Section 1

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)

Somatic 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.
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 predominant (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.

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

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 ± 2°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 ± 2°C for callus induction. Only N₆ media (Chu et al., 1975) with minor modifications (Appendix II) was used for anther culture.
Different media combinations tried were:

1. N₆ + 1 mg/l NAA + 0.5 mg/l Kinetin + 6 % maltose.
2. N₆ + 1 mg/l NAA + 0.5 mg/l Kinetin + 6 % Sucrose
3. N₆ + 2 mg/l 2,4-D + 6 % Sucrose
4. N₆ + 2 mg/l NAA + 1 mg/l Kinetin + 6 % Sucrose
5. N₆ + 1 mg/l 2,4-D + 0.5 mg/l IAA + 0.5 mg/l BAP + 6 % Sucrose
6. N₆ + 2 mg/l NAA + 0.5 mg/l Kinetin + 6 % Sucrose
7. N₆ + 2 mg/l PAA + 0.5 mg/l Kinetin + 6 % Sucrose
8. N₆ + 2 mg/l PAA + 1 mg/l Kinetin + 6 % Sucrose
9. N₆ + 2 mg/l PAA + 6 % Sucrose
10. N₆ + 1 mg/l PAA + 0.5 mg/l IAA + 0.5 mg/l BAP + 6 % Sucrose
11. N₆ + 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.
REGENERATION RESPONSE IN THE CALLUS CULTURES OF POKKALI

Fig. 1

a. Two months old

b. Three months old

c. Four months old
d. Six months old

% of regeneration

Media

Green plantlets
Rooting only
REGENERATION RESPONSE IN THE CALLUS CULTURES OF MI 48

Fig. 2

a. Two months old

b. Three months old
c. Four months old
d. Six months old

- Green plantlets
- Rooting only
REGENERATION RESPONSE IN CALLUS CULTURES OF ANNAPOORNA

a. Two months old

b. Three months old

c. Four months old

d. Six months old

Media

% of regeneration

Green plantlets
Rooting only
Fig. 4

REGENERATION RESPONSE IN CALLUS CULTURES OF JYOTHI

a. Two months old

<table>
<thead>
<tr>
<th>Media</th>
<th>% of regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

b. Three months old

<table>
<thead>
<tr>
<th>Media</th>
<th>% of regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

c. Four months old

<table>
<thead>
<tr>
<th>Media</th>
<th>% of regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

d. Six months old

<table>
<thead>
<tr>
<th>Media</th>
<th>% of regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

Legend:
- Green plantlets
- Rooting only
## Media used for Regeneration

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS + 0.5 mg/l 2,4-D + 2 mg/l Kinetin</td>
</tr>
<tr>
<td>2</td>
<td>MS + 0.5 mg/l Kinetin + 0.5 mg/l BAP + 0.5 mg/l IAA</td>
</tr>
<tr>
<td>3</td>
<td>MS + 0.1 mg/l 2,4-D + 2 mg/l Kinetin</td>
</tr>
<tr>
<td>4</td>
<td>MS + 1 mg/l Kinetin</td>
</tr>
<tr>
<td>5</td>
<td>MS + 0.5 mg/l NAA + 2 mg/l Kinetin + 10 mg/l ABA for two days and transferred to ABA free MS medium.</td>
</tr>
</tbody>
</table>
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 ANOTHER 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 Annapoorna, 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, i.e., 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

% of callusing

Media

Six days cold treatment
Eight days cold treatment
Ten days cold treatment
CALLUS INDUCTION IN ANThER CULTURES OF ANNAPOORNA

Fig. 7

% of callusing

Media

Six days cold treatment
Eight days cold treatment
Ten days cold treatment
CALLUS INDUCTION IN ANther CULTURES OF JYothI

Fig. 8

% of Callusing

Media

Six days cold treatment
Eight days cold treatment
Ten days cold treatment
REGENERATION OF DIRECT GREEN PLANTLETS IN ANther CULTURES OF RICE

Fig. 9

Rice varieties

Pokkali  MI 48  Annapoorna  Jyothi

% of green plantlet regeneration

Medium 1  Medium 6  Medium 11
REGENERATION OF ALBINO PLANTLETS IN ANther CULTURES OF RICE

Fig. 10

Pokkali MI48 Annapoorna Jyothi

Rice varieties

% of albino plants

Media 1
Media 6
Media 11
## Media used for anther culture

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N₆ + 1 mg/l NAA + 0.5 mg/l Kinetin + 6% maltose.</td>
</tr>
<tr>
<td>2</td>
<td>N₆ + 1 mg/l NAA + 0.5 mg/l Kinetin + 6% Sucrose</td>
</tr>
<tr>
<td>3</td>
<td>N₆ + 2 mg/l 2,4-D + 6% Sucrose</td>
</tr>
<tr>
<td>4</td>
<td>N₆ + 2 mg/l NAA + 1 mg/l Kinetin + 6% Sucrose</td>
</tr>
<tr>
<td>5</td>
<td>N₆ + 1 mg/l 2,4-D + 0.5 mg/l IAA + 0.5 mg/l BAP + 6% Sucrose</td>
</tr>
<tr>
<td>6</td>
<td>N₆ + 2 mg/l NAA + 0.5 mg/l Kinetin + 6% Sucrose</td>
</tr>
<tr>
<td>7</td>
<td>N₆ + 2 mg/l PAA + 0.5 mg/l Kinetin + 6% Sucrose</td>
</tr>
<tr>
<td>8</td>
<td>N₆ + 2 mg/l PAA + 1 mg/l Kinetin + 6% Sucrose</td>
</tr>
<tr>
<td>9</td>
<td>N₆ + 2 mg/l PAA + 6% Sucrose</td>
</tr>
<tr>
<td>10</td>
<td>N₆ + 1 mg/l PAA + 0.5 mg/l IAA + 0.5 mg/l BAP + 6% Sucrose</td>
</tr>
<tr>
<td>11</td>
<td>N₆ + 2 mg/l NAA + 0.5 mg/l Kinetin + 6% Maltose.</td>
</tr>
</tbody>
</table>
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 redifferentiation 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 up to 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 up to 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°C, 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.