tissues to increase disease resistance. Application of BABA as foliar spray is more effective and gives quick reponse in terms of numerous biochemical changes such as induction of reactive oxygen species and glycolate oxidase, which tightly linked to defense. Cohen *et al*. (2011) reported that the spraying of BABA to the plant surface induced an oxygen burst in their cells and that will facilitated the cross-linking of BABA-to the cell wall matrix. Siegrist *et al*. (2005) reported that local foliar spray of BABA activated defence system in tobacco plants through the accumulation of salicylic acid.

4.3. *IN VITRO* FLOWERING

4.3.1. Physical factors

4.3.1.1. Effect of photoperiod on *in vitro* flowering

Photoperiod was found to be important factor that influences the flowering phenomenon in shoot cultures of bamboo. Shoot cultures incubated at 12/12 hand 14/10 h showed shortening in newly formed buds. However, a positive correlation between floral spike formation and photoperiod was observed (Table **75**). Significant increase in floral spike number was observed in shoot cultures under 16/8 h. Continuous exposure of the shoot cultures to the 16/8 h photoperiod induced flowering in all the 5 species (*B. tulda:* 40 d., *B. balcooa:* 42 d*., B. nutans:* 46 d*., D. longispathus:* 52 d. and *P. stocksii:* 48 d.). The 14/10 h photoperiod also induced flowering in all these species, but only after 70 d of incubation (*B. balcooa*: 71 d., *B. tulda*: 74 d. (Fig.**77**), *B. nutans:* 82 d., *D. longispathus*: 95 d. and *P. stocksii*: 75 d.). It was observed that shoot culture incubated under ≤12 h, photoperiod had no influence on floral bud development. Different light treatments however did influence the vegetative development in all species tested. Shoot cultures showed an etiolating effect in darkness, but growth promotion was also detected under extended dark period. Shoot cultures under 12/12 h photoperiod were found to be smaller.

Table 75: Effect of photoperiod on *in vitro* flowering in bamboo

Photoperiod (light/dark)	12/12	10/14	14/10	16/8	8/16
Species					
B. balcooa	0	0	1.34 ± 2.63^{d}	2.02 ± 0.58^{a}	0
B. tulda	0	0	1.06 ± 1.09^{d}	2.11 ± 1.73^{b}	0
B. nutans	0	0	1.26 ± 0.24^{d}	1.98 ± 1.22^{c}	0
D. longispathus	0	0	1.11 ± 0.26^{d}	1.65 ± 1.69^{d}	0
P. stocksii	0	0	1.08 ± 2.02^{e}	1.36 ± 1.15^{b}	0

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d. of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Plants have evolved to adapt to the various fluctuations and shifts in environmental conditions through different sensory mechanisms. This adaptation helps them adjust with local environmental demands and this is crucial for their reproductive success.

Fig. 77: Photoperiod induced *in vitro* flowering in shoot cultures of *B. tulda*.

The sensitivity of plants to photoperiod is one of such mechanisms that play an important role in reproductive physiology, primarily being responsible for the shift from vegetative to the reproductive phase. Vaz *et al.* (2004) reported that the incubation of shoot cultures to long day period induced the accumulation of carbohydrates in the shoot tips. This accumulation was due to photo oxidative stress responses such as destruction of the chloroplasts (Jensen and Veierskov, 1998). In *Arabidopsis thaliana*, long photoperiod accelerate the flowering function through the activation of *FLOWERING LOCUS T(FT)* protein, one of the main component of florigen, the systemic floral inducing substrate (Song *et al.,* 2013). Lin *et al*. (2010) reported that many bamboo homologs of *Arabidopsis* flower-related genes were identified in *B. oldhamii* during its *in vitro* flowering stage and those included *LFY, FT, AP1, EMF, FCA* etc. and suggested that it is likely that bamboo also have similar mechanisms that have been accelerated during floral transition.

4.3.1.2. Stress Factors

4.3.1.2.1. Effect of water stress on *in vitro* flowering

The comparison of flower induction efficiency on the MS media with BAP (15 µM) and 3% sucrose with selective agents revealed that the water stress can induce flowering under long photoperiod (16/8 h) conditions (Table **76**). *In vitro* flowering was observed in all species during second subculture period (within 38-40 d.). Flowering frequency between 0.09 % - 3.63 % was observed at different concentrations of agar at different photoperiods. Shoot cultures at 10/14 h photoperiod exhibited the lowest flowering incidence in all species tested (0.09 in *B. balcooa*, 0.38 in *B. tulda*, 1.00 in *B. nutans*, 0.44 in *D. longispathus* and 0.74 in *P. stocksii*). Similar results were obtained in the case of the other two chemicals tested. These results indicate that, the long d. period has strong influence on flowering in bamboo and it strongly interact with water stress induced signals. Flowering was first observed on 32nd d (10 % PEG *B. nutans* at 16/8 h) of inoculation (Fig: **78**), followed by *B. balcooa* in 10 % agar on 35th d. of inoculation (16/8 h). All flowering events occurred within 60th d. of inoculation. After this period, the shoot cultures were seen to undergo severe damage and tissue necrosis.

Fig. 78: Stress induced (PEG) *in vitro* flowering in shoot cultures of *B. nutans* at photoperiod16/8 h.

Amendment of culture media with these chemicals adversely affect shoot proliferation and health of shoot cultures during the first passage itself. Shoot necrosis was minimum on media with 5 % PEG and < 8 % agar. All other combinations caused severe tissue damage. Besides, they brought about shoot abnormalities such as shortening and shoot tip browning in the newly formed shoots. New growth found in all shoot cultures of bamboo has very small leaves in a curled up condition. It also reduced the multiplication rates of shoots in all ranges tested.

It is known that stress due to the insufficient availability of water to the plants induces major physiological changes in the vegetative growth. Toole *et al*. (1984) reported the effect of water potential on stomatal resistance, transpiration rate, net photosynthesis rate, canopy temperature etc. Plants under water stress reduced their photosynthetic capacity by reduced photosynthetic leaf area controlled by hormonal signals generated by roots (Munns, 2002).

This mechanism could be applied to control plant growth (Layne and Tomlinson, 1993; Johnson *et al*., 1997). Alteration of the plant hormonal level might be the reason for flower development in *Eriobotrya japonica* (Cuevas *et al*., 2007). There are other reports that support the link between bamboo flowering and stress (Rai and Dey, 2012; Peng *et al.,* 2013; Ge *et al*., 2016). Peng *et al*. (2013) reported that overall expression level of general stress responsive genes involved in ABA, ethylene, sugar metabolism and Ca^{+2} dependent signaling pathway were 11.1-fold higher than that of the flowering genes in *Phyllostachys heterocyclea* during its flowering period. DNA binding with one finger (Dof) transcription family was up-regulated in the floral transcriptome and it was about 16-fold in the flowering tissues of *P. heterocycla* collected from a drought affected area. Similarly, 28 unigenes related to Dof were detected in the floral transcriptome of *P. edulis* (Gao *et al*., 2014).

4.3.1.2.2. Effect of mineral depletion induced by culture duration

In the experiments where the subculture was extended, a noticeable effect of photoperiod on induction of flowering was visible. Under the photoperiod of 10/14 h, subculture duration of 4 wks could not induce flowering in shoot cultures of any species (Table. **77**). Four wks of culture was not enough to induce a nutrient stress in

shoot cultures and the periodical shifting into the fresh media eliminate the effects of shock evolved within first 4th wk. In the second treatment with 6 wk passages, flowering was induced at a very low rate (≤ 0.8) after third subculture period (after 126 d). Incubation of shoot cultures up to 42 d. (6 wks) in the same media cause transition in the newly emerged shoots whereas immediate transferring of shoot cultures into the fresh media hindered the further development of floral meristem. In the third treatment, where the subculture duration was of 8 wk, flowering was induced in all shoot cultures within the 3rd passage (~ 110 d.) under the 16/8 h photoperiod except in *D. longispathus* where flowering occurred only after 120 d. in all the different photoperiods. This may due to juvenility of the shoots since the culture was developed from germinated seeds. In adult shoot culture, the prolonged incubation in same media upto 56 d., formation of flower bud was seen after 40th d of incubation and their retention in the same media ensure the further development as flower buds. Transferring the culture into fresh nutrient media helped the maturation of floral meristem and converted it into complete flower buds. And it was found that, the rate of flowering in long duration i.e. 6 and 8 wk induced similar in all treatments in all species tested at photoperiod 16/8 and 14/10 h.

Induction of in *vitro* flowering in media with reduced mineral nutrient availability has been reported earlier by Kolar and Senkova (2008) in *Arabidopsis thaliana* and Behera *et al.* (2017) in *Oldenlandia umbellate.* Dixon and Paiva, (1995) reported that poor nutrition increases the PAL activity. Subculture duration was also found to be an important factor for *in vitro* flowering in *Swertia chirayita* (Sharma *et al*., 2013) and importance of subculture duration has been demonstrated by Wang *et al*. (2002) in roses. Gielis *et al.,* (1997) observed that bamboo under stress conditions of high light intensities and drought flowered, while those in more favorable conditions grew vegetative. They also found that superoxide dismutase, ascorbate peroxidase, and catalase, which are generally formed during oxidative stress, were higher in reproductive tissues than in vegetative tissues. Shoot cultures are under stress when overcrowded.

Table 76: Effect of water stress on *in vitro* flowering in bamboo

Treatment		**Agar (%)**			**PEG (%)**			**Mannitol (%)**		
Species		**8**	**9**	**10**	**5**	**10**	**15**	**8**	**9**	**10**
B. balcooa	**10/14**	0.0±0.0	0.0±0.0	0.09±0.1^{e}	0.0±0.0	0.0±0.0	0.38±0.6^{d}	0.0±0.0	0.0±0.0	0.58±0.9^{d}
	14/10	1.08±0.2^{c}	2.01±1.2^{b}	2.16±1.2^{b}	0.0±0.0	0.46±0.7^{d}	1.32±7.2^{c}	0.0±0.0	1.52±1.7^{c}	1.64± 0.2^{c}
	16/8	1.29±0.1^{c}	2.06±0.2^{b}	2.27±0.4^{b}	2.12±0.6^{b}	1.37± 0.6^{c}	3.25±0.5^{a}	1.02±1.8^{d}	1.86±1.2^{d}	1.92±1.0^{bc}
B. tulda	**10/14**	0.0±0.0	0.38±0.6^{d}	1.02±4.2^{c}	0.0±0.0	0.0±0.0	0.78±1.1^{d}	0.0±0.0	0.0±0.0	0.89±0.8^{d}
	14/10	1.16±1.2^{c}	1.25±1.9^{c}	1.76±1.0^{c}	0.0±0.0	1.34±0.6^{c}	1.62±0.2^{c}	1.15±1.2^{d}	1.38±0.4^{c}	1.51±1.0^{c}
	16/8	1.23±0.5^{c}	1.41±1.7^{c}	1.88±0.5^{c}	1.42±0.4^{c}	1.99±0.1^{c}	2.15±0.7^{b}	1.32±0.9^{d}	1.55±0.1^{c}	1.95±0.1^{bc}
B. nutans	**10/14**	0.0±0.0	1.00±1.9^{c}	1.86±0.2^{c}	0.0±0.0	0.0±0.0	0.56±0.3^{d}	0.0±0.0	0.0±0.0	0.86±0.1^{d}
	14/10	1.11±0.2^{c}	1.23±0.2^{c}	3.22+1.6^{a}	1.22±0.2^{c}	2.04+0.3^{b}	2.29+5.1^{b}	1.02±0.4^{c}	1.26±0.1^{c}	1.58±0.3^{c}
	16/8	2.27±1.2^{b}	2.43±1.2^{b}	3.63±0.2^{a}	1.66±0.2^{c}	2.78+1.6^{b}	2.86±0.6^{b}	1.18±0.4^{c}	1.47±0.2^{c}	1.62±0.5^{c}
D. longispathus	**10/14**	0.0±0.0	0.00±0.0	0.44±1.4^{d}	0.0±0.0	0.0±0.0	1.13±1.3^{c}	0.0±0.0	0.0±0.0	0.46±0.2^{d}
	14/10	0.84±2.6^{d}	1.25±0.2^{c}	1.29±1.6^{c}	1.06±0.5^{c}	1.26±0.0^{c}	1.49± 0.1^{c}	2.25±0.2^{b}	1.36±0.1^{c}	1.64±0.1^{c}
	16/8	1.69±1.3^{c}	2.44±1.6^{b}	2.91±1.2^{b}	1.48±1.0^{c}	1.86±0.7^{c}	1.92±1.6^{c}	1.74±0.1^{d}	3.02±0.2^{a}	1.69±1.6^{c}
P. stocksii	**10/14**	0.0±0.0	0.74±1.9^{d}	1.06±2.7^{c}	0.0±0.0	0.0±0.0	1.28±0.1^{c}	0.0±0.0	0.00±0.0	0.56±0.2^{d}
	14/10	1.45±1.9^{c}	1.28±2.0^{c}	1.32±0.4^{c}	1.02±0.3^{c}	1.21± 0.6^{c}	1.64±0.4^{c}	1.28±1.6^{c}	1.46±1.0^{c}	1.62±2.3^{d}
	16/8	1.89±0.6^{c}	1.92±1.1^{c}	1.73±1.2^{c}	1.18±0.1^{c}	1.26±0.2^{c}	1.82±0.1^{c}	1.42±3.1^{c}	1.64±0.4^{d}	1.79±0.2^{d}

* All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Table 77: Effect of subculture duration on *in vitro* flower induction in bamboo shoot cultures

Treatment*		**Subculture duration (weeks)**		
Species		**4**	**6**	**8**
B. balcooa	**10/14**	0.0± 0.08^{b}	0.5± 0.05^{c}	1.0± 0.16^{b}
	14/10	0.9± 0.09^{b}	1.5± 0.06^{b}	1.8 ± 0.73^{ab}
	16/8	1.6± 0.07^{a}	2.2 ± 0.5^{a}	2.7 ± 0.61^{a}
B. tulda	**10/14**	0.0± 0.00	0.6± 0.36^{c}	1.0± 1.26^{b}
	14/10	0.9 ± 0.03^{c}	1.1± 0.08^{b}	1.7± 0.04^{b}
	16/8	1.3± 0.09^{b}	1.6 ± 0.06^{ab}	2.1± 0.06^{a}
B. nutans	**10/14**	0.0± 0.00	0.8± 0.26^{c}	0.9± 1.02^{c}
	14/10	1.3 ± 0.02^{c}	0.6± 0.09^{c}	1.4± 0.04^{ab}
	16/8	1.4 ± 0.04^{c}	1.4± 0.06^{ab}	2.1± 0.07^{a}
D. longispathus	**10/14**	0.0± 0.00	0.6± 0.08^{c}	0.9± 1.23^{bc}
	14/10	1.0± 0.07^{a}	1.5± 0.07^{b}	1.7± 0.04^{ab}
	16/8	1.7± 0.07^{b}	1.9± 0.05^{b}	2.1± 0.09^{a}
P. stocksii	**10/14**	0.0± 0.00	0.2± 0.62^{c}	0.6± 0.72^{c}
	14/10	0.4± 0.03^{a}	0.8± 0.08^{c}	1.3± 0.08^{b}
	16/8	1.3 ± 0.02^{c}	1.9± 0.03^{b}	2.4± 0.04^{a}

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

The rise in shoot proliferation observed with progressive subculture could lead to the development of too many shoots in vessels. Seedling tissues of bamboo are also known to proliferate rapidly, but the changes in their rate of shoot proliferation with *in vitro* flowering have not been reported (Ramanayake *et al*., 2001). Once flowering was induced, there was a drop in shoot proliferation in shoot cultures also reported.

4.3.1.2.3. Effect of mineral depletion on *in vitro* flowering through use of low-salt basal media

The effect of nutrient stress was investigated by abrupt reduction of mineral nutrition on *in vitro* flowering of bamboo. When shoot cultures grown in full strength MS media for several subculture periods were transferred to a diluted medium, the time taken for flower bud appearance was significantly reduced. This result was influenced by photoperiod and the age of the plant material. The bud break was found early in cultures established from axillary buds of adult field grown bamboo which

were incubated under shorter photoperiod. Media dilution of 1/10 was not able to induce flowering in any of the shoot cultures under photoperiod 10/14 h whereas, at other two photoperiods, this dilution effectively induced flowering. Exposure of shoot cultures to 1/1000 dilution enhanced the rate incidence of flowering (5.6) (Table **78**) and reduced the time taken for flowering (within 4 wks) (Fig.**79**).

Nutrient stress generally accelerated bud induction in all species tested and this acceleration was more pronounced in long days. Stressed shoots developed flower buds within 30 d. in the 1/1000 dilution under 16/8 h photoperiod. After transferring into diluted media (1/1000), the shoot cultures exhibited symptoms of nutrient deficiency such as smaller leaf area, greenish young leaves, within 10 d., in all species tested. A delayed appearance of the same symptoms were observed in shoot cultures in other dilutions also. The older leaves become yellow and the length of the shoots decreased to <1.5 cm. Nutrient stress also substantially reduced the rate of leaf formation especially in short d. (10/14) cultures. A reduced multiplication rate was observed in all dilutions except the control. In the control treatment, length of the shoots became ≥ 3.5 cm and shoot multiplication rate was ~ 4.5. Shoot cultures in 1000 diluted nutrient solution had much smaller biomass, leaf area and length at flowering than other treatments. Nevertheless, most of the shoots were able to develop flower buds. Dickens and Van-Staden (1988) demonstrated the inhibitory

Fig. 79: Stress induced (1/1000 dilution of MS media) *in vitro* flowering in shoot cultures of *B. tulda* at photoperiod16/8 h.

Table 78: Effect of low salt strength on *in vitro* flower induction in bamboo shoot cultures

Treatment*		Dilution of the MS Basal media		
Species		**1/10**	**1/100**	**1/1000**
B. balcooa	**10/14**	0.0 ± 0.1^{f}	0.7 ± 0.3^{f}	2.2 ± 0.4^{d}
	14/10	1.2 ± 0.6^{e}	3.3 ± 2.13^{c}	4.5 ± 0.5^{b}
	16/8	2.6 ± 1.15^{d}	4.2 ± 0.21^{b}	5.6 ± 0.28^{a}
B. tulda	**10/14**	0.0 ± 0.5^{f}	1.6 ± 0.4^{e}	2.1 ± 0.2^{d}
	14/10	1.4 ± 0.02^{e}	2.1 ± 0.4^{d}	4.6 ± 0.2^{b}
	16/8	3.0 ± 2.61^{c}	$4.2 \pm 0.4^{a\,b}$	4.9 ± 1.12^{b}
B. nutans	**10/14**	0.0 ± 0.3^{f}	2.1 ± 0.4^{d}	3.7 ± 0.3^{c}
	14/10	1.4 ± 0.1^{e}	2.7 ± 0.1^{d}	4.5 ± 0.7^{b}
	16/8	2.3 ± 0.1^{d}	4.6 ± 0.1^{b}	5.2 ± 0.03^{a}
D. longispathus	**10/14**	0.0 ± 0.0^{f}	1.0 ± 0.0^{e}	2.8 ± 0.3^{d}
	14/10	2.22 ± 0.74^{d}	3.8 ± 0.0^{c}	4.2 ± 0.2^{b}
	16/8	3.33 ± 0.62^{c}	4.22 ± 0.64^{b}	5.6 ± 1.13^{a}
P. stocksii	**10/14**	0.0 ± 0.0^{f}	1.2 ± 3.0^{e}	3.2 ± 0.4^{c}
	14/10	2.56 ± 1.08^{d}	3.78 ± 0.94^{c}	4.6 ± 0.3^{b}
	16/8	3.17 ± 0.32^{c}	4.60 ± 0.70^{b}	5.8 ± 1.51^{a}

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 ds of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$

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action on *in vitro* flowering by higher concentration of nitrogen in the form of ammonium nitrate and potassium nitrate in *Kalanchoe blossfeldiana*. Franklin *et al.* (2000) revealed that ammonium nitrate at lower levels induced *in vitro* flowering and higher concentrations induced the vegetative growth in *Pisum sativum*.

4.3.2. Chemical factors

4.3.2.1. Effect of cytokinins on induction of in *vitro* flowering

Table **79** shows the effect of cytokinins on *in vitro* flowering in bamboo. Incidence of flowering increased with the concentration of BAP. A delay in flowering (by more than 70 d.) was observed at 15 µM of BAP. At 90 µM it induced flowering within 30 d. but the further incubation in the same media resulted the tissue damage and death of the culture. Flowering was induced in MS media supplemented with 45 µM BAP after 6 wks of cultures in all the bamboo species under all photoperiods except 10/14

h and 8/16 h (Fig. **79** & **80**). Maximum numbers of flower (5/culture) were obtained at this condition in *B. balcooa*. All the concentrations of BAP induced flowering in *B. balcooa* at photoperiod 16/8 h. The results indicated that there was a correlation between day length and concentration of cytokinin especially BAP.

Fig. 79: Shoot cultures of *B. balcooa* with *in vitro* flower (MS+ BAP 45µM)

Fig. 80: Spikelet of *B. balcooa* (MS+ BAP 45µM)

Application of TDZ resulted in highest number of shoots (19.07 shoots/shoot clump), but the shoots were dwarfed (2.50 cm being the maximum length attained) and vitrified (glassy shoots). TDZ induced shoot shortening at higher concentrations but also at the lower concentrations in third passage. TDZ induced flowering at all concentrations added at a range of 1.25 of spikelet/cluster. Flowering was observed in all species in all photoperiod in the second passage in higher concentration of TDZ (5 µM). Most of the flower buds induced on this concentration were wilted and a very few were opened. During the flower induction period, vegetative growth of the shoot culture became abnormal with thick and short shoots. Shoot cultures maintained in media with lower levels of TDZ (1 and 2.5 µM) exhibited flowering during their third and fourth passage of the culture along higher shoot multiplication rate (Fig. **79**). However, the prolonged exposure of the shoot cultures of bamboo to the lower

concentration of this cytokinin also induced some affects on their vegetative growth. This indicates that the lower concentration of TDZ is required for the effiecient flower evocation in bamboo shoot cultures.

Fig. 79: Shoot cultures of *D.longispathus* with *in vitro* flower induced by TDZ

It can be seen from these experiments that cytokinin included in a medium play an essential role in the mechanism of flowering in bamboo shoot cultures. They stimulate flowering under conditions of long photoperiods and induced flowering even under completely non-inductive conditions (10/14 h) at higher concentration. Galoch *et al*. (2002) reported that the phytohormones influenced the plant growth correlations within the meristem of an apical shoot and the role of cytokinins is to initiate the cell divisions during flowering in *Pharbitis*. Herbert *et al*. (1992) demonstrated the active cell division was occurred during flowering in *Pharbitis.* Application of exogenous cytokinin in apical meristems of *Sinapsis alba* triggered the mitotic cycle that commonly precedes flowering in some other species also (Bernier *et al*., 1977 and 1988). Jumin and Nito (1996) found that cytokinin applied to branch internodes of flowering plants of *Fortunella hindsii* could induce flowering *in vitro*. Srinivasan and Mullin (1978) revealed that exogenous cytokinin especially BAP stimulated flowering by acting on the endogenous cytokinin in the ascending xylem sap in grapevine through the Scanning electron micrographs. They also explained the influence of concentrations cytokinins and described that with

increasing concentrations of BAP there was a progressive increase in the number of branches and in the dry weight of inflorescences but higher concentrations were found to be toxic.

4.3.2.2. Effect of auxin on induction of *in vitro* flowering

Multiple shoots were rooted when they were transferred to the medium containing auxins. IBA at 4.9 µM induced only rooting and no reproductive growth was found in any shoot cultures. While NAA at three concentrations applied induced both rooting as well as flowering and high rooting and flowering ratio was achieved at 53.8 µM NAA. After 6 wks culture, flowering was occurred in 15 % of shoot cultures and after 8 wks of culture, 50 % of cultures were flowering (Table **80**).

Flower induction property of auxins are comparatively lower than cytokinins and the presence of NAA negatively affected the flower bud formation when cultures were supplemented with cytokinins (Lin *et al*., 2003). By keeping all the parameters such as sucrose concentration and photoperiod as optimum, NAA and IBA induced flowering along with root induction in all shoot cultures. Among the auxins, NAA was found to be more efficient than IBA in induction of *in vitro* flowers; 70% of shoot cultures flowered. *In vitro* flowering by auxins were reported in other species such as *Vigna radiata* (Avenido and Haulea, 1990). Addition of NAA was influenced the flowering in *in vitro* cultures of 'German Red'carnation (Sankhla *et al*., 1994) and *Pisum sativum* (Franklin *et al*., 2000).

Tisserat and Galletta (1988) reported that it is it is essential to add NAA or IBA in the medium for flower induction in *Pisum sativum.* Lin *et al.* (2003) reported the effect of NAA and IBA on *in vitro* flowering in shoot cultures of *Bambusa edulis* and found out that the flowering was occurred during rooting and the flowering ratio increased with incubation period. Reinhardt *et al*. (2000) reported that local treatment with IAA induces flower primordia on inflorescence apices of the *Arabidopsis* Mutant pin1^{-1}

Table 79: Effect of cytokinins on *in vitro* flower induction in bamboo shoot cultures

Treatment*		**Cytokinins**							
Species		**BAP(µM)**					**TDZ(µM)**		
		15	**30**	**45**	**60**	**90**	**1**	**2.5**	**5**
B. balcooa	10/14	0	0	2.02 ± 0.58^{d}	2.16± 1.72^{ab}	1.03± 1.03^{e}	1.00± 0.03^{e}	1.46± 0.28^{b}	1.89± 0.57^{a}
	14/10	2.34± 2.63^{d}	3.46 ± 1.15^{c}	3.48 ± 0.21^{c}	2.06± 0.02^{d}	1.21± 1.73^{b}	1.23± 1.26^{e}	1.68± 1.09^{e}	1.92± 1.06^{a}
	16/8	5.02 ± 0.58^{a}	5.68 ±6.98^{a}	5.82 ±6.85^{a}	2.24 ± 0.62^{ab}	1.42 ± 0.51^{a}	2.01±1.32^{a}	1.94 ±1.21^{a}	1.48± 0.29^{e}
B. tulda	10/14	0	0	2.34± 0.58^{d}	2.11± 1.02^{b}	1.02± 1.94^{e}	0.98± 1.26^{f}	1.24± 0.02^{c}	1.38± 0.09^{e}
	14/10	2.06± 1.09^{d}	3.67± 1.15^{c}	3.98 ± 0.21^{c}	2.21± 0.92^{ab}	1.26± 0.05^{b}	1.16± 0.03^{e}	1.28± 4.63^{e}	1.46± 0.44^{ef}
	16/8	4.81± 1.73^{b}	5.02 ±6.98^{a}	5.52 ±0.42^{a}	2.36 ± 0.02^{a}	1.46± 0.01^{a}	1.98±0.46^{a}	1.96 ±0.76^{a}	1.34± 0.52^{e}
B. nutans	10/14	0	0	2.12± 0.58^{d}	2.00± 0.85^{d}	1.11± 6.02^{bc}	1.12± 0.27^{e}	1.39± 0.02^{b}	1.21± 0.34^{e}
	14/10	1.96± 0.24^{e}	2.98 ± 1.15^{d}	3.72 ± 0.21^{c}	2.23± 1.92^{a}	1.18±0.68^{bc}	1.16± 0.53^{e}	1.32± 1.09^{b}	0.99± 0.18^{f}
	16/8	3.98 ± 1.22^{c}	4.37±6.98^{b}	5.25 ±6.85^{a}	2.37 ± 0.08^{a}	1.25 ± 0.51^{b}	2.01±0.18^{a}	1.46 ±0.87^{b}	1.02± 0.04^{e}
D. longispathus	10/14	0	0	2.26 ± 0.58^{d}	2.22± 0.18^{ab}	1.00± 0.04^{e}	0.86± 1.21^{f}	1.02± 0.06^{e}	1.17± 0.38^{e}
	14/10	2.11± 0.26^{d}	2.98 ± 1.15^{d}	3.48 ± 0.21^{c}	2.14± 0.67^{b}	1.27± 0.63^{b}	1.16± 0.01^{e}	1.29± 0.03^{c}	1.10± 1.02^{e}
	16/8	2.65± 1.69^{d}	3.24 ±6.98^{c}	5.16 ±6.85^{a}	2.25 ± 3.47^{a}	1.32 ± 0.09^{ab}	1.93±0.67^{a}	1.92 ±0.42^{a}	1.08± 0.01^{e}
P. stocksii	10/14	0	0	2.19 ± 0.58^{d}	2.12± 0.46^{b}	1.10± 0.06^{bc}	0.88± 0.16^{f}	1.25± 1.00^{c}	1.16± 1.01^{ab}
	14/10	1.78± 2.02^{e}	2.93 ± 1.15^{d}	3.48 ± 0.21^{c}	2.22± 1.02^{ab}	1.29±0.75^{b}	1.08± 1.26^{e}	1.14± 0.32^{d}	1.51± 0.45^{g}
	16/8	4.36 ± 1.15^{b}	4.56 ±6.98^{b}	5.16 ±6.85^{a}	2.43 ± 0.19^{a}	1.30 ± 0.01^{ab}	1.79±0.36^{b}	1.88 ±0.26^{a}	1.36± 0.11^{ef}

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Table 80: Effect of auxins on *in vitro* flower induction in bamboo shoot cultures

	Treatment*	Auxins					
Species		**IBA(µM)**			**NAA(µM)**		
		4.9	**24.6**	**49.3**	**5.4**	**26.9**	**53.8**
B. balcooa	10/14	0	0	1.26 ± 0.26^{d}	1.21 ± 0.01^{e}	1.58 ± 0.49^{e}	2.04 ± 0.26^{e}
	14/10	0	1.0 ± 1.1^{d}	3.9 ± 0.2^{bcd}	2.8 ± 0.3^{bcd}	2.5 ± 0.3^{bcd}	3.0 ± 0.3^{bc}
	16/8	0	2.4 ± 0.3^{bcd}	4.4 ± 0.2^{cd}	1.4 ± 0.1^{cd}	2.1 ± 0.2^{bcd}	1.0 ± 0.0^{d}
B. tulda	10/14	0	0	2.34 ± 0.58^{d}	0.98 ± 1.26^{f}	1.24 ± 0.02^{e}	1.38 ± 0.09^{e}
	14/10	0	1.7 ± 0.5^{d}	4.8 ± 0.7^{a}	2.6 ± 0.3^{bcd}	1.8 ± 0.31^{d}	1.1 ± 0.04^{e}
	16/8	0	3.7 ± 0.3^{ab}	5.3 ± 0.7^{a}	2.0 ± 0.3^{bcd}	1.6 ± 0.80^{d}	0.9 ± 0.07^{e}
B. nutans	10/14	0	$0.0 + 0.5^{e}$	2.4 ± 0.47^{c}	4.2 ± 0.37^{d}	3.3 ± 0.79^{a}	2.1 ± 0.07^{ab}
	14/10	0	2.0 ± 0.47^{b}	2.6 ± 0.69^{c}	2.5 ± 2.03^{b}	2.9 ± 0.05^{b}	0.9 ± 0.1^{e}
	16/8	0	1.7 ± 0.67^{d}	4.2 ± 1.47^{a}	5.01 ± 1.86^{a}	6.6 ± 0.07^{d}	1.2 ± 0.02^{e}
D. longispathus	10/14	0	0.7 ± 1.66^{e}	1.2 ± 2.15^{c}	1.0 ± 0.0^{b}	1.1 ± 0.03^{b}	0.9 ± 0.07^{e}
	14/10	0	1.23 ± 1.54^{e}	3.8 ± 0.31^{b}	3.3 ± 0.79^{a}	0.7 ± 0.07^{b}	5 ± 1.86^{a}
	16/8	0	4.2 ± 0.37^{a}	4.6 ± 1.45^{a}	3.3 ± 0.79^{a}	0.7 ± 0.07^{b}	4.7 ± 0.5^{a}
P. stocksii	10/14	0	0.3 ± 2.8^{d}	1.5 ± 0.04^{c}	4.2 ± 0.37^{d}	4.2 ± 0.37^{d}	4.2 ± 0.37^{d}
	14/10	0	1.6 ± 0.79^{d}	4.2 ± 0.44^{b}	2.5 ± 2.03^{b}	2.5 ± 2.03^{b}	2.5 ± 2.03^{b}
	16/8	0	3.3 ± 1.6^{a}	5.1 ± 1.02^{a}	5.01 ± 1.86^{a}	5.01 ± 1.86^{a}	5.01 ± 1.86^{a}

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

and they found out that the phenotype of the *pin*1^{-1} mutant formed broad leaf primordia due to the lack of auxin in the apical inflorescence meristem, which prevents flower formation.

4.3.2.3. Different carbohydrate sources on induction of *in vitro* flowering

The effects of different carbon sources on adventitious and floral bud formation were summarized in Table **81**. In the case of glucose, the effects were more or less similar to those obtained with fructose at concentration 30g l^{-1}. The maximum rates of floral bud formation were obtained with sucrose, followed by glucose and fructose. Flowering rates in glucose and fructose were found to be half of the flower induction that induced by sucrose in all shoot cultures. The stimulatory effects of maltose on floral bud formation and its development were rather weak as compared with those of sucrose, glucose and fructose whereas lactose was totally ineffective.

The carbohydrates varied in their ability to support the transition to flowering and found to be necessary for floral induction (Nguyen *et al*., 2006). In general, sucrose was best closely followed by glucose, maltose and fructose were also effective for formation of flowering shoots. Sucrose is not only a source of carbon and energy for plant growth and development, but also has a signaling function and modulates expression of genes that encodes enzymes, transporter and storage proteins. Sharma *et al.* (2013) reported that carbohydrate source was found to be an important factor for flower initiation and maturation in *Swertia chirayita*. This view finds support in the study by Roldan *et al.* (1999) on *Arabidopsis thaliana* in which the flowering was promoted by presence of sucrose in its aerial parts.

Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by moving between shoot and root (Corbesier *et al*., 2002). The interaction of sucrose with light to promote *in vitro* flowering has been reported in a number of species (Jumin and Nito, 1996).

Table 81: Effect of different carbohydrates sources on *in vitro* flower induction

Species		Sucrose	Glucose	Fructose	Maltose	Lactose
B. balcooa	10/14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	14/10	2.34± 2.63^{d}	1.21± 0.28^{e}	1.24± 0.39^{e}	1.01± 0.12^{e}	0.00 ± 0.00
	16/8	5.02 ± 0.58^{a}	1.46± 6.22^{e}	1.52± 0.78^{e}	1.22± 0.35^{e}	0.00 ± 0.00
B. tulda	10/14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
	14/10	2.06± 1.09^{d}	1.04± 0.42^{e}	1.20± 2.02^{e}	1.00± 0.98^{e}	0.00 ± 0.00
	16/8	4.81± 1.73^{b}	1.13± 0.46^{e}	1.41± 0.24^{e}	1.05± 0.06^{e}	0.00 ± 0.00
B. nutans	10/14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
	14/10	1.96± 0.24^{d}	1.00± 0.02^{e}	1.26± 0.01^{e}	0.98± 1.03^{f}	0.00 ± 0.00
	16/8	3.98 ± 1.22^{c}	1.29± 1.06^{e}	1.38± 0.06^{e}	1.04± 0.96^{e}	0.00 ± 0.00
D. longispathus	10/14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
	14/10	2.11± 0.26^{d}	1.06± 1.31^{e}	1.22± 0.02^{e}	0.91± 9.00^{f}	0.00 ± 0.00
	16/8	2.65± 1.69^{d}	1.21± 0.64^{e}	1.37± 1.03^{e}	1.12± 0.06^{e}	0.00 ± 0.00
P. stocksii	10/14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
	14/10	1.78± 2.02^{e}	1.21± 0.28^{e}	1.02± 0.04^{e}	1.20± 0.08^{e}	0.00 ± 0.00
	16/8	4.36 ± 1.15^{b}	1.46± 6.22^{e}	1.11± 0.02^{e}	0.99± 0.02^{f}	0.00 ± 0.00

All shoot cultures were grown on liquid MS media with BAP (15 µM) + Sucrose 3 %. Data represented the number of opened flowers after 90 d of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

In a comparison between different carbon sources, sucrose was found to be best for the development of mature spikelet in *Zea mays in vitro* than fructose and glucose (Parredy and Greyson, 1985). Mohammed *et al.* (2016) too reported that among four different sugars viz. cane sugar, fructose, glucose and sucrose tested at 3 %, sucrose was found to be the best followed by cane sugar and glucose for *in vitro* flower induction in *Andrographis lineata.*

4.3.2.4. Effect of high sucrose levels in media on induction of *in vitro* flowering

Floral bud formation was observed in all shoot cultures with concentrations of sucrose ranging from 30 g l^{-1} to 80 gl^{-1} at photoperiod 14/10 and 16/8. Flowering incidences exhibited a correlation with the concentration of sucrose and maximum flowering was obtained at 5 % of sucrose. All the shoot cultures exhibited flowering within 45 d in all shoot cultures cultured in 5 % sucrose at photoperiod 16/8 (Table

82). Maximum flowering (≥ 4.81) and the maximum flower induction is in *B. nutans* (5.51). Shoot cultures at photoperiod 10/14 exhibited flowering in media with sucrose of concentration 4 % to 7 % and the flowering frequency was observed between 1.68 and 0.01. Incidence of *in vitro* flowering was decreased from 6 % sucrose and effected the shoot morphology. At the highest sucrose concentration (10 %), flowering was totally inhibited in all the shoot cultures. This media adversely effected the axillary bud formation and the shoot cultures rapidly turned brown or red in it.

Significance of sucrose concentration on floral meristem maturation in *Zea mays* was demonstrated by Parredy and Greyson (1985) and they demonstrated that highest flowering efficiency can be achieved applying 100 mg l^{-1} sucrose concentration whereas 0.2 M sucrose was significantly inadequate for overall growth for both vegetative and reproductive stage. Marcelina and Oktawia (2016) stated that the best sucrose concentration to obtain maximum of flowering plants (75 %) with the greatest number of flower per plant (2.6) was 5 % of sucrose for *Celosia argentea.* High frequency of *in vitro* flowering (82.1 %) and maximum number of flowers (14 flowers/shoot) in *Andrographis lineata* was obtained 3 % sucrose (Mohammed *et al.,* 2016).

Table 82: Effect of different concentrations of sucrose on flower induction in *in vitro* shoot cultures of bamboo

		Sucrose (%)							
Species	**Photo-period**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
B. balcooa	10/14	0.00 ± 0.00	2.03 ± 0.58^{d}	2.12 ± 0.58^{d}	2.16 ± 0.62^{d}	1.48 ± 1.15^{bc}	0.01 ±0.24^{f}	0.00 ±0.00	0.00 ± 0.00
	14/10	2.34± 2.63^{d}	2.39 ± 1.15^{d}	2.56 ± 0.21^{d}	2.49 ± 1.02^{d}	1.63± 0.57^{bc}	0.79 ± 0.73^{f}	0.06 ± 0.02^{f}	0.00 ± 0.00
	16/8	3.06 ± 0.27^{c}	3.98 ± 1.22^{c}	5.02 ± 0.58^{a}	4.92 ± 0.45^{b}	2.98± 0.02^{ab}	1.63 ± 1.03^{ab}	1.30± 0.29^{ab}	0.00 ± 0.00
B. tulda	10/14	0.00 ± 0.00	2.00 ± 0.02^{d}	2.54 ± 0.26^{d}	2.25 ± 0.17^{d}	1.13 ± 0.92^{bc}	0.54 ± 0.04^{f}	0.00 ±0.00	0.00 ±0.00
	14/10	2.06± 1.09^{d}	2.42± 0.48^{d}	3.46± 0.09^{c}	2.84± 0.16^{d}	1.98± 0.01^{b}	0.62± 0.67^{f}	0.12± 0.15^{f}	0.00 ± 0.00
	16/8	2.76± 0.03^{d}	3.89± 1.48^{c}	4.81± 1.73^{b}	4.64 ± 0.92^{b}	2.88 ± 0.02^{ab}	1.27 ± 1.64^{ab}	1.09 ± 0.02^{b}	0.00 ± 0.00
B. nutans	10/14	0.00 ± 0.00	1.97 ± 1.27^{e}	2.41 ± 0.01^{d}	2.16 ± 0.04^{d}	1.19 ± 0.73^{bc}	0.04 ± 0.08^{g}	0.00 ±0.00	0.00 ±0.00
	14/10	1.96± 0.24^{e}	2.59 ± 0.56^{d}	3.84 ± 1.11^{c}	2.67 ± 0.73^{d}	1.82 ± 0.01^{b}	0.79 ± 0.37^{f}	0.03 ± 0.16^{f}	0.00 ± 0.00
	16/8	3.91 ± 1.86^{c}	4.02±0.01^{b}	5.51 ± 0.20^{a}	4.38 ±0.36^{b}	2.56 ± 0.02^{ab}	1.23 ± 0.42^{ab}	1.00 ±0.11^{a}	0.00 ± 0.00
D. longisp.	10/14	0.00 ± 0.00	2.12 ± 3.02^{d}	2.36± 0.03^{d}	2.19 ±0.07^{d}	1.24 ± 0.76^{b}	0.68 ± 0.02^{f}	0.00 ±0.00	0.00 ±0.00
	14/10	2.11± 0.26^{d}	2.78± 0.47^{d}	3.48± 0.39^{c}	3.48±0.39^{c}	2.51± 0.06^{ab}	1.01± 0.43^{ab}	0.11± 0.02	0.00 ± 0.00
	16/8	2.65± 1.69^{d}	3.59± 0.13^{c}	5.03 ± 0.14^{b}	3.72±0.02^{c}	2.54 ± 0.27^{ab}	1.48 ± 0.29^{ab}	1.08± 0.09^{b}	0.00 ± 0.00
P. stocksii	10/14	0.00 ± 0.00	1.68 ± 1.62^{e}	2.79± 0.02^{d}	1.84±0.02^{a}	1.99 ± 0.61^{b}	1.99 ± 0.61^{ab}	0.00 ±0.00	0.00 ±0.00
	14/10	1.78± 2.02^{e}	2.58± 0.43^{d}	3.46 ±0.82^{c}	2.62± 0.39^{d}	1.78± 0.02^{b}	1.78± 0.02^{ab}	0.08± 0.16^{f}	0.00 ± 0.00
	16/8	2.69± 0.72^{d}	3.79 ±0.11^{c}	4.99 ± 1.15^{b}	4.00 ± 0.23^{b}	3.01 ± 0.17^{a}	2.02 ± 1.0^{a}	1.00 ± 1.07^{b}	0.00 ± 0.00

*****All shoot cultures were grown on liquid MS media with BAP (15 µM). Data represented the number of opened flowers after 90 d. of incubation. Mean separation was analyzed by ANOVA and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

CONCLUSION

5. Summary and Conclusions

In an effort to develop an efficient micropropagation procedure in the first objective of this study, a step-wise comparison of the two bamboo species, *P. ritcheyi* and *P. stocksii* in plant regeneration through axillary bud proliferation and somatic embryogenesis was done. An efficient micropropagation protocol was developed from nodal explants of field grown culms of *P. stocksii*. Multiple shoots were induced on a modified Murashige and Skoog's solid medium supplemented with 8.88 µM BAP and 2.5 µM NAA and further multiplication obtained through serial subcultures to MS medium with 10 µM BAP and 1 µM NAA and plantlets regenerated through rooting and rhizome induction on 10 µM BAP and 2 µM NAA. Axillary bud proliferation in *P. ritcheyi* was best achieved by inoculating explants on solid MS with 4.44 µM BAP, 1 µM TDZ and 1.25 µM NAA. Solid media was found the best for early bud break in *P. stocksii* but rapid shoot proliferation (6.17 shoots/shoot cluster) was achieved in MS liquid medium supplemented with 4.5 µM TDZ and 1.25 µM NAA. Meta-topolin was effective for overcoming the season-induced dormancy especially in the monsoon for both species, thus enabling the use of nodes throughout the year. Rooting in *P. stocksii* was achieved by *ex vitro* rooting. *P. ritcheyi* proved to be recalcitrant to all the rooting treatments attempted, indicating that novel strategies have to be adopted to overcome the hurdle.

Somatic embryogenesis (95.7 %) was successfully induced on MS supplemented with 2.7 µM IBA and 6.6 µM BAP from calli derived from leaf sheath explants (85 %) and from nodal segments (100 %) in *P. stocksii* on MS media with 2, 4-D (12 µM) and kinetin (9.3 µM). In all of the explants from *P. ritcheyi,* callus induction was obtained but consisted of non-embryogenic fiber like cells. *P. stocksii* was found to be relatively easier to propagate, with plantlet regeneration through both axillary bud proliferation and somatic embryogenesis. In *P. ritcheyi,* shoot necrosis was the major problem during shoot multiplication, which could be overcome by adjusting the pH of the media to the acidic range of 4.5. Further refinement in the procedure and optimization is required to develop a protocol for this species which is recalcitrant to conventional vegetative propagation as well and *in vitro* culture.

Endophytic contamination in the shoot cultures which resulted in latent contamination, was tackled by adopting two strategies – incorporating antimicrobial principles in the media and through activation of the innate plant defense mechanism. The season in which highest incidence of fungal contamination from endophytes in the three selected species, *B. balcooa, P. stocksii* and *P. ritcheyi* was determined to be June-August period, coinciding with the monsoon rains and the lowest during the summer months of March-May. Altogether 73 endophytic microbial isolates were isolated from the three bamboo species and 11 were found to cause latent contamination. *Bacillus subtilis* and *Fusarium oxysporum* were predominant as latent contaminants throughout the year in all the bamboo species. Total eradication of the endophytes from shoot cultures of *D. longispathus* with antibiotic treatment was shown to adversely affect the growth and survival of the shoot cultures and the beneficial effect was demonstrated to be the production of IAA by the endophyte, *Sporosarcina pasteruii*. Therefore retaining the beneficial endophytes in *in vitro* culture is recommended as a general strategy that could be adopted in micropropagation.

In a novel approach to combating endophytes, the incorporation of chemical preservatives used in the food and pharmaceutical industry in tissue culture media was demonstrated to significantly reduce the levels of exogenous contamination as well as latent contamination. Methyl paraben, a common preservative used in cosmetics, gave good control over contamination when added at 0.03 % with the contamination rate reduced to below 2 % in all the bamboo species. Thimerosal, a preservative in vaccines, also gave > 98.97 % aseptic cultures in the three species with < 3.2 % latent contamination. Likewise, benzalkonium chloride was found to be effective for the control of contamination with < 1.6 % fungal and bacterial growth during culture initiation and < 2.81 % in later stages of bamboo tissue culture.

Plant defense activation was found to be a successful strategy for control of latent contamination in shoot cultures of the three bamboo species. Prophylactic treatment of plants mother plants with chitosan at 1.75 % reduced the exogenous contamination of explants from 7.3 % to 3.01 % in *P. ritcheyi;* 8.16 % to 3.04 % in *P. stocksii* and 7.25 %

to 3.35 % in *B. balcooa* and latent contamination from 57.8 to 63.12% to a range of 1.59 % to 2.76 % in *B. balcooa, P. stocksii* and *P. ritcheyi*. Inoculation of unopened nodal explants into the media with 1.75 % chitosan for 48 h. reduced exogenous contamination from 7.22 to 0.2 % in *P. ritcheyi*, 8.16 % to 1.07 % in *P. stocksii* and 7.25 % to 1.03 % in *B. balcooa*. This treatment enhanced the number of shoot cultures without endophytic contamination to 99.82 %, 99.68 % and 98.22 % in *P. ritcheyi, P. stocksii* and *B. balcooa* respectively. When nodal explants with sprouted axillary buds were cultured after prophylactic treatment with 1.75 % chitosan 2 d. before collection, exogenous contamination was reduced from 54.2 % to 2.20 % in *P. ritcheyi*, 56 % to 1.32 % *P. stocksii* and 62 % to 4.26 % *B. balcooa*. Application of exo-polysaccharides isolated from endophytic bacteria to mother plants, 24 h. before explant collection reduced the latent contamination in all three bamboo species studied. Treatment of secondary branches consisting of axillary buds with 3 % H_2O_2 for 5 h. reduced the exogenous contamination to between 0.98 % to 1.11 % and latent contamination to between 0.57 % to 1.88 % in *B. balcooa, P. stocksii and P. ritcheyi* respectively. Application of β -amino benzoic acid (30 µg ml^{-1}) on the leaves of mother plant, one day prior to the collection of nodes, was effective against all the exogenous (0.57 % - 2.01 %) and endogenous (0.28 % - 1.01 %) contaminants in all three bamboo species. This chemical was found to be more effective against fungal contaminants. This procedure had the added advantage that it facilitated the use of sprouted buds for culture initiation especially in the case of *P. ritcheyi*. All these treatments diminished the contamination to < 3 % in all the species during culture establishment, shoot multiplication and root induction stages.

The study on three bamboo species revealed that the presence of endophytes could be beneficial to prevent the emergence of other microorganisms as well as for the growth of *in vitro* shoot cultures followed by better success during acclimatization. Rather than eliminate the endophytes totally, the use of thimerosal, methyl paraben and lactic acid as biostatic additives in the media was found to be a relatively safe strategy for the control of microbial contamination. The second approach of triggering the plant defense mechanism chitosan, exo-polysaccharides constitutes a novel solution to the hurdles faced in establishing sterile cultures in many bamboo species. The present studies have

served to emphasize what has been revealed in recent years of covert organisms that reside in plant tissues and their impact on micropropagation and offered some approaches to manage them. These conclusions are however based on a relatively short-term study and the effects of prolonged culture in the presence of these additives needs to be ascertained before they can be made a standard practice in micropropagation. Only culture indexing with a combination of culture and molecular identification methods can reveal the extent of endophyte presence in cultured tissues especially the covert non-culturable ones.

The final objective dealt with the unique phenomenon of *in vitro* flowering in multiplying shoot cultures of five bamboo species (*B. balcooa, B. tulda, B. nutans, D. longispathus* and *P. stocksii*). Photoperiod was found to be an important factor for inducing *in vitro* flowering in bamboo with the highest incidence occurring under the 16/8 h light/dark period within 40 d. in *B. tulda*, 42 d. in *B. balcooa*, 46 d. in *B. nutans*, 52 d. in *D. longispathus* and 48 d. in *P. stocksii*. The next best photoperiod of 14/10, induced flowering in all these species, but only after 70 d of incubation (*B. balcooa*: 71 d., *B. tulda*: 74 d., *B. nutans:* 82 d., *D. longispathus*: 95 d. and *P. stocksii*: 75 d.). It was observed that incubation of shoot culture for less than 12 h. did not result in floral bud development. The effect of nutrient stress on *in vitro* flowering of bamboo in shoot cultures was investigated by increasing the length of each passage (through delayed subculture) of the shoot cultures and through the abrupt reduction of mineral nutrition by transfer to low salt media. Prolonged incubation of shoot cultures without sub culturing resulted in the depletion of all nutrients in the media. All the shoot cultures studied except *D. longispathus* developed flower buds in the first subculture after the prolonged incubation period of 8 wks. in 16/8 h. photoperiod. Shorter sub culture periods did not evoke any flowering response in any shoot cultures. Their shifting into the fresh media induced only fresh vegetative growth. When shoot cultures were grown in full strength MS media for several passages and then transferred to a diluted medium, the time taken for bud appearance was significantly reduced. A combination of cytokinin and optimum photoperiod induced flowering in all species studied. All concentrations of BAP (15-90 µM) induced *in vitro* flowering in *B. balcooa* at a photoperiod of 16/8 h., but the

incidence of flowering increased at higher levels of BAP. BAP at 90 µM induced flowering within 30 d. and the further incubation in the same combination resulted in the tissue damage and death of the culture. Flowering was delayed by more than 70 d. at the lower levels i.e. 15 µM of BAP. Similarly, TDZ also induced flowering under 16/8 h. at 1 µM and 2.5 µM within 45 d and 30 d respectively. Application of individual auxins at different concentrations had no influence on *in vitro* flowering in bamboo whereas, a combination of NAA and IBA (10 µM and 13 µM respectively) with the photoperiod of 10/14 h. induced flowering in all shoot cultures within 45 d. All of the different carbon sources added at 3 %, except lactose gave *in vitro* flowering in the 16/8 h. photoperiod. Sucrose was best for flower induction in bamboo cultures followed by glucose, maltose and fructose

All the parameters tested viz. photoperiod, stress factors, PGRs and C/N ratio for induction of *in vitro* flowering demonstrated their role in the control of the flowering phenomenon in all the bamboo species. In general stress in *in vitro* cultures is seen to be the important factor that triggers flowering. The implication of the results is perhaps relevant only to the phenomenon of sporadic flowering in some bamboo species and not the gregarious inter-mast flowering in bamboo that seems to be regulated by a biological clock like mechanism that works at long intervals.

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6. REFERENCES

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