Generation of variability for salt tolerance in rice using tissue culture techniques

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CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Generation of variability for salt tolerance in rice using tissue culture techniques "** is based on the original research done by Miss Swapna T S, under my guidance and supervision at the Department of Biotechnology and no part there of has been included in any other thesis for the award of any degree.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	Benzyl adenine
IAA	Indole-3-acetic acid
NAA	Napthalene acetic acid
ABA	Abscissic acid
PAA	Phenyl acetic acid
EDTA	Ethylene diamine tetra acetic acid
KI	Potassium iodide
NBT	Nitroblue tetrazolium
PMS	Phenazonium methosulphate
G_6PDH	Glucose 6- phosphate dehydrogenase

Introduction

INTRODUCTION

Rice is the staple food of more than half of the world's population. It provides 20% of calories and 13 % of protein for human consumption, on a worldwide basis. Rice production has mostly been observed to be affected by soil salinity and in India saline soil covers nearly 4 million ha of land under rice production (Paul and Ghosh, 1986).

The management of drought, salinity and acidity in soils are all energy – intensive agricultural practices in both developing and developed countries. In modern agriculture, it is important to produce plants with increased fitness for available environments. So it is imperative to understand, and to be able to manipulate, the various responses of plants to salt stress. To breed a tolerant line one must be aware of crop genetics and physiology and armed with an efficient screening system based on stable selection criteria extending over to genetic and physiological endowments.

On the basis of growth responses to saline conditions, two groups of plants can be distinguished. The halophytes (salt lovers) that are capable of completing their life cycle at salt concentrations exceeding 300 mM (Flowers *et al.*, 1977), and glycophytes (sugar lovers) which cannot complete their life cycle at salt concentrations exceeding 300 mM. However at low salt concentrations (20 mM to 200 mM) there is an overlap of growth responses between the two groups (Greenway and Munns, 1980).

Halophytes under saline conditions accumulate Na^+ or Cl^- in excess (Flowers and Yeo, 1981). They accumulate salts at concentrations equal to or in excess of seawater in their leaves. Ninety percentage of the total Na^+ absorbed by the plant is present in the shoot, of which eighty percent is in the leaves. Further, they have

similar concentrations of ions in old and young leaves (Flowers and Hall, 1978).

Halophytes exhibit certain morphological and anatomical features in response to salinity (Jennings, 1976). These include increase in leaf succulence, thickening of cuticle, increased lignification, changes in number and diameter of xylem vessels and presence of salt glands which are effective devices for the secretion of salt from the plant body (Helder, 1956; Scholander, 1968). The internal osmotic environment is balanced against the external one and the plants do not suffer physiological drought by osmotic loss of water to the soil medium. However, in order to counteract the salt accumulation mechanism, halophytes must have an internal tolerance of NaCl level, which would be directly toxic to normal plants (Maathuis *et al.*, 1992).

Glycophytes, on the other hand, respond primarily by salt exclusion and they tend to exclude salt from leaves but may accumulate high levels of it in their roots and stems (Flowers, 1975; Flowers and Yeo, 1981). Older leaves may accumulate more than younger leaves and buds. The reasons for such differences between halophytes and glycophytes are not known. The observable effect of salinity in glycophytes, as a consequence of the osmotic effect is reduced growth (United States Salinity Lab Staff, 1954). Extreme salinity results in death, preceded by loss of ionic control in the roots, chlorosis, wilting and leaf necrosis. At sublethal salinity, growth processes, although slowed, are not prevented and developments follow the normal pattern with no visible cell damage (Mass and Nieman, 1978).

An important mechanism for salt tolerance is the accumulation of Na^+ in the vacuole. This is possible by reduction in the cytoplasmic Na^+ level and the osmotic potential of cell sap whereby salt stress avoided. Accumulation of salt in the vacuole does not interfere with plant function physiologically, whereas its accumulation in the

cytoplasm has varied deleterious effects. Oertli (1968) observed that salt injury was caused by the ion accumulation in the apoplast leading to cellular dehydration. Non halophytic species which exhibit some degree of tolerance, avoid excess ions in the cytoplasm or can adapt to high salt concentrations or can produce other osmotica to prevent osmotic water loss (Greenway and Munns, 1980). Non salt tolerant plants, then, would be those that can not restrict cytoplasmic salt build up or modify cytoplasmic components to accommodate accumulation (Raghavaram and Nabors, 1985).

Several reports have shown that in salt tolerant and halophytic species a tonoplast Na^+/H^+ antiport system can be induced to drive Na^+ accumulation in the vacuole (Blunwald and Poole, 1985; Matah *et al.*, 1989). In salt sensitive plants this transport system was not present or it could not be induced (Jacoby and Rudich, 1985). Allen *et al.*, (1997) correlated concentration of Na^+ and Cl^- in leaf with salt tolerance.

SALT STRESS IN CROP PLANTS

Both wet and dry parts of the world have salinity problems. In terms of plant production, salinity can be defined as the excessive concentrations of soluble salts in soils (Richards, 1954). If the salt concentration is high enough to lower the water potential appreciably (as in NaCl stress), the stress will be called a salt stress (Levitt, 1980).

Salinity has a wide range of effects on productivity in different plant families, and legumes are found to be most sensitive and survive only in minimal salt concentrations. Cereals can withstand medium salt levels; whereas forage plants can

survive high levels of salt (Gorham and Hardy, 1990).

In many crops, resistance to salinity is greater during seed germination, than at emergence and during early or later stages of growth and development (Meiri and Shalhevet, 1973; Unger, 1974; Berstein, 1975). Suppression of growth might be attributed to a decreased concentration of cytokinins in leaves and xylem exudates (Ben-Zioni *et al.*, 1967; Itai *et al.*, 1968). However, this alone could not be suggested as a cause for growth suppression as external application of cytokinins had little effect. It may be necessary to consider salt related cytokinin effect in connection with other growth regulating substances (Itai and Vaadia, 1971).

Direct exposure to salt affects several enzymes adversely. Malate dehydrogenase from a halophyte *Suaeda maritima* has two molecular forms. The high molecular weight form remained more active at higher salt concentration than the low molecular weight counterpart. While *in vitro* studies of the effects of salt on enzymes are important, they need to be complemented with experiments to determine whether *in vivo,* the enzymes are exposed to salt (Raghavaram and Nabors, 1985).

There are reports that tissue variability (Harrington and Alm, 1988; Eberhardt and Wegmann, 1989) and banding patterns of isozymes, particularly of peroxidase (Edreva *et al.*, 1989), acid phosphatase (Dubey and Sharma, 1989) and esterase (Gangopadhayay *et al.*, 1995; Harsanein, 1999) were changed in tissues due to different stresses. These effects might result from direct or indirect exposure to NaCl depending on the extent of salt accumulation in the cytoplasm. Enzymes like superoxide dismutase, catalase, peroxidase and glutathione reductase showed significantly higher activities under NaCl stress and chilling stress (Gosset *et al.*, 1994; Lee and Lee, 2000).

Ramanjulu *et al.*, (1993) reported an increase in inorganic salt content with increased salinity. Nucleic acid content decreased sharply with prolonged exposure to salinity. RNA is more sensitive to salt stress than DNA. The ribonuclease activity in susceptible variety was much higher than in the tolerant one (Renu and Dubey, 1991). The protein to RNA ratio was similar in tolerant and susceptible varieties, indicating similar effectiveness of their RNA in protein synthesis. The ratio of RNA to DNA was higher in the tolerant variety. Salinity causes decrease in chlorophyll-a content, increase in chlorophyll b and a marked decrease in nitrogen, phosphorus and potassium content (Parasher and Verma, 1987).

Under stress various types of organic solutes have been suggested to accumulate, which play a role in osmoregulation. Certain solutes are generally produced in plants under salt, drought or osmotic stress (Jefferies 1980; Wyn Jones, 1980; Wyn Jones and Gorham, 1983). The most important of these organic solutes are sugars, free amino acids, methylamines and polyhydric alcohols. Unlike salts these do not inhibit *in vitro* enzymes even at a concentration of 500 mM (Greenway and Munns, 1980). Saline stress in vegetative stages of cereals and pulses causes an increase in polyamine content and concomitantly an enhancement of arginine decarboxylase activity (Ghosh *et al.*, 1991). Contrary to this, Kao (1997) reported that polyamine levels are not associated with stress. Plants regenerated from NaCl adapted callus showed higher endogenous free proline (Sangita *et al.*, 1997).

According to Singh *et al.*, (1989) NaCl adapted cells exhibit several physiological and biochemical changes. Salt tolerant cultivars of pepper and cotton accumulate much of the Na^+ in leaves and sensitive ones avoid Na^+ accumulation (Cornillon and Palloix, 1997; Leidi and Saiz, 1997). Leidi and Saiz (1997) postulated

that higher tolerance is the result of several traits such as a higher Na^+ uptake and water content. Higher K^+/Na^+ ratio in leaves of the tolerant variety indicates its greater capacity for adaptation to salinity. Adaptation through adequate, but tightly controlled ion uptake, typical of some halophytes, matched with efficient ion compartmentation and redistribution, would result in an improved water uptake capacity under salt stress and lead to maintenance of higher growth rates (Niazi *et al.*, 1992).

When plants or plant cells experience changes in environmental conditions, a system of physiological responses is set up that involves integrated changes in nutrient, water, carbon and hormonal balances. These responses are mediated by the expression of specific genes, which leads to an improved adaptation to environmental changes. A number of studies on regulation of gene expression during the imposition of a stress indicate similar gene products, which can also be induced by the exogenous application of abscisic acid (Skriver and Mundy, 1990). Alterations in gene expression were demonstrated by observing the synthesis of several novel proteins including osmotin in the adapted cells. The synthesis of osmotin was found to be induced by exogenous abscissic acid in tobacco (Singh *et al.*, 1989).

EFFECT OF SALINITY ON RICE GROWTH AND TOLERANCE MECHANISMS

The effect of salinity on the growth of rice was found to be related to a number of factors (Akbar *et al.*, 1986). Many reports showed that, there were marked differences in response to salinity at different growth stages. Rice was found to be tolerant to salinity at the germination stage. Apart from a delayed germination, salinity was found to reduce the germination percentage (Akbar and Yabuno, 1974).

Early seedling stage of rice was found to be highly sensitive to salinity (Kaddah and Fakhrys, 1961). Tolerance of rice seedlings to salt stress was found to be progressively increase from 1 to 6 weeks of age. At the tillering stage, parameters such as plant height, tiller number, straw weight and dry weight of roots were affected by salinity. In the flowering stage, however, grain yield was more affected than vegetative growth. The tillering stage is more sensitive to salinity than grain filling stage and adverse effect of salt stress is reflected on grain yield (Gill and Singh, 1989). Salinity reduces length of roots, height of seedlings, emergence of new roots and cell division (Akbar *et al.*, 1986; Sultana *et al.*, 2000), suppresses leaf elongation and formation of new leaves and reduces DNA and RNA content (Renu and Dubey, 1991). But salinity causes accumulation of stress induced proteins (Moons *et al.*, 1995) and soluble sugars (Derbey and Singh, 1999). Change in activities of enzymes like malate and glutamate dehydrogeneses in salt sensitive varieties, might be one of the possible reasons for decreased growth of rice plants under stress (Kumar *et al.*, 2000).

Salt tolerant wild rice (*Oryza coarctata*) accumulated large amounts of amino acids under salt stress (Bal, 1975), higher level of dry matter, chlorophyll and protein as compared to cultivated varieties of paddy (Gill and Dutt, 1976), and produced stress proteins at different developmental stages (Parrek *et al.*, 1999).

Higher salt tolerance in rice might be associated with high electrolytic content in root and shoots, which can be achieved by a balance between roots and the soil. Accumulation and retention of ions against transfer to shoots could have probably enabled an osmotic balance by generating harmless soluble organic substances like proline (Storey and Wyn Jones, 1977) glycine and betaine (Krishnamoorthy *et al.*,

1987). With the increase in salt concentration the accumulation of proline increased, and this increase was higher in salt susceptible varieties than in salt tolerant varieties. Proline accumulation might possibly be only a plant response or symptom of stress and need not be indicative of osmotic tolerance (Berstein, 1961). In rice, accumulation of proline was reported to be more in salt tolerant than in salt sensitive cultivars and has been implicated to confer tolerance to salinity stress (Prakash and Padayatty, 1989). Tolerant cultivars showed higher protease activity, which increased further under salt treatment whereas higher salinity was inhibitory to the enzyme in susceptible cultivars (Dubey and Rani, 1987).

BREEDING FOR SALT TOLERANCE

Traditionally, the development of new varieties has depended on the efforts of plant breeders. Conventional approaches involving selection and hybridization have been important for breeding salt tolerant rice varieties. Experienced breeders have selected some useful mutants and hybridized them to combine the characteristics of both parents in the progeny. The most significant example is the semi dwarf gene of rice utilized in China, (Huang *et al.*, 1960) and at IRRI (Chang *et al.*, 1965) resulting in the green revolution. Although 50 % of the crop yield increase during the past 50 years has been attributed to genetic improvement, the efficiency of conventional breeding is low because of the long term of selection and back crosses, the large scale of field trials and the labour intensity (Evans and Sharp, 1983). Some of the existing tolerant crops have come accidentally into agricultural use as a result of breeding experiments for other characters. The identification and isolation of salt tolerant germplasm in related species of existing crop plants is a basic requirement in a

breeding program for salt tolerance (Akbar *et al.*, 1972; Akbar and Yabuno, 1974).

Genetic differences for salt tolerance have been reported in tomato, wheat, barley and rice (Rush and Epstein, 1976; Epstein *et al.*, 1980; Abo-Elenin *et al.*, 1981; Flowers and Yeo, 1981).

Among the cereal crops, barley is found to be most tolerant to salinity followed by wheat and oats. Maize is less tolerant when compared to others. Rice is included under moderately tolerant species with considerable variations in its tolerance depending on variety (FAO/UNESCO, 1973). Of the 60,261 rice varieties screened for salt tolerance, 10,369 varieties, including several traditional varieties such as Getu, Pokkali and Kalarata, showed this character (Ponnamperuma, 1984).

Breeding efforts involving the traditional rice variety Pokkali have met with moderate success. The first variety developed in Vyttila Rice Research Station, Vyttila, Kerala, was a selection from Chuttipokkali called Vyttila-1 and subsequently four saline tolerant rice varieties viz., Vyttila-2, 3, 4 and 5 were produced. Vyttila- 2 also was evolved through selection and Vyttila 3 and 4 were evolved through hybridisation. Vyttila- 3 is a hybrid of Vyttila- 1 and Taichung Native- 1 and having yield potential of 4-4.5 tons/ha. with a flowering duration of 115 days. Vyttila- 4 is a hybrid of Chettivirippu and IR.4630-22-2-17 having a yield potential of 4.5 - 5.5 tons/ha. with a duration of flowering of 120 days. Vyttila - 5 is a mutant of Mashuri with grain quality same as Mashuri and yield potential of 4.5 - 5.5 tons/ ha. It showed salt tolerance similar to Pokkali.

Novel techniques like tissue culture and somaclonal variation offer great potential for crop improvement. For a long time, tissue culture has been recommended as an extremely potential method for vegetative propagation of plant species.

However, due to the frequently observed variability in plant populations raised through tissue culture, it appears that, tissue culture may not be a safe method for cloning plant species, while it may be a rich and novel source of variability with great potential in crop improvement.

Cultivars with increased salt tolerance can potentially be isolated by several methods, apart from conventional and mutational breeding.

a. Tissue cultures, on solid or liquid medium, with or without specific selection procedures as a population base for isolation of spontaneous or induced variants.
b. Anther culture and protoplast culture as a source of somaclonal variants.
c. Techniques of gene isolation and gene transfer from one cultivar, species or genus to another for increased tolerance (Raghavaram and Nabors, 1984).

Compared to the normal back cross program, somaclonal and gametoclonal variations provide scope for broadening of variation and the acceleration of the breeding process (Kuksova *et al.*, 1997). The term somaclonal variation, used to indicate variability manifested in regenerated plants due to genetic modification, was coined by Larkin and Scowcroft (1981). Earlier reports claim a clonal uniformity among plants regenerated as a rule, but of late, clonal uniformity is mostly recognised as an exception rather than a rule (Scowcroft, 1985). Larkin and Scowcroft (1981) were of the opinion that genetic variability in the tissues of original explant and/or that passages through tissue culture itself caused mutations, rearrangements, deletions, enhanced somatic recombination, chromosome loss, polyploidisation etc. These plants regenerated at random from culture must be examined in the field for useful characters and selected or added to breeding populations for increasing genetic

variation. Further, variations occurring in cultured cells have enhanced the widely heralded expectations for genetic improvement of crop plants by application of cell culture screening methodology.

Haploids play an important role in the rapid development of homozygous lines and in genetic manipulation especially in isolation of desirable mutants in the very first generation. The production of new cultivars by haploid breeding reduces the breeding cycle, raises the efficiency of selection and saves space and labour in the experimental field. Anther culture of rice has been carried out since 1968, when Niizeki and Oono first recovered plantlets from anther culture of rice. Now large number of haploids and spontaneously doubled haploids can be obtained from many cultivars and hybrids. Investigation of pollen plants showed abundant variation (Sathish *et al.*, 1995). The character segregation of pollen plant population provides diversity in genotypes and thus frequency of variation in anther culture is very high (Limei-Fang *et al.*, 1981; Antoinemichard and Beckert, 1997). Breeding for inter varietal hybridization has been improved using anther culture techniques. Doubling the chromosomes of haploid pollen plants has been used for early stabilization of segregated character in hybrid progeny (Limei–Fang *et al.*, 1981; Yang, 1997).

The application of anther culture technique for improvement of rice varieties is hampered by the difficulty in inducing morphogenesis, either directly from cultured microspores or indirectly from callus derived from microspores. Further, the callus produced frequently loses its plant regeneration ability with time in culture, making studies on selection of cell mutants difficult when longer periods of *in vitro* culture are required. Another difficulty is that when cultured *in vitro*, all rice varieties do not respond equally in producing callus and in regenerating plants. This could be due to

genetic and environmental characteristics of different varieties (Guha, 1973). The critical factor affecting androgenesis of rice, is the genotype (Chen *et al.*, 1986). The order of culture ability (% of callus initiation x % of regeneration) was japonica/japonica - indica/japonica - indica hybrid - indica/indica (Shen *et al.*, 1983). Even within subspecies, e.g., japonica rice, the culture ability is different among cultivars. Some problems, which hinder the wider application of anther culture technique to breeding, especially in indica rice, are low frequencies of callus initiation and plant regeneration, high frequency of albino plants and high frequency of haploids. So it is necessary to pay more attention towards improving the methodology of callus culture, regeneration of plants and anther culture. Callusing response of anthers is controlled by various factors like medium, genotype, pre treatment of anthers etc. (Chen *et al.*, 1986: Karsai and Bedo, 1997; Xie *et al.*, 1997; Bishnoi, 2000).

Salinity affects many aspects of plant metabolism, and accumulation of various organic solutes that contribute to turgor maintenance. The relative rates of protein synthesis and the stability and expression of specific proteins are altered when cells are exposed to salinity stress (Ramagopal, 1986). Available information suggests that the characterization of biochemical-genetic markers for salt resistance might aid breeding programs for the development of salt resistant plants, by reducing the time needed for screening. Selection of plants, based on gene product level, may increase the accuracy of genetic selection due to reduced involvement of environmental factors, affecting the conventional selection.

The occurrence of isozymes came under extensive investigation after the development of zymogram technique by Hunter and Markert (1957). This technique

involves electrophoretic separation, followed by demonstration of zones of enzyme activity using specific histochemical staining procedures applied directly to the electrophoretic medium i.e., poly acrylamide. Isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring within the same organism, and since every enzyme is encoded by a gene and every gene might mutate to produce functional alleles, every enzyme may eventually exist in isozymic forms. Further, changes in isozyme patterns reflects the changing patterns of gene activation and repression (Markert and Moller, 1959). Isozymes were perhaps the best for probing the detailed metabolic activities of cells in discharging their physiological functions, because alternative isozymes like alternative alleles, allow us to examine the normal condition by contrast with a variant condition. In fact, since alleles are commonly expressed as isozymes, both a genetic and biochemical comparison is possible (Markert and Mollar, 1959).

Isozymes were used as markers for somaclonal variation (Bergman and Mann, 1974; Bassiri, 1976), embryogenesis (Ahn *et al.*, 1985; Fransz *et al.*, 1989; Alves *et al.*, 1994), regeneration (Samantaray *et al.*, 1999) and developmental variation (Scandalios, 1974; Rao *et al.*, 1992). Gangopadhayay *et al.*, (1995) used the banding pattern of certain isozymes like esterase, peroxidase and acid phosphatase in *Brassica juncea* to identify some ubiquity of stress markers among different stresses. Changes in isozyme banding pattern were studied in hybrid rice progenies (Zhang and Tang, 1986).

OBJECTIVES OF THE PRESENT STUDY

From the literature, it is understood that biotechnological methods such as callus culture and anther culture could be used to generate salt tolerant variants in rice varieties as alternatives to conventional breeding programs. Further, use of isozyme markers could facilitate rapid identification of salt tolerant variants for study and use in breeding programs.

In this context the present study primarily addresses two major areas of concern with respect to development of salt tolerant rice varieties in South India.

1. Probability of generation of somaclonal and androclonal variants,
2. Use of isozymes as markers for salt tolerance in rice cultivars.

In the present study four rice varieties namely, Pokkali (Vytilla-1) MI 48, Annapoorna and Jyothi were used. Pokkali is one of the most salt tolerant rice variety identified for the acid saline conditions (Ponnamperuma, 1984). The variety is grown in the coastal belt of Kerala in an unique system of paddy cultivation known locally as 'Pokkali cultivation' or 'saline farming', which covers an area of 22,000 ha in the Ernakulam and Alappuzha districts. The salient characteristic of the rice fields in this belt is that rice can be cultivated only during the monsoon season (low saline phase) which lasts from June to mid-October. From November onwards, salinity builds up and high salinity levels in water makes paddy cultivation impossible (George, 1993).

MI 48 is a highly salt sensitive variety, which has similar vegetative characters as that of Pokkali. Annapoorna and Jyothi are salt sensitive varieties but have good yield. Attempts were made to generate somaclonal and androclonal variants in the selected rice varieties and to identify suitable isozyme markers for salt tolerance.

Specific objectives of the present study include the following:

- To generate salt tolerant variants from callus and anther cultures of rice.
- To study changes in isozyme banding pattern during developmental stages in different rice varieties in order to find a suitable isozyme, which can be used as a molecular marker for salt tolerance in rice.
- To investigate the variations found in plants regenerated from callus culture and anther culture using isozymes.
- To detect isozyme variations in the plants from F1 generation, obtained by hybridization between salt tolerant variety and sensitive varieties.

This study is being presented in two sections for ease of expression. The first section deals with the generation of somaclonal and androclonal variants while second section includes work done in identification of a isozyme marker for salt tolerance and testing of somaclones and F1 hybrids using this marker.

Section I

Generation of Somaclonal and Androclonal variants

Review of Literature

1.1 REVIEW OF LITERATURE

1.1.1 TISSUE CULTURE AND SOMACLONAL VARIATION

Regeneration of plants from cells, especially from cereal plant cells, has proven to be difficult. However, regeneration is a necessary step when tissue culture methods are used for crop improvement. It appears that the materials themselves, rather than medium components, is more instrumental in callus induction and green plantlet regeneration (Abe and Futsuhara, 1986).

Callus can be induced from almost any part of the rice plant. For instance, rice plants have been regenerated *in vitro* from roots (Abe and Futsuhara, 1986), coleoptile tissue (Oinam and Kothari, 1995), shoot (Tsai *et al.*, 1978), leaf sheath (Abdullah *et al.*, 1986), etc. Search for promoting factors continue, especially with regard to those capable of promoting regeneration. These include various organic additives, plant hormones, as well as amino acids. Chowdhry *et al.*, (1993), reported the promotive effect of proline and tryptophan and Singh *et al.*, (1993) reported the promotive effect of tryptophan and riboflavin on the frequency of callusing and regeneration. Abscisic acid also had a promotive effect in embryogenesis (Yang *et al.*,1999: Guzman and Aris, 2000)

Somaclonal variants with increased salt tolerance have been produced in many species (Evans and Sharp, 1983; Bhattacharya, 1991; Miah *et al.*, 1991; Gosset *et al.*, 1994; Tal, 1996). Phenotypic variation in plant height, maturity, panicle length and number of grains per panicle (Mandal and Gupta, 1997) and NaCl adapted callus of a salt sensitive scented indica variety of rice has been reported (Sangita, *et al.*, 1997).

1.1.1.1 Origin of somaclonal variation

Some of the variations existing in regenerating plants might reflect pre-existing heterogeneity in the explant. However, most of the variations occur during the culture process. This is obvious in the changes in frequency of chromosomal abnormalities with time in culture. Meiotic analysis in maize have shown that there were no chromosomal abnormalities in plants regenerated after 3 to 4 months in culture, while those regenerated after 8 to 9 months were cytologically abnormal (Lee and Philips, 1987).

1.1.1.2 Chromosomal abnormalities

The most frequent cause of somaclonal variation is chromosomal rearrangement. The types of rearrangements are deletions, fusions, and interchanges as well as changes in ploidy level (Larkin, 1987). These aberrations cause changes in phenotypic expression of one or more genes. One of the most dramatic example of changes in chromosome size, number and DNA content is in *Scilla siberica* (Deumling and Clemont, 1989). The cause of high frequency of chromosomal abnormalities during culture is yet to be understood. According to one suggestion, chromatin diminute before the cells become competent to regenerate. Another theory suggests that chromosome breaks may be induced in culture by the late replication of heterochromatin (Johnson *et al.*, 1987; Lee and Philips, 1987).

1.1.1.3 Gene amplification and diminition

In plant genome, with repetitive sequences being present as both tandem arrays and dispersed sequences, low copy number sequences are present. Changes during the process of culture and regeneration could be in both, the number of copies of a sequence as well as in its state of modification. The ribosomal RNA genes are

examples of a tandemly repeated set of genes, which are frequently altered in culture. Changes in this family have been demonstrated in flax (Cullis and Cleary, 1986), triticale (Brettell *et al.*, 1986) and pea (Cullis and Creissen, 1987).

1.1.1.4 Transposable elements

One of the consequences of a chromosome breakage in maize crosses is the activation of transposable elements (Mc Clintok, 1951). Apparent activation of transposable elements has been found in studies with maize (Peschke *et al.*, 1987) and alfalfa (Groose and Bingham, 1986). It is still not clear whether the activation of transposable elements was the major cause of somaclonal variation in systems, other than maize and alfalfa, or even in maize and alfalfa themselves.

Not all genotypes within a given species might respond to culture in the same way. The response of flax genotypes to a cycle of culture was very variable, both at the phenotypic level and at the level of genomic responses (Cullis and Cleary, 1986). Some lines varied in a range of DNA families, while others appeared to be completely stable. The stable forms were also those which had the lowest DNA amount of the repetitive sequence families.

The major significant demonstration of the potential value of somaclonal variation was provided by Shepard *et al.*, (1980) in case of potato, where somaclones with more resistance to *Alternaria solani* and *Phytophthora infestans* were recovered. Somaclonal variation affects many important characters and shows promise for improvement of varieties particularly those with single defects (Larkin and Scowcroft, 1981). Palit and Reddy (1990) reported selection of calli resistant to *Pyricularia oryzae* in rice. Somaclones with aluminium tolerance in maize (Moon *et al.*, 1997) and herbicide tolerance in wheat (Bozorgipour and Snape, 1997) have also been

reported.

Somaclonal variants for a number of characters like tiller number, height, etc., were recovered in many cereals and seed propagated crops (Oono, 1978; Larkin *et al.*, 1983; Pring *et al.*, 1981; Mandal and Gupta, 1997). Genetic nature of herbicide resistance and salt tolerance was studied by Kinoshita and Mori (1991). Promising stress tolerant somaclones were produced from an indigenous cultivar Pokkali (Mandal *et al.*, 1999). Bajaj *et al.*, (1980) and Bajaj and Bidani (1980) have reported variations in chromosome numbers from 11 to 60 and also changes in ploidy levels.

Primary regenerants of rice were reported to express phenotypic variants. The variants observed were in traits-plant height, number of fertile tillers per plant, panicle length, number of fertile seeds, and flag leaf length (Nishi *et al.*, 1968; Henke *et al.*, 1978; Mohmad and Nabors, 1990). Morphological variants such as branched spikes, basal tillering, compact multiple branched spike, split spike and branched tiller were confined in regenerated plants of rice (Padmaja, *et al.*, 1993). Regeneration potential of callus from various rice varieties were studied by Seraj *et al.*, (1997).

1.1.2. ANTHER CULTURE AND ANDROCLONAL VARIATION.

Haploids are of great value in agriculture as inbred lines can be directly produced by chromosome doubling and these are useful for the study of mutagenesis, since there is only one set of chromosomes in haploid and no masking of dominant allele. The benefits of doubled haploids for plant improvement include the rapid achievement of homozygosity and in consequence, the rapid incorporation of new genes into breeding material and the increase of selection efficiency. Chinese scientists extensively used these techniques in plant breeding.

Variations in seed fertility, plant height, heading date, morphology and chlorophyll deficiency were observed in homozygous lines of diploid seed callus (Oono, 1983; 1984). Genetic variations for short stature in anther derived doubled haploid rice were reported (Schaeffer *et al.*, 1983; 1984). Variability in quantitative characters like plant height, number of productive tillers, grain yield, flowering and plant dry matter of anther derived plants (Sathish *et al.*, 1995), variations in ploidy levels of rice plants (Chen *et al.*, 1983; Mercy and Zapata, 1986), variations for blast resistance (Kucherenco, 1984) and salt tolerance (Sathish *et al.*, 1997) were also reported.

High frequencies of somaclonal variation in agronomic characters were reported (Kucherenco and Mammaeva, 1979; Davoyan, 1983; Suenoga *et al.*, 1982 and Abrigo *et al.*, 1985) with more than one varied trait. Percentage of anthers producing callus and organogenesis showed wide range of variation. Response of anther depends on genotype, stage of pollen, media composition, pre treatment etc., (Zagorske *et al.*, 1997 and Rakoczytrojanowskam *et al.*, 1997; Immonen and Robinson, 2000). Stress at induction stage appeared to be the best in inducing embryogenesis by reducing the total time required to regenerate plants. This induces a switching over from the gametophytic to sporophytic development. An additional non-gametophytic nuclear division is known to occur during the cold shock treatment whereby facilitating induction process in the culture medium. Cold treatment alters pollen grains in different ways. It triggers the pollen mother cell to produce two identical nuclei instead of one vegetative and one generative nucleus and production of specific proembryo inducers. It synchronize the cells and maintain a higher percentage of viable pollen, slowing down the senescence of somatic tissues

(Sunderland and Roberts, 1979).

Cistue *et al.*, (1994) reported production of large numbers of doubled haploid plants from barley anthers pretreated with high concentration of mannitol. Pretreatment of fresh anthers in 0.3 M mannitol solution for 3 days was shown to be a potential substitute for 28 days cold treatment in barley microspore culture (Kasha *et al.*, 1992). Even substitution of sucrose with maltose promotes androgenesis (Glaszmann, 1999; Guo and Pulli, 2000). Faruque *et al.*, (1999) studied variations in green plant regeneration response in various indica and japonica varieties of rice.

The best pollen development stage for producing callus in rice was studied by Sun (1978). In anthers inoculated at binucleate stage most pollens were not responsive. In anthers inoculated at the early, mid and late uninucleate stages, the rates of viable pollen grains were relatively higher. The calli recovered from differentiating pollen grains at early and mid-uninucleate stages showed an excellent capacity to regenerate green plants, with a minimum number of albino plantlets. Calli arising from microspores in the late uninucleate stage appeared less capable of plant regeneration. When pollen was at first mitotic division, only albino plants were obtained (Lee *et al.*, 2000; Afza *et al.*, 2000). The switch towards embryoid /callus development seems to occur more readily when the process of nuclear division has already been initiated, than when it has to be initiated in culture. The difficulties with the older stages of the pollen appear to be due to already committed stage of differentiation into male gametophyte or may be because the accumulation of starch grains in the late stages hampers pollen cell division (Sun, 1978).

The chromosome number in the cells of anther derived callus varied widely and changed during subculture (Niizeki and Oono, 1968). At the first passage of

anther culture, many kinds of calli consisting of different ploidy or mixed ploidies were reported. Among them, 24 % of callus were non haploid, and after 17 passages of subculture, it was found that haploid cells were eliminated from all cultures and diploid or tetraploid cells become predominent (Chen and Chen 1980).

Somaclonal variations, were also found in plants regenerated from ovary (Liu and Zhou, 1984), seed (Oono, 1978), seed embryo (Davoyan, 1983), immature endosperm (Davoyan and Smetanin, 1979), immature inflorescence (Sun *et al.*, 1983), and mature embryo (Padmaja *et al.*, 1993). It seems that the somaclonal variation within pollen plants is greater than that within plants regenerated from other explants.

1.2 MATERIALS AND METHODS

1.2.1 TISSUE CULTURE

Callus cultures were established from mature embryo explants of the selected rice varieties namely, Pokkali, MI 48, Annapoorna and Jyothi. Source of seeds and characteristics of each varieties are given in the table below.

Rice varieties used	Source of seeds	Characteristics
Pokkali (Vyttila 1)	Rice Research Institute, Vyttila	Moderately salt tolerant, tall, long duration of flowering
MI 48	Central Salinity Research Institute, Lucknow	Salt sensitive, tall, long duration of flowering
Annapoorna	Kerala Agricultural University , Mannuthy	Salt sensitive, short, short duration of flowering, good grain yield
Jyothi	Kerala Agricultural University , Mannuthy	Salt sensitive, short, short duration of flowering, good grain yield

Callus was also induced from mature embryo explants of F1 hybrids obtained from crosses between Pokkali and the sensitive rice varieties.

1.2.1.1 Callus induction

Dehusked mature rice seeds were surface sterilized with 0.1 % (w/v) mercuric chloride with a drop of Tween 20 surfactant for 10 min. The seeds were then rinsed 4 to 6 times with sterile distilled water. Explants thus prepared were inoculated, under

aseptic conditions on agar solidified callus induction medium such that the endosperm of the seed was within the medium and the embryo exposed on the surface. Three seeds were inoculated per tube and hundred seeds of each variety were inoculated.

The callus induction medium was composed of the inorganic constituents of Murashige and Skoog (1962) (MS) medium, (Appendix I), supplemented with 2 mg/l glycine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 0.1 mg/l thiamine, 100 mg/l inositol, 100 mg/l tryptophan, 3 % sucrose, 2 mg/l 2,4 -D and 0.5 mg/l kinetin.

Cultures were incubated in the dark at 25 $\pm$ 2 ^{0}C. After one month the calli were subcultured in fresh MS media with low level of auxin, (1 mg/l 2,4-D). For determining frequency of callus induction, 100 seeds of each variety was inoculated each time and the experiment was performed in triplicate.

1.2.1.2 Plant regeneration

For plant regeneration, calli of different ages (2, 3, 4 and 6 months old) were transferred to plant regeneration media of the following hormonal combinations.

1. MS + 0.5 mg/l 2,4-D + 2 mg/l Kinetin
2. MS + 0.5 mg/l Kinetin + 0.5 mg/l BAP + 0.5 mg/l IAA
3. MS + 0.1 mg/l 2,4-D + 2 mg/l Kinetin
4. MS + 1 mg/l Kinetin
5. MS + 0.5 mg/l NAA + 2 mg/l Kinetin + 10 mg/l ABA for two days and transferred to ABA free MS medium.

In each experiment calli were transferred to 50 tubes and the experiment was repeated thrice. The number of calli that produced green plantlets and only roots was recorded .

1.2.1.3 Hardening of regenerated plants

More than 100 plants were regenerated from every rice varieties. Regenerated plants were separated and taken out of the culture tubes. The plants were-kept under laboratory conditions for one week with the roots immersed in a solution containing one tenth of MS organic and inorganic constituents without any hormones. This solution was changed every day. After one week the plants were taken out and kept in shade with fungicide spray (1 % dithane twice a week). These plants were used for further studies.

1.2.2 ANTHER CULTURE

Attempts were made to find out suitable hormonal combination, duration of low temperature treatment, and carbon source for anther culture of selected rice varieties.

Inflorescences were collected at boot leaf stage around 9 AM at which time more microspores were found to be at mid uninucleate stage, which is the suitable stage for anther culture. These were given a cold pretreatment of 10°C for 6, 8 and 10 days and the effect was studied. The middle florets of the panicle, which had anthers with uninucleate microspores, were detached, surface sterilised with 0.1 % mercuric chloride and washed 4 to 6 times with sterile distilled water. Anthers were dissected out under aseptic conditions and inoculated on the media with different hormonal combinations. The cultures were incubated in the dark at 25 $\pm$ 2 ^{0}C for callus induction. Only N_6 media (Chu *et al.*, 1975) with minor modifications (Appendix II) was used for anther culture.

Different media combinations tried were:

1. N_6 + 1 mg/l NAA + 0.5 mg/l Kinetin + 6 % maltose.
2. N_6 + 1 mg/l NAA + 0.5 mg/l Kinetin + 6 % Sucrose
3. N_6 + 2 mg/l 2,4-D + 6 % Sucrose
4. N_6 + 2 mg/l NAA + 1 mg/l Kinetin + 6 % Sucrose
5. N_6 + 1 mg/l 2,4-D + 0.5 mg/l IAA + 0.5 mg/l BAP + 6 % Sucrose
6. N_6 + 2 mg/l NAA + 0.5 mg/l Kinetin + 6 % Sucrose
7. N_6 + 2 mg/l PAA + 0.5 mg/l Kinetin + 6 % Sucrose
8. N_6 + 2 mg/l PAA + 1 mg/l Kinetin + 6 % Sucrose
9. N_6 + 2 mg/l PAA + 6 % Sucrose
10. N_6 + 1 mg/l PAA + 0.5 mg/l IAA + 0.5 mg/l BAP + 6 % Sucrose
11. N_6 + 2 mg/l NAA + 0.5 mg/l Kinetin + 6 % Maltose.

Between 10 - 14 anthers were inoculated in a tube and 40 tubes were inoculated at a time and the experiment was done in triplicate. The percentage of callusing (number of anthers which showed callusing out of total number of anthers inoculated), direct green plantlet regeneration, and number of albinos (number of plantlets regenerated out of total number of anthers showing callusing) were estimated. Calli were later transferred to various regeneration media mentioned in section 1.2.1.2. Approximately 50 regenerated plants of every variety were hardened and used for further studies.

1.3 RESULTS

1.3.1 CALLUS CULTURE

1.3.1.1 Callus induction

Friable white embryogenic callus was induced from the scutellum of mature seeds within two weeks of inoculation in all the rice varieties. Varietal differences with respect to frequency of callus induction were observed. Of the four varieties tested MI 48 recorded high percentage of callus induction (97 %), followed by Annapoorna (95 %), Jyothi (94 %) and Pokkali (85 %).

1.3.1.2 Regeneration response of callus cultures

Results presented in the Fig. 1, 2, 3 and 4 suggest that, in general increase in the age of callus resulted in a decrease in the percentage of regeneration of green plantlets, and an increase in the browning of callus and of calli producing only roots.

1.3.1.2.1 Regeneration in Pokkali

Of the various age groups of calli tested during the study, three month old calli supported maximal plant regeneration (67 %) in medium No.3 and in general an increase in age of calli showed a decrease in plant regeneration, irrespective of the culture media tried. Among the different culture media tested medium No.3, containing 0.1mg/l 2,4-D and 2 mg/l kinetin induced higher number of green plantlets while fewer cultures had brown callus with roots (Fig.1, Appendix III A).

While medium No. 3 produced 66 % of plant regeneration in two-month-old calli, medium No.1 with 0.5 mg/l 2,4 - D and 2 mg/l kinetin induced only 58 %. Six month old calli of Pokkali showed only poor regeneration ability. Medium No. 2 containing 0.5 mg/l BAP, 0.5 mg/l IAA and 0.5mg/l kinetin yielded only 15 %

regeneration while inducing 50.3 % browning and rooting in the two month old calli. Medium No. 5 containing 0.5 mg/l NAA, 2 mg/l kinetin and 10 mg/l ABA also induced more rooting and browning (30 %) than regeneration (5.3 %).

1.3.1.2.2 Regeneration in MI 48

Calli of MI 48 showed reduction in regeneration ability with increase in age (Fig.2, Appendix III B)). Upto 3 months, calli of MI 48 showed higher regeneration ability (68 %) in the medium No.3, while four month old calli showed 63 % regeneration in medium No.1. Regeneration efficiency decreased drastically in six month old calli. Medium No.2 produced only 25 % regeneration, and 40 % browning and rooting in three-month-old calli. Medium No.4 containing 1mg/l of kinetin without any auxin produced 51 % plant regeneration in two and three month old calli, while medium No.5 produced only 18 % and 14.6 % in two and three month old calli respectively.

1.3.1.2.3 Regeneration in Annapoorna

Results presented in Fig. 3, (Appendix III C) indicated that Medium No.3 was suitable for green plantlet regeneration in 2 and 3 month old calli, which showed 70 % regeneration. Regeneration efficiency decreased drastically in six-month-old calli. Medium No.4 showed 45 % regeneration and 28.3 % browning and rooting in two month old calli, and 42 % regeneration and 30.3 % browning and rooting in three month old calli. Two and three month old calli showed 25.3 % and 23.3 % regeneration respectively and 40 % browning and rooting in medium No.2. Medium No. 5 produced only 12.3 % regeneration while it showed 30 % browning and rooting in two month old calli.

Fig. 1

REGENERATION RESPONSE IN THE CALLUS CULTURES OF POKKALI

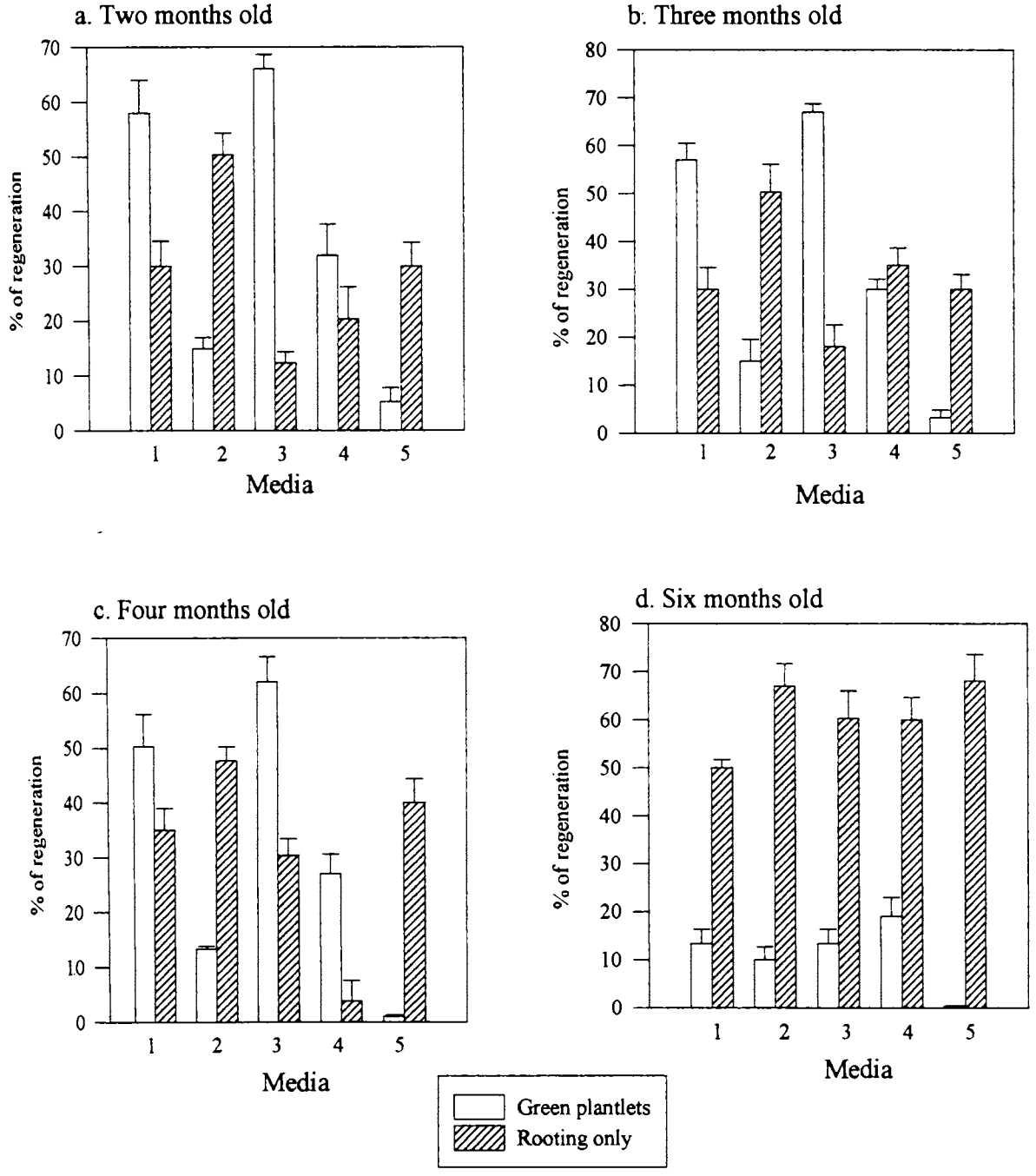


Fig. 2

REGENERATION RESPONSE IN THE CALLUS CULTURES OF MI 48

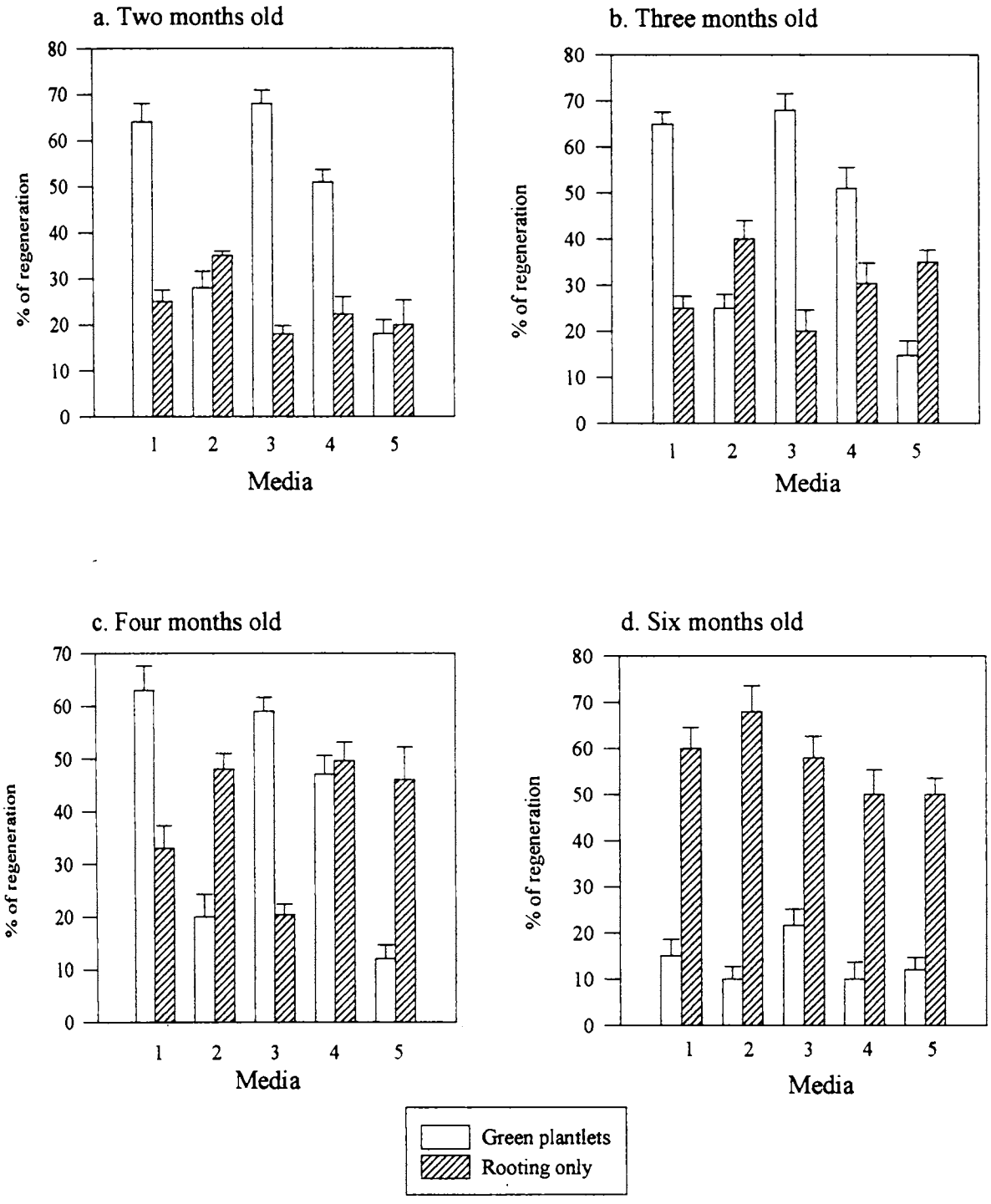


Fig. 3

REGENERATION RESPONSE IN CALLUS CULTURES OF ANNAPOORNA

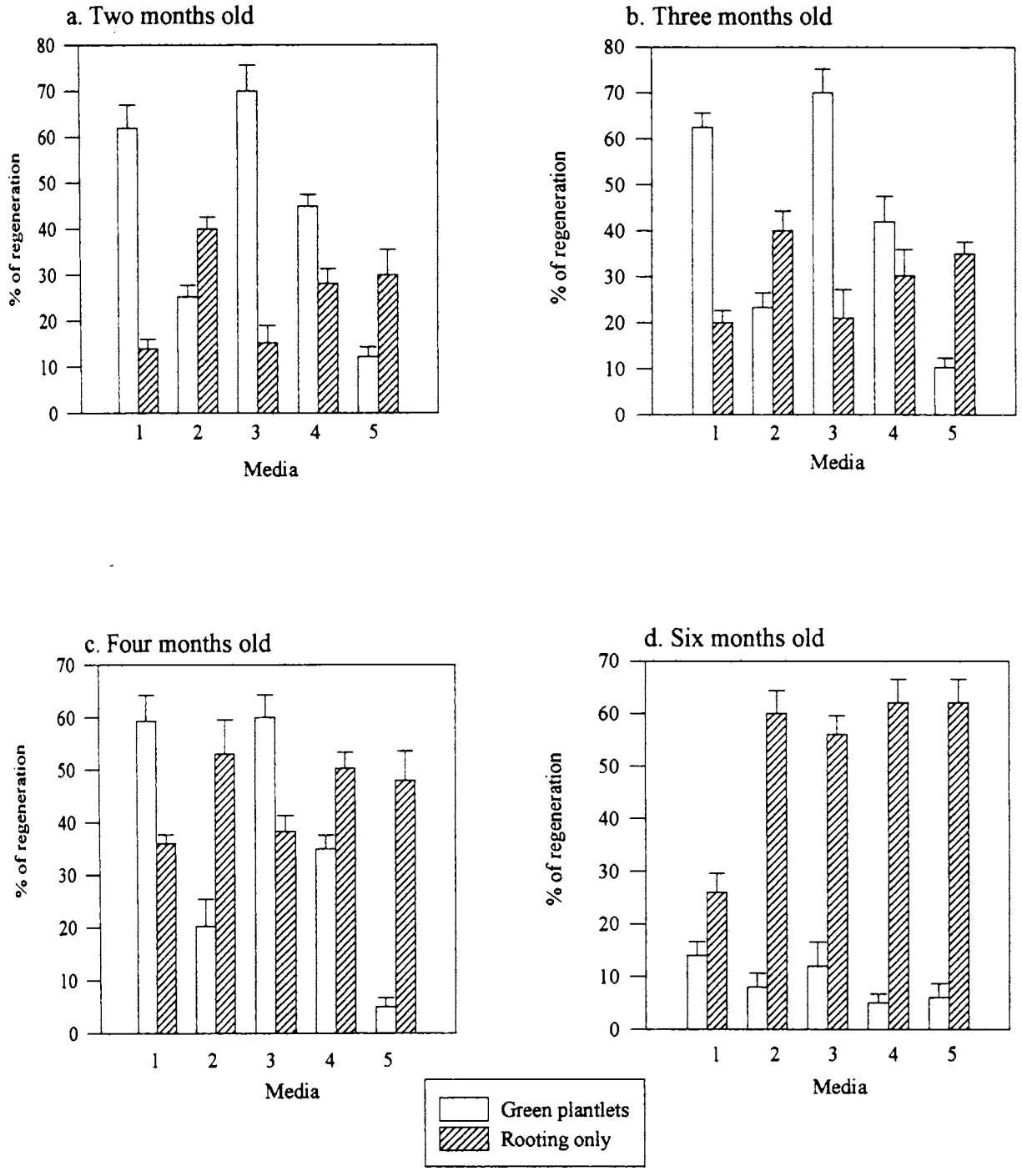
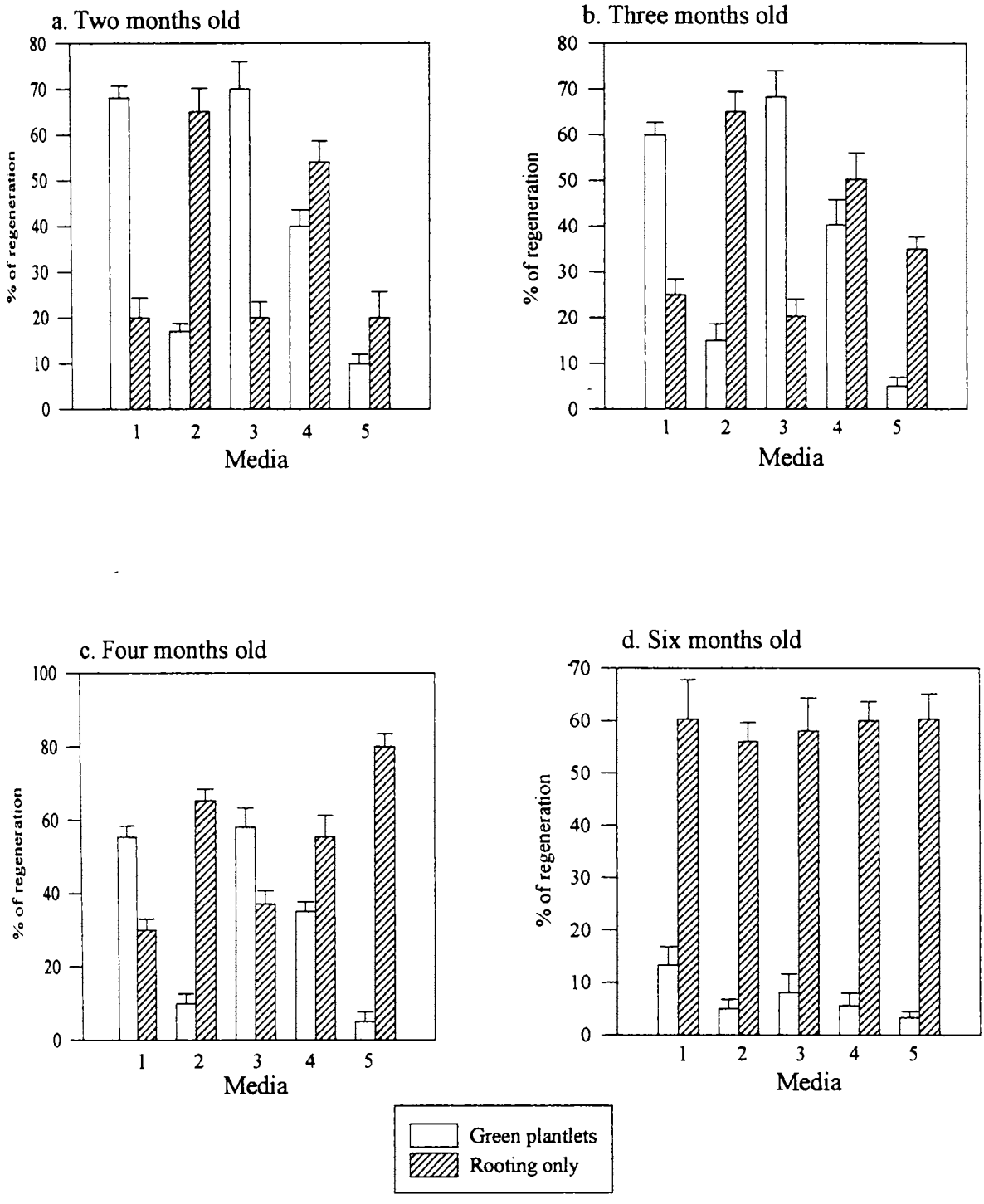


Fig. 4

REGENERATION RESPONSE IN CALLUS CULTURES OF JYOTHI



Media used for Regeneration

Medium No.	Composition
1	MS + 0.5 mg/l 2,4-D + 2 mg/l Kinetin
2	MS + 0.5 mg/l Kinetin + 0.5 mg/l BAP + 0.5 mg/l IAA
3	MS + 0.1 mg/l 2,4-D + 2 mg/l Kinetin
4	MS + 1 mg/l Kinetin
5	MS + 0.5 mg/l NAA + 2 mg/l Kinetin + 10 mg/l ABA for two days and transferred to ABA free MS medium.

1.3.1.2.4 Regeneration in Jyothi.

It could be seen from Fig.4, (Appendix III D) that medium No.3 promoted regeneration in two and three month old calli of Jyothi (70 % and 68.3 % respectively). Whereas medium No.2 containing 0.5 mg/l BAP, 0.5 mg/l IAA and 0.5 mg/l kinetin supported only 17 % regeneration in two month old calli while browning and rooting was 65 %. Medium No. 4 and 5 were also found to produce more browning and rooting than regeneration. Medium No.4 induced 40 % regeneration and 54 % rooting while medium No.5 10 % regeneration and 20 % rooting in two month old calli.

1.3.2 ANTHER CULTURE

1.3.2.1 Callusing response in Pokkali

Of the eleven media tried, medium No. 1 and 11 (containing 1mg/l NAA and 0.5 mg/l kinetin, and 2 mg/l NAA and 0.5 mg/l kinetin, respectively), containing maltose as carbon source showed considerable callusing response (Fig.5, Appendix IV A). Anthers subjected to 10 days of cold treatment supported a maximum of 23.7 % callusing in medium No.11, while medium No.1 produced only 14 %. With 8 day cold treatment medium No.1 and 11, supported only 18.5 % and 21.3 % callusing respectively. All the other media produced only very low callusing response. Medium No.6 containing 2 mg/l NAA, 0.5 mg/l kinetin with sucrose as carbon source supported only 3.3 % callusing with 10 days cold pretreatment.

1.3.2.2 Callusing response in MI 48

Results presented in Fig. 6 (Appendix IV B), indicated that maximal callusing response in MI 48 could be obtained with medium No. 6, and 10 days of cold

treatment (21.1 %), whereas 8 day cold treatment supported only 3.7 % callusing. Medium No.5 containing 1 mg/l 2,4-D, 0.5 mg/l IAA, 0.5 mg/l BAP and 6 % sucrose induced 17.9 % callusing in anthers subjected to 10 day cold treatment. Medium No. 1 and 11, which were most suitable for Pokkali, were not suitable for MI 48, since maltose as a carbon source was not effective in inducing callus in MI 48. These media induced only 8.2 and 10.9 % callusing respectively after 10 days of cold treatment.

1.3.2.3 Callusing response in Annapoorna

Annapoorna showed callusing in most of the media tested, except in medium No.7, 8, 9 and 10, which contained PAA as auxin, compared to Pokkali and MI 48 (Fig. 7, Appendix IV C). Anthers of Annapoorna subjected to 10 day cold treatment, showed good response in medium No.1 containing NAA 1 mg/l, kinetin 0.5 mg/l and 6 % maltose (16.6 %). While anthers given an 8 day cold treatment, showed good response in medium No.6 containing NAA 2 mg/l, 0.5 mg/l kinetin and 6 % sucrose (16.5 %). Maltose was not found to be necessary for better response in Annapoorna.

1.3.2.4 Callusing response in Jyothi

Callusing response in Jyothi was similar to that of Annapoona, and was comparatively higher than in Pokkali and MI 48 in most of the media tested. Results presented in Fig. 8 (Appendix IV D), indicate that anthers subjected to 10 days of cold treatment showed 13.7 % callusing in medium No.6, (with 2mg/l NAA, 0.5 mg/l kinetin and 6 % of sucrose). Nevertheless anthers given 10 day cold treatment produced 11.67 % callusing in medium No.1, 11.9 % callusing in medium No. 11 and 8.2 % callusing in medium No.5 containing 1 mg/l 2,4-D, 0.5 mg/l IAA, 0.5 mg/l BAP with 6 % sucrose. Whereas anthers given 8 days of cold treatment produced 7.3

% callusing in medium No. 4 (containing 2 mg/l NAA, 1mg/l kinetin with 6 % sucrose).

1.3.2.5 Direct green plantlet regeneration

Direct green plantlet regeneration from anther culture of four rice varieties Pokkali, MI 48, Annapoorna and Jyothi was observed in the three media, ie., 1, 6 and 11 containing 1mg/l NAA, 0.5 mg/l kinetin and 6 % maltose; 2 mg/l NAA, 0.5 mg/l kinetin and 6 % sucrose; and 2 mg/l NAA, 0.5 mg/l kinetin and 6 % maltose respectively (Fig.9, Appendix IV E). The rice varieties differed in their response in the three media. While Pokkali showed higher direct green plantlet regeneration in medium No.1 (21.33 %), MI 48, Annapoorna and Jyothi showed 31 %, 20.66 % and 26.33 % direct green plantlet regeneration respectively in medium No.11. It was also observed that Medium No.6 supported 14.66 % direct green plantlet regeneration in the case of Jyothi alone.

1.3.2.6 Regeneration of albino plants

From the results documented in Fig 10, it was noted that, percentage of regeneration of albino plants was higher in Pokkali (39.66 %), when compared to other rice varieties in medium No.1, even though the same medium showed considerable percentage of green plantlet regeneration. Pokkali produced 26.67 % albino regeneration in medium No.11. All the other varieties showed lesser albino regeneration in medium No.1, 6 and 11. Jyothi showed relatively a higher albino regeneration percentage (14.33 %), when compared to MI 48 and Annapoorna.

Fig. 5

CALLUS INDUCTION IN ANTHER CULTURES OF POKKALI

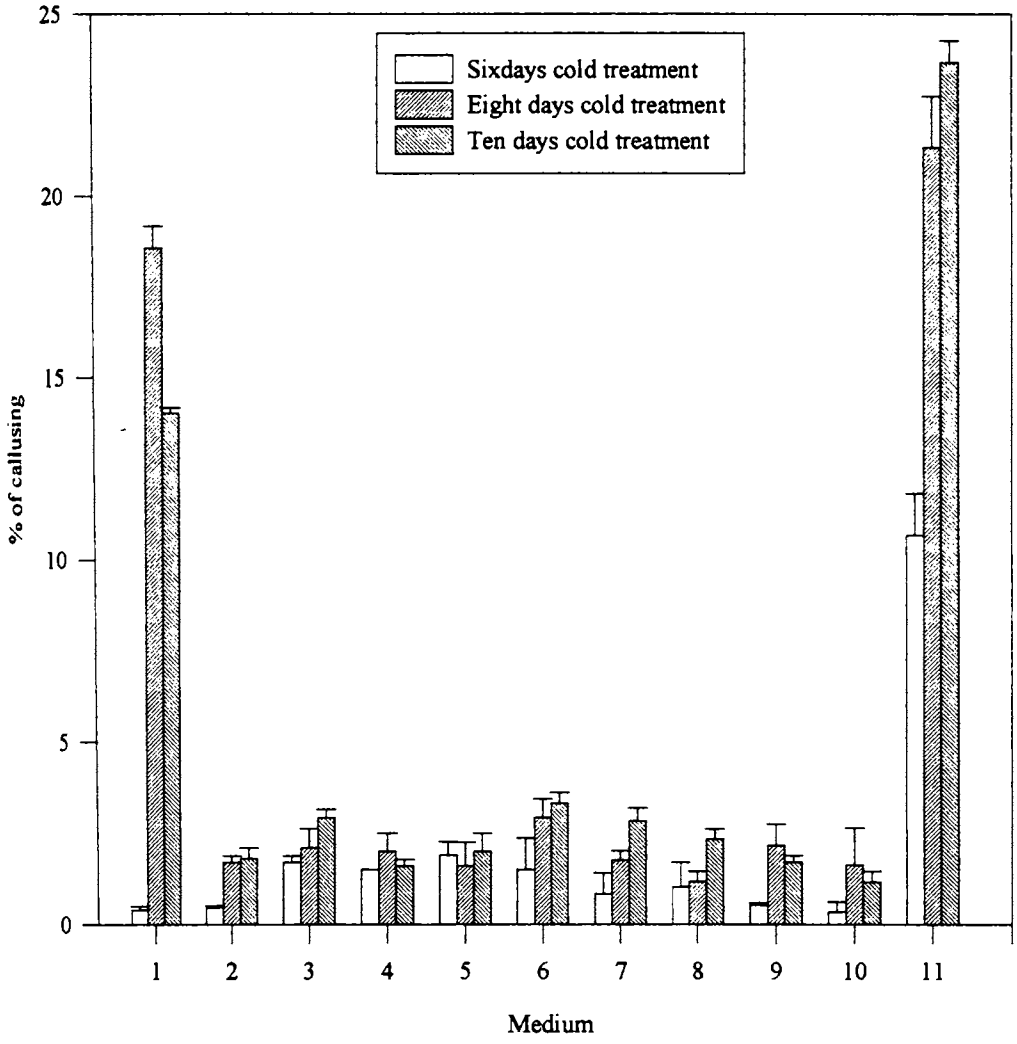


Fig. 6

CALLUS INDUCTION IN ANTHER CULTURES OF MI -48

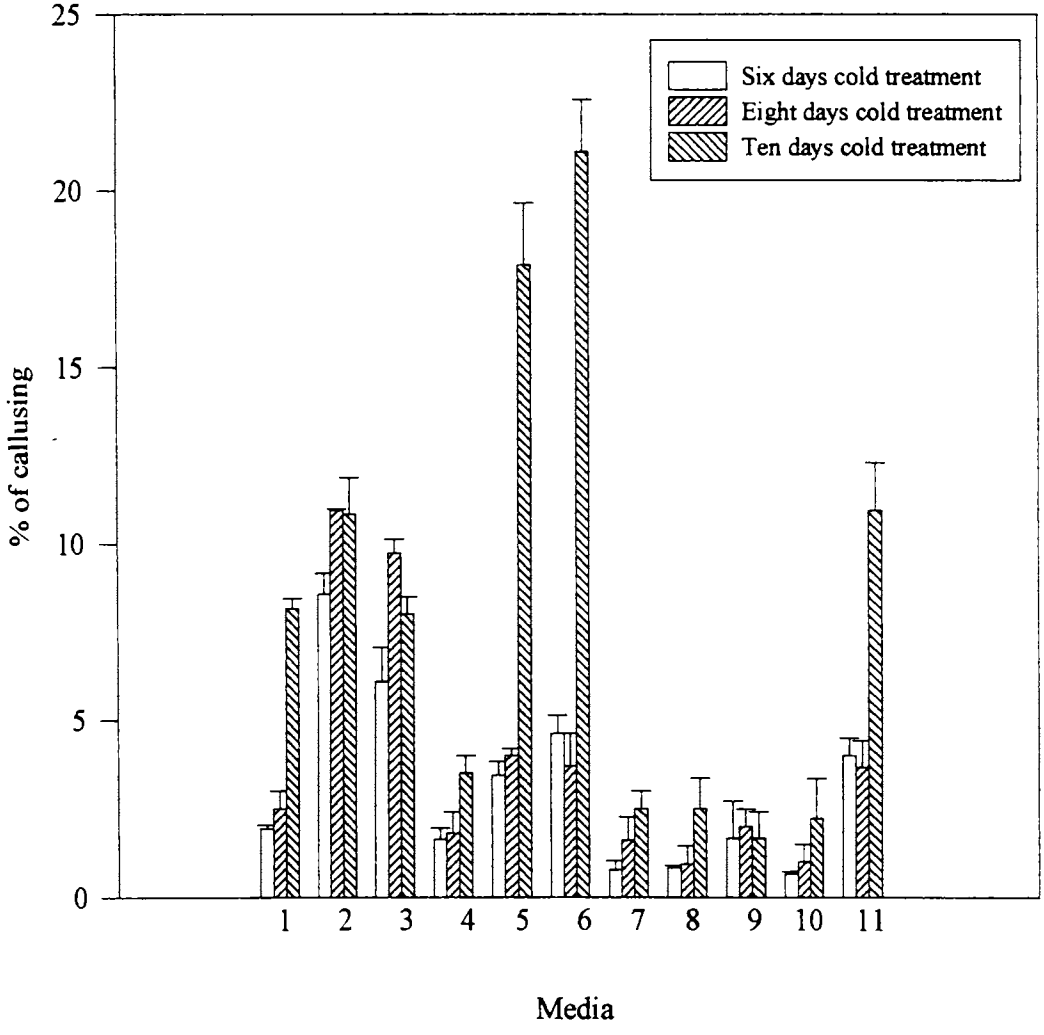


Fig. 7

CALLUS INDUCTION IN ANTHER CULTURES OF ANNAPOORNA

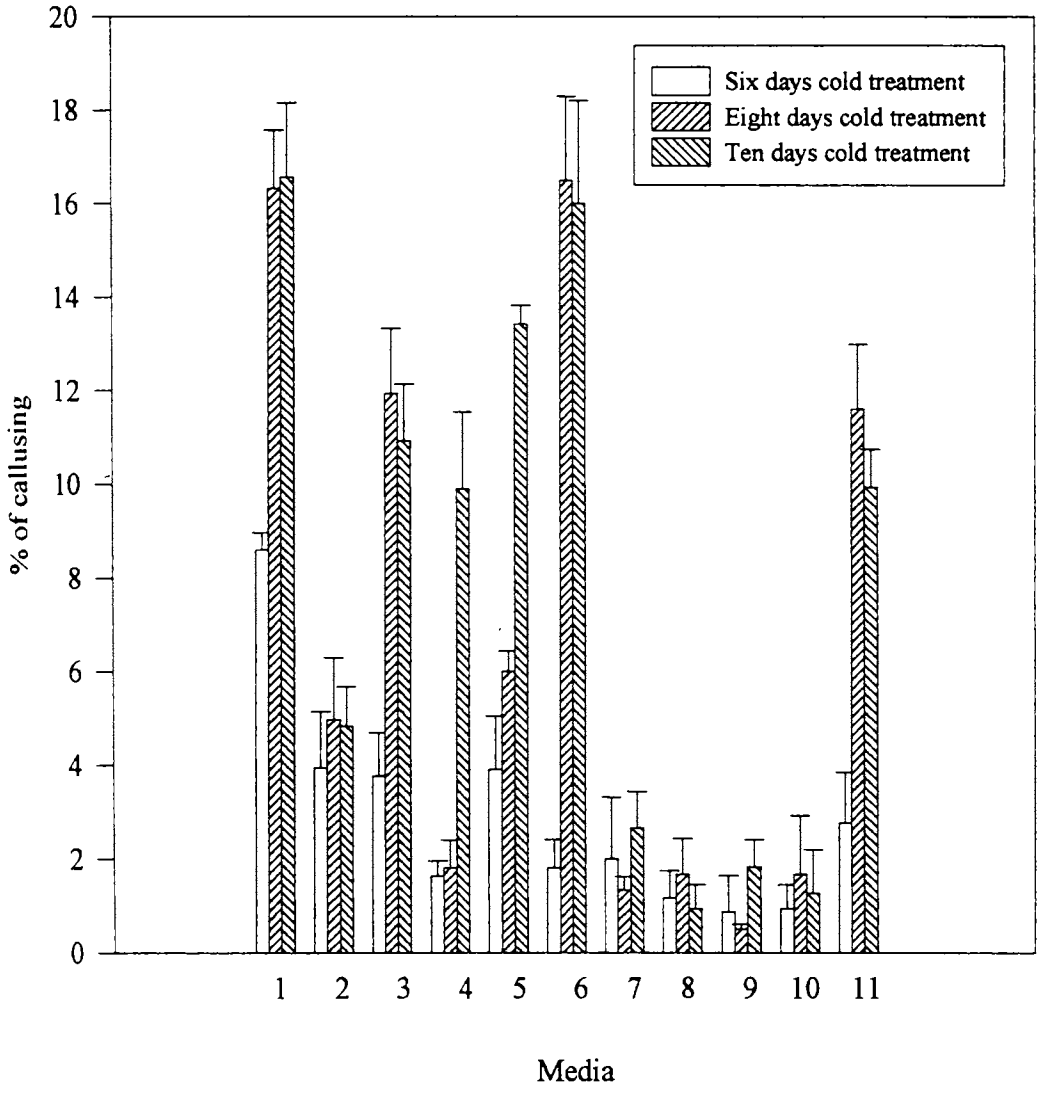


Fig. 8

CALLUS INDUCTION IN ANTHER CULTURES OF JYOTHI

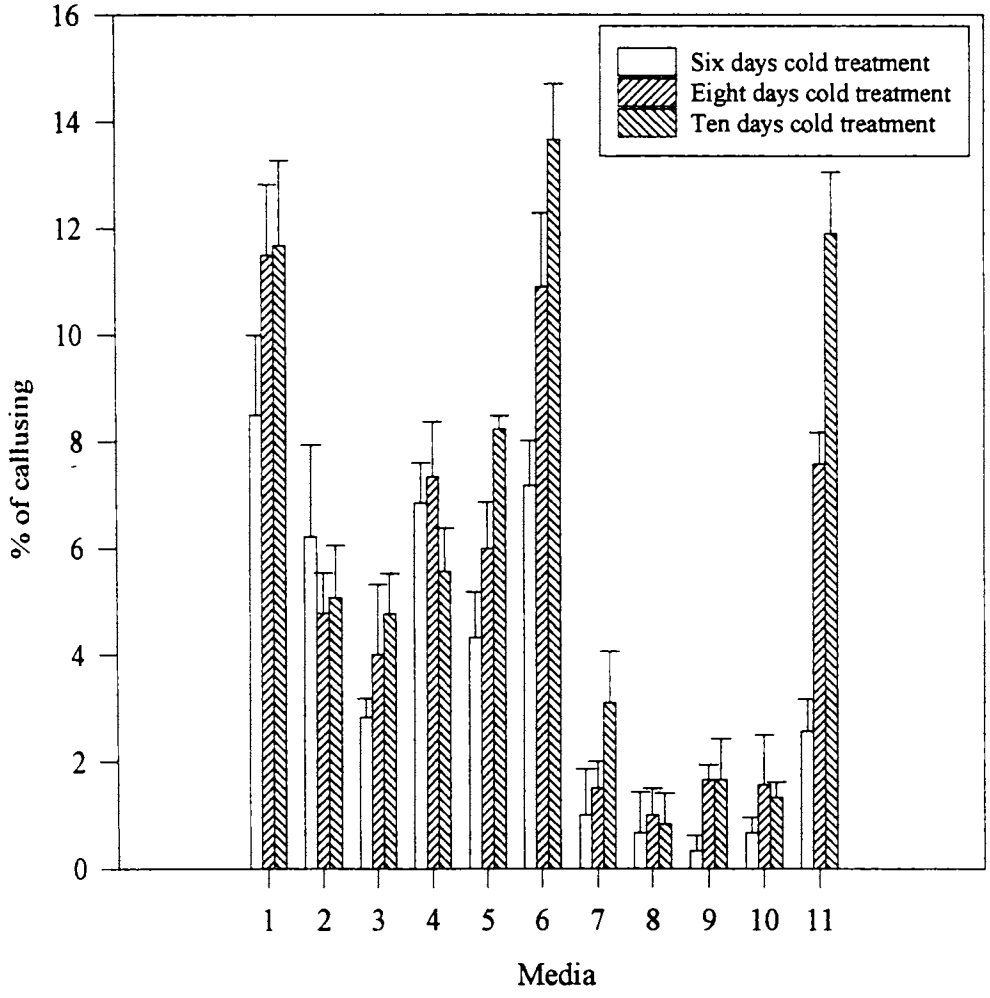


Fig. 9

REGENERATION OF DIRECT GREEN PLANTLETS IN ANTHER CULTURES OF RICE

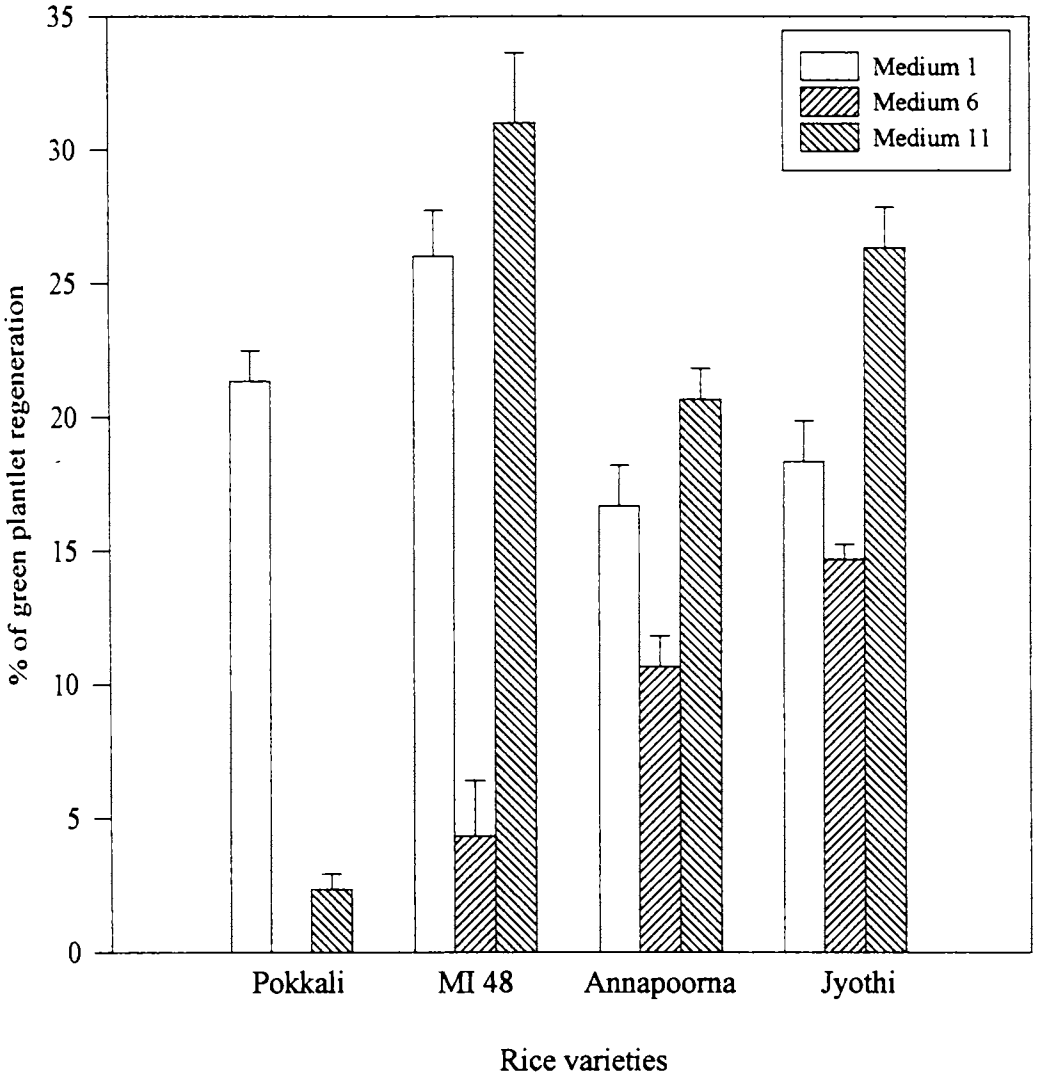
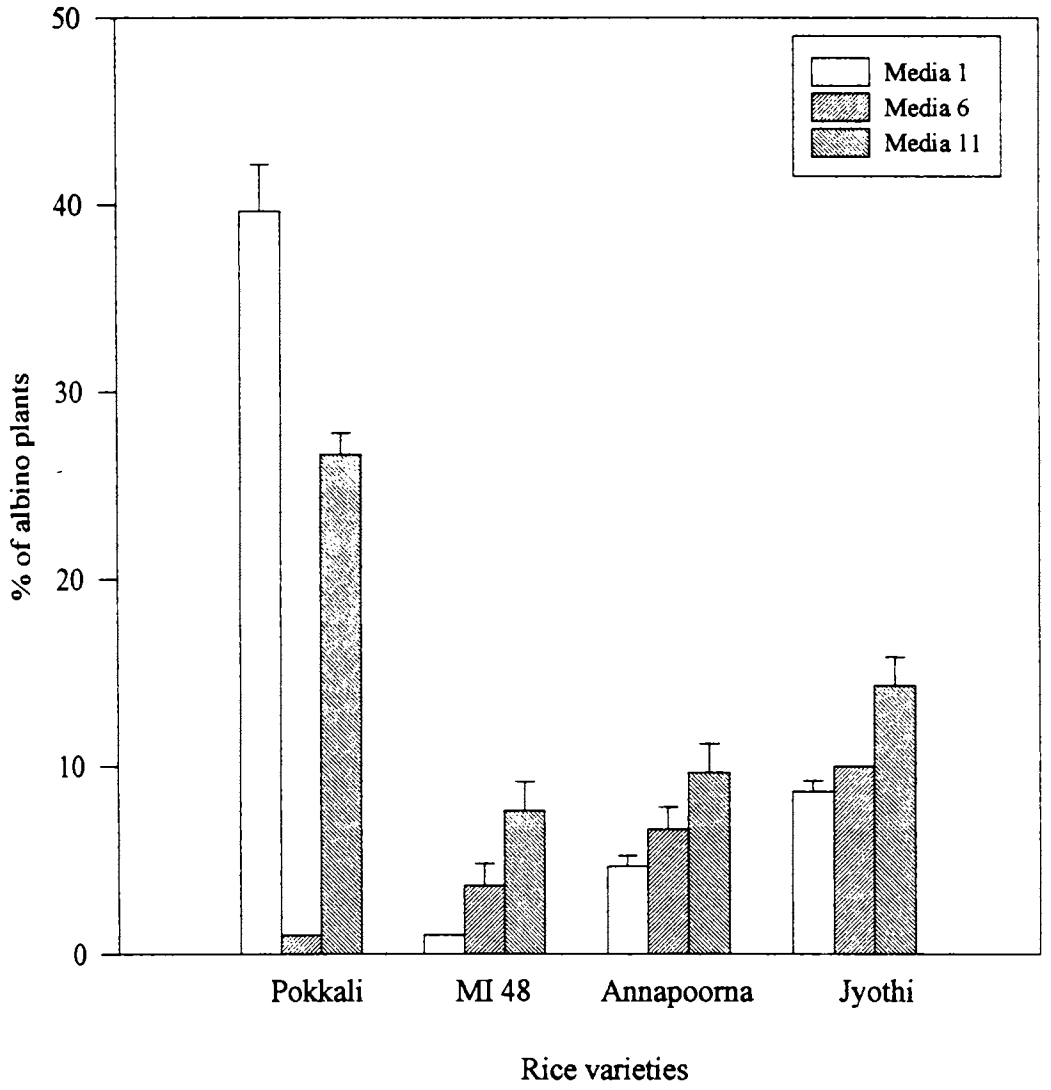


Fig. 10

REGENERATION OF ALBINO PLANTLETS IN ANTHER CULTURES OF RICE



Media used for anther culture

Medium No.	Composition
1	N_6 + 1 mg/l NAA + 0.5 mg/l Kinetin + 6% maltose.
2	N_6 + 1 mg/l NAA + 0.5 mg/l Kinetin + 6% Sucrose
3	N_6 + 2 mg/l 2,4-D + 6% Sucrose
4	N_6 + 2 mg/l NAA + 1 mg/l Kinetin + 6% Sucrose
5	N_6 + 1 mg/l 2,4-D + 0.5 mg/l IAA + 0.5 mg/l BAP + 6% Sucrose
6	N_6 + 2 mg/l NAA + 0.5 mg/l Kinetin + 6% Sucrose
7	N_6 + 2 mg/l PAA + 0.5 mg/l Kinetin + 6% Sucrose
8	N_6 + 2 mg/l PAA + 1 mg/l Kinetin + 6% Sucrose
9	N_6 + 2 mg/l PAA + 6% Sucrose
10	N_6 + 1 mg/l PAA + 0.5 mg/l IAA + 0.5 mg/l BAP + 6% Sucrose
11	N_6 + 2 mg/l NAA + 0.5 mg/l Kinetin + 6% Maltose.

1.4 DISCUSSION

1.4.1 VARIETAL SPECIFIC DIFFERENCES EXIST IN RICE WITH RESPECT TO CALLUS INDUCTION AND REGENERATION RESPONSE

The capability of callus induction and plant regeneration depends considerably on the genotype (Abe and Futsuhara 1986), and also on the original parts of a plant which is used as explants (Li and Heszky 1986), nutrient media, hormones, age of explant, physiological status of the donor plant and passage in culture (Inque and Maeda, 1980; Vasil, 1982). Raman *et al.*, (1994) while studying regeneration potential of twenty two genotypes of rice, used immature inflorescences and embryos which provided a higher frequency of callus induction and plant regeneration, although they were not available all year around. Suprasana *et al.*, (1995) reported that mature seed embryos as explant, exhibits embryogenic potential with high frequency of plant regeneration. In the present study, induction of embryogenic callus from scutellum of mature seeds was highest in MI 48 and lowest in Pokkali.

The role of hormones and sugars in plant regeneration is well known (Akins and Vasil, 1985). But other components may also influence the regeneration capability of *in vitro* cultures since nutrition affects endogenic levels of phytohormones in higher plants (Marschner, 1986). Of the different media tested for plant regeneration from embryogenic calli, medium No.3 containing 0.1 mg/l 2,4-D and 2 mg/l kinetin was found to be most suitable for regeneration in all rice varieties in two, three and four month old calli. Even though medium No.3 showed higher percentage of regeneration, medium No.1 containing 0.5 mg/l 2,4-D and 2 mg/l kinetin, was also found to be suitable for regeneration in all rice varieties. Medium

No.4 containing only 1 mg/l kinetin as growth hormone was found to induce 19 % regeneration in six month old calli of Pokkali, which was higher when compared to other media. Medium No.4 was found to be better than medium No.2 even though it could not produce as much regeneration as in medium No.1 and 3. After comparing the result obtained from three media i.e., medium No.1, 2 and 3, it is inferred that, 2,4-D as auxin in media No.1 and 3 could have promoted better result than medium No. 2 which contained IAA as auxin. Further medium No.4, which did not contain any auxin, produced only lesser percentage of regeneration compared to medium No.1 and 3, which contains auxin. So it is speculated that the presence of auxin, in low level is necessary for better regeneration in different rice varieties.

There is an increasing interest in the role of abscisic acid as a possible promoter of plant regeneration *in vitro*. Abscisic acid stimulated adventitious bud formation in protoplast derived calli of potato, and shoot vigour (Shepard, 1980). Stimulation of shoot bud and plantlet formation by a two step method using abscisic acid, followed by kinetin treatment, was demonstrated in rice somatic callus cultures (Inque and Maeda, 1981; Yang, *et al*,. 1999; Guzman and Aris, 2000). Torrizo and Zapata (1986) observed that media containing 10 mg/l ABA has a stimulatory effect on rice plantlet regeneration. Increase in efficiency of plantlet regeneration was observed not during the ABA treatment itself but upon transfer of the calli to ABA free medium. In the present study medium No.5 used for regeneration of plantlets comprised of MS medium with 0.5 mg/l NAA, 2mg/l kinetin and 10 mg/l ABA. Calli were kept in the above medium for two days and then transferred to an ABA free medium. Contrary to the various reports, in the present study ABA did not show any positive effect on plant regeneration in rice. Percentage of regeneration was lesser

when compared to other media used in the study, especially in two and three month old calli, where the regeneration percentage was very low when compared to other media in all the rice varieties. Six month old calli of Pokkali did not regenerate in medium No.5 but other varieties like MI 48, Annapoorna and Jyothi produced low percentage of plantlet regeneration, which was however higher than other media. Thus it may be concluded that ABA treatment reduces the frequency of regeneration in younger calli, but shows better regeneration response in older calli.

1.4.2 FREQUENCY OF REGENERATION DECREASES WITH AGE OF CALLUS

The response in terms of plantlets regenerated in rice is, however, poor in comparison to dicots and in general, indica lines have shown a low regeneration potential as compared to japonica lines (Suprasana *et al.*, 1995). The low rate of plant regeneration in cereal tissue culture, particularly those derived from mature embryos, could be explained by the fact that embryogenic callus usually make up only a small fraction of the callus. Also, most media which select for rapidly growing callus usually favours the growth of larger non- embryogenic cells which forms, friable and sometimes crystalline- appearing calli typical of cereal tissue cultures (Nabors *et al.*, 1983). Selecting embryogenic callus during every passage of subculture (She *et al.*, 1984) and optimising the medium components and culture conditions (Raghavaram and Nabors 1984, 1985; Davoyan 1986) are by far the most common and effective methods for callus redifferenciation after long term subculture. In the present study although callus of one to six months old showed regeneration potential, it was noted that the callus of age up to three months alone maintained considerable efficiency for regeneration. Aged calli showed a drastic reduction in regeneration efficiency. In

Pokkali, two to four month old calli produced over 60 % regeneration whereas in six month old calli it was reduced to a mere 13 %. Similar behaviour was also observed in the other rice varieties.

1.4.3 CALLUS INDUCTION FROM ANTHERS IS DEPENDENT ON THE DURATION OF COLD PRETREATMENT AND CULTURE MEDIUM USED

Response in anther culture depends on many factors. Stress induces switching over from the gametophytic to sporophytic development. A cold shock treatment synchronizes pollen division and reduces the total time required to regenerate plants (Sunderland and Roberts, 1979). Zhou and Yang (1980) pointed out that when cold treatment duration exceeded a certain limit, induction frequency decreased markedly. Zhou and Yang (1981) observed that cold treatment increased green plantlet production. In the case of barley anther culture, a 28 day cold pretreatment showed higher regeneration ability, and spikes could be stored in cold environment upto six weeks without reducing anther culture responses (Powell, 1988).

In the present study it was found that the effect of cold treatment differed in different rice varieties. Results indicated that impact of the duration of cold shock on callusing and regeneration varied depending on the media used for inducing callus. In case of Pokkali, 10 days cold treatment was effective in medium No. 3, 6, 7, 8 and 11 and 8 day cold treatment was effective, in medium No. 1, 4, 9 and 10. In MI 48 regeneration was higher with 10 day cold treatment in most of the media tried (medium No. 1, 4, 5, 6, 7, 8, 10 and 11). Generally 6 day cold treatment showed lesser percentage of callusing compared to 8 day and 10 day cold treatment. In case of Annapoorna both 8 and 10 day cold treatment was effective in inducing callus. Eight

day cold treatment was effective in medium No.6 and 10 day treatment was effective in medium No.1. Six day cold treatment was more effective than 8 day cold treatment in medium No.7 and 9. Ten day cold treatment was effective for Jyothi in most of the media and also percentage of callusing was higher when compared to 8-day cold treatment. Eight-day cold treatment produced better response only in medium No.4 and 10. Interestingly 6-day cold treatment gave higher percentage of callusing than 8 and 10 days in medium No.2.

1.4.4 MALTOSE AS A CARBON SOURCE WAS EFFECTIVE FOR ANDROGENESIS IN POKKALI WHILE SUCROSE WAS EFFECTIVE IN THE OTHER RICE VARIETIES

Choice of carbohydrate used as carbon source and the osmoticum in the medium may play an important role in callus induction. Lower sucrose content gave better callus development while a high concentration of it improved somatic embryogenesis and subsequent plant regeneration (Ling and Yoshida, 1987). High concentration of sucrose (6 % and above), in general, reduced callus induction frequency in seed cultures. Six percent of sucrose was found to be most suitable for panicle culture and anther culture. Moreover, high concentration of sucrose in callusing medium improved plant regeneration from panicle as well as seed derived calli (Singh *et al.*, 1993). Tiwari and Rahimbai (1992), compared glucose, sucrose and maltose for isolated microspore culture *of Hordeum vulgare* L. and maltose was found to be suitable. Hunter (1987), reported that decisive prerequisite for high regeneration of plants from isolated barley spores was the replacement of sucrose by maltose in the medium. The beneficial effect of maltose could be associated either with its ability to stabilize the initial culture medium osmolality or with a slow rate of maltose

degradation to sucrose (Kuhlmann and Foroughi-Wehr 1989). The effect of maltose in promoting androgenesis was also documented in rice anther culture (Zhuang 1993), wheat microspore culture (Stephen *et al*,. 1993) and rye microspore culture (Guo and Pulli, 2000).

From the present study, it could be inferred that the positive effect of maltose on androgenesis was dependent on the genotype. Percentage of callusing in Pokkali was higher in media containing maltose as carbon source (medium No.1 and 11). In all the other media containing sucrose as carbon source, frequency of regeneration was below 5%. But on supplementation with maltose, percentage of regeneration increased sharply upto 23%. Hence, it was inferred that for anther culture of Pokkali, maltose was essential. But this effect was not observed in the other rice varieties. In MI 48, media containing sucrose as carbon source supported a much higher percentage of callusing (medium No.6). But in medium No.11, which differed from medium 6 only in carbon source (6 % maltose), callus induction was only 10 %. This indicates that maltose does not have a inducing effect in androgenesis in MI 48. In Annapoorna and Jyothi, both maltose and sucrose showed similar result. Hence, the results of the present study suggest that effect of maltose and sucrose on androgenesis is variety specific.

1.4.5 NAA PROMOTES ANDROGENESIS IN ALL RICE VARIETIES

Callus initiated in NAA containing media were more efficient for regenerating green plantlets than those induced in 2,4-D. In rice anther culture, higher concentrations of 2,4-D often leads to nonembryogenic calli while weaker auxins like NAA and IAA promote embryo formation (Reynolds, 1986). In the present study,

NAA was found to be more effective for inducing callus from rice anthers in all the four rice varieties tested. Further, plant regeneration could be achieved by transferring pollen derived calli to regeneration medium. MS medium was found to be suitable for plant regeneration in all the four selected rice varieties. It was however, also observed that not all pollen calli could regenerate shoots.

Results of the present study indicate that NAA had a positive effect on androgenesis, since media supplemented with NAA induced much higher rate of callusing than 2,4-D, IAA, BAP or PAA supplemented media. In Pokkali androgenesis was supported only by NAA, whereas in MI 48, media containing 2,4-D, IAA and BAP produced almost a similar effect to those containing NAA. In Annapoorna and Jyothi, even though androgenesis was effective in media containing 2,4-D, IAA and BAP, NAA supported higher percentage of callusing.

Ziauddin *et al.*, (1992), reported improved plant regeneration from wheat anther and barley microspore culture using PAA. Regeneration of green plants increased three times in the presence of PAA in the induction medium. In the present study, however, presence of PAA in the medium did not showed any inducing effect on androgenesis in different varieties of rice. Jyothi showed better callusing in most of the media although its response was lower in media containing PAA.

1.4.6 DIRECT POLLEN PLANTS WERE INDUCED BY MALTOSE

Unlike *Nicotiana* or *Datura*, where pollen embryoids develop directly into plantlets - passing through stages similar to those of zygotic embryos - in rice, pollen plantlet development involves an intervening callus phase. Callus formation not only involved problems of plant regeneration but also of genetic instability. Attempts made

to induce direct plants in rice met with some success (Ooyang *et al.*, 1983; Chu *et al.*, 1986) but frequency of response remained very low. Liu *et al.*, (1980) made elaborate studies involving mainly auxins and cytokinins. The green plantlet regeneration seemed to be mainly affected by the growth regulators used in regeneration medium. Maltose was reported to have no significant effect on green plantlet regeneration. Only the frequency of albino plant regeneration increased. Process of transfer of anther callus to regeneration media could be avoided, if there was higher percentage of green plantlet regeneration (Xie *et al.*, 1995).

In the present study, direct green pollen plants were produced only in medium No. 1, 6 and 11 in the four rice varieties tested. For Pokkali, medium No. 1 was ideal for callus induction and direct green plantlet regeneration, while, medium No. 11 was found to be suitable for the other rice varieties. Comparing the three media, direct green plant regeneration was found to be less in medium No.6 which contained sucrose as carbon source. It may be inferred that, maltose is better suited for direct green plantlet regeneration, compared to sucrose.

1.4.7 POKKALI SHOWED HIGHER FREQUENCY OF ALBINO REGENERANTS THAN OTHER RICE VARIETIES

One of the problems causing concern in rice anther culture is the occurrence of albino plants in large numbers. No definite relationship could be established between the occurrence of albinos and the media components. However the temperature during incubation seems to affect production of albinos. When calli were incubated at 24 to 25^0C, albinos produced were lesser in number and its frequency increased with increase in temperature. Low temperature resulted in albino plant production mainly

during the early stages of divisions in the pollen. Perhaps temperature affected orientation of the first division in pollen, which could be significant in that the embryo initial so produced, could be deficient in cytoplasmic contents (Song *et al.*, 1978). Pokkali produced higher percentage of albino plants when compared to other rice varieties in both medium No.1 and 11. However in other rice varieties, medium No.11 produced more albino plantlet regeneration.

Section II

Identification of an isozyme marker for salt tolerance

2.1. REVIEW OF LITERATURE

2.1.1 MOLECULAR MARKERS.

Stress causes metabolic alterations. Several proteins are synthesized and accumulated in plant tissues under a range of stress conditions. Information on stress proteins is with minor modifications important for several reasons. Assessment of the sum total of alterations in cellular proteins/transcripts provides a measure of the stress response (Gulick and Dvarak, 1987; Ramagopal, 1987)

Several studies have been undertaken in the recent past to analyze gene/protein alterations induced in rice in response to salt stress, water stress, cold stress and high temperature stress (Mundy and Chua 1988; Hahn and Walbot, 1989; Claes *et al.*, 1990; Parrek *et al.*, 1995). SDS gel electrophoretic profiles of uninduced and salt stressed seedlings of salt sensitive and salt tolerant cultivars of rice showed similar protein alterations, while two tolerant cultivars showed difference in relative levels of certain proteins in stress (Ashwani *et al.*, 1998).

Molecular markers have significant advantage compared to traditional phenotypic markers. They are phenotypically neutral because normal DNA or protein molecules were used to score the genetic material. The most common types of markers used today are isozymes, Restriction Fragment Length Polymorphism (RFLP) and Randomly Amplified Polymorphic DNA (RAPD).

Hunter and Markert (1957) developed the zymogram technique to identify separated enzymes, by coupling the starch gel electrophoresis technique of Smithies (1955) with histochemical staining procedures, which is characterized by great resolving power for the identification of many enzymes.

As an alternative to other time consuming biochemical procedures simple and rapid identification of salt tolerance might be established by isozyme analysis (Fransz *et al.*, 1989). Isozymes are increasingly being utilized in rice genetics and breeding. Poly Acrylaminde Gel Electrophoresis (PAGE) of isozymes of plant tissues is a very useful biochemical index reflecting changes in metabolic activity during growth, development, differentiation and under stress (Pramanik *et al.*, 1996).

Isozyme analyses have been used to detect somaclonal variation at molecular level. In the early 1970s, electrophoretic pattern of alpha amylase isozyme in rice calli was studied (Saka and Maeda, 1973, 1974). Ishikaewa *et al.*, (1987) compared the electrophorograms of 14 isozymes in the calli of 36 strains of rice with that of seed germinated plants. It was found that the loci of some isozymes were stable during callus culture, while the electrophorograms of some others were changed.

Isozyme polymorphism was used for identifying and assessing genetic variation (Huang *et al.*, 1997). Investigations on genetic organization of isozyme variations in rice based on polymorphic isozyme loci revealed that selection of multi locus gene complexes was largely responsible for maintenance of extensive isozyme variation with in the species (Li and Rutger, 2000). Genetic diversity and genetic structure were studied using isozyme loci in *Oryza sativa* L. (Suh *et al.*, 1997), wild rice *Oryza rufipogon* Griff. (Gao and Hong, 2000) and barley (Liu *et al.*, 1999). Isozymes like esterase, isocitric dehydrogenase, glutamate oxaloacetate transaminase were tried as isozyme marker linked to incompatibility locus (Boskovic *et al.*, 2000).

A classical genetic map of rice has been developed consisting of 200 phenotypic trait markers, which include primary loci for morphological mutants, disease resistance and isozymes (Kinoshita, 1995). RFLP and RAPD markers may be

employed for assessment of somaclonal variation (Haluskova and Cellarova, 1997), drought tolerance (Price and Tomos, 1997), temperature tolerance (Mackill and Hei, 1997) and salt tolerance (Brouwer and Osborn, 1997 and Foolad *et al.*, 1997). Das *et al.*, (2000) compared AFLP (Amplified Fragment Length Polymorphism), RAPD and RFLP markers for measuring genetic diversity and AFLPs showed highest efficiency in detecting polymorphism. Genetic map of wild rice *Zizania palustris* L. showed 121 RFLP markers (Kennard *et al.*, 2000). RAPD finger printing of the inbred parental lines and of the resulting hybrid was proposed as a convenient tool for the identification, protection and parentage determination of plant hybrids (Wang *et al.*, 1994). The RAPDs may also be used to quantify the most favourable genetic diversity among rice lines proposed for the production of heterosis. Microsatellites are a powerful tool for discriminating between closely related accessions, and therefore provide a valuable source of genetic markers to complement the use of RFLPs in genetic analyses and marker assisted breeding (McCouch *et al.*, 1997; Cordeiro *et al.*, 2000). Quantitative trait loci were used to study phylogenetic relation among populations (Bartish *et al.*, 2000), drought tolerance (Subudhi *et al.*, 2000), low potassium stress tolerance (Wu *et al.*, 1998) and disease resistance (Zou *et al.*, 2000). Inter simple sequence repeat polymorphism (ISSR) in rice was used to study genetic diversity and phylogenetic relationship in rice (Joshi *et al.*, 2000)

2.2 MATERIALS AND METHODS

The main objective of the present study was to identify an isozyme for use as a molecular marker for salt tolerance and to use the same to detect salt tolerant somaclonal and androclonal variants in rice. Four rice varieties Pokkali (moderately salt tolerant), Jyothi and Annapoorna (salt sensitive) and MI 48 (highly salt sensitive) were used for this study. Isozyme patterns, in the presence and absence of salt stress, was studied in embryo, 14 day old seedling, tillering and flowering stages. The patterns were also studied in undifferentiated callus cells. Callus cultures were initiated from mature seeds of the four rice varieties and their F1 hybrids. Anther cultures were initiated in the four rice varieties, as described in section I. Plants were regenerated from three month old callus culture and anther culture of four rice varieties and callus culture of F1 hybrids. Tillering stage of regenerated plants were used to study isozyme variation and Na^+ content.

Isozyme analysis were carried out using Poly Acrylamide Gel Electrophoresis (PAGE) following essentially, the method of Davis (1964) and using the buffer systems and staining procedures suggested by Brewbaker *et al.*, (1968) and Shaw & Prasad (1970). In order to make definitive comparison among the patterns obtained from different tissues, the Rf value was calculated for each band based upon the migration of the band relative to the front.

2.2.1 Sample preparation for isozyme analyses

Seeds were soaked in water and upon initiation of germination embryos were dissected out. Only shoot portions were selected in the case of other stages. Half of the plants were separated and subjected to salt stress by immersing roots of the plants in NaCl (100mM) solution for 48 hours. Dissected plant parts were immediately

transferred to ice. Tissue samples were weighed and ground using chilled mortar and pestle with acid washed sand in buffer containing 0.1M Tris HCl, pH 6.8, 0.25M sucrose, 1% Poly vinyl pyrrolidone, 0.1% ascorbic acid, 0.1% cysteine hydrochloride, 1mM EDTA, 1mM MgCl2 at pH 6.8 at 4°C (Pramanik *et al.*, 1996). 1 ml of buffer was used for grinding 0.5 g of tissue and the slurry was centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken out and again centrifuged at 10,000 rpm for 10 minutes to get a clear supernatant. Protein in supernatant solution was estimated using Bradford method (Bradford, 1976). Only fresh samples were used for isozyme studies.

2.2.2 Poly acrylamide gel electrophoresis (PAGE)

Vertical slab gel electrophoresis was performed in 16 x14 (cm) slab gels of 1.5 mm thickness. Sample extracts of isozymes were electrophoresed at a low temperature (4-8^{0}C). A constant voltage of 100 V was applied until sample entered resolving gel followed by electrophoresis at a constant voltage of 150 V for 5 hours.

2.2.2.1 Stock solutions for electrophoresis

1. *Acrylamide-bisacrylamide* (30:0.8) was prepared by dissolving 30 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100 ml water and stored refrigerated in amber coloured bottles.
2. *TEMED (N,N,N,N- tetramethylethylenediamine)* was used as supplied, and was stored refrigerated in amber coloured bottle.
3. *Ammonium persulphate* (1.5% w/v) : 0.15 g of ammonium persulphate was dissolved in 10 ml water. This was made fresh just before use.

4. *Buffer stocks*:

Stacking gel buffer stock: 0.5M Tris HCl (pH 6.8)

6 g of Tris buffer was dissolved in approximately 40 ml water and pH was adjusted to 6.8 using 1M HCl and made upto 100ml.

Resolving gel buffer stock:

36.3 g of Tris buffer was dissolved in about 48 ml of water and pH was adjusted to 8.8 by 1M HCl and then made upto 100 ml.

Reservoir gel buffer stock:

3 g of Tris buffer and 14.4 g of glycine dissolved in distilled water and made upto 1 litre.

2.2.2.2. Preparation of gel

Only 7.5 % gel was used for electrophoresis of isozymes.

Recipe for gel preparation: (discontinuous buffer system)

Stock solution (as mentioned above)	Stacking gel (ml)	Resolving gel (ml)
Acrylamide-bis acrylamide	2.5	7.5
Stacking gel buffer stock	5	-
Resolving gel buffer stock	-	3.75
Ammonium persulphate (1.5%)	1	1.5
Distilled water	11.3	16.65
TEMED	0.03	0.03

2.2.2.3 Sample loading

150 µg (about 150 µl) of protein was used for loading a well. To this sample 10 µl of 0.1% bromophenol blue was added as a marker dye, just before loading.

2.2.2.4 Protein Estimation

Total Protein in samples was estimated using method of Bradford (1976). 100mg of Bradford dye (Coomassie Brilliant Blue G250) was dissolved in 50 ml of 95 % ethanol. 100ml of concentrated phosphoric acid were added and the volume was made up to 200 ml with distilled water. The solution was stored in amber bottles under refrigeration.

To 1ml of the sample, 5 ml of diluted Bradford dye (1:4 in distilled water) was added, mixed well and absorbance was measured immediately at 595 nm in a UV visible spectrophotometer. A standard curve was prepared with known concentration of protein (0.2, 0.4, 0.6,0.8, and 1 mg/ml of Bovine Serum Albumin (BSA) and using distilled water as blank and the protein concentration in the sample was calculated from the standard curve.

2.2.3 STAINING FOR ISOZYMES

Different isozymes namely esterase, isocitric dehydrogenase, peroxidase, superoxide dismutase, catalase, phosphogluco isomerase, alcohol dehydrogenase, malate dehydrogenase and hexokinase were studied.

2.2.3.1 Staining procedure for esterase:

Esterase activity was localized on the gels according to Shaw and Prasad (1970), with minor modifications suggested by Pramanik *et al.*, (1996). 40 mg of α-Naphthyl acetate was dissolved in 1 ml of acetone and the volume made upto 100 ml with 100 mM phosphate buffer (pH 6). Then 76 mg of fast blue RR salt was dissolved in it by vigorous stirring. Gels were incubated in this solution at 35 ^{0}C for 20 minutes, then washed with distilled water. Brown coloured bands of esterase appeared almost

immediately on a clear background.

2.2.3.2 Staining for isocitric dehydrogenase:

100 mg of DL-isocitric acid dissolved in 10ml of 0.5 M Tris HCl buffer, (pH 8.5), added with 3 ml of 0.1M $MgCl_2$ and 8.5 mg of NADP, and the solution was made upto 50 ml. Just before use added 1% aqueous nitroblue tetrazolium and 0.5 ml of 1 % aqueous phenazonium methosulphate. Gel was incubated in dark for 30 minutes at 40 ^{0}C. Presence of isocitric dehydrogenase was detected by observing dark blue bands (Shaw and Prasad, 1970).

2.2.3.3 Staining for superoxide dismutase:

Superoxide dismutase activity was localized on the gels according to Geburuck and Wang (1990) with minor modifications (Pramanik *et al.*, 1996). The gels were immersed in 40 ml of 0.2M Tris HCl, pH 8, 0.5mM EDTA buffer containing 0.2 ml of 0.5 M $MgCl_2$, 1ml of 1% aqueous nitroblue tetrazolium and 0.5 ml of 1 % aqueous phenazonium methosulphate. The gels were exposed to strong fluorescent light for 15 min. followed by one-hour incubation in the dark. Superoxide dismutase was detected as light coloured bands (negatively stained) on a dark blue background.

2.2.3.4 Staining for peroxidase:

20 mg of 3- amino 9-ethyl carbazole was dissolved in 2.5 ml of N, N-dimethyl formamide, and then added with 1 ml of 0.1 M $CaCl_2$ solution, and 5 ml of 1M acetate buffer (pH 4.65). The solution was then made upto 50 ml using distilled water. Just before use 0.7 % of hydrogen peroxide solution was added. Gel was incubated in a refrigerator for 60 minutes. Reddish brown coloured bands in the gel indicated presence of peroxidase enzymes (Shaw and Prasad, 1970).

2.2.3.5 Staining for catalase:

Staining of gels for catalase was performed as per the procedure of Glaszmann *et al.*, (1988). 0.7 % of hydrogen peroxide solution was poured on to the gel. After three minutes the gel was rinsed with water. Then KI solution (1.5 %) with 1 ml of acetic acid was poured onto the gel. White bands on blue background (negatively stained) indicated presence of catalase.

All the gels were photographed immediately after staining.

2.2.3.6 Staining for phosphoglucose isomerase

Staining of gels for phosphoglucose isomerase was done according to procedure of Glaszmann *et al.*, (1988). 5 ml of Tris-HCl buffer (0.5 M, pH 8.5) containing 50 mg fructose-6-phosphate, 0.1 MgCl, 5 mg NADP 10 Units of glucose6-phosphate was mixed with 10 mg of nitroblue tetrazolium and 1 mg phenazine methosulphate just before use. Gels were incubated in this solution for 30 min at 40 ^{o}C.

2.2.3.7. Staining for alcohol dehydrogenase

5 ml of Tris-HCl buffer (0.5 M, pH8.5) containing 0.25 ml ethanol, 10 mg NAD was made up to 50 ml and just before use added 10 mg of nitroblue tetrazolium and 1mg of phenazine methosulphate. Gels were incubated in this solution for 20 min at 40 ^{o}C in dark (Glaszmann *et al.*, 1988).

2.2.3.8. Staining for Malate dehydrogenase

20 ml of Tris- HCl (0.5 M, pH 8.5) containing 250 mg malic acid , 0.1 M $MgCl_2$ and 5 mg NADP was mixed with 10 mg nitroblue tetrazolium and 1 mg phenazine methosulphate just before use. Gels were incubated for 2 h at 40 ^{o}C in dark (Glaszmann *et al.*, 1988).

2.2.3.9 Staining for Hexokinase

50 ml of Tris-HCl buffer (0.2M, pH 8) containing 0.1 M $MgCl_2$, 0.25 g ATP, 5g of glucose, 80 NAD U of G_6PDH and 20 mg of NAD was mixed with 5 mg each of nitroblue tetrazolium and phenazine methosulphate just before use. Gels were incubated in dark at 40°C for 30 min (Glaszmann *et al.*, 1988).

2.2.4 ENZYME ASSAYS:

2.2.4.1 Assay for esterase

Esterase activity was assayed by Huggins and Lapides method (1947). 1 ml of tissue extract, to be assayed was mixed with 2 ml of M/15 phosphate buffer and 5 ml of water and allowed to come to temperature in a water bath at 25°C. 2 ml of, accurately adjusted substrate (0.66 µl of P-nitrophenol) solution was added, the tube was placed in the spectrophotometer, and observed the reading at 400nm. After 20 minutes final reading was obtained. From the standard curve of p- nitrophenol in phosphate buffer the amount of p-nitrophenol in micromoles was read and this value minus the blank control for non-enzymatic hydrolysis was multiplied by the dilution factor. One unit of esterase activity is defined as that amount of enzyme liberating 1 millimol of p-nitrophenol in 20 min at 25 ^{0}C and pH 7 in phosphate buffer when the substrate is at a concentration of 0.666 micromol. The units are expressed per ml of the undiluted fluid.

2.2.4.2. Assay for isocitric dehydrogenase:

Isocitric dehydrogenase activity was assayed by Wolfson and Ashman (1957) method. 2 ml of Tris-Manganous chloride solution (5 volume of 0.01 M manganous chloride in 0.15 M NaCl solution with 15 volume of 0.1 M Tris HCl buffer pH 7.5)

and 0.5 ml of 0.001 M NADP were measured into a small test tube, added 0.5 ml of tissue extract and placed in a water bath at 25°C. 0.1 ml of substrate (0.1 M Sodium dl isocitrate) was added mixed and transferred to a spectrophotometer cuvette and read against a blank at 1-min interval for 5 minutes at 340 nm. Activity was expressed in terms of micromols of $NADPH_2$ formed per litre of sample per hour at 25^{0} C. Enzyme activity was expressed as Units per litre.

2.2.4.3 Assay for superoxide dismutase:

Superoxide dismutase activity was assayed by Marklund and Marklund method (1974). 0.2 mM of pyrogallol was added to 10 ml of 50 mM Tris HCl buffer pH 8.2, containing 1 mM EDTA. 1 ml of tissue extract was added and increase in absorbance at 420 nm was measured. Rate of pyrogallol auto oxidation at 420 nm is 0.02/min. in the absence of superoxide dismutase. A Unit of enzyme is generally defined as the amount of enzyme, which inhibits the pyrogallol autooxidation by 50 %. Activity was expressed as Units per ml.

2.2.4.4 Assay for peroxidase:

Peroxidase activity was measured using procedure of Putter, (1974). 3 ml of 0.1 M Phosphate buffer, 0.05 ml of 20 mM Guaiacol solution, 0.1 ml of tissue extract and 0.03 ml of 12.3 mM hydrogen peroxide solution was mixed in a cuvette and read in spectrophotometer at 436 nm. Noted the time required in minutes to increase the absorbance by 0.1. The peroxidase activity was measured as rate of formation of guaiacol dehydrogenation product, and expressed as Units per litre. Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of extract is calculated as

$$\text{Enzyme activity units per litre} = \frac{3.18 \text{ X } 0.1 \text{ X } 1000}{6.39 \text{ X } 1 \text{ X } t \text{ X } 0.1} = \frac{500}{t}$$

2.2.4.5 Assay for catalase:

For assaying catalase, decomposition of hydrogen peroxide by catalase was measured (Luck, 1974). 3 ml of hydrogen peroxide-phosphate buffer (0.067 M phosphate buffer pH 7 and 10 % w/v hydrogen peroxide) was mixed with 0.01ml of tissue extract. The time required for a decrease in absorbance from 0.45 to 0.4 was measured at 240 nm and activity expressed as Units per ml.

Activity of enzymes before and after giving salt stress was studied. Effect of NaCl on enzyme activity under *in vivo* and *in vitro* conditions was also studied. Roots of plants were immersed in 100 mM of NaCl solution for 48 hours and shoot portions were used to study the effect of NaCl on enzyme activity in vivo. 50 mM and 100 mM NaCl was added directly to the sample just before measuring enzyme activity to study the effect of NaCl *in vitro*.

2.2.5 NA^+ CONTENT IN DIFFERENT RICE VARIETIES

In case of tillering and flowering stage sixth leaf from the terminal bud was selected as sample. Sample was cut into very small pieces and stored over night below 70°C and centrifuged at 15,000 rpm. Extract was taken out and Na^+ ion content was determined using a flame photometer.

2.2.6 ISOZYME STUDIES AND STUDIES ON NA^+ CONTENT IN REGENERATED PLANTS

Variations in isozyme banding patterns of esterase and isocitric dehydrogenase, and activity were studied in plants regenerated from three month old calli and also from anther cultures and were compared with seed germinated plants. Mature leaves from regenerated plants in the tillering stage were harvested and divided into two portions. One portion was used to study isozyme-banding pattern and activity studies (protocols as mentioned above). The other portion was used to study Na^+ content.

2.2.7 ISOZYME STUDIES IN F1 GENERATION

Variations in banding pattern and the activities of different isozymes of F1 hybrids were studied as described under section 2.2.6.

2.3 RESULTS

2.3.1 ISOZYME ANALYSIS

Changes in isozyme activity in different tissues were detected by pattern shifts on zymograms subsequent to electrophoresis. In order to make definitive comparison among the patterns obtained from different tissues, a Rf value was calculated for each band. The mean of observations from three gels was used to compute Rf value for each band. Only the isozymes moving towards anode were studied. Isozyme bands were numbered in the order of their migration, such that band with lowest Rf value was numbered as band 1.

2.3.1.1. Banding patterns of esterase

All the four rice varieties showed variation in banding patterns depending on the developmental stages (Fig.11). Staining of esterase zymograms showed ten bands with Rf values 0.37, 0.4, 0.43, 0.47, 0.5, 0.57, 0.6, 0.65, 0.73 and 0.77 respectively. Bands 3, 5, and 8 with Rf values 0.43, 0.5, and 0.65 respectively were found in every stage of all rice varieties, irrespective of the presence or absence of NaCl. Band 7 was present only in Pokkali, which was faint in tillering stage and flowering stage. Under stress, this band became further fainter. Band 7 was found to be absent in all salt sensitive rice varieties, irrespective of the developmental stage.

Salt stress had an impact on expression of isozymes of esterase. It caused appearance of certain bands, like bands 1 and 2 in flowering stage of Pokkali, and band 9 in tillering stage of MI 48 and embryo of Pokkali under stress. Salt stress also resulted in disappearance of bands in MI 48 like band 2 in embryo, band 5 in callus and band 10 in tillering stage.

Variations in banding patterns with respect to the developmental stages were

observed. In embryo stage, Pokkali showed all bands except 1 and 10. Band 7 was thicker compared to other bands, except band 8. After NaCl treatment band 6 was slightly thinner. In all the sensitive rice varieties, band 7 was absent. In Annapoorna and Jyothi, band 6, was thicker. However in MI 48, band 6 was absent. Band 9 was present in Pokkali in presence of stress whereas MI 48 showed band 9 both in presence and absence of NaCl.

In the case of 14-day-old seedling stage, all varieties showed bands 3, 4, 5 and 8 but bands 1 and 2 were absent in all rice varieties. In MI 48 band 3 and 5 were thinner when compared to other varieties. Pokkali, as in embryo stage, showed an extra band 7, which was absent in salt sensitive varieties.

In tillering stage band 6 and 7 were faint even in Pokkali. Band 4 was thin in all salt sensitive varieties, while in Pokkali it was thicker. Further Pokkali showed band 7 which was absent in other varieties, whereas band 10 was absent in Pokkali, while present in all salt sensitive rice varieties. All the bands in sensitive varieties were slightly thicker in presence of stress.

In flowering stage also there was a slight reduction in the thickness of bands on exposure to stress. As in other stages, bands 3, 4, 5 and 8 were present in all rice varieties. While band 6 was present in Annapoorna and Jyothi, Pokkali had a faint band 7. Pokkali in the presence of salt stress showed band 1 and 2. In MI 48 band 9 was present which was very faint in both stressed and unstressed condition.

Rice callus, showed a slight change in banding pattern compared to the various developmental stages. In MI 48, band 5 was absent in callus, although it was present in all other stages. Also bands 1 and 2 were absent in all rice varieties. Band 6 in Annapoorna, Jyothi and Pokkali, and band 7 in Pokkali were found to be thicker,

similar to the bands in embryo and 14 day old seedlings. Band 9 was absent in MI 48, but present in all other varieties.

2.3.1.2. Banding patterns of isocitric dehydrogenase

Zymograms of isocitric dehydrogenase showed three bands in different varieties of rice with Rf values 0.29, 0.34, and 0.4 (Fig.12). Of these, band 3 with Rf value 0.4 was present only in Pokkali. A slight variation was seen in some developmental stages. Bands 1 and 2 were present in all the rice varieties, at all developmental stages, with and without salt stress, except in embryo stage of Pokkali in the absence of salt stress. In certain stages, bands were thick and difficult to resolve into distinct bands. A slight reduction in band thickness was found in response of NaCl stress in most developmental stages.

In embryo stage Pokkali showed only two bands, i.e., 2 and 3 and band 1 was missing. In presence of stress this band is however present. All other varieties showed 2 bands, 1 and 2, but a decrease in their thickness was observed in the presence of stress.

In 14-day-old seedling stage, Pokkali showed 3 bands and all other varieties showed two bands 1 and 2. Stress caused decrease in thickness of bands in all rice varieties.

In tillering stage MI 48 which is a highly salt sensitive variety showed only one band ie. band 2. In other sensitive varieties bands 1 and 2 were present and in Pokkali band 3 was also present. Stress caused a slight decrease in thickness of bands. Band 1 in Pokkali was very thin in presence of stress.

In flowering stage, Pokkali showed 3 bands while other varieties showed only 2 bands, irrespective of presence or absence of stress. In MI 48, stress caused an

increase in thickness of band 1.

In callus, the banding pattern of isocitric dehydrogenase was similar to that in flowering stage. However bands were thick and, the resolution was poor.

2.3.1.3 Banding patterns of superoxide dismutase

Superoxide dismutase showed only three bands in all the four rice varieties and there was no difference in the banding pattern irrespective of the development stage and presence or absence of salt stress.

2.3.1.4 Banding patterns of peroxidase

Peroxidase zymograms had only a single band in all varieties, irrespective of the developmental stage and stress.

2.3.1.5 Banding patterns of catalase

The single band of catalase, which was observed, did not show any variation in different developmental stages or under stress. No varietal specific variation could be observed.

2.3.1.6 Banding patterns of phosphogluco isomerase

Phosphogluco isomerase showed only one band, which did not change under stress or in different developmental stages of the four varieties studied.

2.3.1.7 Banding patterns of Malate dehydrogenase

Malate dehydrogenase also had a single isozyme, which did not show any variation in different developmental stages and under stress.

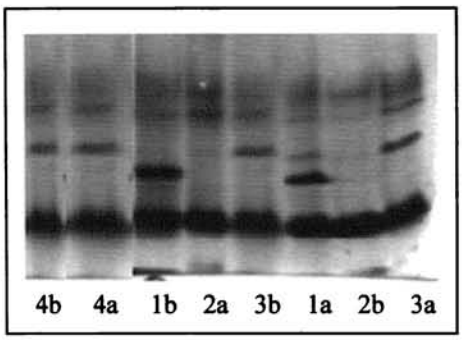
2.3.1.8 Banding patterns of Hexokinase

Zymograms of Hexokinase showed two bands. However they did not show any variation in different developmental stages, irrespective of exposure to salt stress.

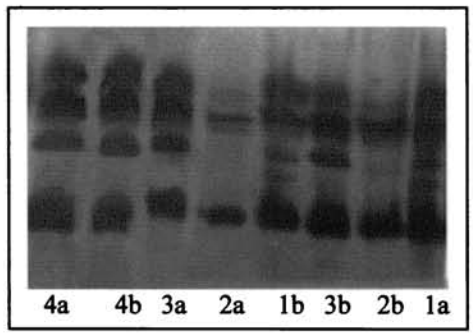
Fig . 11

ESTERASE BANDING PATTERN IN FOUR RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS BEFORE AND AFTER NaCl TREATMENT

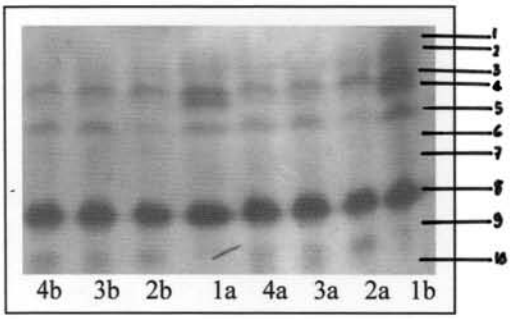
Embryo



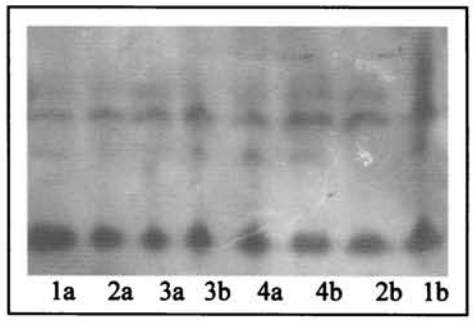
14 day old seedling



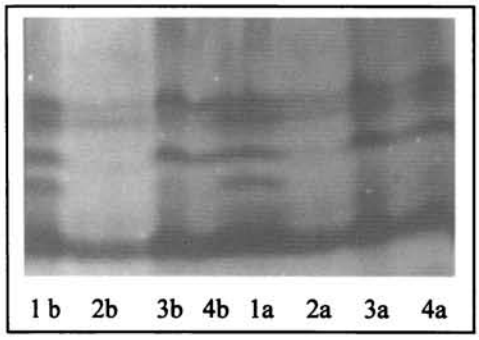
Tillering



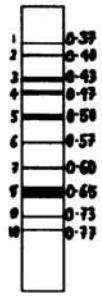
Flowering



Callus



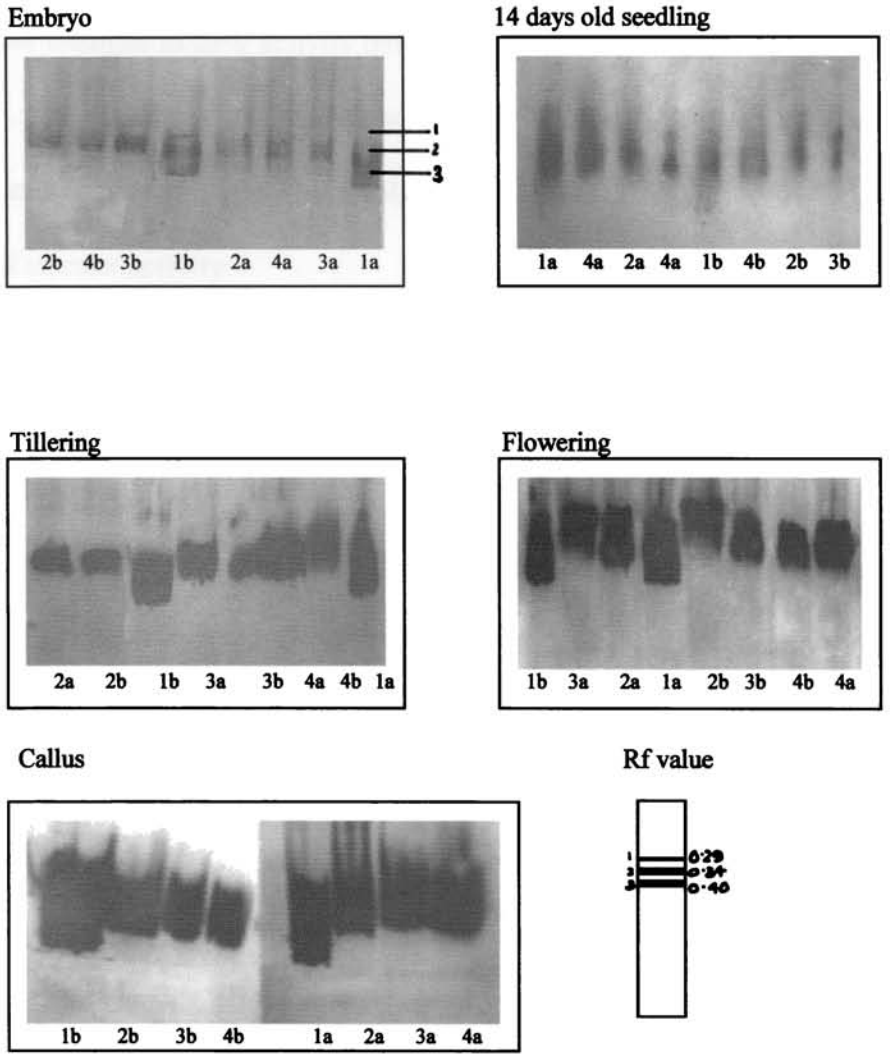
Rf value



1.Pokkali
2. MI 48
3 Annapoorna
4 Jyothi
a Before NaCl treatment
b After NaCl treatment

Fig. 12

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN FOUR RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS BEFORE AND AFTER NaCl TREATMENT



1 Pokkali
2 MI 48
3 Annapoorna
4 Jyothi
a Before NaCl treatment
b. After NaCl treatment

2.3.2 ENZYME ACTIVITY

From among the eight enzymes analyzed for banding pattern, esterase, isocitric dehydrogenase, superoxide dismutase, peroxidase and catalase were further studied for changes in their activity before and after salt treatment, in all the four rice varieties. The activity was observed both under *in vitro* (i.e., NaCl was added to the plant extract) and *in vivo* conditions (plant subjected to stress).

2.3.2.1 Esterase activity

Activity of esterase, in different rice varieties, was higher in embryo stage when compared to other stages and showed a significant reduction in activity in the presence of stress (Fig.13, Appendix V A).

In the absence of salt stress, in Pokkali, esterase activity decreased from embryo to tillering stage and increased in flowering stage. Callus of Pokkali showed a higher esterase activity compared to the different developmental stages (212.6 U/ml), except embryo stage. In MI 48, 14-day-old seedlings showed lesser activity than other stages, and a sharp increase in activity was observed from 14-day-old seedling stage to flowering stage. In Annapoorna, tillering stage showed lower esterase activity than all other stages (78 U/ml). Callus showed higher esterase activity (200.6 U/ml) than other stages, except embryo stage. Esterase activity in Jyothi was similar to Annapoorna, but flowering stage in Jyothi showed higher esterase activity than 14-day-old seedling and tillering stages.

Effect of NaCl *in vivo* on esterase activity was found to differ in the different developmental stages (Fig.13). In Pokkali, esterase activity decreased in presence of salt stress in embryo and 14 day old seedlings, but it increased in other stages.

Esterase activity in MI 48 decreased under salt stress, at embryo and flowering stage, and in callus, but increased slightly in 14 day old seedling and tillering stage. In Annapoorna, embryo stage and callus showed a decrease in esterase activity under stress condition, but in Jyothi flowering stage also showed a decrease in esterase activity. Esterase activity increases sharply in tillering stage of Annapoorna under stress (from 78 U/ml to 203.3 U/ml). Callus of Pokkali showed an increase in esterase activity under stress, but other varieties showed a reduction in its activity.

2.3.2.2 Isocitric dehydrogenase activity

In general, in the absence of stress isocitric dehydrogenase activity was significantly higher in callus compared to the developmental stages (Fig.14, AppendixV B). In Pokkali, isocitric dehydrogenase activity increased from embryo to the flowering stage whereas in MI 48 and in Annapoorna isocitric dehydrogenase activity decreased from embryo to tillering stage and then increased in the flowering stage. 14-day-old seedlings of Jyothi showed higher enzyme activity than other developmental stages (266.8 U/ml).

Salt stress *in vivo* lead to a reduction in the isocitric dehydrogenase activity in most developmental stages. However, embryo stage of Pokkali, Annapoorna and Jyothi showed an increase in activity, whereas MI 48, a highly sensitive variety, showed decrease in isocitric dehydrogenase activity under stress condition.

2.3.2.3 Superoxide dismutase activity

Superoxide dismutase activity in Pokkali was different from other rice varieties (Fig.15, Appendix V C). Superoxide dismutase activity was higher in embryo stage (196.6 U/ml) and decreased in the 14-day-old seedling stage. However it again increased in tillering stage. But in all the other varieties superoxide dismutase activity

was lower in embryo stage. In MI 48, 14-day-old seedling showed higher enzyme activity, which decreased gradually towards flowering stage. In Annapoorna and Jyothi, tillering stage showed highest activity (403.5 U/ml and 295 U/ml respectively).

Effect of NaCl *in vivo* on superoxide dismutase activity *in vivo* varied in different stages of the rice varieties. Generally, stress caused an increase in superoxide dismutase activity. But tillering stage of all rice varieties showed a decrease in superoxide dismutase activity. Superoxide dismutase activity in the callus of Pokkali and flowering stage of Annapoorna decreased under stress conditions.

2.3.2.4 Peroxidase activity

In Pokkali, Annapoorna and Jyothi, peroxidase activity increased from embryo to 14-day-old seedling. However it decreased in tillering stage and again increased in flowering stage. In MI 48, flowering stage showed the highest peroxidase activity (3012 U/ml). Salt stress *in vivo* caused an increase in peroxidase activity except in the flowering stage of Annapoorna (Fig.16, Appendix V D).

2.3.2.5 Catalase activity

In Pokkali, tillering stage showed maximum catalase activity. Whereas in all salt sensitive varieties, flowering stage showed higher catalase activity (Fig.17, Appendix V E).

Effect of salt stress *in vivo* on catalase activity differed in different stages of the rice varieties. In Pokkali, while embryo stage and callus showed an increase in activity under stress, other stages showed a decrease. Stress caused a reduction in catalase activity in every developmental stage of MI 48. But Annapoorna showed an increase in catalase activity under stress, except in flowering stage. Embryo of Jyothi

Fig. 13

EFFECT OF NaCl TREATMENT ON ESTERASE ACTIVITY IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

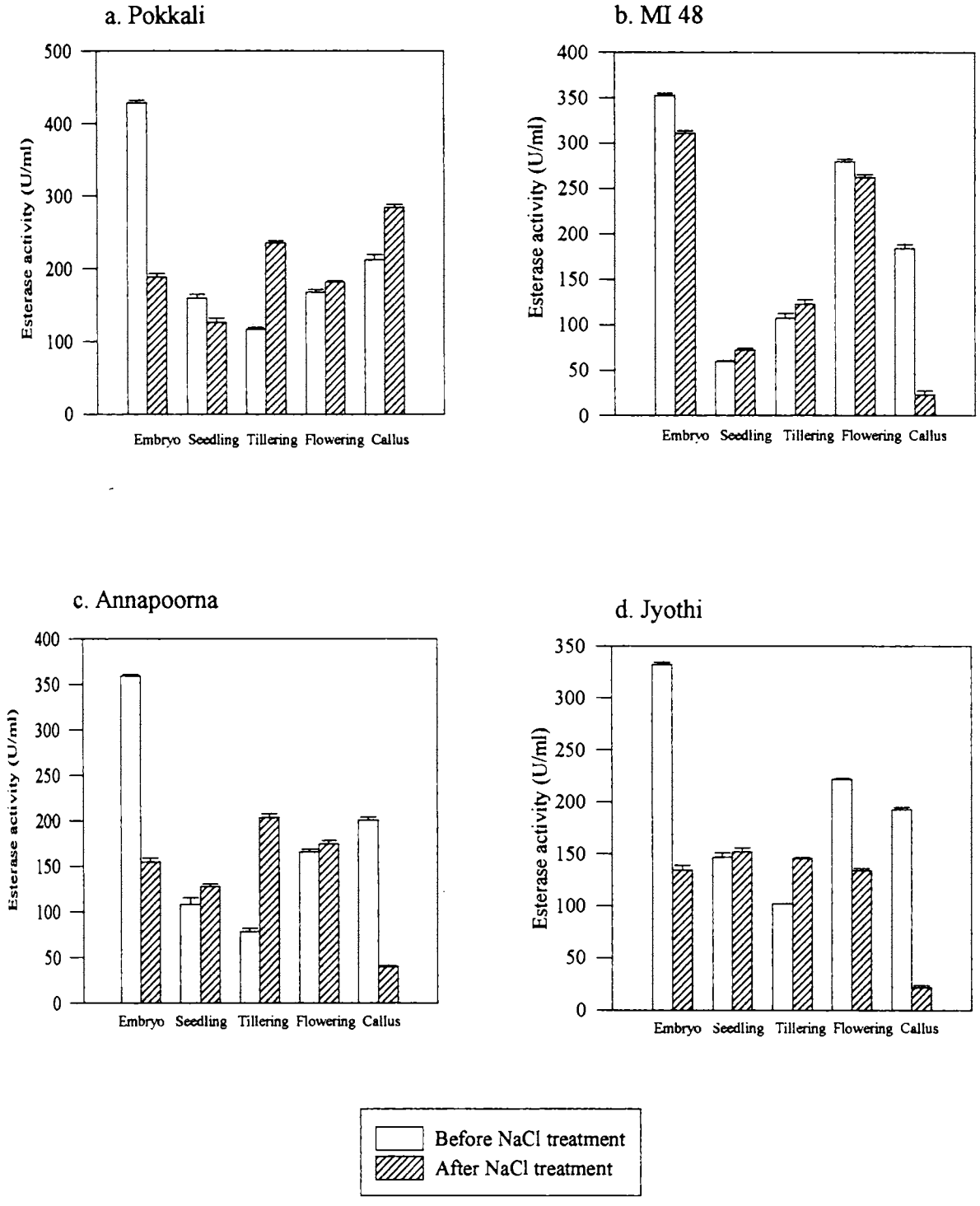


Fig. 14

EFFECT OF NaCl TREATMENT ON ISOCITRIC DEHYDROGENASE ACTIVITY IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

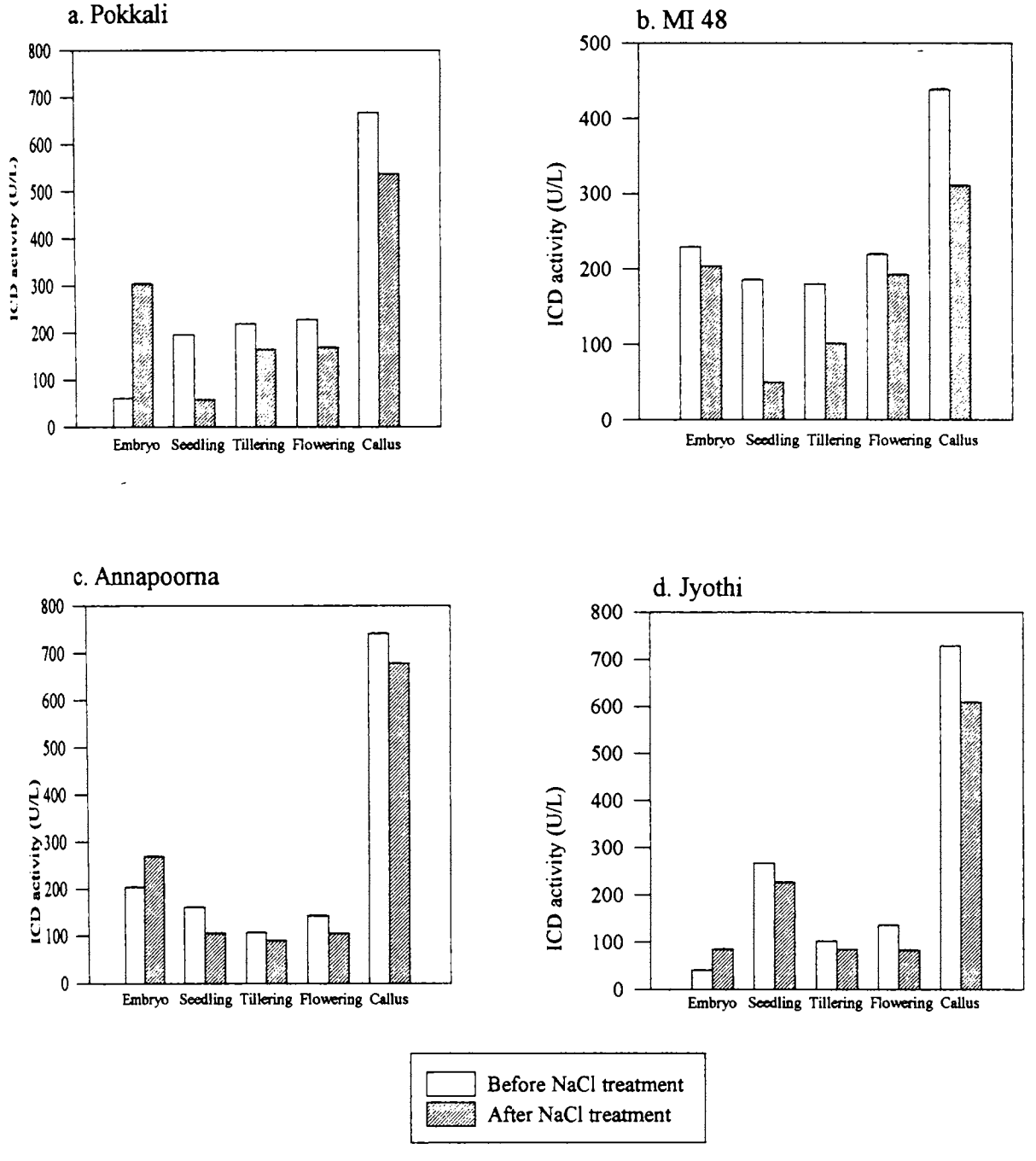


Fig. 15

EFFECT OF NaCl TREATMENT ON SUPEROXIDE DISMUTASE ACTIVITY IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

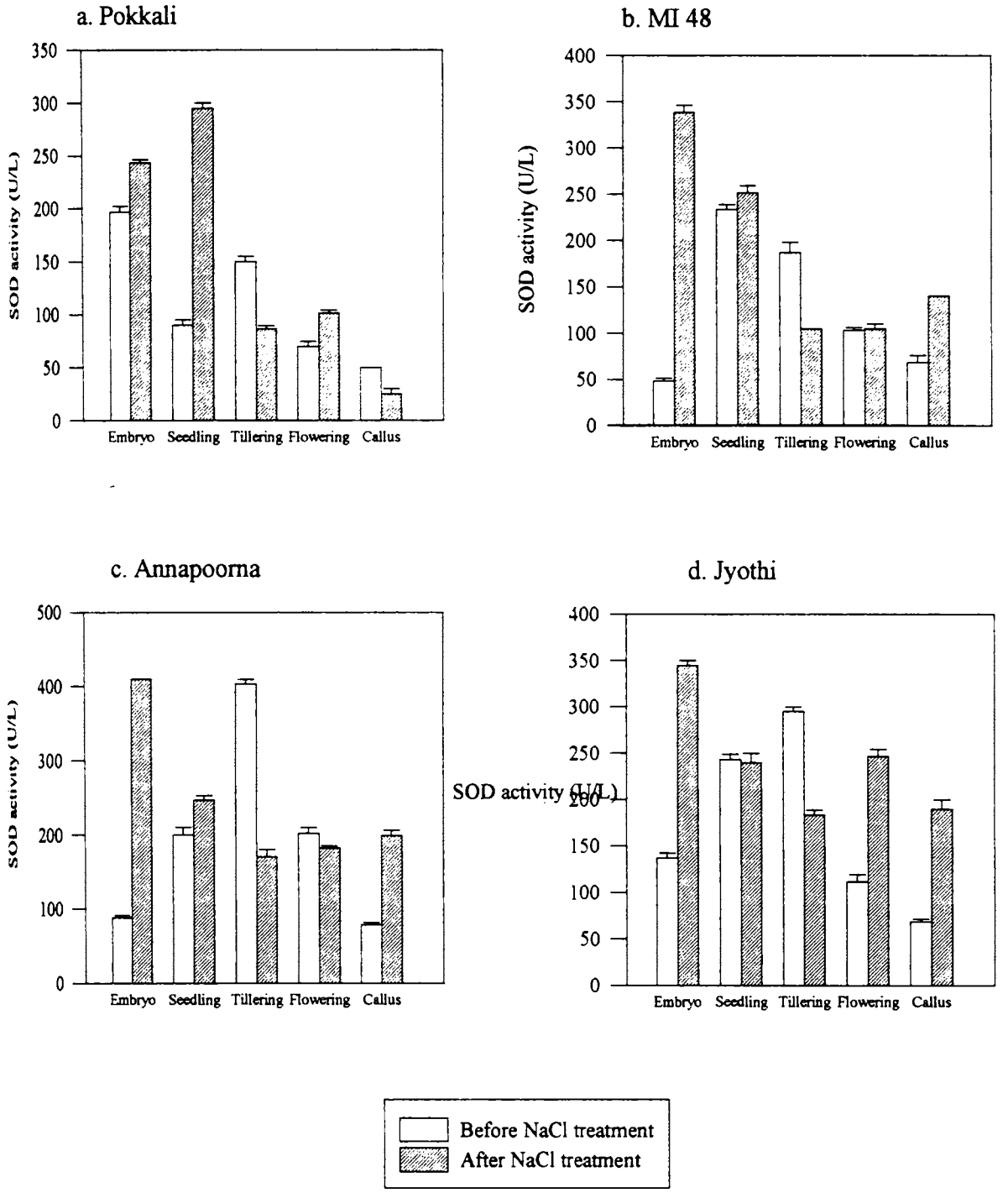


Fig. 16

EFFECT OF NaCl TREATMENT ON PEROXIDASE ACTIVITY IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

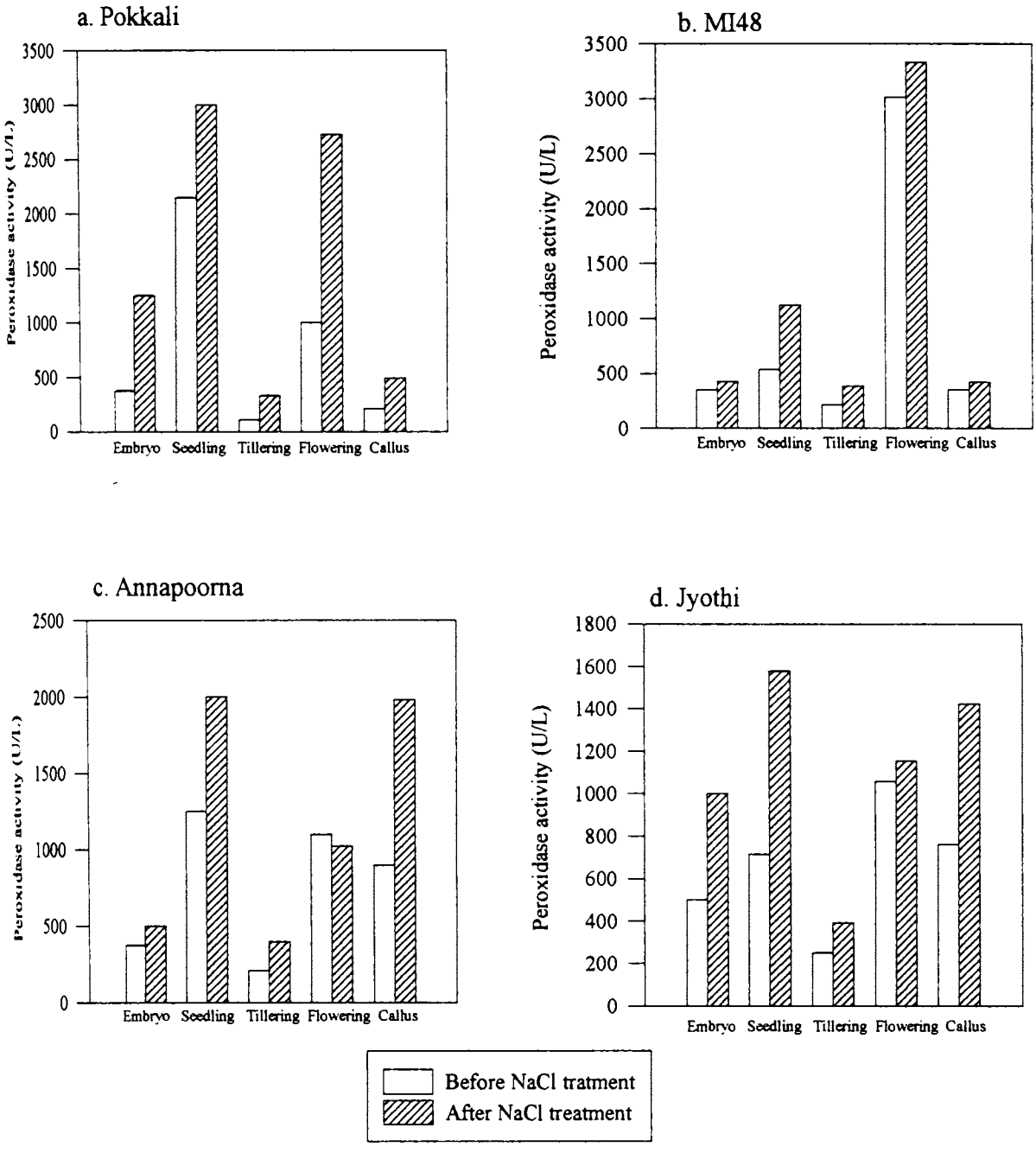
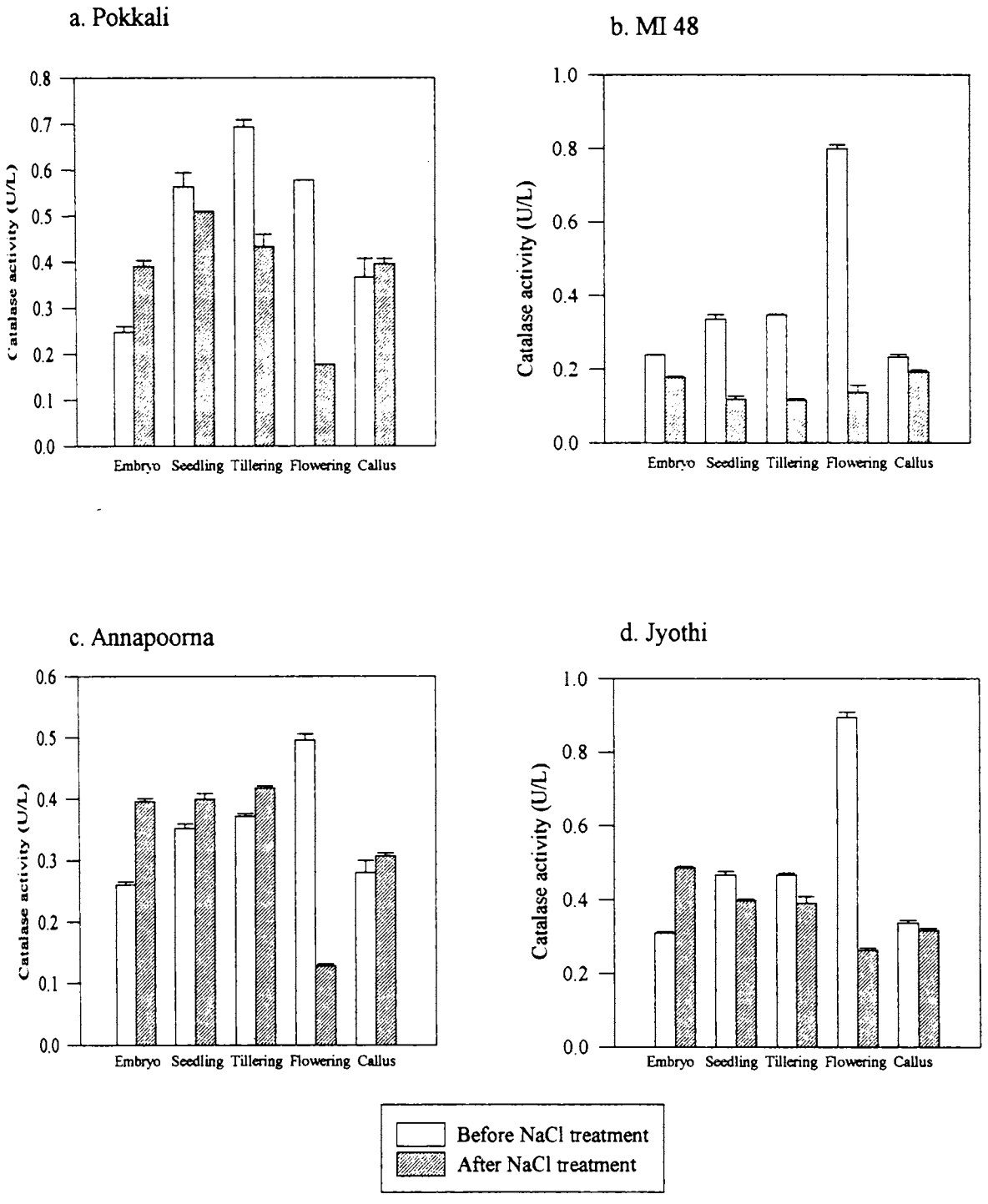


Fig. 17

EFFECT OF NaCl TREATMENT ON CATALASE ACTIVITY IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS



showed an increase in catalase activity while in the other stages a decrease in activity was recorded.

2.2.3 EFFECT OF NaCl ON ENZYME ACTIVITIES *IN VITRO*

2.3.3.1 Effect of NaCl on esterase activity *in vitro*

Presence of NaCl *in vitro* caused a slight reduction in esterase activity in most cases (Fig.18, Appendix VI A). A sharp decrease in enzyme activity was noticed in the embryo stage of Pokkali and MI 48 in the presence of 50 mM NaCl. 100 mM NaCl caused a further reduction in enzyme activity. Tillering stage of Annapoorna and Jyothi showed an increase in esterase activity in the presence of 50 mM NaCl. In all the other stages there was a decrease in enzyme activity in 50 mM NaCl and 100 mM NaCl.

2.3.3.2 Effect of NaCl on superoxide dismutase activity *in vitro*

In embryo stage of Pokkali, there was an increase in superoxide dismutase activity in presence of NaCl when it was added directly to the sample. There was no significant change in superoxide dismutase activity in embryo stage of MI 48 and Annapoorna. In Jyothi, 50 mM NaCl did not cause any change but 100-mM NaCl caused a reduction in enzyme activity. In the callus of Pokkali also there was not much change in superoxide dismutase activity. Whereas, in tillering stage, 50 mM NaCl resulted in a decreased enzyme activity. However, 100 mM NaCl did not cause any further reduction. In all other stages 50 mM NaCl caused a slight reduction and 100 mM NaCl caused a further decrease in all rice varieties (Fig.19, Appendix VI B).

2.3.3.3 Effect of NaCl on peroxidase activity *in vitro*

Effect of NaCl *in vitro* on peroxidase activity was insignificant when compared to the other enzymes (Fig.20, Appendix VI C). Reduction in peroxidase activity in the presence of NaCl was found only in embryo stage of all rice varieties. 14-day-old seedling stage, flowering stage and callus of MI 48 and tillering of Jyothi did not show much change in enzyme activity. In the other stages of all rice varieties, there was a slight increase in peroxidase activity.

2.3.3.4 Effect of NaCl on catalase activity *in vitro*

Effect of NaCl *in vitro* on catalase activity varied in different stages (Fig.21, Appendix VI D). In Pokkali and MI 48 there was not much change in catalase activity in embryo stage, in the presence of NaCl. Whereas, in Annapoorna there was a reduction in enzyme activity due to NaCl. 14-day-old seedlings of Pokkali showed a decrease in activity due to stress *in vitro*. However, there was an increase in activity in tillering stage. In flowering stage of Pokkali, 50 mM NaCl caused a reduction in activity. In 14 day old seedlings of MI 48, Annapoorna and Jyothi, catalase activity decreased at 50 mM NaCl, showed a slight increase at 100mM NaCl. Flowering stage of three salt sensitive varieties showed no reduction in enzyme activity, but recorded a slight increase. Callus stage of MI 48 and Jyothi did not show any change in activity, but in Annapoorna salt stress caused a decrease in activity.

2.3.4 NA^+ CONTENT IN RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES

In Pokkali levels of Na^+, quantitatively was higher in every stage both in the presence and absence of NaCl when compared to the salt sensitive varieties (Fig.22,

Fig. 18

EFFECT OF NaCl ON ESTERASE ACTIVITY *IN VITRO* IN RICE VARIETIES IN DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

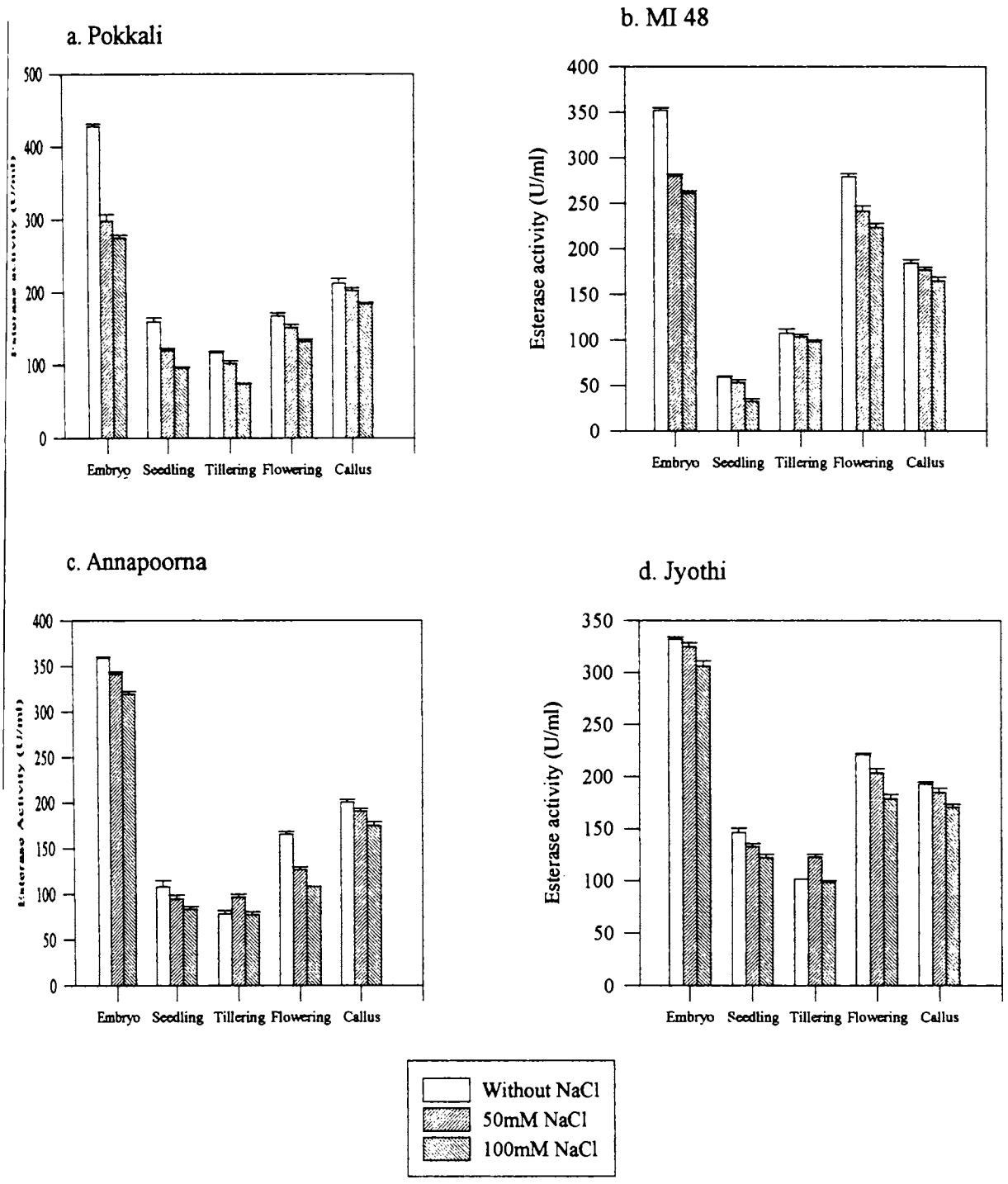


Fig. 19

EFFECT OF NaCl ON SUPEROXIDE DISMUTASE ACTIVITY *IN VITRO* IN RICE VARIETIES IN DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

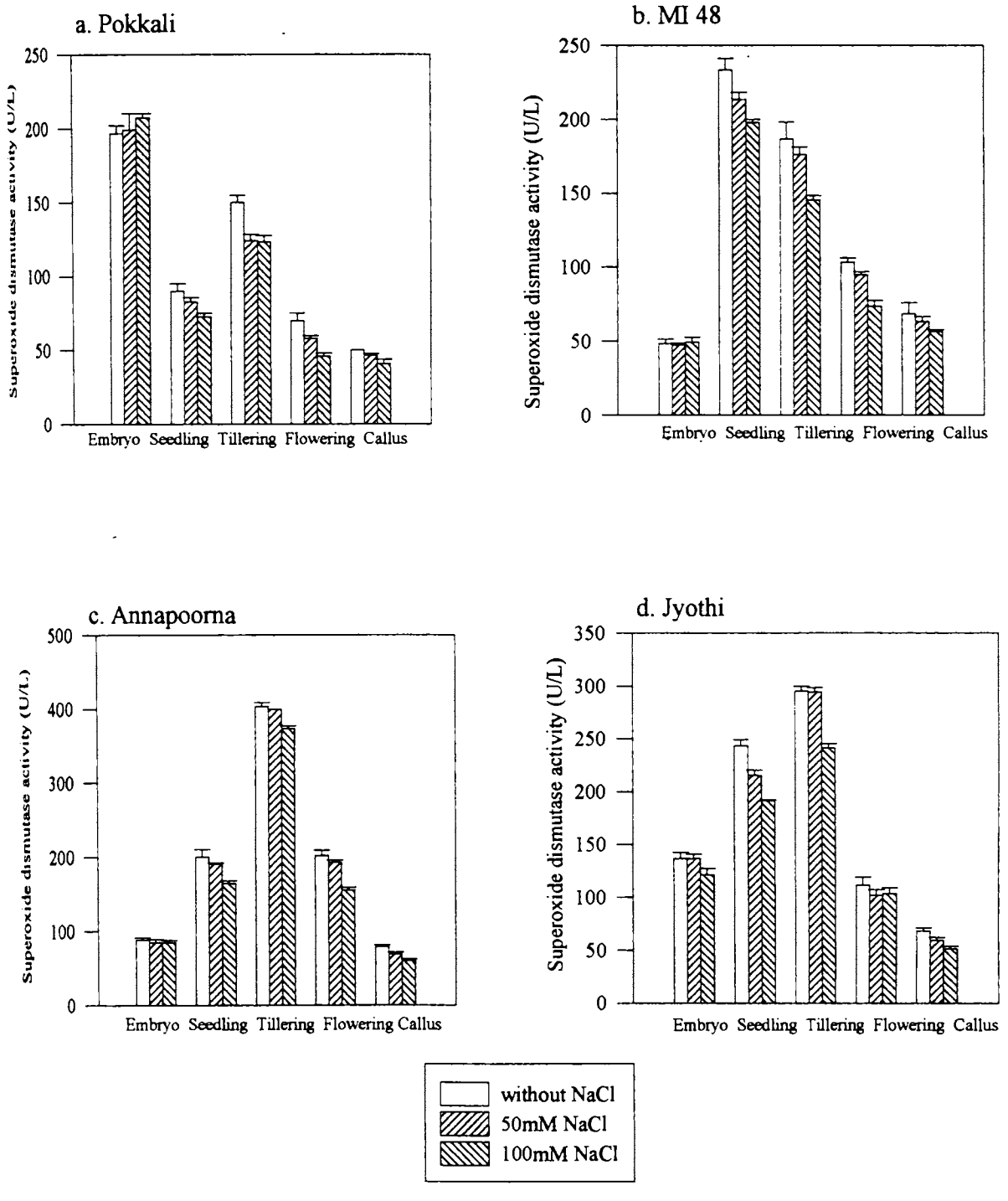


Fig. 20

EFFECT OF NaCl ON PEROXIDASE ACTIVITY *IN VITRO* IN RICE VARIETIES IN DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

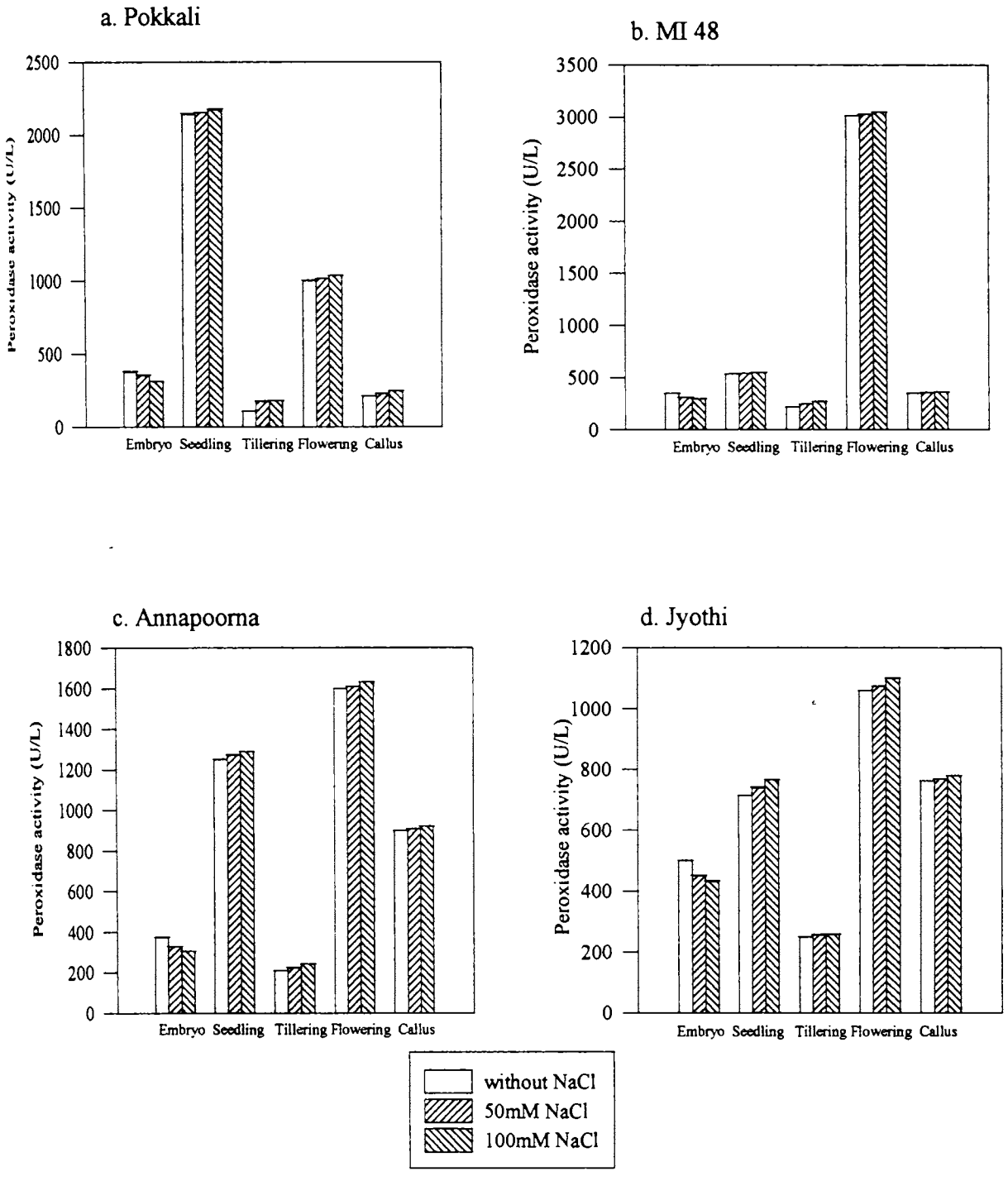


Fig. 21

EFFECT OF NaCl ON CATALASE ACTIVITY *IN VITRO* IN RICE VARIETIES IN DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS

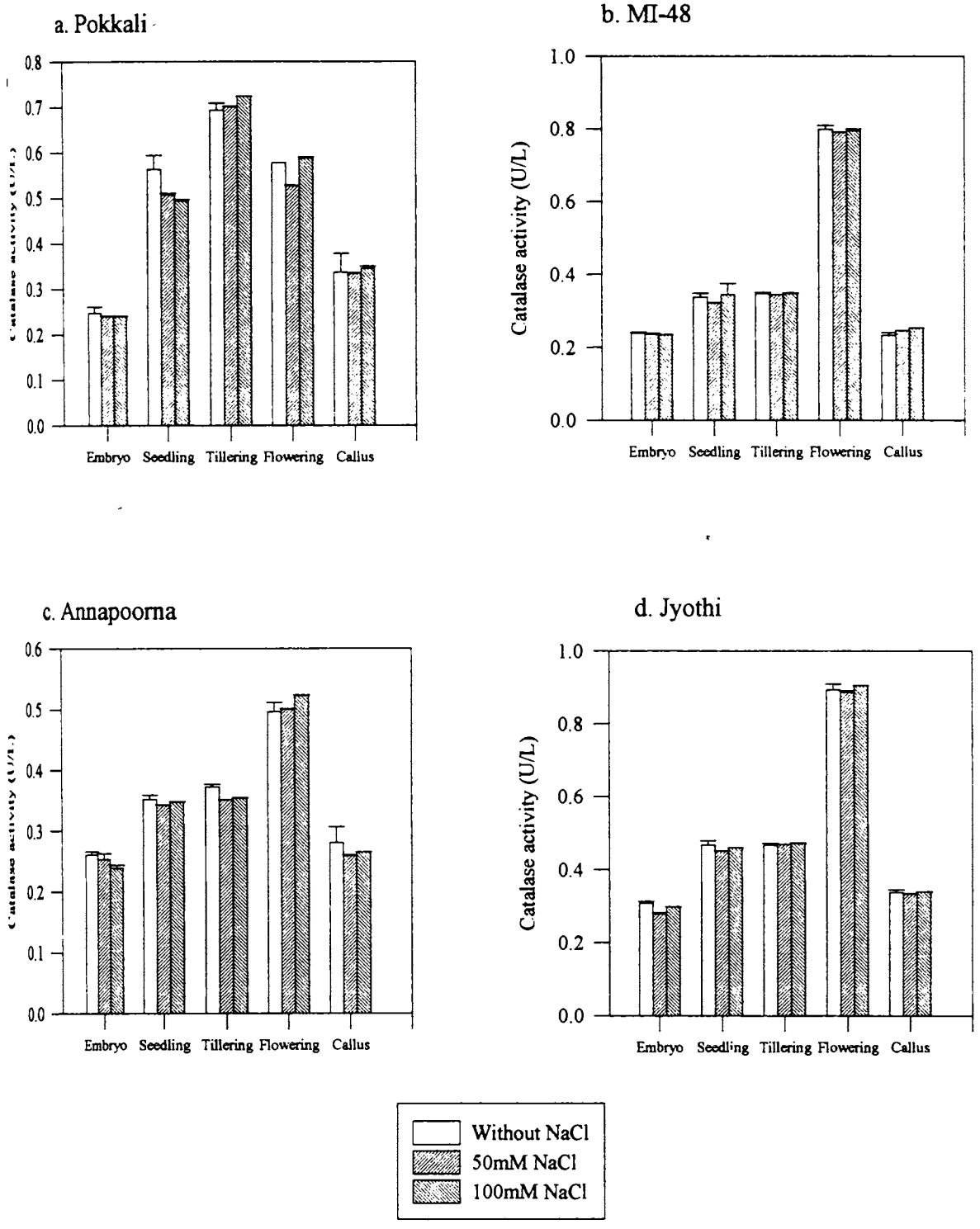
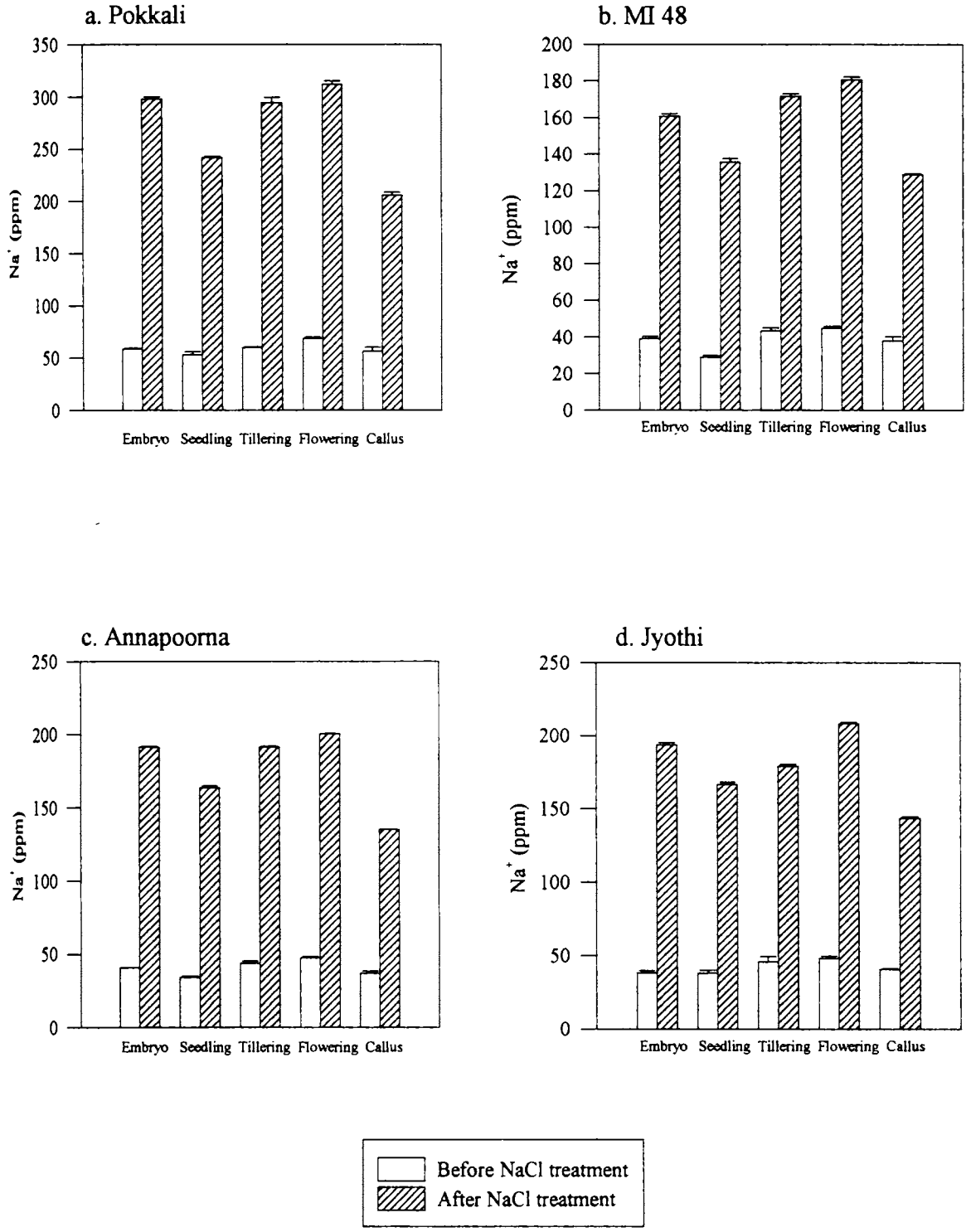


Fig. 22

Na^+ CONTENT IN DIFFERENT RICE VARIETIES AT DIFFERENT DEVELOPMENTAL STAGES AND IN CALLUS BEFORE AND AFTER NaCl TREATMENT



Appendix VII). In the absence of NaCl, flowering stage of Pokkali showed higher Na^+ contents (68.73 ppm) than the other developmental stages and callus. MI 48 had the lowest Na^+ content in its plant body, compared to other rice varieties. Compared to MI 48 and Annapoorna, Jyothi showed higher Na^+ content, in most stages except, the embryo stage.

Under stress condition Pokkali showed almost three-fold increase in Na^+ in older leaves, but only two-fold increase was noted in sensitive varieties. Increase in Na^+ content under stress was almost similar in sensitive varieties.

2.3.5 ESTERASE BANDING PATTERN IN REGENERATED PLANTS.

Variations in esterase banding patterns and activity were observed in regenerated plants of the rice varieties tested.

2.3.5.1 Variations in regenerated plants of Pokkali

Results presented in Fig.23 indicate that many of the regenerated plants of Pokkali showed variations in esterase banding patterns. While some showed slight variation in banding pattern, others showed variation in thickness of bands and corresponding variations in enzyme activity. Seventy-five plants were regenerated and out of these, 39 plants were studied for esterase variation and Na^+ content. Of these, 11 plants corresponding to lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 showed an additional band between the bands 2 and 3, which was absent in seed germinated plants. Bands 6 and 7 were faint in these groups of plants, and band 9, which was present in seed germinated plants, was found to be absent. In plants corresponding to lane 14, 15, 20, 21, 25 and 26, band 6 and 7 were thicker than in other regenerated plants and seed germinated plants. Increase in thickness of bands showed a

corresponding increase in enzyme activity. Generally, regenerated plants showed more esterase activity than seed germinated plants. This increase in thickness and activity of enzymes was found to be correlated with Na^+ content of regenerated plants. Plants in lane 38 and 39 showed thinner bands when compared to bands in other lanes and correspondingly their activity and Na^+ content was similar to seed germinated plants (Table.1). Plants with an additional band between 2 and 3 did not show any increase in enzyme activity or Na^+ content. Plants with thicker band 6 and 7 showed highest esterase activity and Na^+ content. Thicker bands 6 and 7 in lane 14, 25 and 26 had higher enzyme activity and Na^+ content.

Seventy-five plants were regenerated from anther cultures, and out of these 38 plants was studied for esterase variation (Fig.24) and Na^+ content (Table.2). Plants in lanes 7, 8, 9, 10, 11 and 12 showed faint bands 3 and 4 and low enzyme activity. Lanes 22, 23, 24 and 25 showed an additional band above the band 1. Similarly plants corresponding to lanes 19, 22, 23, 24 and 25 have an additional band between band 4 and 5. These four plants with two additional bands showed higher esterase activity and Na^+ content. Plants corresponding to lanes 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35 had an additional band between band 5 and 6. But these plants did not show a significant increase in enzyme activity and Na^+ content compared to plants having two additional bands (between 4 and 5, and above 1) and thick bands 6 and 7.

2.3.5.2 Variations in regenerated plants of MI 48

In MI 48, 85 plants were regenerated from callus culture and out of these 39 plants was studied for variation in esterase and Na^+ content. The regenerated plants of MI 48 showed variation in band thickness (Fig.25), mainly in band 8, and a corresponding variation in esterase activity and Na^+ content (Table.3). Generally

regenerated plants showed higher enzyme activity and Na^+ content.

Out of 90 plants regenerated from anther cultures of MI 48, 51 were studied and showed wide variation in esterase banding pattern and activity (Fig.26). All the bands from 1-8 including faint bands 6 and 7, which were absent in seed germinated plants, were present in plants corresponding to lane 1, 2, 3, 4 and 5. These plants showed higher enzyme activity and Na^+ content which was almost similar to Pokkali (Table.4). Lane 21 showed a faint band 7 and an increase in enzyme activity. Plant in lane 39 also had additional bands 6 and 7, and higher enzyme activity and Na^+ content.

2.3.5.3 Variations in regenerated plants of Annapoorna

Out of 90 plants regenerated from callus cultures of Annapoorna, 39 were studied and they did not show wide variation in esterase banding pattern, but there were some change in band thickness (Fig.27). Plants corresponding to lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 26, 27 and 28 showed additional faint band 7, which was present only in Pokkali. Interestingly, these plants showed much higher esterase activity and Na^+ content (Table.5). All the regenerated plants had bands 1 and 2, but band 9, which was present in seed germinated plants, was missing in regenerated plants.

Ninety plants were regenerated from anther culture of Annapoorna, and out of these 39 were studied for esterase variation. These plants showed significant variation from seed germinated plants in banding pattern and also there were changes in band thickness (Fig.28). In some plants like those in lane 1, 2, 3, 4, 11, 12, 13, 14 to 32 showed a faint band 7 which was present only in seed germinated plants of Pokkali. Bands 1 and 2 were present in most of the anther culture regenerated plants. Plants

with band 7, which was absent in seed germinated plants showed higher esterase activity and Na^+ content (Table.6).

2.3.5.4 Variations in regenerated plants of Jyothi

Ninety plants were regenerated from callus cultures of Jyothi and in 39 plants studied variations in banding pattern and band thickening were observed (Fig.29). Two regenerated plants, corresponding to lanes 1 and 2, showed faint band 7, which was absent in seed germinated plants and present only in Pokkali. These plants had high esterase activity and Na^+ content (Table.7). In plants, corresponding to lanes 15, 16, 17, 18, 19, 20, 21, 22 and 23, bands 1 and 2 were absent. Their esterase activity and Na^+ content was lower when compared to other plants.

Out of 90 plants regenerated from anther culture of Jyothi, 39 were studied and showed wide range of variations in esterase and Na^+ content (Fig.30). All the bands from 1 to 10 were present in the anther culture regenerated plant corresponding to lane 7, which also had high esterase activity and Na^+ content (Table.8). Plants in lane 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26 showed bands from 1 to 8, but bands 9 and 10 were missing, although they showed an additional band between bands 4 and 5. These plants also showed corresponding increase in enzyme activity. Plants in lanes 27 and 39 had bands 2 to 8 and bands 1, 9 and 10 were missing. Interestingly plants in lane 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 and 38 had band 7 which was normally absent in seed germinated plants, while band 6 which was normally present in seed germinated plants was faint. Further their esterase activity and Na^+ content were high.

Fig. 23
ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI

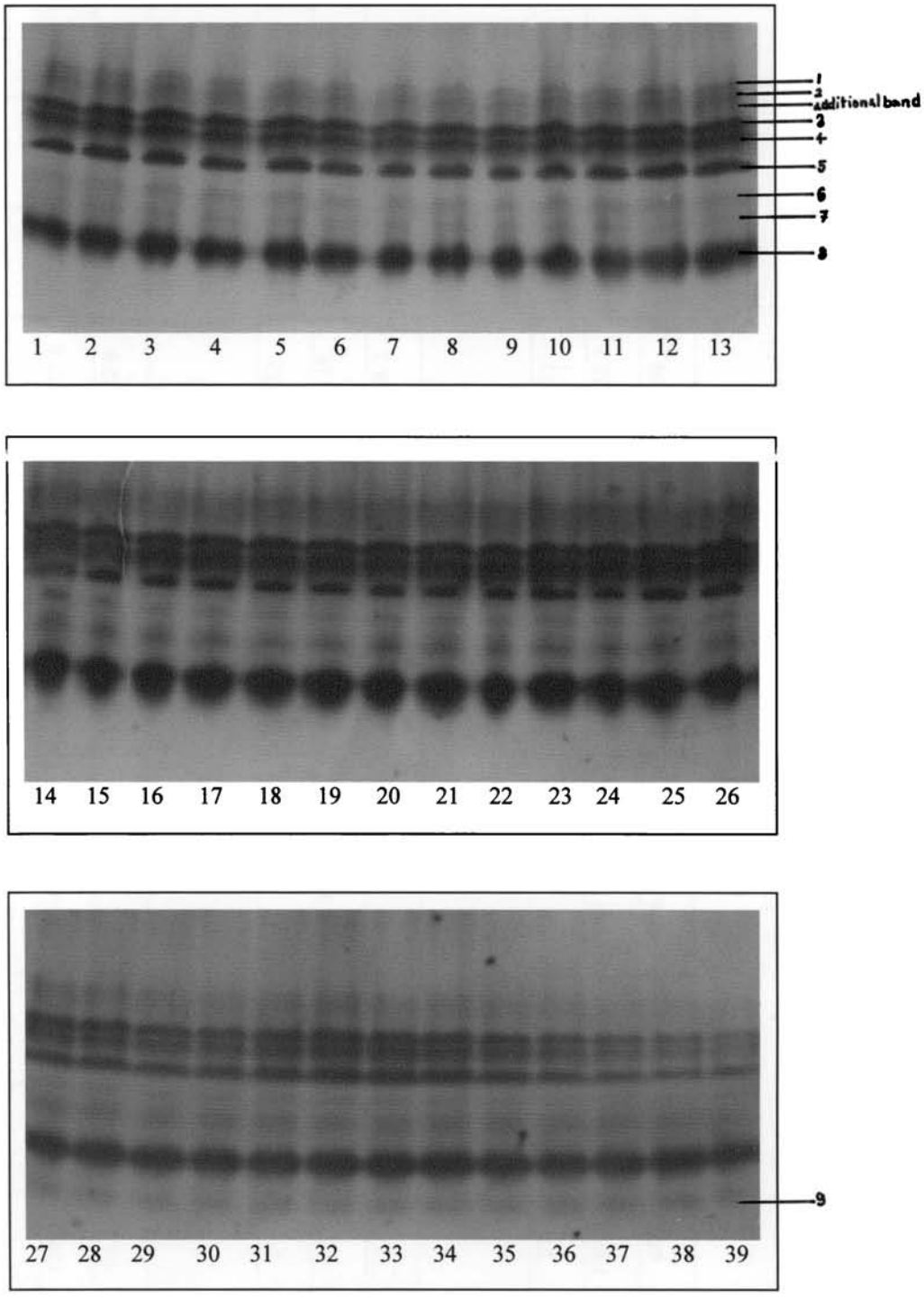


Fig. 24
ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF POKKALI

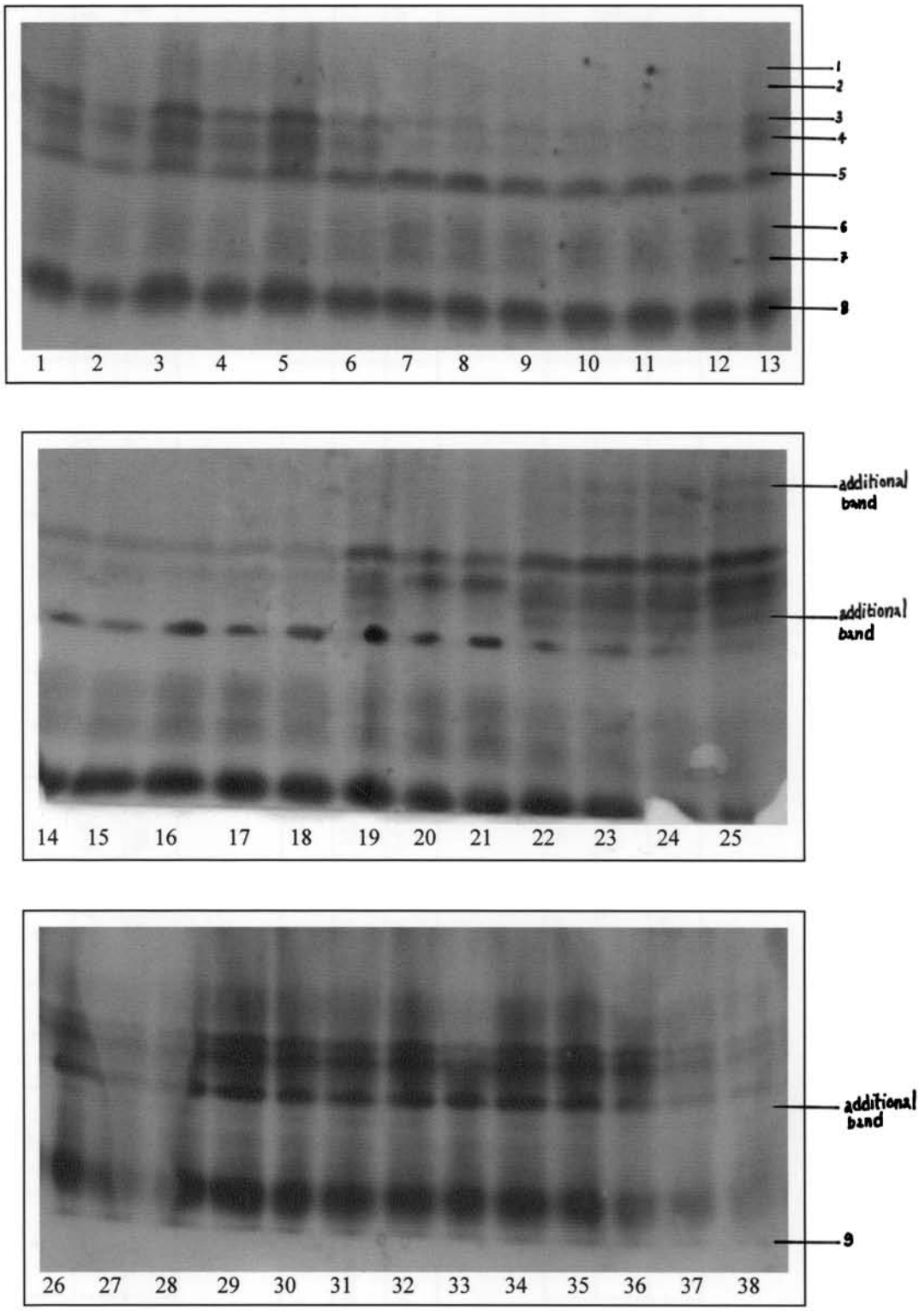


Fig. 25
ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF MI 48

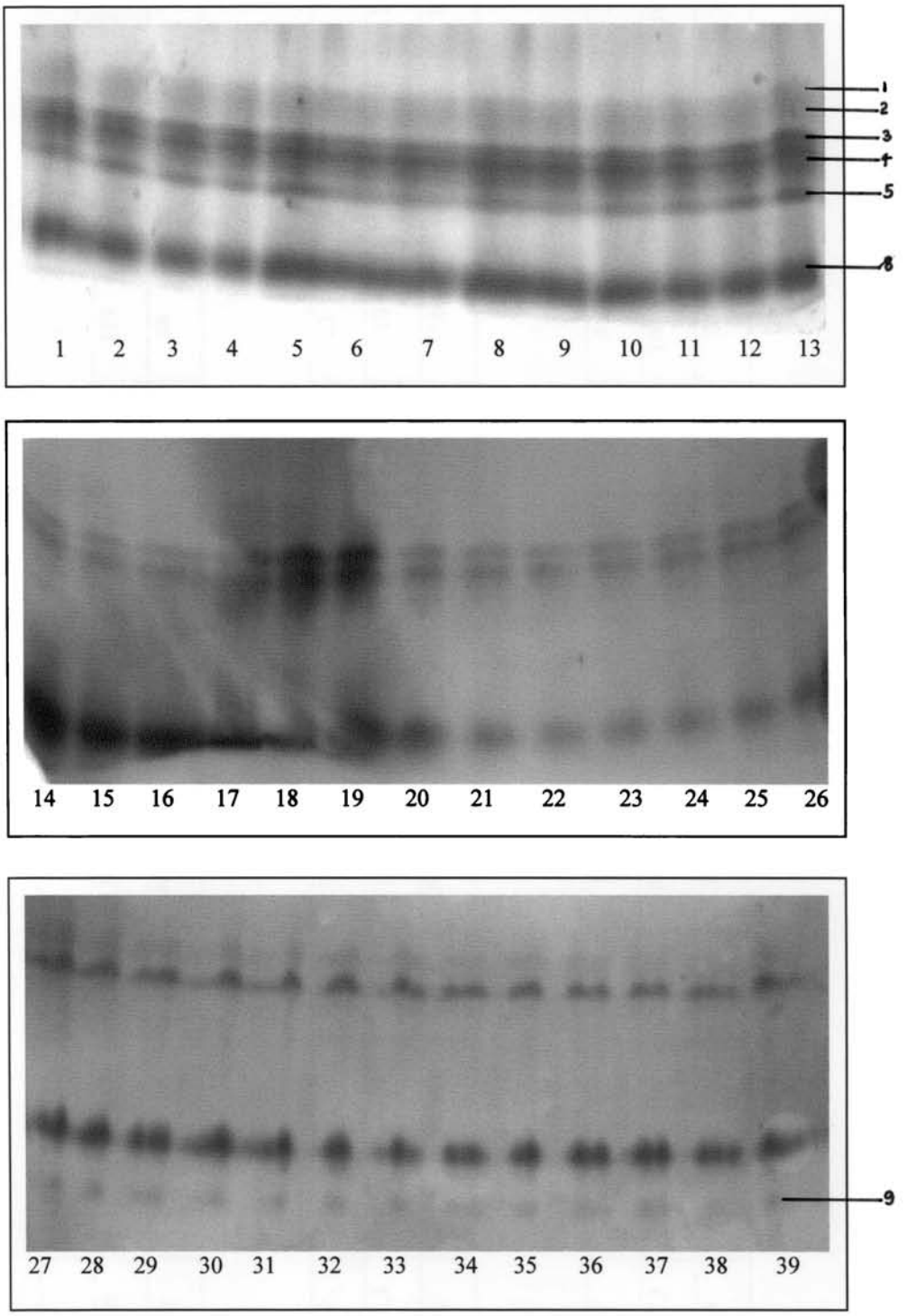


Table 3

sterase activity and Na+ content in plants regenerated from callus cultures of MI 48.

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	120	122	122	121.3	123.3	122.6	121	122.6	121.2	123	120	122	122.6
Na+ Content (ppm)	46	47.6	47	46.6	48.3	48	47.3	47	47.6	48	46	47.6	48

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	107.3	107.6	107	109.3	115.6	116.3	109.3	112.3	111.6	110	110	113.3	114
Na+ Content (ppm)	36	35	34	35.6	39.3	41.6	37.3	38.6	38	37.6	38	38.6	38.6

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	107.3	106	106	106	108.3	107.6	107.6	108.3	107	107.3	106.6	106	109.3
Na+ Content (ppm)	35	36	36	34	35	34	34	34	33	33	32.6	33	35

Fig. 26
ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF MI 48

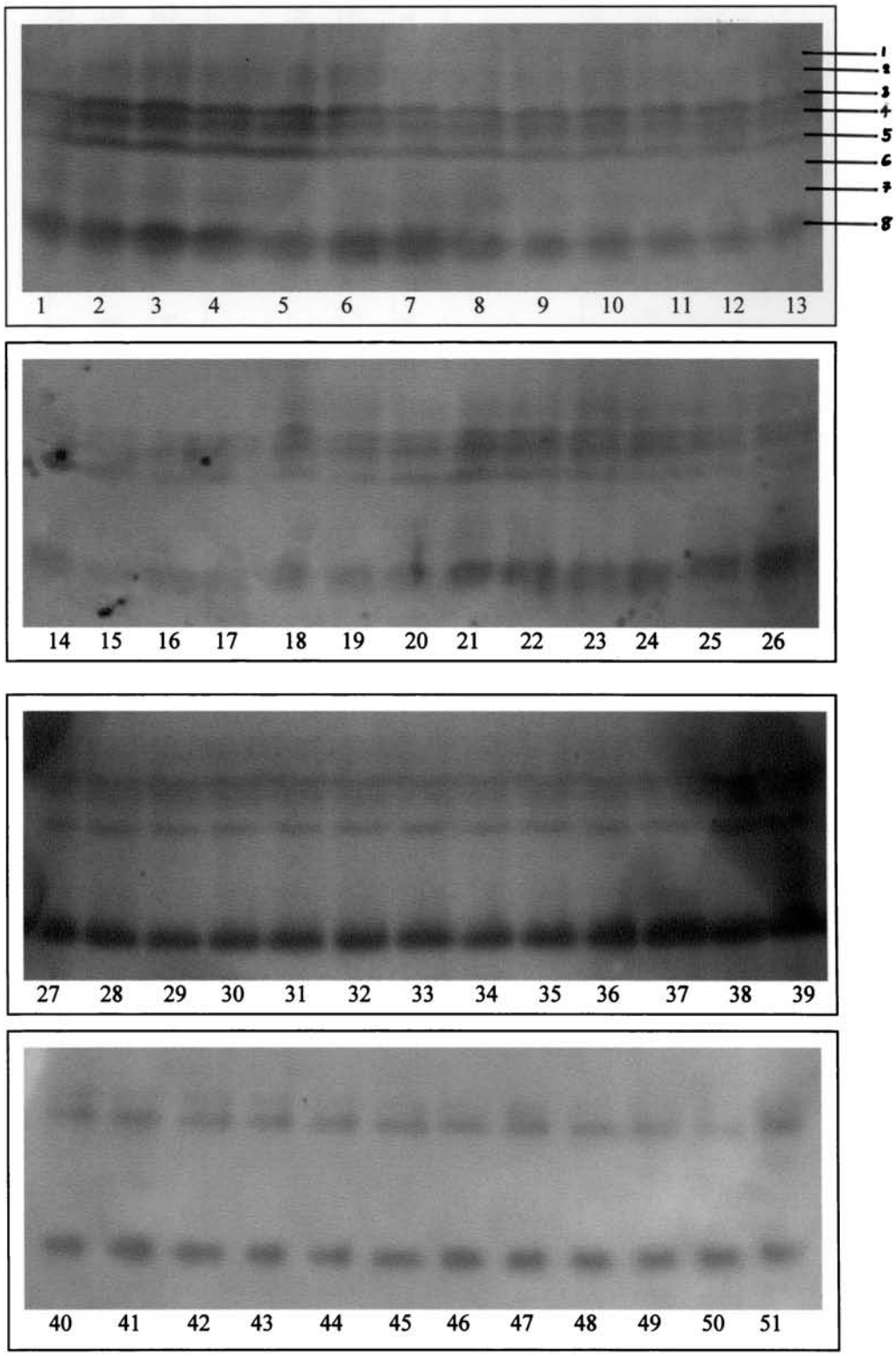


Table 4

Esterase activity and Na+ contents in plants regenerated from anther culture of MI 48

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	140	142.3	142.3	141.6	141	131	130.6	120	119	120	121	121.6	122
Na+ Content (ppm)	56.3	58	57	56.6	56	48.3	48	45	44	45.6	46	46	47.6

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	115	115.3	115	117.6	118.3	117	117.6	120	119.3	118.3	118	117	120
Na+ Content (ppm)	42.6	42	42.3	44	44	43	42.6	47	45.2	43.6	44	44	44.3

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	118.3	116	116	115	116.3	116.3	117	114	112.3	114.6	113.3	118.3	132.6
Na+ Content (ppm)	43.6	42	42	42	42.3	43	43.6	39.6	39	41	40.3	45.6	55.3

Fig. 27

CHANGES IN ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF ANNAPOORNA

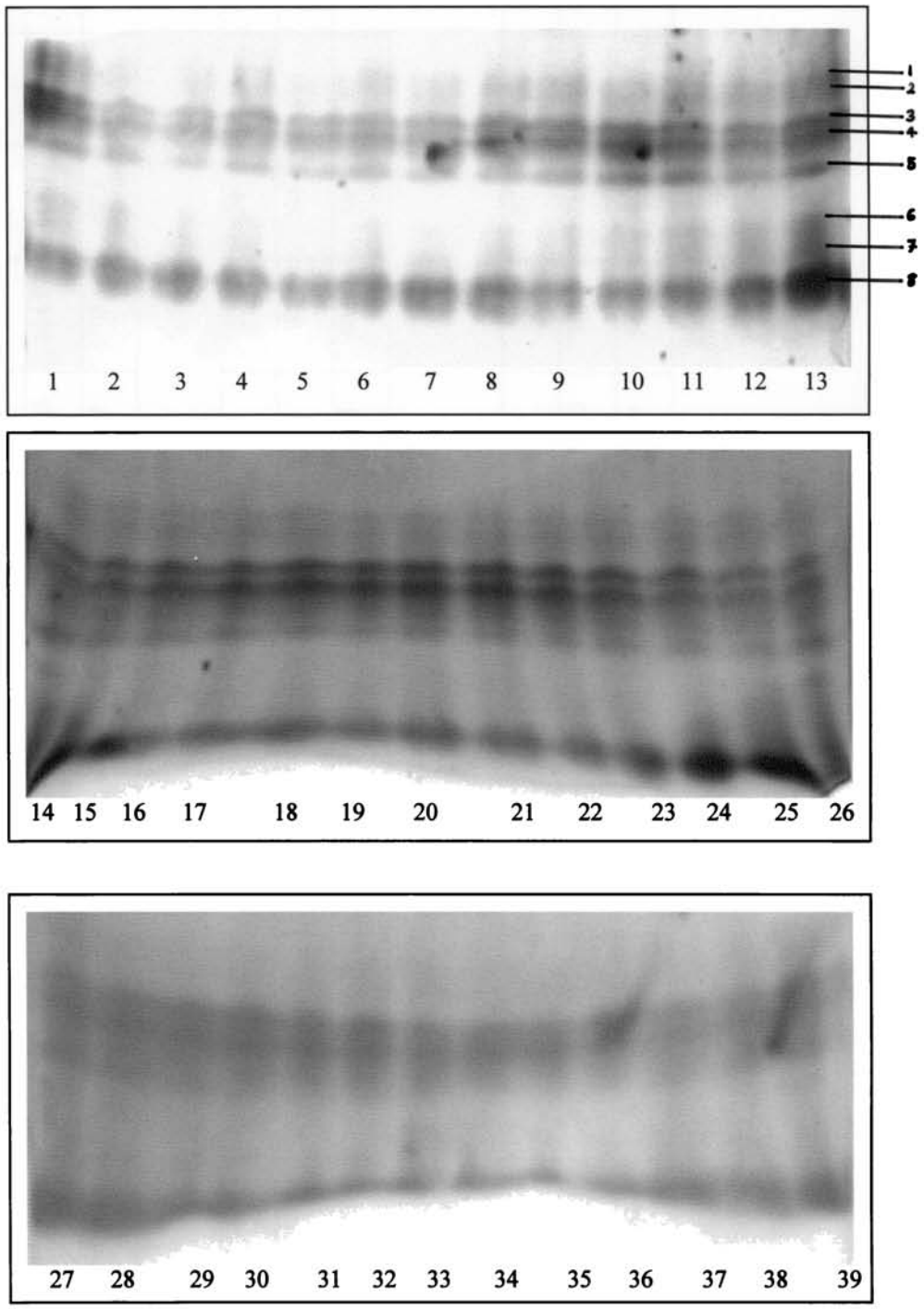


Table 5

Esterase activity and Na+ content in plants regenerated from callus cultures of Annapoorna

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	131.6	108.6	109.6	108	109	108.6	110.3	112.3	113.6	114.6	112.3	110.3	112
Na+ Content (ppm)	53	42	42.6	42	42.3	42	43	43	43.3	43	42.3	41.6	43

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	134	103.3	106.3	107	108	105.3	107	106.6	105	105	105	106	138
Na+ Content (ppm)	59.3	39	40.3	40.3	41	40	41	40.6	40.3	40.3	40	40	51.6

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	138	137.6	107	107	106.6	105	106.3	105	105.3	106	103.3	104	115.3
Na+ Content (ppm)	51.3	51	41	41	40	40	40	39	39.6	40.6	38.3	39	42

Fig. 28

ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF ANNAPOORNA

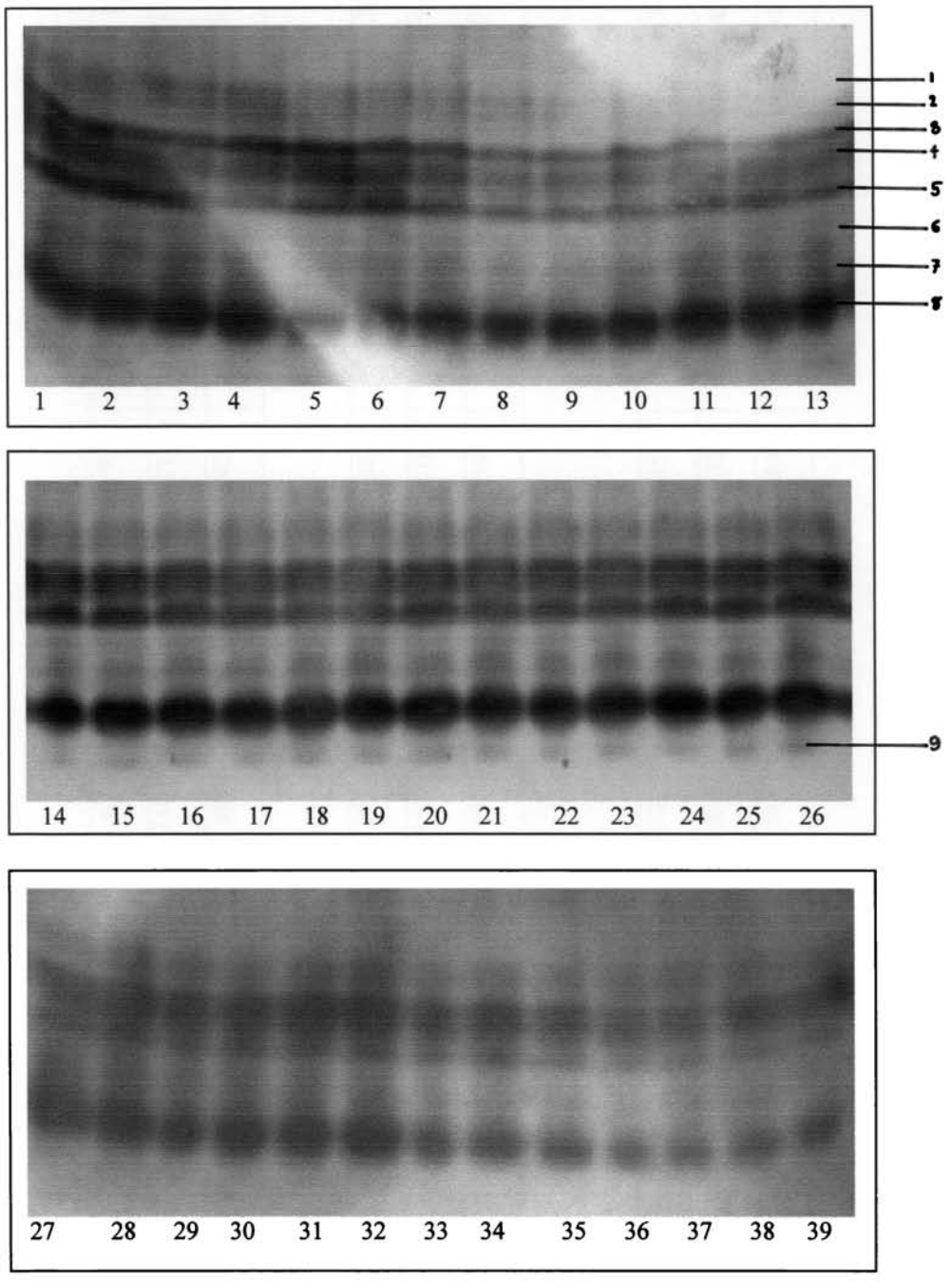


Table 6

Esterase activity and Na+ content in plants regenerated from anther cultures of Annapoorna

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	132.3	131.3	124	125.6	116.3	116	115.3	114.3	113.3	113	124	124.3	125
Na+ Content (ppm)	54.6	54	46	46	43.6	43	43	42.6	42	42	48.6	48	48.6

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	130	139.6	138.3	137.6	137	137	138	138.3	138	136.6	138	138	131.3
Na+ Content (ppm)	56	55.6	56	55	55	55	55.6	56	56	54.3	56	56.3	57.3

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	136	136	126.6	127.3	128.3	120.6	116.3	116.6	115	114	113	113	112.3
Na+ Content (ppm)	50	50	52	52.3	53	53	48.3	48	48.6	45.6	44	42.6	42.6

Fig. 29

CHANGES IN ESTERARE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF JYOTHI

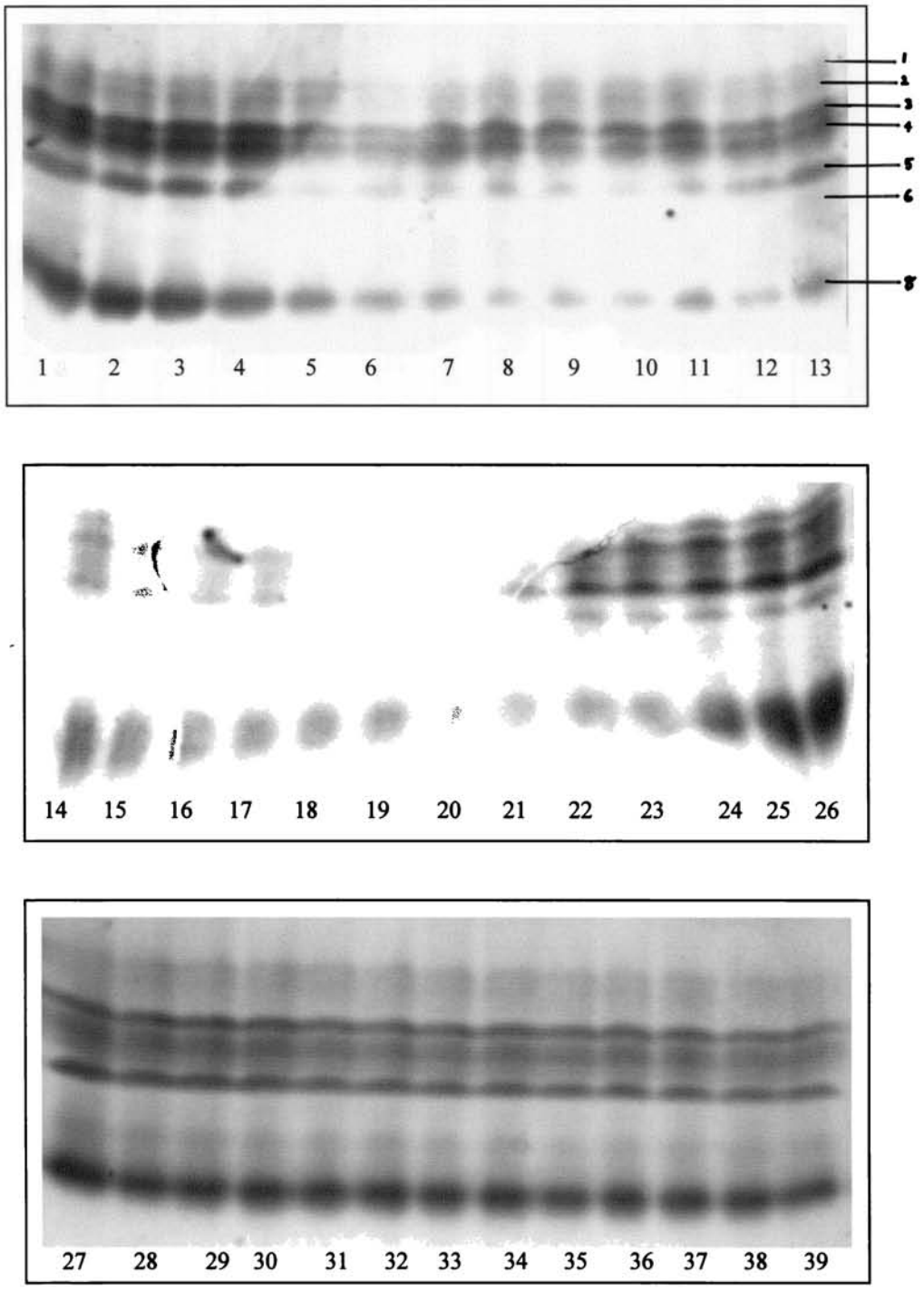


Table 7

Esterase activity and Na+ content in plants regenerated from callus cultures of Jyothi

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	128	125.3	125	128.6	117.3	115.3	116.6	116	115	115	116	117.3	117
Na+ Content (ppm)	52	51	51	52	46.6	46	46.6	46.3	46	45.6	46	47	47.3

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	115.3	114.6	114	113.6	111	100	102	103	104.6	104	104.3	103	106
Na+ Content (ppm)	45.6	43	43.3	42.6	42	37	37.6	38.3	39	39	39.6	39	40

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	126.3	125	123.6	122	123	123.6	125.3	123.6	122	122	121	122.6	126.3
Na+ Content (ppm)	52	52	51	51	51.6	51	51	50	50	50	48	50	52

Fig. 30

ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF JYOTHI

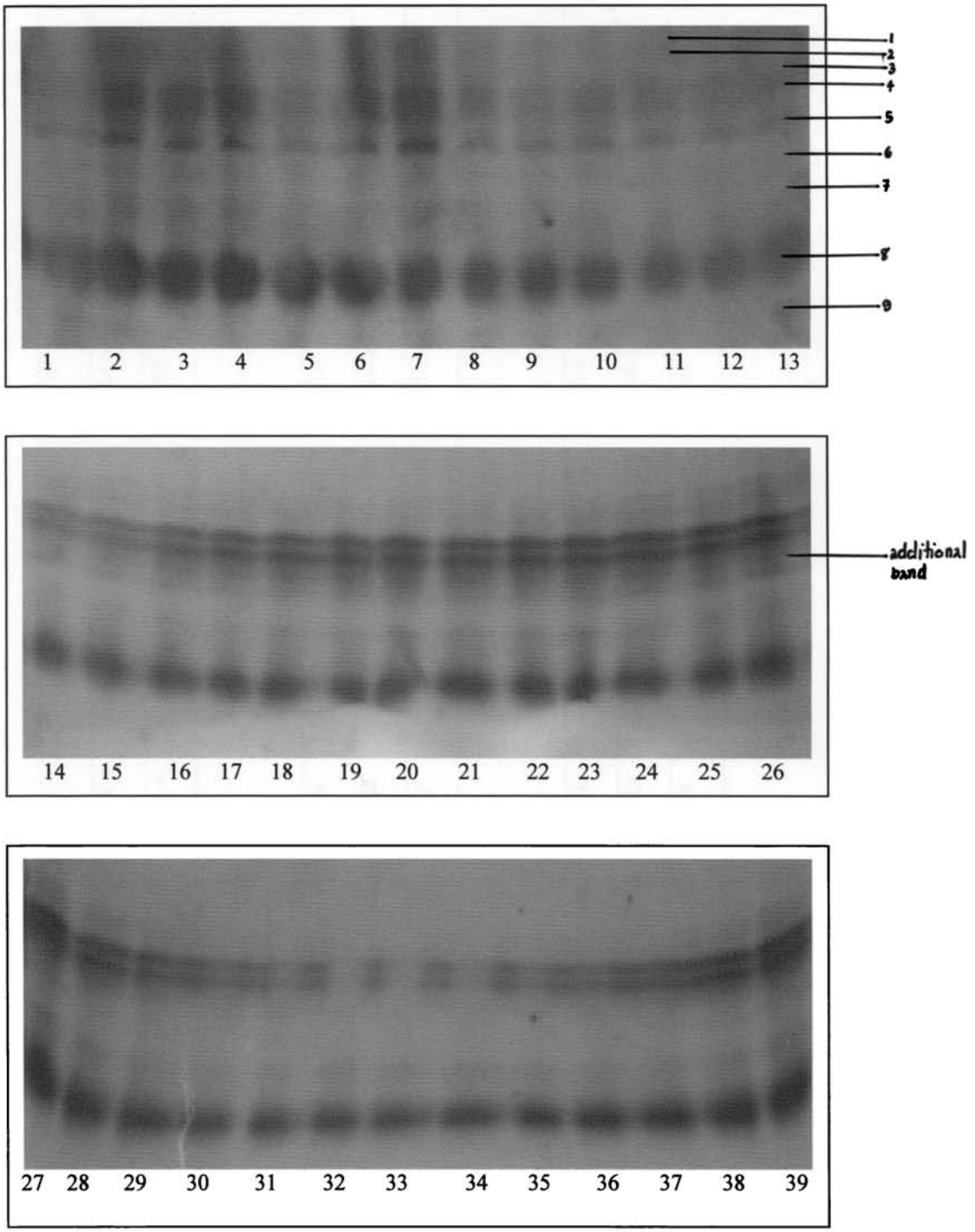


Table 8

Esterase activity and Na+ content in plants regenerated from anther cultures of Jyothi

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	108	127.3	114.6	115.6	109.3	123.3	135.6	106.3	104.6	105	104	103.3	103
Na+ Content (ppm)	43.3	56.3	45	45	43.6	48	55	42	42	42.6	42	41.3	41

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	126	127.3	120	108.3	128	127.6	129	127	128.3	128.6	127.3	127.3	120
Na+ Content (ppm)	49	49	51.6	51	51	50.3	51	50.6	51	51.6	51.6	51	52.6

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	132.3	127.3	125	124.6	122	121	120	121.3	199.6	121.3	113.6	115	120.3
Na+ Content (ppm)	52.3	50	49	49	48	48	48	49	47	48	43	45	48

2.3.5.5 Variations in esterase banding patterns in F1 hybrids

2.3.5.5.1 Pokkali x MI 48 hybrids

One hundred and twenty plants were regenerated from callus cultures of hybrid Pokkali X MI 48, and out of these 63 were studied. A wide range of variation was observed in these hybrids (Fig.31). Plants showed bands corresponding to both parents and also bands unique to the hybrid. Banding pattern similar to Pokkali was seen in plants corresponding to lanes 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 39and 40, and had higher enzyme activity and Na^+ content than those showing banding pattern similar to MI 48. Out of 63 plants studied, 28 plants banding pattern similar to MI 48. However very faint bands 6 and 7, were present and enzyme activity and Na^+ content were higher than seed germinated MI 48 plants (Table 9).

2.3.5.5.2 Pokkali x Annapoorna hybrids

Out of 100 plants regenerated 52 plants was studied for esterase variation and Na^+ content. Bands of both parents' and an additional band between bands 4 and 5 were observed in Pokkali x Annapoorna hybrid (Fig.32) although most of the plants showed bands similar to that of Pokkali. Bands 9 and 10 were missing in all the regenerated hybrid plants. Band 7 was absent in lanes 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38 and 39 and both bands 6 and 7 were missing in lanes from 48, 49, 50, 51 and 52. Enzyme activity and Na^+ content were similar to that of Pokkali (Table.10).

2.3.5.5.3 Pokkali x Jyothi hybrids

Ninety plants were regenerated from callus cultures of hybrid Pokkali X Jyothi and 52 plants were studied for esterase variation and Na^+ content. Here also various combinations of the bands of two parents were observed although most of the plants

Fig. 31

ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x MI 48 HYBRIDS

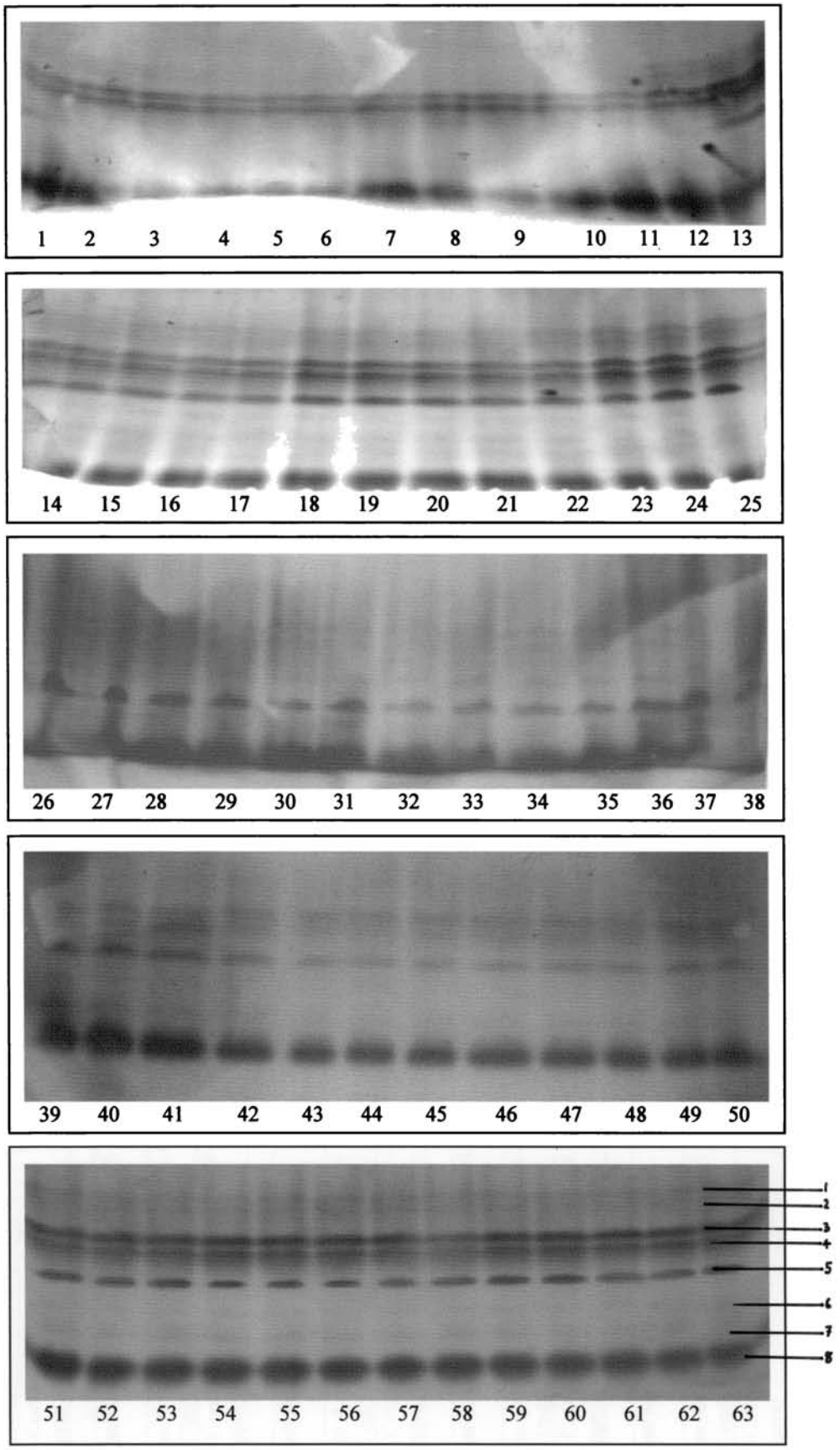


Table 9

Esterase activity and Na+ content in plants regenerated from callus cultures of Pokkali x MI 48 hybrids

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	116.6	114.3	108	108	107.3	108.6	110	112.6	109.6	108.3	108.6	117.3	125.3
Na+ Content (ppm)	52	48.3	44.3	44	44	44.6	45	45.6	41.3	39.6	39	58	65.3

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25
Esterase Activity (U/ml)	121	121.6	122	122.6	127.3	127	128.3	122.3	125	125	126.2	129
Na+ Content (ppm)	56	57	58	58	63.3	63	62.6	58.6	59	59	62.6	64.3

Lane No.	26	27	28	29	30	31	32	33	34	35	36	37	38
Esterase Activity (U/ml)	132	132.6	132	131.6	130	130.6	120	118.6	119.6	118	121.3	124.6	127
Na+ Content (ppm)	64	64	63	62.6	60	59.6	57	55.3	55.6	55	58	60	62

Lane No.	39	40	41	42	43	44	45	46	47	48	49	50
Esterase Activity (U/ml)	137.3	134.3	124	120	119	118.6	116	118.3	117.6	119.6	122.3	123
Na+ Content (ppm)	68.3	64	58	53.3	52	52	50	53	52	52	53	54

Lane No.	51	52	53	54	55	56	57	58	59	60	61	62	63
Esterase Activity (U/ml)	128.3	127.3	127	130	130.3	129.3	128	128	129.3	130	131.3	129.3	128
Na+ Content (ppm)	64.3	62	62	65	65	64.3	63.3	63	63.6	64	65	63.6	63

Fig. 32

ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x ANNAPOORNA HYBRIDS

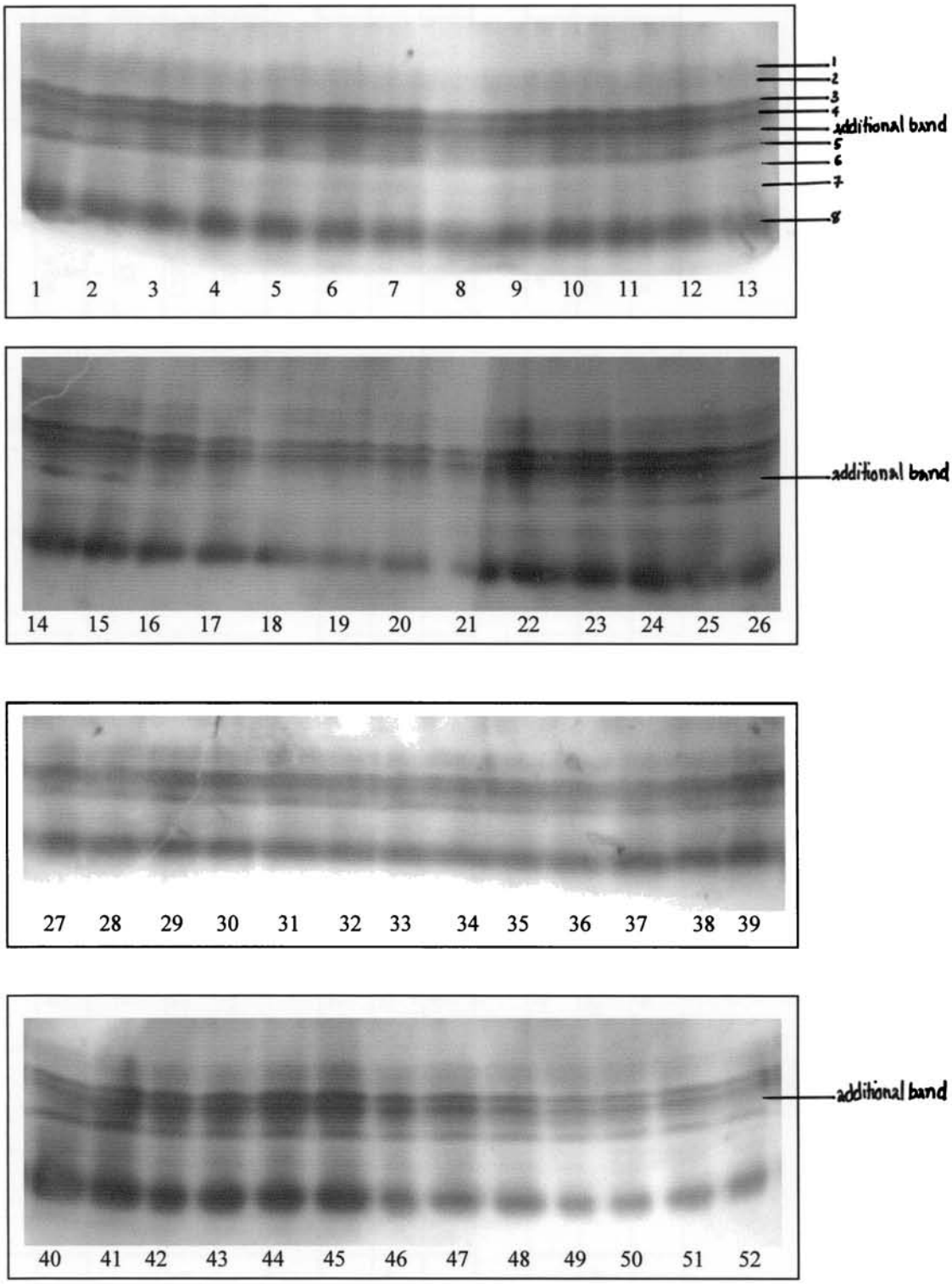


Table 10

Esterase activity and Na+ content in plants regenerated from callus cultures of Pokkali X Annapoorna hybrids

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	130.3	124.3	123.3	125	125	122.6	123.3	114.3	124.6	126.6	127.3	129.6	120.3
Na+ Content (ppm)	65.6	59	59	60	60	57	57.6	52	59	60.6	61	63.6	57

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	129	128.6	127.3	126.3	120.6	120	122	119	127.3	129.6	130	130.3	125.3
Na+ Content (ppm)	64.3	63	62.6	62	57.3	57	59.3	56	62	63	64	64	60.3

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	125.3	122	120	122.3	123.6	124.3	122	121.6	120.6	119.6	122	122.3	126.3
Na+ Content (ppm)	60	58.6	57	59.1	59.6	60	57	56	55.6	55	59	59.3	60

Lane No.	40	41	42	43	44	45	46	47	48	49	50	51	52
Esterase Activity (U/ml)	131.6	130	129.3	129.6	130	130.6	128.3	129.6	127.3	127.3	124	123.6	123
Na+ Content (ppm)	65	64	63	63.3	63	64	62	62	63	61.3	59	59	59

Fig. 33

ESTERASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x JYOTHI HYBRIDS

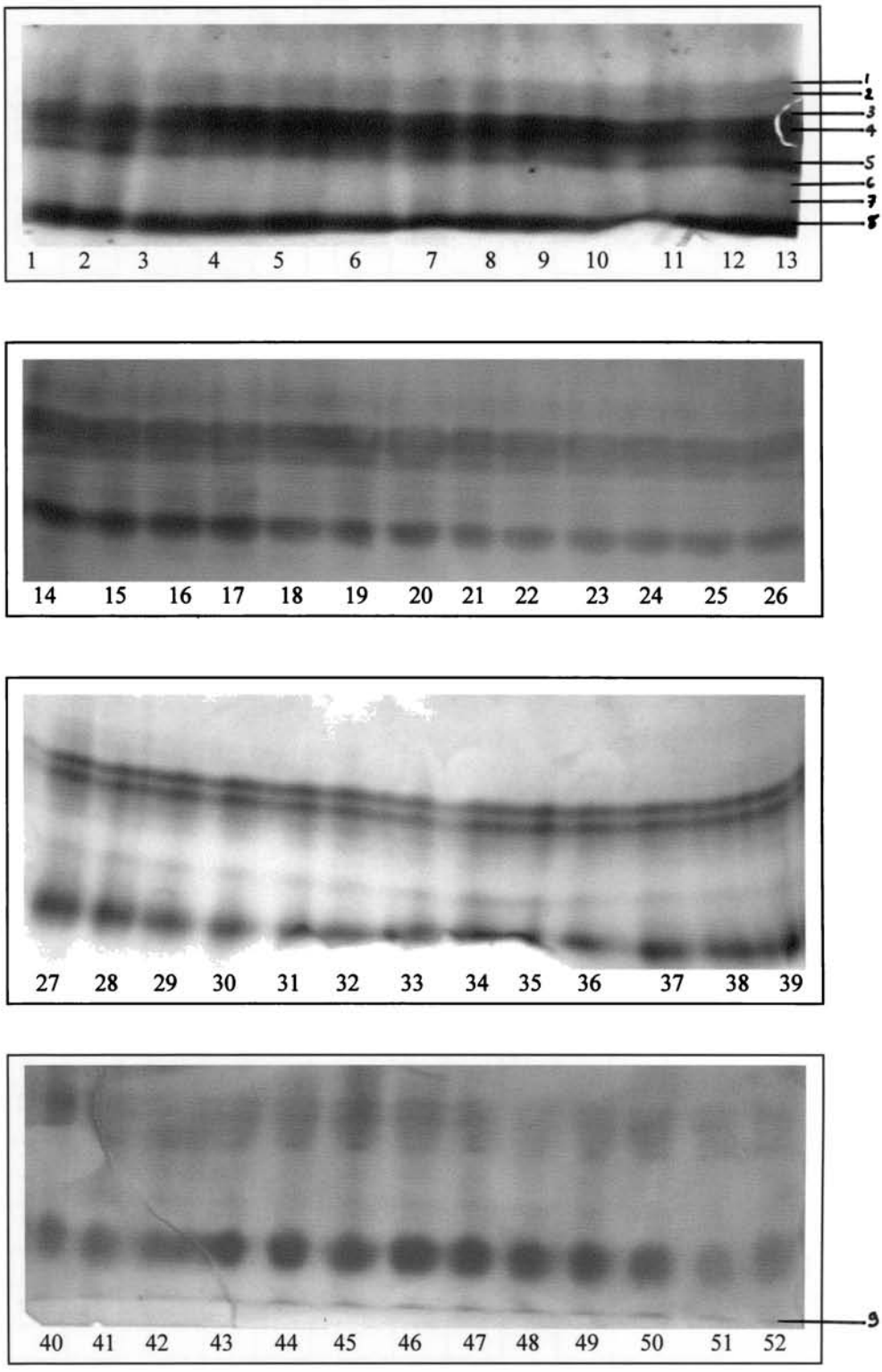


Table 11

Esterase activity and Na+ content in plants regenerated from callus cultures of Pokkali X Jyothi hybrids

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Esterase Activity (U/ml)	135.3	136.6	126.6	127.6	128.3	129.3	128.6	128	129	129	129.3	137.3	132.3
Na+ Content (ppm)	63.6	65	55.3	57	57.6	58.6	58	58	59	58	58.6	66	61

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
Esterase Activity (U/ml)	136	133.3	134	138.6	132	132.3	133.6	134	133.6	132	132	132.3	133.6
Na+ Content (ppm)	73.3	70.6	71	71.6	67.6	69	66.3	63.3	62.6	61	61	61	61.6

Lane No.	27	28	29	30	31	32	33	34	35	36	37	38	39
Esterase Activity (U/ml)	130.3	129.6	127.3	118.3	119.6	118	118	119.6	118.3	117	117.3	116	115
Na+ Content (ppm)	60.3	60	65.3	45.6	48	46.6	49	49	48	45	45	44.3	44

Lane No.	40	41	42	43	44	45	46	47	48	49	50	51	52
Esterase Activity (U/ml)	137.3	130.6	132	122	120	136.3	134.3	133.3	132	129.6	130.3	129.3	128.6
Na+ Content (ppm)	56	60.3	68	60	61	70	62.6	62	61	60.3	60	58.6	58

showed banding pattern similar to that of Pokkali (Fig.33). Samples in lanes 1, 2 and 13 showed faint bands 6 and 7, and had high enzyme activity and Na^+ content (Table.11). In lanes 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26, plants showed exactly similar banding pattern as in Pokkali and had all the bands from 2 to 9. Plant in lane 14 showed highest esterase activity and Na^+ content. In plants corresponding to lanes 29, 30, 31, 32, 33, 34, 35, 36, 37, 38 and 39 bands 1 and 2 were missing. Lane 45 had all the bands except band 10 and this plant also showed high activity and Na^+ content.

2.3.6. VARIATIONS IN ISOCITRIC DEHYDROGENASE BANDING PATTERN IN REGENERATED PLANTS

2.3.6.1 Variations in regenerated plants of Pokkali

Thirteen plants regenerated from callus cultures of Pokkali were studied for isocitric dehydrogenase variation and Na^+ content. Regenerated plants showed changes in band thickness and corresponding changes in enzyme activity (Fig.34). Na^+ content did not show much correlation with enzyme activity (Table.12). The sample in lane 13 had thicker bands and high isocitric dehydrogenase activity. It also showed higher Na^+ content. Lane 1 had thinner bands and low isocitric dehydrogenase activity and Na^+ content.

Thirteen plants regenerated from anther cultures of Pokkali were studied, and they did not show much variation in isocitric dehydrogenase and Na^+ content (fig.35). In lane 1 bands were faint, and enzyme activity and Na^+ content were lower (Table.13). At the same time bands in lane 3 were slightly thicker and enzyme activity and Na^+ were higher.

2.3.6.2 Variations in regenerated plants of MI 48

Twenty-six plants regenerated from callus culture and 26 from anther culture of MI 48 were studied. Isocitric dehydrogenase bands in MI 48 were very thin when compared to Pokkali (Fig.36). There was only one band and there was no visible variation in banding pattern. Anther culture regenerated plants showed changes in thickness of bands (Fig.37). There was an increase in enzyme activity and Na^+ content with increase in thickness (Table.14 and 15).

2.3.6.3 Variations in regenerated plants of Annapoorna

Twenty-six plants regenerated from callus culture and 26 from anther culture of Annapoorna were studied for isocitric dehydrogenase variation and Na^+ content. Isocitric dehydrogenase bands showed wide range of variations in thickness and a slight variation in enzyme activity (Fig.38) Those with thicker bands showed higher isocitric dehydrogenase activity and Na^+ contents (Table.16). Anther culture regenerated plants did not show much variation (Fig.39).

2.3.6.4 Variations in regenerated plants of Jyothi

Twenty six plants regenerated from callus culture and 26 from anther culture of Jyothi were studied for isocitric dehydrogenase variation and Na^+ content. With varying thickness in isocitric dehydrogenase bands, Jyothi showed changes in enzyme activity (Fig.40). But isocitric dehydrogenase activity did not appear to be correlated with Na^+ content (Table.18). Two thick bands were present in most of the regenerated plants of Jyothi. Plant in lane 26 showed only very faint bands and very low enzyme activity. Among anther culture regenerated plants, lanes 5 and 9 showed three bands, which were corresponded with those of Pokkali (Fig.41). Enzyme activity was also very high in these plants. However Na^+ content was not very high (Table.19).

Fig. 34

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI

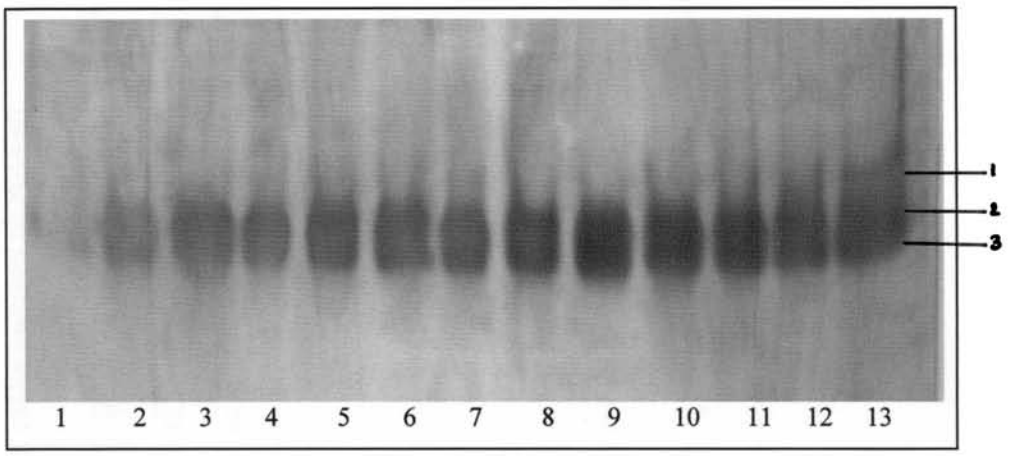


Fig. 35

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF POKKALI

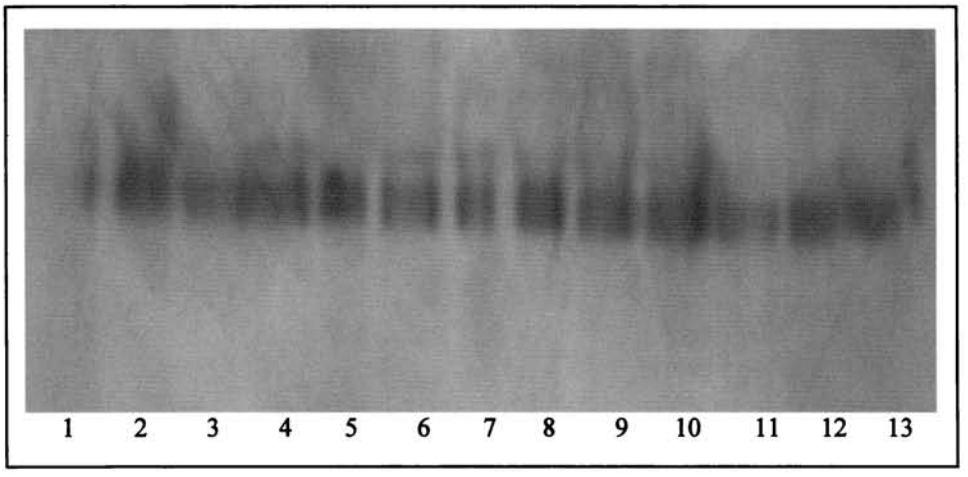


Table 12

Isocitric dehydrogenase activity and Na+ content in plants regenerated from callus cultures of Pokkali

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	165. 78	176.28	182.35	175.36	198.55	188.5	175.66	177.58	182.35	180.79	179.35	178.5	222.08
Na+ Content (ppm)	65.6	66.3	68	68	68.6	67	63.3	64	64.6	65	65	65	68.6

Table 13

Isocitric dehydrogenase activity and Na+ content in plants regenerated from anther cultures of Pokkali

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	163.54	188.92	172.8	174.55	173.8	173.3	173.8	175	175.89	182.55	177.65	172.88	175.92
Na+ Content (ppm)	63.3	64.3	60	60.3	64	63.3	62	63.6	63	64	63	62.3	63

Fig. 36

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF MI 48

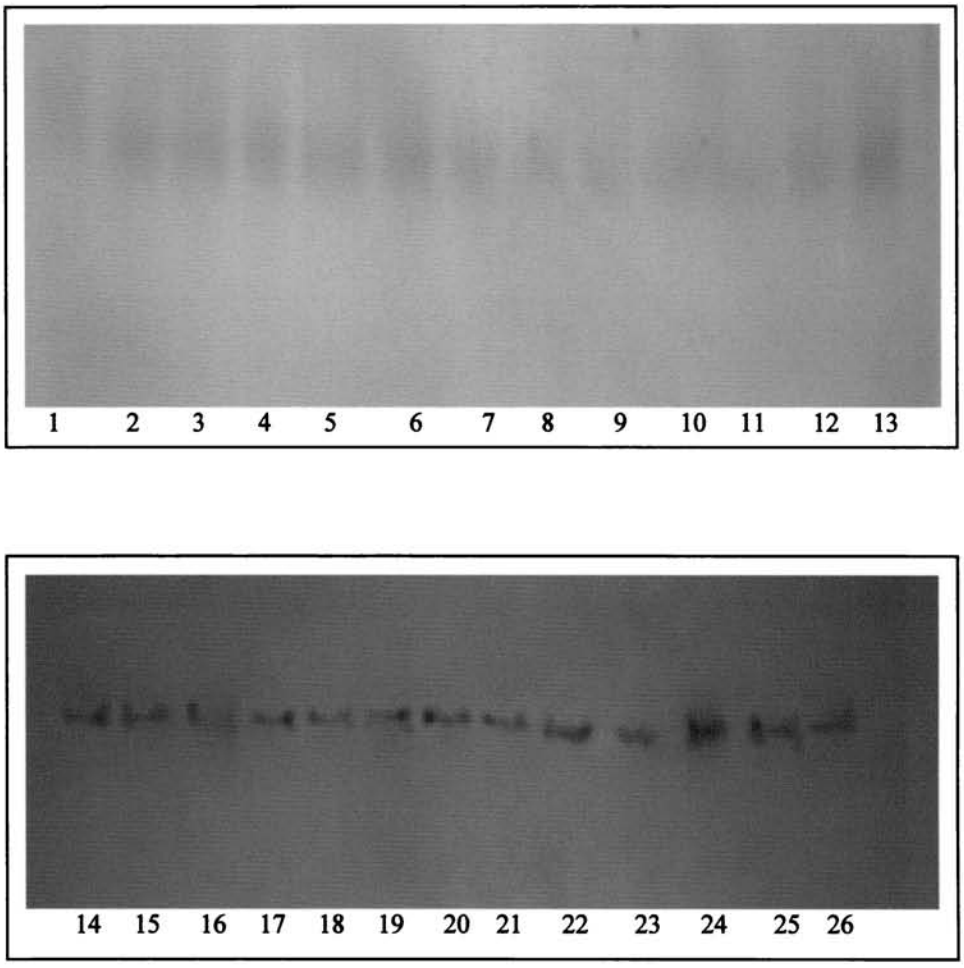


Table 14

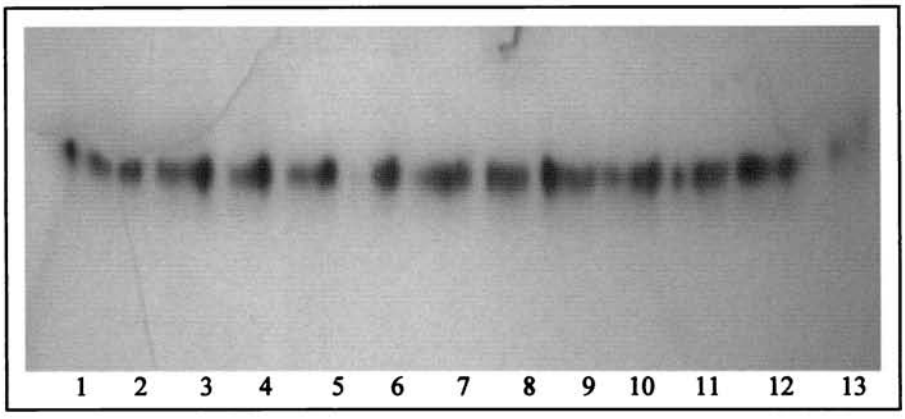
Isocitric dehydrogenase activity and Na+ content in plants regenerated from callus cultures of MI 48

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	169.54	168.33	170.39	167.82	165.67	170.39	174.59	166.9	165.82	164.39	163.69	162.5	178.36
Na+ Content (ppm)	42	42.6	45	44	42	42	42.6	39	39	41.3	40	40	39.3

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
ICD Activity (U/L)	172.3	173.3	173.93	166.92	165.35	168.92	162.59	163.68	172.54	179.39	169.54	169.39	163.86
Na+ Content (ppm)	40.3	40	41	40	38	38	35.3	36	40	42	40	40	37

Fig. 37

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF MI 48



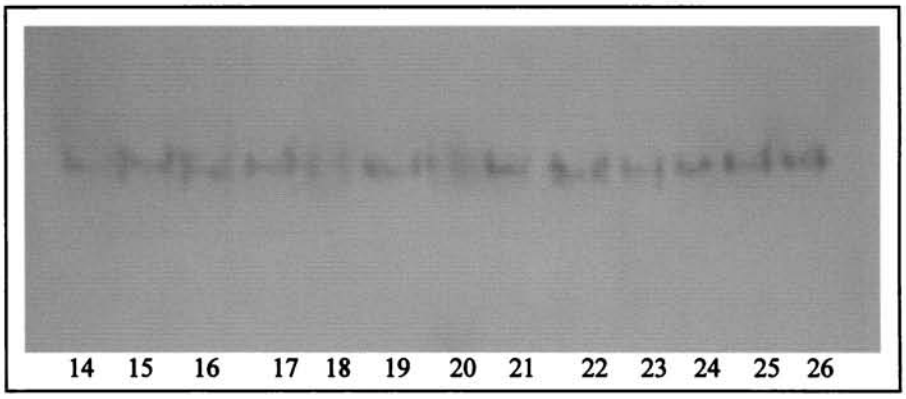
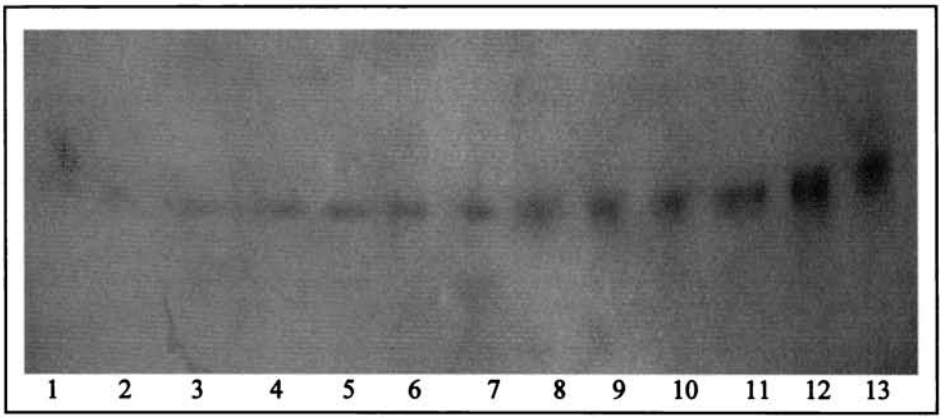


Fig. 38

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF ANNAPOORNA



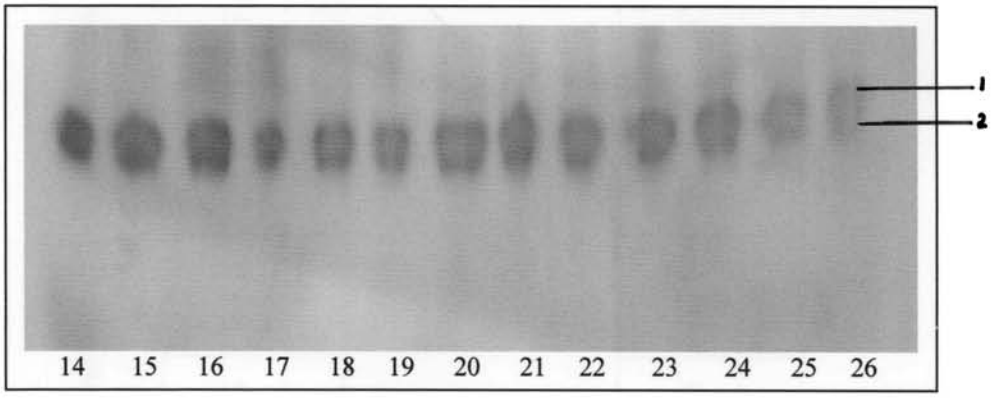


Table 16

Isocitric dehydrogenase activity and Na^+ content in plants regenerated from callus cultures of Annapoorna

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	107.55	103.29	112.45	108.97	103.59	112.4	116.82	118.09	118.55	117.96	118.35	119.96	120.82
Na+ Content (ppm)	43.6	44	43	46	43	48	49	49.6	49	49	49.3	49.6	50

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
ICD Activity (U/L)	120.69	117.67	118.93	116.2	114.95	115.35	116.67	118.93	116.4	115.39	116.9	112.34	110.68
Na+ Content (ppm)	49	48	48	44	44	44	44.3	48	48	48	48	47	43

Fig. 39

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF ANNAPOORNA

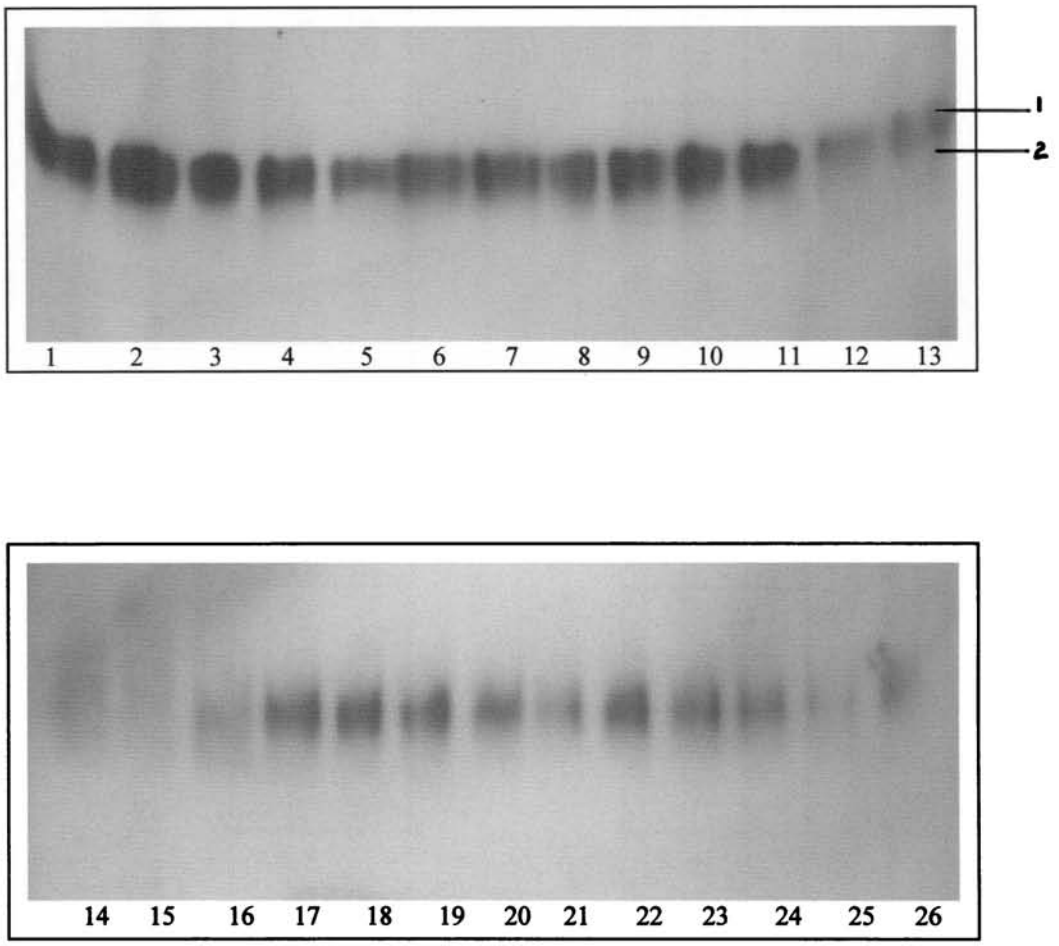


Table 17

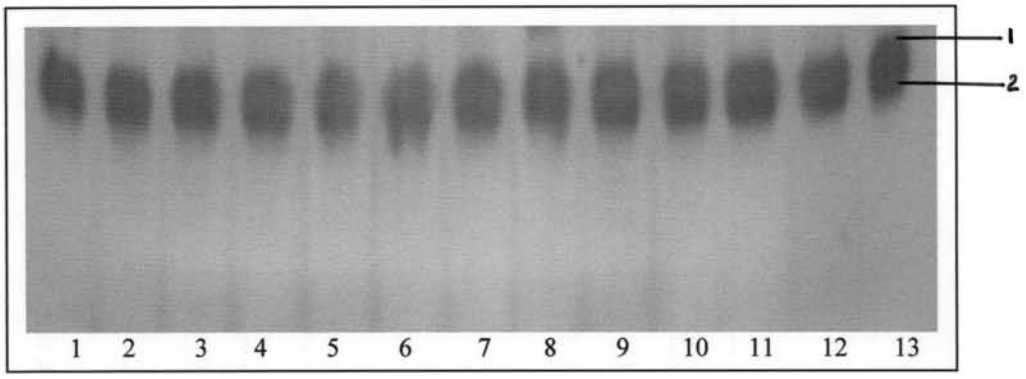
Isocitric dehydrogenase activity and Na+ content in plants regenerated from anther cultures of Annapoorna

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	123.62	124.38	123.9	122.54	120.99	121.35	120.99	119.73	120.38	120.92	120.35	118.9	117.39
Na+ Content (ppm)	49	49.6	49	49	48	49	48.6	47	47.3	48	48.6	45	43.3

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
ICD Activity (U/L)	108.43	106.9	109.79	114.35	115.62	116.33	115.9	113.78	118.28	116.32	115.5	110.67	111.73
Na+ Content (ppm)	40.3	40	40.3	41.6	41	42	42.3	42	46.3	46	45	41.3	40

Fig. 40

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF JYOTHI



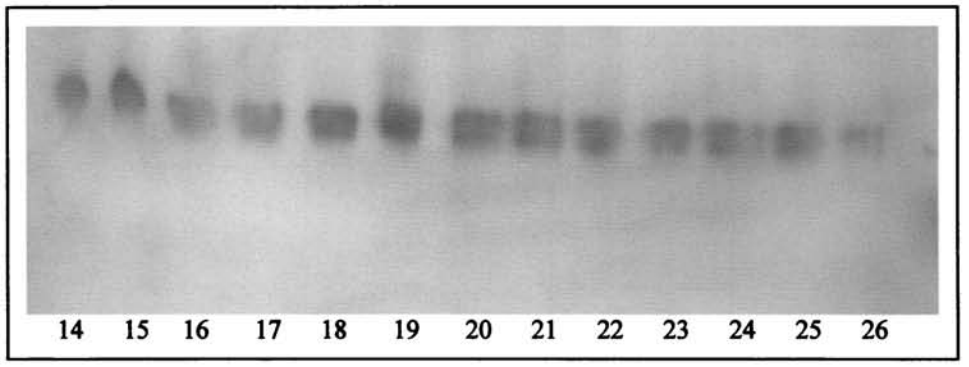


Table 18

Isocitric dehydrogenase activity and Na^+ content in plants regenerated from callus cultures of Jyothi

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	115.32	116.95	115.95	114.35	117.33	118.5	116.39	115.96	114.28	114.93	115.96	114.54	116.39
Na+ Content (ppm)	40.3	42.3	40	40	42.6	42.6	41.6	41	41	41.3	42.3	42	42.3

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
ICD Activity (U/L)	107.69	112.69	108.67	114.24	113.96	114.67	113.41	112.89	111.67	110.38	110.69	109.96	105.28
Na+ Content (ppm)	39.3	40	40	40.3	40.3	42	41	41	41	36	42	38.3	37

Fig. 41

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM ANTHER CULTURES OF JYOTHI

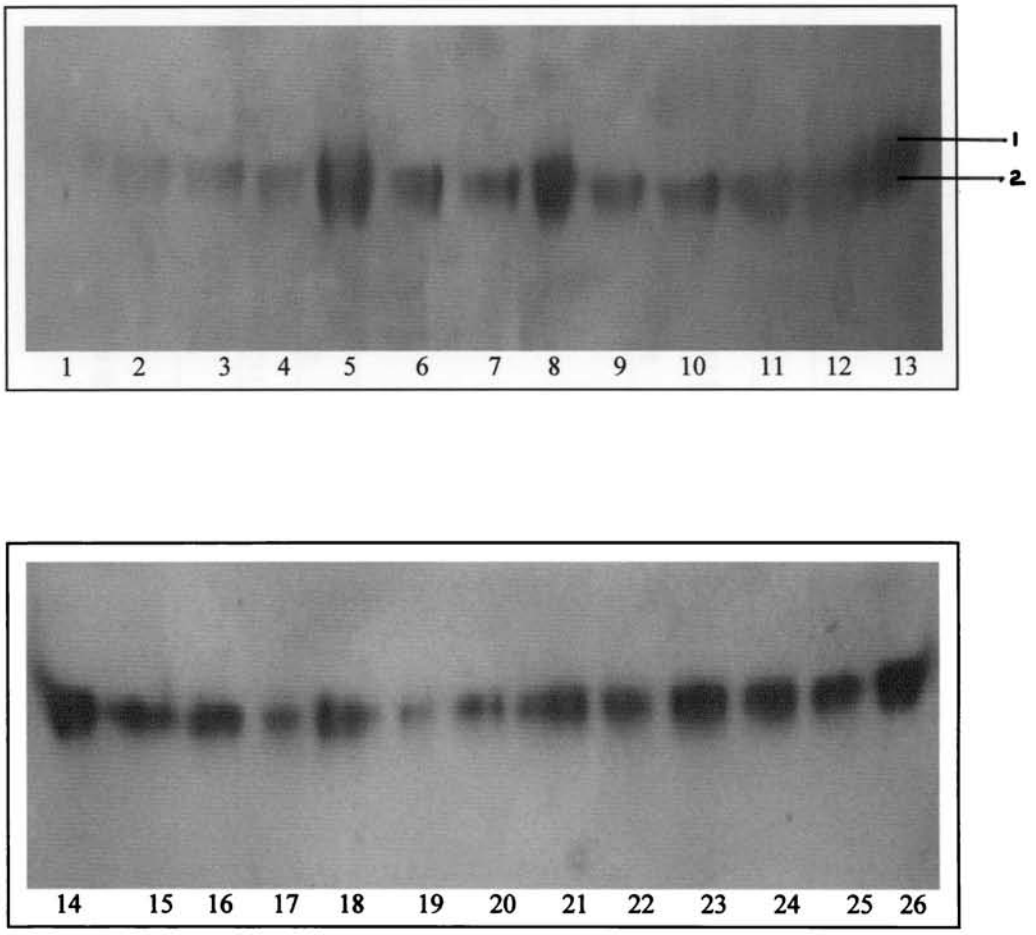


Table 19

Isocitric dehydrogenase activity and Na+ content in plants regenerated from anther cultures of Jyothi

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	108.57	109.35	112.48	113.72	189.62	114.74	113.76	183.46	112.46	113.79	114.38	116.59	118.65
Na+ Content (ppm)	36.3	38.6	40	40	42	40	40	46	40	40	40	41.3	44.6

Lane No.	14	15	16	17	18	19	20	21	22	23	24	25	26
ICD Activity (U/L)	116.96	112.46	111.35	108.6	110.92	103.45	108.72	112.78	113.05	114.26	112.42	110.69	117.62
Na+ Content (ppm)	41	40.6	40	39.6	40	39	40	40	42.3	42	40	40	44.6

2.3.6.5 Variations of isocitric dehydrogenase in hybrids

2.3.6.5.1 Pokkali x MI 48 hybrids

Thirty plants were studied among regenerated plants of hybrid Pokkali X MI 48. Plants corresponding to lanes 14, 15, 19,20, 21 showed banding pattern similar to that of MI 48. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 21, 23, 24, 29 and 30 had two bands, 2 and 3. Whereas, lanes 22 and 25 showed three bands which were similar to Pokkali (fig.42). They showed higher Na^+ ionic content compared to MI 48 (Table.20).

2.3.6.5.2 Pokkali x Annapoorna hybrids

Out of 28 plants studied, most hybrid plants showed banding patterns similar to that of Pokkali with high enzyme activity (Fig.43). In lanes 22, 25, 26 and 28, bands 1 and 3 were very faint. Na^+ content of the plant was not correlated with isocitric dehydrogenase activity. Lanes 12, 13, 14, 15, 17, 18, 19, 21 and 27 showed banding pattern of Pokkali and in all others the banding pattern typical of seed germinated plants of Annapoorna was observed. In lanes 17, 19 and 27 band 3 was very faint. Enzyme activity corresponded to thickness of bands. While Na^+ content was found to be comparable with Pokkali (Table. 21).

2.3.6.5.3 Pokkali x Jyothi hybrids

Eighteen plants were studied and combinations of characters of both parents were observed (Fig.44). Out of 39 plants studied 12-showed banding pattern of Jyothi, while others were similar to Pokkali. Plants in lanes from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14 and 17 showed banding pattern similar to Jyothi while others showed similarity to Pokkali. Enzyme activity and Na^+ content was more similar to Pokkali (Table.22)

Fig.42

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x MI 48 HYBRIDS

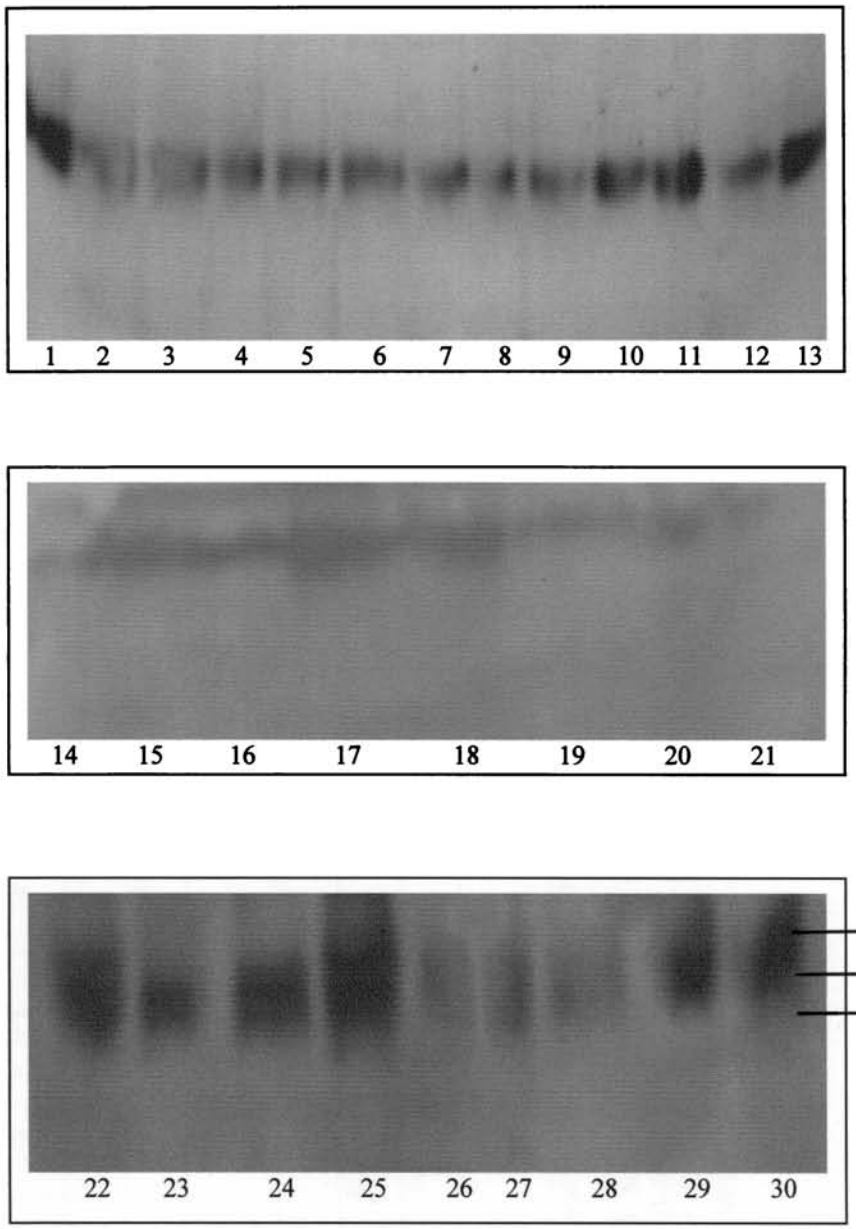


Table 20

Isocitric dehydrogenase activity and Na+ content in plants regenerated from callus cultures of Pokkali x MI 48 hybrids

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	198.57	152.39	155.67	150	150.92	157.69	160.98	162.3	160.65	160.35	162.92	162.33	175.5
Na+ Content (ppm)	60.3	40.3	41.6	40	40	42.3	46	44.3	44	44	43	43	45.6

Lane No.	14	15	16	17	18	19	20	21
ICD Activity (U/L)	162.39	162.59	165.67	166.35	166.25	160.95	165.35	158.89
Na+ Content (ppm)	44	44	43	43	43.6	42	42.3	40

Lane No.	22	23	24	25	26	27	28	29	30
ICD Activity (U/L)	200.65	185.65	189.36	209.67	163.92	162.89	160.92	190.56	196.74
Na+ Content (ppm)	53.6	48.3	48	49.6	43	43	44	50	50.6

Fig. 43

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x ANNAPOORNA HYBRIDS

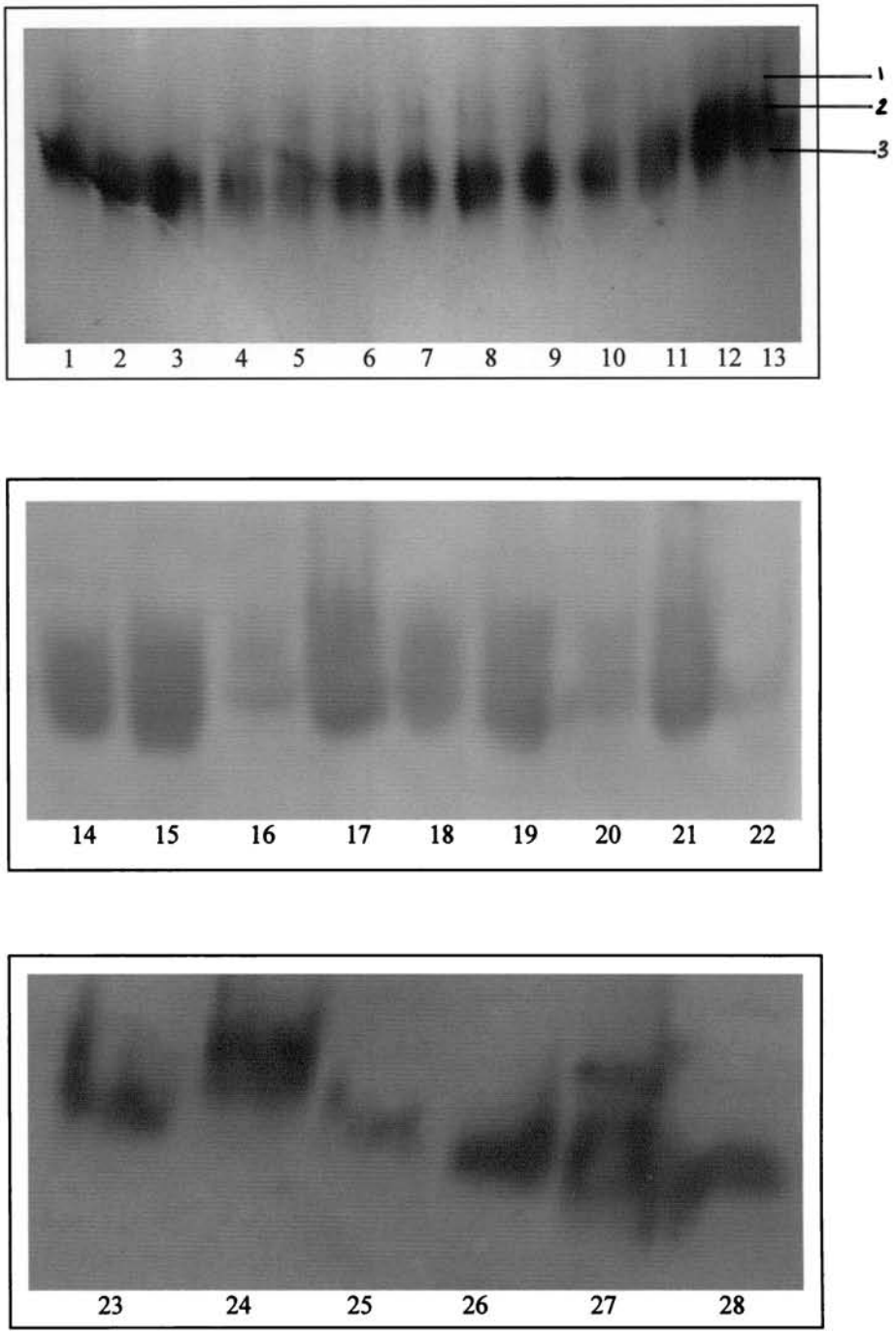


Table 21

Isocitric dehydrogenase activity and Na^+ content in regenerated plants from callus cultures of Pokkali x Annapoorna hybrids

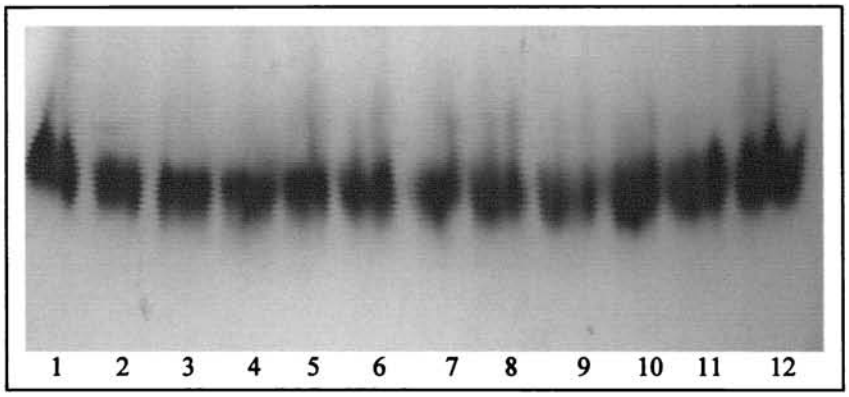
Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13
ICD Activity (U/L)	180.35	182.69	190.56	189.68	186.92	188.68	189.64	190.4	179.65	180.1	188.6	195.3	193.62
Na+ Content (ppm)	58.3	59	60.6	60	60	60	60.6	61.6	59	60	60.3	61.6	61

Lane No.	14	15	16	17	18	19	20	21	22
ICD Activity (U/L)	192.39	194.92	154.25	119.26	178.95	190.26	165.92	180.62	153.65
Na+ Content (ppm)	60.3	62.3	54.3	62	59.3	60	55.3	58.6	52

Lane No.	23	24	25	26	27	28
ICD Activity (U/L)	178.69	190.55	108.25	184.77	195.82	180.94
Na+ Content (ppm)	59	60.3	38.3	60.6	62.3	58.3

Fig. 44

ISOCITRIC DEHYDROGENASE BANDING PATTERN IN PLANTS REGENERATED FROM CALLUS CULTURES OF POKKALI x JYOTHI HYBRIDS



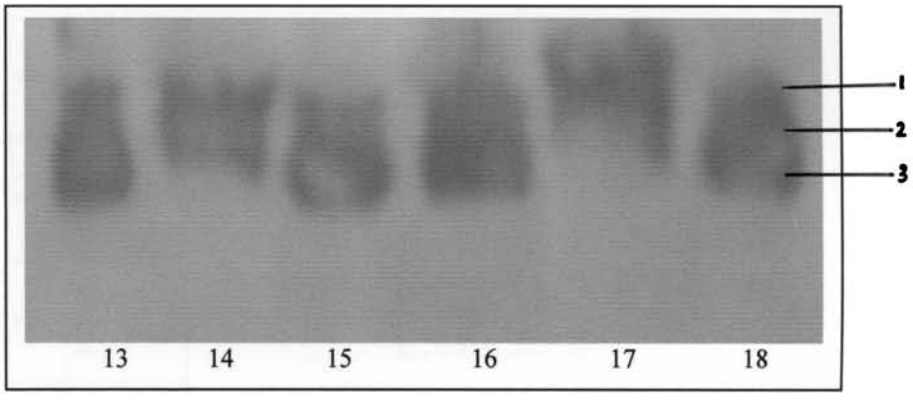


Table 22

Isocitric dehydrogenase activity and Na^+ content in regenerated plants from callus cultures of Pokkali x Jyothi hybrids

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12
ICD Activity (U/L)	168.39	165.35	162.9	120.68	121.35	158.7	160.96	165.32	160.19	163.65	168.79	170.24
Na+ Content (ppm)	52.3	52	52	48.3	50.3	51	52.6	52	53	48.3	50.3	54

Lane No.	13	14	15	16	17	18
ICD Activity (U/L)	192.69	188.92	192.05	199.46	190.82	189.69
Na+ Content (ppm)	60.3	57.3	60	62.3	60	60

Discussion

2.4 DISCUSSION

From a group of regenerated plants, it is advantageous to detect the variants at an early stage. Isozymes are being increasingly utilized as a useful biochemical marker reflecting changes in metabolic activities, corresponding to molecular events associated with growth and development or stress tolerance (Pramanik *et al.*, 1996). Banding patterns expressing differential intensity indicate the status of an enzyme activity affected by stress, and the affected proteins can be considered as injury markers of a particular stress which can be simultaneously used for identification of tolerant lines (Gangopadhyay *et al.*, 1995).

2.4.1 Esterase isozymes could probably be used as a molecular marker for salt tolerance.

In the present study, the feasibility of using isozymes, as a molecular marker for salt tolerance has been investigated. Many isozymes were tested and two isozymes showing varietal variations and developmental stage specific variations were selected. These were esterase and isocitric dehydrogenase.

In rice, the esterase zymogram showed ten bands, of which three were consistently present in all the rice varieties in all developmental stages. All the other bands showed variations. Pokkali, which is a salt tolerant variety, showed a band 7 with a Rf value of 0.6 which was absent in all the other rice varieties irrespective of the developmental stage and presence or absence of stress but faint in tillering and flowering stage. This band showed a slight variation in thickness under stress, indicated that proteins appearing as the band were possibly susceptible to stress injury. Consequently this particular isozyme, esterase, is probably linked with salt tolerance of this particular variety and may possibly be used as a marker for salt

tolerance although further studies beyond the scope of the present one, are required to establish this. Other varieties selected in this study were MI 48, Annapoorna and Jyothi. In Annapoorna and Jyothi bands 3, 4, 5, 6 and 8 were consistently present irrespective of their developmental stages. Band 6 showed slight variation in thickness under stress. In MI 48 bands 3, 4 and 8 were consistent. Band 5 was present in all other stages of MI 48, except in callus. Enzyme activities were also tested in different rice varieties. In all of the developmental stages except flowering stage Pokkali showed higher esterase activity when compared to other varieties.

Rice showed three isozymes of isocitric dehydrogenase and of these two were present in all varieties and band 3 was characteristic of Pokkali. Stress caused a slight change in enzyme activity but it could not cause any noticeable change in banding pattern. So isocitric dehydrogenase could not be used as a molecular marker for stress tolerance, although varietal polymorphism exists for the isozyme.

2.4.2 Isozymes show developmental stage specific variation.

Scandalous (1974) has listed 46 isozyme systems in which the pattern of gene expression varied with developmental condition. The enzyme activity of individual isozymes in a specific tissue is dependent on maturity and cellular environment. The specificity of an enzyme pattern implies the role of specific enzyme and isozymes in plant development and differentiation. During development the variation appears to be two folds, involving the number of bands and their relative intensities. Rao *et al.*, (1992) suggested that several structural and regulatory genes might be involved in the production of different isozyme bands at various stages of development. Gene duplication followed by subsequent mutation at the duplicated loci is a likely mechanism which can lead to enzyme multiplicity and to divergent physiological

functions (Scandalios, 1974). However, the possibility that the control ... may vary from tissue to tissue, which might also contribute to the differential expression of the genes cannot be ruled out (Johri *et al.*, 1977). In the absence of genetic data, it is difficult to say whether the appearance of new bands is the result of differential activation of genes in different cell population or the product of the same gene modified secondarily in each tissue to meet the specialized requirements for growth and development.

Esterase isozymes have been reported to perform a particular physiological action at a specific development stage. Certain specific esterase isozymes had been correlated with photoperiod sensitivity, grain shape and grain weight. Chauhan and Nanda, (1987) made a comparative study of variations in esterase isozymes during progressive stages of development of induced mutants and the parent variety of rice. They correlated the differences in isozymes with the morphological differences of the genotypes and with developmental stages.

In the present study, bands 3, 4, 5 and 8 of esterase were present in all rice varieties in developmental stages like embryo, 14 day old seedling, tillering and flowering stage. In embryo, bands 1and 2 were present in all rice varieties. Band 9 was present in embryo of Pokkali and MI 48. In Annapoorna and Jyothi band 9 was absent in embryo stage. In 14 day old seedling stage, bands 1 and 2 were absent in all rice varieties and band 9 was present in Annapoorna and Jyothi. In tillering stage, band 2 and 9 were present in all varieties. Band 1 was present only in Pokkali but band 10 was present in all rice varieties except Pokkali. In flowering stage, bands 1 and 2 were present only in Pokkali. In callus Pokkali showed all the esterase bands from 3 to 10. Bands 1 and 2 were absent in calli of all rice varieties and in MI 48 band

5 which was present in all other rice varieties was missing in calli. The rice varieties thus showed developmental stage specific banding pattern.

In Pokkali, higher activity of esterase was present in embryo stage. This activity decreased up to tillering stage and then increased in flowering stage. In MI 48 esterase activity decreased drastically in 14 days old seedling stage and increased in tillering and flowering stage. Annapoorna and Jyothi showed a pattern similar to Pokkali.

In the case of isocitric dehydrogenase, in embryo stage, only Pokkali showed band 3. Band 1, which was present in all other varieties, was absent in embryo stage of Pokkali. In all the other stages Pokkali showed three isocitric dehydrogenase bands. Other varieties showed only bands 1 and 2. In tillering stage MI 48 produced only one band, that is band 2. In all the other stages MI 48 showed two isocitric dehydrogenase bands. In case of flowering stage and calli Pokkali showed three bands and all sensitive varieties produced only two bands. In the callus, bands were too thick to differentiate.

Callus of all rice varieties showed higher isocitric dehydrogenase activity than the various developmental stages studied. Isocitric dehydrogenase activity was lower in embryo of Pokkali, and this increased in 14-day-old seedling, tillering and flowering stages. In MI 48 and Annapoorna embryo had higher activity, except in callus and enzyme activity decreased in other developmental stages. In Jyothi isocitric dehydrogenase activity became high in 14-day-old seedling stage, and then decreased in tillering stage. Even though isocitric dehydrogenase did not show variation in banding pattern, it showed changes in thickness of bands, and activity with developmental stages.

2.4.3 Stress induced variation in isozyme banding pattern.

Edreva *et al.*, (1989) and Dubey and Sharma, (1989) reported that banding patterns of isozymes changed in tissues under stress conditions. Gangopadhyay *et al.*, (1995) reported that esterase plays an important role in overcoming the stress effects, particularly salt stress, since certain isozymes were seemed to lost and some others seemed to declined their intensity under stress. They also reported variation in banding pattern of peroxidase under stress, whereas in the present study it was observed that peroxidase did not show any variation and hence cannot be used as a marker. Mostly stress caused a difference in thickness of certain bands in the isozyme profile. Band 6 of esterase was thinner under stress condition, in embryo stage of Pokkali, Annapoorna and Jyothi. In 14-day-old seedling stage, esterase bands in MI 48 was thicker in presence of stress but it was thinner in Pokkali. In tillering and flowering stages also stress caused a decrease in band thickness. Band 7 in Pokkali also showed a decrease in thickness under stress. In calli stress caused an additional band 10 in Pokkali, Annapoorna and Jyothi.

Generally esterase activity decreased under stress. In embryo and callus esterase activity decreased under stress although 14-day-old seedling and tillering stages showed an increase in enzyme activity under stress. Flowering stage showed increase in enzyme activity in Pokkali and Annapoorna, and decrease in enzyme activity in MI 48 and Jyothi. Since salt stress induces significant changes in banding pattern and enzyme activity, esterase could possibly be used as a marker for salt tolerance.

Isocitric dehydrogenase bands generally showed a decrease in band thickness under stress. In embryo of Pokkali, band 1 was not visible normally, but it appeared

under stress. In tillering stage of Pokkali, band 1 became faint under stress. Band 3 was not significantly affected by stress. Isocitric dehydrogenase activity showed a reduction under stress, except in embryo stage of Pokkali, Annapoorna and Jyothi.

2.4.4 Esterase activity is not significantly inhibited by *in vitro* NaCl treatment.

In vitro treatment with 50 mM and 100mM of NaCl produced a change in esterase activity. Sharp change was noticeable only in embryo of Pokkali and MI 48. Flowering stage was sensitive to *in vitro* NaCl treatment. Tillering of Annapoorna and Jyothi showed an increase in enzyme activity at 50 mM NaCl. Results of *in vitro* NaCl treatment were different from *in vivo* treatment, and these enzymes were stable at 50 and 100 mM NaCl for some time.

2.4.5 Na^+ content is higher in Pokkali than the other rice varieties under study

Quantitative analysis of Na^+ content showed that Pokkali, which is a salt tolerant variety had more Na^+ content in its plant body than the salt sensitive varieties, and it can accumulate Na^+ content, under stress in older leaves to about three times higher than normal without causing death of the plant. However, in the sensitive varieties, Na^+ accumulation was only 2 fold. Older leaves showed higher amount of sodium ions. High K^+/Na^+ ratio in shoot of tolerant rice genotypes or low Na^+/K^+ ratio was suggested as a criterion for salt tolerance (Hedge and Joshi, 1974; Allen *et al.*, 1997). There is a significant difference in the partitioning of ions between the shoot and the root in the different rice varieties. The tolerant rice cultivar, Pokkali has a higher Na^+/Ca^{++} ratio and a lower K^+/Na^+ ratio in the root compared to the shoot indicating higher retention of Na^+ ions in the root and greater translocation of K^+ to the shoot. On comparing the outer and inner leaves of Pokkali it was noted that the inner leaves maintain a higher K^+/Na^+ ratio indicating higher translocation of K^+ to

the metabolically more active younger leaves or a greater retention of Na^+ in the older leaves (Thomas and Nambisan, 1999).

From the present study it was evident that, Na^+ content in older leaves of Pokkali, which was a salt tolerant variety, was higher when compared to sensitive varieties. This increase could be correlated with esterase activity in most stages, especially in tillering stage. There appears to exist a direct relation between Na^+ content and esterase. However, no correlation could be made for Na^+ content directly with isocitric dehydrogenase activity.

2.4.6 Isozyme **markers could be used for the identification of somaclonal and androclonal variation**

Exogenously supplied hormones affected the isozyme banding pattern and it varied frequently in cultured cells as well as intact plant (Lee, 1972). In the present study variations in esterase banding pattern in regenerated plants included slight variations in banding patterns and band thickness. There was a direct relation between thicker bands and increase in enzyme activity. When band 7, which was present only in Pokkali, showed an increase in thickness, increase in enzyme activity was much higher than seed germinated plants.

In Pokkali, regenerated plants showed variation in band thickness although there was not much change in Na^+ content. In Pokkali, an isozyme band, which was present only in callus, was found in a regenerated plant. 13 regenerated plants from anther culture, showed band 9, which was present only in seed germinated plants under stress. Plants regenerated from anther culture of Pokkali showed wide variation. Plants with two additional bands, between 4 and 5, and 5 and 6 showed a slight increase in esterase activity and Na^+ content.

In MI 48 most of the plants showed banding pattern similar to seed germinated plants however some plants showed faint bands 6 and 7, which were absent in seed germinated plants. Na^+ content in these plants was also higher in the variants. Plants regenerated from anther culture, with additional band 6, which was absent in seed germinated plants of MI 48 but present in Pokkali, showed high esterase activity and Na^+ content. Plant corresponding to lane 39 also had band 7 and had high enzyme activity and Na^+ content.

In Annapoorna, regenerated plants showed similar characters of seed germinated plants, although in all of them certain minor bands like 1, 2 and 9, which were present in seed germinated plants, were missing, and six plants out of 39 studied, showed a faint band 7, which was present only in salt tolerant Pokkali. In these plants a corresponding increase in esterase activity and Na^+ content was recorded. Twenty-eight plants regenerated from anther culture of Annapoorna showed a faint band 7 and a high esterase activity and Na^+ content. Plants with similar banding pattern as that of Annapoorna showed only low enzyme activity and Na^+ content.

In regenerated plants of Jyothi, out of 39 plants studied, 2 plants showed band 7 which was thicker and these plants showed higher esterase activity and Na^+ content. Plants with a faint band 7 showed a slight increase in esterase activity and Na^+ content. Thirteen anther culture regenerated plants of Jyothi, showed band 7, while band 6, which was normally present in seed germinated plants, was absent in these plants. In plant corresponding to lane 27 both bands 6 and 7 were present, and showed higher esterase activity and Na^+ content.

Thus, from the present study it may be concluded that plants regenerated from callus culture and anther culture showed somaclonal and androclonal variation and

these variants can be identified using esterase as isozyme marker. The results also suggest that increase in thickness and presence of additional bands could be correlated with an increase in enzyme activity, and a slight increase in Na^+ content. When band 7 was present or thickness of band 7 increased, there was a corresponding increase in esterase activity and Na^+ content. Band 7, which was present in Pokkali, might possibly be related to salt tolerance. Regenerated plants of salt sensitive varieties, showing an additional band 7, had higher esterase activity and Na^+ content. So this particular band could putatively be used as a marker for identification of moderate salt tolerance although further studies are required. An attempt to establish the inheritance of this isozyme was made- Pokkali was crossed with salt sensitive rice varieties and the isozyme profiles of the F_1 hybrids were studied. However banding patterns in the F_1s was complex- while some of the bands were similar to Pokkali, others corresponded to those of the salt sensitive parent. Band 7 of Pokkali was not consistently present in the F_1s. A typical codominant inheritance pattern was not observed, thus it appears that the parents selected were not homozygous for the esterase loci.

In the case of isocitric dehydrogenase, all the rice varieties showed only a slight variation in thickness of bands and correspondingly there was a change in isocitric dehydrogenase activity. But this could not be correlated with Na^+ content.

2.4.7 Variations in F1 hybrids

One advantage of the use of isozymes as marker systems for hybrids is that this technique allows an estimation of the relative contribution of each parental genome to the hybrid pattern. Plants regenerated from calli of F1 hybrids showed banding pattern characteristic of either parent, or also a combination of both parents.

In the case of the hybrid of Pokkali and MI 48, band 5 was absent in 12 regenerated plants studied. Esterase activity and Na^+ content in the plant were very high when compared to MI 48. This kind of variation was present only in hybrid plants. Some regenerated plants of Pokkali x Jyothi hybrids showed all the 10 esterase bands, and this condition was absent in the two parental rice varieties. Variations in esterase banding pattern and activity was higher in hybrid plants when compared to the parents. These variants may possibly be detected using esterase banding pattern, esterase activity and Na^+ content of plant body. Isocitric dehydrogenase bands in hybrids also showed various combinations of banding patterns of salt tolerant and salt sensitive varieties. Even though it could not be correlated directly with salt tolerance, it could be used to distinguish varietal differences in rice. Further studies are required to confirm the relationship of esterase with salt tolerance.

Conclusions

CONCLUSIONS

Salinity is a problem in coastal environments and generation of salt tolerant rice varieties with good grain quality and yield is very essential for states such as Kerala, where rice cultivation is done near backwater areas. Pokkali is a moderately salt tolerant variety, but has poor grain quality and yield. On the other hand Annapoorna and Jyothi have high yield but tolerance level is low. A combination of these characters is desirable. Genetic variability is the basis of crop improvement. Somaclonal and androclonal variation can be effectively used for this purpose. Suitable culture media and other cultural conditions were standardized towards this objective. Media containing 0.1-mg/l 2,4-D and 2 mg/l kinetin was found to be beneficial for regeneration. Calli induced from mature embryo explants maintained its regeneration ability for up to three months, which then decreased drastically. Conditions favourable for androgenesis varied for different rice varieties. N_6 media containing 2 mg/l NAA and 0.5 mg/l kinetin with sucrose or maltose was ideal for androgenesis. Maltose as a carbon source was effective in inducing androgenesis in Pokkali but sucrose induced high percentage of callusing in other rice varieties. This media induced direct green plantlet regeneration with higher frequency. Alternatively, callus may be transferred to regeneration media.

Characterization of biochemical markers for salt tolerance might aid breeding programs for developing salt tolerant plants by reducing the time needed for screening. Isozymes have been used as markers for somaclonal variation, embryogenesis, stress tolerance etc. in several crop plants. In the present study, eight isozymes were studied and esterase and isocitric dehydrogenase was found to have

varietal specific, developmental stage specific and stress specific banding pattern in rice. Under salt stress thickness of bands and enzyme activity showed changes. Pokkali, a moderately salt tolerant variety, had a specific band 7, which was present only in this variety and showed slight changes under stress. This band was faint in tillering and flowering stage. Band 7 may possibly be used as a marker for identifying salt tolerance in rice. Somaclonal and androclonal variations in regenerated plants were detected using these isozymes. A wide range of variations could be seen. Certain new bands which were not present in seed germinated plants were found in regenerated plants. But this increase in number of bands did not cause much change in enzyme activity. Nevertheless the presence of band 7 and increase in its thickness resulted in an increased enzyme activity. Pokkali plants had greater Na^+ content in their plant body compared to other varieties tested, and under stress it increased up to 3 fold. Regenerated plants with variation in esterase bands and activity also showed variation in Na^+ content. Plants with band 7 of esterase showed higher Na^+ content in its body.

Based on the results obtained in the present study it is suggested that esterase could possibly be used as an isozyme marker for salt tolerance in rice. Varietal differences and stage specific variations could be detected using esterase and isocitric dehydrogenase. Moreover somaclonal and androclonal variation could be effectively detected using isozyme markers.

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