DISCUSSION

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Callus induction was noticed from mature rice embryos from 5th day onwards on L.S. medium supplemented with 2,4-D (Linsmaeir and Skoog, 1965). 2,4-D has been found to be the best source of auxin for callus induction in many cereals (Nambisan and Chopra 1992; Padmaja *et al.*, 1992; Sabu *et. al.*, 1995). The concentration of 2,4-D required for callusing varies from species to species as well as explant to explant (Henke *et. al.*, 1978; John and Prathapasenan, 1999). Requirement of 2,4-D for callus induction in rice was demonstrated by Wu and Li (1971). In the present study also 2,4-D has been found effective in callus induction. The maximum production of callus was observed at 2.5 mg/l of 2,4-D. Promotion of callusing by 2,4-D has been reported in *Coix aquatica* Roxb. (Katiyar and Chandel, 1998). *Heteropogan contortus* (L.) P. Beauv (Purohit and Kukda, 1996) and wheat embryos (Mohamand, 1993). The regeneration potential of monocot cultures has been found to be highly dependent on the level of 2,4-D in the callus induction medium (Bhaskaran and Smith, 1990).

Auxins are generally associated with promotion of growth, proliferation of callus and root induction (Cano *et.al.*, 1999). 2,4-D has been used widely for the proliferation of the induced callus in a variety of plant species especially in monocots (Hutchinson *et. al.*, 1997). The callus obtained after 30 days of culture was loose and was yellow-creamish in colour. Under the influence of NaCl there was a reduction in the dry weight of the callus compared to that of the control. A similar reduction in dry weight of rice calli was observed by Chauhan and Prathapasenan (1999), Reddy and Vaidyanath (1986) and Cushman *et. al* (1990) in embryo derived calli of rice. The reduction in callus growth may be due to high osmotic pressure of the culture medium as well as due to the channelisation of

energy for salt resistance (Cushman *et al.*, 1990). The reduced growth on salanization may also due to the suppression of nutrient absorption by NaCl. The slow growth of the callus observed under the influence of NaCl may be indicative of the adaptation cells of the callus. This was indicated by the gradual increase of the callus from zero week to fourth week which then become stationary. According to Basu *et. al.* (1997a) the retarded growth of rice callus (Basmati 370) was due to a low tissue viability of cells in the adapted tissues.

Regeneration of plants in tissue culture systems is the most important tool in biotechnology. A reliable tissue culture system with a high capacity of regeneration is a prerequisite for use of biotechnology for the improvement of plant species. Such improvement through *in vitro* culture technology depends on callus induction, growth and differentiation (Abe and Futsuhara, 1991). For many years, *in vitro* culture of plant tissues has been a useful technique to study salt tolerance mechanisms at the plant cell level (Lutts *et. al.*, 1999). In the present study maximum differentiation of callus cultures was obtained (68%) in MS medium supplemented with IBA (1.5 mg/l) and KN (0.5 mg/l). NaCl in the regeneration medium had a detrimental effect on plant regeneration. Several authors have reported a strong NaCl induced decrease in plantlet regeneration in rice (Subhashini and Reddy, 1991; Bhattacharya 1991; Binh *et. al.*, 1992). Here also, under the influence of NaCl (LD_{50}) the regeneration frequency of adapted callus was reduced to 40% compared to the control. A ratio between auxin and cytokinin is necessary for regeneration in many plants (Centeno *et. al.*, 1999). Kavikishor *et. al.* (1999) got 42 and 50% of regeneration in two varieties of rice Tellahamsa and Bala respectively under the influence of 100 µM of NaCl. Lutts *et al.* (1999) reported the improvement of regeneration of rice callus under various

doses of NaCl (0, 50, 100 mM). Even though NaCl strongly decreased the regeneration frequency in all varieties tested the survival of the regenerants slightly increased. John *et al.* (1997) reported regeneration of the three rice varieties from NaCl (LD_{50}) adapted callus cultures. NaCl adapted callus of a salt sensitive indica variety of rice (*Oryza sativa* var. Basmati 370) showed 55% regeneration in modified B5 medium supplemented with IAA and KN. Regeneration was low at 85 mM NaCl and a concentration of 128 mM was inhibitory (Basu *et. al.*, 1997a).

Rice callus generally shows a greater capacity to initiate roots than shoots (Maeda and Saka, 1973). The omission of auxin or reducing the level of auxin from the culture medium initiates the reduction of rhizogenesis in many plants. Mascarenhas *et. al.* (1975) found that callus cultures of wheat seedling segments could be completely converted into a mass of roots merely by changing physical state of the medium. The callus induction medium and the hormones used for the proliferation of the callus modulate the callus morphology which may be embryogenic or non-embryogenic (Raval and Chatoo, 1993). From the present study it is clear that the increase or decrease in the level of 2,4-D during the subculture period can alter the nature of the callus from embryogenic to rhizogenic. In the present study 100% of the cultures showed rhizogenesis when a high level of 2,4-D (4 mg/l) was used in the subculture medium. 2,4-D appears to be necessary for the induction of callus formation and for the onset of somatic embryogenesis. The embryogenic callus was maintained in the presence of 2,4-D, but after lowering the concentration or its removal, somatic embryos will mature and develop to fertile plants when cultured appropriately (Green, 1983). In *Pennisetum americanum* (Vasil and Vasil, 1982) either omitting the level of auxin specially 2,4-D or lowering the level of 2,4-D leads to the formation embryogenic cultures.

In the present study also a medium without 2,4-D lead to the formation of embryogenic callus. The stimulation of totipotent cells to become embryogenic and the isolation of embryogenic calli are critical for embryogenic cell cultures (Katiyar and Chandel, 1998). Formation of embryogenic callus is a well documented phenomenon in many cereals (Brettel *et. al.*, 1980, Chen *et. al.*, 1988; Eapen and Rao, 1995). Under the influence of NaCl and a high concentration of 2,4-D (2.5 mg/l of 2,4-D) the embryogenic callus showed extensive rooting. Kinizios *et. al.* (1997) reported that the callus induced from mature wheat embryos produced extensive rhizogenesis under 6 g/l of NaCl. Roots induced at lower NaCl levels were significantly longer, thinner, light coloured compared to those formed at a high level of NaCl. Embryogenic callus grown under the influence NaCl was slightly brown in colour, friable in nature while embryogenic callus grown in salt free medium was compact, pale white and nodular in nature.

Komai *et. al.* (1996a) reported that auxins, preferentially 1-napthalene acetic acid (10 μM) gave the best results in the induction of embryogenic callus in spinach cultures. Embryogenic cultures from mature seeds were obtained in many graminaceous species (Smith and Bhaskaran, 1986; Purohit *et. al.*, 1992). In many reported systems 2,4-D can be used to induce embryogenesis and its removal will help in embryo germination (Kackar and Shekhawat, 1991). The occurrence of green spots in cereal callus cultures has been frequently noted, and a positive correlation between the presence of such spots in the embryogenic callus and regeneration has been reported by Ben Amer (1997) in wheat. In the present study it was interesting to note that maximum number of green spots are observed in the embryogenic callus and not even a single green spot is observed in non

embryogenic calli. Piqueras *et. al.* (1996) isolated NaCl (170 mM) resistant embryogenic callus cultures of lemon (*Citrus lemon* L. Burm f. cv. Verna).

Somatic embryogenesis from callus or cell suspension cultures is an efficient method for high frequency of plant regeneration (Choi *et. al.*, 1999). Suspension cultures consists of rapidly dividing group of cells with an enriched cytoplasm. The liquid suspension protocol is relatively efficient because individual embryos can easily be handed, manipulated and they may be suitable for further biotechnological applications like genetic transformation, screening of somaclonal variants, cell selection for desirable traits, clonal propagation and production of artificial seeds (Anbazhagan and Ganapathi, 1999). Establishment of embryogenic cell suspensions is the most important milestone in rice biotechnology (Rani and Reddy, 1996). In the present study fine suspensions have been obtained from the embryogenic callus within ten days of culture on LS basal medium. In a similar manner, Singh *et. al.* (1997) established uniform embryogenic cell suspensions in barley (*Hordeum vulgare* cv. Schooner) within a week. Suspension culture derived under the influence of LD_{50} concentration of NaCl showed slight browning after two days compared to control. The cells from adapted embryogenic cultures contained mostly single cells or cell aggregates. If these cultures are kept in the same medium for more than 30 days they turned dark, necrotic and died. It is difficult to establish cultures of well dispersed embryogenic cells of *Oryza sativa* L. in suspensions (Ozawa *et. al.*, 1996). Dix and Street (1975) were able to establish cell lines which were capable of growth in media containing 20 g/l NaCl using inoculum from the cultures growing in l0 g/l NaCl. Rangan and Vasil, (1983) used inoculum of cells growing in 10^{-1} M NaCl medium to establish cell lines that can grow in the presence of 2×10^{-1} M NaCl.

Somatic embryogenesis has been proposed as the most common pathway of plant regeneration in cereals and grasses (Kothari and Varshney, 1998). The potential use of somatic embryogenesis in developmental studies, crop improvement and genetic transformation has been widely recognized and the number of species displaying the embryogenic potential is constantly increasing. Induction and development of somatic embryos are controlled by growth regulators (Korac and Neskovic, 1999). A low percentage of embryogenesis was observed in the medium devoid of growth regulators. Auxin alone when tried failed to bring about embryogenesis. However, BAP alone could induce embryogenesis in a small percentage of cultures. These results clearly indicate the requirement of growth regulators in inducing somatic embryogenesis. Xio and Branchard (1993) applied high concentration of IAA (48.52 μm) and GA_3 (10μm) for initiation of embryogenic callus and then lowered IAA level for improving the development of somatic embryogenesis in spinach. It is evident from the present studies that somatic embryogenesis is favoured by a combination of IAA and BAP as well as NAA and 2 ip. Among the various combinations tried IAA (0.1 mg/l) and BAP (0.5 mg/l) was found to be more effective in inducing somatic emrbyogenesis. George *et. al.*, (1989) reported that the transfer of embryogenic calli to MS medium alone or with reduced level of auxin resulted in production of somatic embryoids. Somatic embryogenesis in pigeon pea and cassava has been shown to be regulated by auxin (George and Eapen 1994, Guohua 1998).

The role of stress treatment on somatic embryogenesis is still obscure (Choi *et. al.*, 1998). Cell suspensions derived from NaCl adapted calli showed induction of embryoids in LS medium supplemented with IAA (0.1 mg/l) and BAP (0.5 mg/l) and LD_{50} concentration of NaCl within 15 days. Along with the embryoids

small clumps of rooted structures were also visible. NaCl induced reduction of rhizogenesis and improvement of somatic embryoids were observed in the present studies. A reduction in embryogenesis under high concentration of NaCl in *Sapindus trifoliatus* was observed by Unnikrishnan *et. al.*, 1993). Salt tolerant embryoids of *Vitis*, wheat and lentil have been isolated by Lebrun *et. al* (1985), Galiba and Yamada (1988), and Ghanem (1995). Here also it was observed that non-adapted embryoids were showing less rhizogenesis compared to adapted embryoids. The globular embryoids induced in 0.25M NaCl developed further in the same medium. In *Vitis*, Lebrun *et. al* (1985) isolated embryogenic cell lines which grow in agitated liquid media containing upto 150mM NaCl. Exposure of embryoids to NaCl (50-100mM) promoted outgrowth of secondary somatic embryoids but inhibited development of plantlets. Galiba and Yamada (1988) reported that incorporation of NaCl and KCl into the medium enhanced the frequency of somatic embryogenesis in wheat callus cultures. Here also the embryoids derived from adapted callus showed improved embryogenesis compared to other treatments. It was observed that the non adapted (NA) embryoids produced elongated roots and abnormal structures were observed rarely (3-4% of the embryoids). Production of normal embryoids have been observed under the influence of NaCl in *Pennisetum* (Rangan and Vasil, 1986). Bingh *et.al.* (1992) isolated embryogenic cell suspension from mature rice embryos which can tolerate NaCl upto 1.5%.

A rise in proline accumulation is always shown to be associated with stress response in a number of plant species. Thus proline is known to render stress tolerance to the plants or their tissues (Yancey *et.al*, 1994). Incorporation of proline into the medium led to an increase in the fomation of embryoids at all

concentrations tried. Many studies revealed the fact that exogeneously applied proline can stimulate somatic embryogenesis in rice (Kavikishor *et al*, 1999;Choudhary *et al.,* 1993; Raval and Chatoo 1993; Ozawa and Komamine, 1989). Similar stimulation of somatic embryogenesis by proline is reported in *Zea mays* (L) (Armstrong and Green, 1985; Vasil and Vasil, 1986). Trigiano and Conger (1987) reported that proline can regulate somatic embryogenesis in cell suspension cultures of *Dactylis glomerata.* Chowdhary *et al.* (1993) reported that 12 mM of proline was optimum in giving 80% of embryogenesis in rice (*Oryza sativa* L. cv. Pusa 169).

Exogeneously supplied proline can act as an osmoprotectant facilitating growth in high saline environment (Yancey, 1994). According to Holme *et al* .(1997) a medium with MS basal salts and 12.5 mM proline increased the formation of embryogenic callus on leaf explants and shoot apices, increased the growth of suspension cultures and increased the plant regeneration from embryogenic callus and suspension cultures in *Miscanthus x Ogiformis* Honda Giganteus.

Hydroxyproline resistant cell lines of rice and cauliflower have been shown to devolop resistance to NaCl however, there is hardly any report on the effect of hydroxyproline on somatic embryogenesis. It was observed that hydroxyproline resistant cell lines are tolerant to NaCl (Van Swaaij *et. al.,* 1986). There are few studies on isolation of hydroxyproline resistant cell lines in rice (Chauhan and Prathapasenan, 1998) and cauliflower (Deane, 1995). From the present study it was observed that hydroxyproline could not improve the somatic embryogenesis as compared to the control, but it was observed that NaCl adapted cultures (especially to 4 and 8mM) showed an increase in percentage of cultures with

embryoids and also mean number of embryoids per culture. Stimulation of callus growth of rice under saline conditions by hydroxyproline has been shown by Chauhan (1998).

Incorporation of putrescine at 0.1 and 0.2mM concentrations improved the percentage of embryogenesis in NaCl adapted cells. However, further rise in the concentration of putrescine resulted in an increase in rhizogenesis. In the present studies it was interesting to note that increasing the concentrations of putrescine (0-0.8mM) increased the percentage of cultures showing rhizogenesis and it reached a maximum (100%)at 0.8 mM. Bagni and Mengoli (1985) isolated carrot cell lines resistant to high level of putrescine. The adapted cultures showed a reduced rhizogenesis. Galston (1983) reported that PAs function as plant growth regulator or secondary messenger of plant growth regulators. So they can play a critical role in regulating somatic embryogenesis. Exogeneous application of PAs did not promote somatic embryogenesis (Fienberg *et. al.*, 1984). Putrescine used to synchronise the process of embryogenesis while spermidine has got a stimulatory effect on plant morphogenesis in long-term indica rice callus. Increasing concentration of putrescine produced extensive roots from the emrbyogenic clumps. Introduction of PAs into tissue culture media or manipulations of their metabolism may favour the change in morphogenetic programmes in *in vitro* cultures. It is interesting to note that the addition of an inhibitor of polyamine (MGBG) is used favoured the germination of embryogenic clumps. Auxins are specific inducers of adventitious root formation and that cytokinins are specific controllers of shoot bud formation and development is being progressively reappraised. Auxins and cytokinins are also implicated in flowering (Bernier *et. al.*, 1993), while in a process like rooting PAs and auxins are indissociable factors

(Gaspar *et. al.*, 1996a, 1997a). From the present observations it can be assumed that PAs especially putrescine may have a role in auxin metablism in the embryogenic clumps of rice. Martin-Tanguy *et. al.* (1997) reported that production of buds, rooting etc. can be oriented using inhibitors of PA metabolism. Presence of NaCl also did not alter the morphology when MGBG is incorporated in the medium. A total inhibition of root formation by MGBG (0.5 mM) was not observed in the present studies. Aribaud (1999) reported that in *Chrysanthemum,* vegetative buds were formed instead of callus on the callus induction medium in presence of 2mM DFMO (difluromethyl ornithine, an inhibitor of polyamine. Koetje *et al.* (1993) reported that the inhibitor of polyamine DFMA (10mM) suppressed culture growth in cell suspension and plant regeneration from callus. Polyamine levels increased about ten fold during embryogenic callus induction and during rapid growth. Yadav and Rajam (1997) demonstrated that in egg plant (*Solanum melangena* L.) MGBG at 0.1 and 0.5mM drastically reduced the number of somatic embryos, which was accompanied by reduced callus growth with browning of the leaf disc without any callus formation. The leaf explants of *Medicago sativa* L. showed a high meristamatic activity and proembryo formation with a higher endogenous levels of free put, spd, spm, cad, and tyramine levels when cultured on embryo inducing medium (Cvikrova *et al.*, 1999). In *Zea mays* a four-fold increase in the number of regenerated plants is obtained after pretreatment with 0.5mM Difluromethyl Arginine (DFMA) thus improving the morphogenetic capacity (Triburcio *et al.*, 1991). In *Brassica napus* 0.35μ M MGBG promoted the shoot regeneration frequency from 50% to 92% (O'Neill *et al.*, 1996).

Precocious germination and rhizogenesis are two serious problems confronted by various workers during the production of embryoids in the members of graminae (Kinizios *et. al.*, 1997; Shayakhmetov 1996). Finkelstein and Crouch (1984) first reported that the abnormal growth of the embryos is due to precocious germination. Precocious germination occurs when there is no separation between mid-embryogenesis and germination, namely late embryogenesis. The above problem is usually controlled by increasing the osmolarity of the medium or by incorporation of ABA. The incorporation of ABA in MS medium has been shown to significantly decreased the sedimentation volume of the cells, endogeneous level of auxin and prevents premature development of root rudiments obstructing the process of embryogenesis (Shayakhmetov, 1996). In the late phase of embryogenesis the embryo gets desiccated and enters into dormancy. This phase separates embryogenesis from events of germination. Precociously germinating somatic embryos of *Vitis* have been attributed to a low level of ABA and IAA Faur *et. al.* (1998).

It was observed in the present study that NaCl at 50 mM level could totally prevent rhizogenesis of embryoids. However, further increase in concentration of NaCl in the medium led to increased rhizogenesis. Promotion of embryogenesis by low levels of NaCl (50 mM and 100mM) was reported in *Sapindus trifoliatus* (Unnikrishnan *et al.*, 1993). They have also reported formation of elongated radicle in *Sapindus* embryoids under the influence of high concentration of NaCl (above 100mM NaCl). Kinizios *et al.* (1997) observed promotion of abnormal rhizogenesis and reduction of root emergence by low level of NaCl in wheat.

In the present study activated charcoal (AC) at 8% was effective in reducing rhizogenesis by 58%. Supression of the formation of morphological

abnormalities in embryo cultures of *Pennisetum glacum* has been shown by Lambe *et.al.* (1999). Incorporation of activated charcoal helps in increasing the osmolarity of the medium. Druart and DeWulf (1993) have shown that addition of activated charcoal increased the osmolarity of the medium due to increased sucrose hydrolysis. Activated charcoal is normally used in *in vitro* cultures for improving morphogenesis particularly somatic embryogenesis. It is summarised that the increased osmolarity in the medium and the resultant stress may be responsible for a low endogeneous level of auxin which is evident from reduced rhizogenesis.

Maturation phase has proven to be one of the most crucial phase representing the late embryogenesis, a stage at which embryoids can under go a period of dormancy, and fully developed germinable somatic embryoids are produced. Single somatic embryos or clumps derived in a suspension culture are blocked in stem meristem formation when transferred to germination medium and their roots grow out precociously before stem meristem. Such an observation was also noticed by Emons *et al.* (1993). Different levels of ABA (Vasil and Vasil 1981), mannitol or sucrose can be used for inducing maturation and dormancy.

Sucrose is the most effective carbon source and osmoticum for somatic embryogenesis (Ammirato ,1983). Depending upon the nature of carbohydrate and its concentration it is also possible to induce non-competent cells to become competent for embryogenesis and to prevent precocious germination (Ammirato and Steward, 1971). In *Coix aquatica* Roxb (Katiyar and Chandel, 1998) it is reported that sucrose at 30 g/l was effective in premoting globular embryoids and their maturation but a concentration of 60 g/l was inhibitory. In the present study it was observed that sucrose at 4% (w/v) level is the best in inducing dormancy and maturation in 100% of embryoids. They remained dormant for 10-15 days and

showed good germination when tried thereafter. From the data it is observed that level of sucrose in the medium is critical for proper maturation of the embryoids. A high concentration of sucrose in the medium cause osmotic dessication in the embryoids. Efficient germination in some species requires a temporary desiccation. This procedure which mimics seed maturation *in vivo*, may be necessary to trigger metabolic process needed for germination and seedling growth. The gradual reduction in osmotic potential through dessication of mature somatic embryos of wheat showed better germination percentage (Kumar, 1999). In maize inbred line A 188 somatic embryos matured on MS medium containing 6% sucrose (Bronsema, 1997).

Mannitol is another osmoticum which is normally employed for obtaining maturity and dormancy of embryoids. Among various concentrations of mannitol tried the best results with respect to maturation of embryoids were obtained at 25 mM. Alfalfa embryos were matured on SH medium containing either mannitol on ABA. Emons *et al.* (1993) showed that mannitol at 8% act as an agent for proper maturation of embryoids in *Zea mays* (L.). The mannitol treated embryoids converted to plantlets at a very low frequency. For *Celery* Somatic embryoids, a combination of mannitol (2 and 3%) and ABA yielded highest maturation process (Fuji *et al.*, 1993).

PEG (Poly-Ethylene Glycol) is another compound which is used as an osmoticum in various biological studies. Cornu and Geoffrion (1990) were the first to report that PEG (MW 6000) encouraged maturation of larch somatic embryos in liquid medium, but the subsequent root development of these embryoids was very poor. Simple sugars and salts were not effective as high molecular weight osmotica such as PEG in enhancing maturation and descication

tolerance of *Aesculus* somatic embryos. Such treatment may lead to the complete maturation and inducing dormancy of somatic embryos (Radojevic, 1995). According to Attree *et al.* (1991b) these osmoticas easily cross the cell wall and cause withdrawal of water from the protoplasts by osmosis, leading to plasmolysis . During such prolonged incubation the plasmolyticum gets absorbed into the symplast of the plant cell. Such absorption leads to adjustment of tissue osmotic potential and deplasmolysis. Attree and Fowke (1993) reported that a concentration of 5-10% PEG (MW 4000) together with ABA lead to a three fold increase in the maturation frequency of white spruce somatic embryos and yielded somatic embryos of superior appearance and well developed cotyledons. These somatic embryos also possessed increased storage reserves and the ability to survive desiccation treatments. In the present investigation, when different concentrations of PEG were used for the maturation of both adapted and non adapted embryoids of rice, it inhibited maturation at all the concentrations tested, and had no effect on their germination. The embryoids showed poor growth, excessive browning and rooting may be due to the PEG induced high osmotic potential of the medium. Kavikishor *et.al.* (1999) reported a high regeneration frequency of plantlets (41%) in calli grown on 2% PEG but a higher concentration (6%) of PEG suppressed the frequency of regeneration.

The comparison of results of germination of embryoids on half and full strength LS medium with sucrose reveals that half strength LS medium is better for germination. Among the different concentrations of sucrose tried maximum germination of embryoids was observed at 5% (w/v). Similar results have been obtained in case of *Zea mays* (L.) by Emons *et.al.* (1993). Sucrose is known to act as an osmoticum as well as a source of carbon and energy.

The nitrogen source used in the germination medium can improve the *in vitro* morphogenesis (Christianson, 1987). Improvement of somatic embryogenesis in callus cultures of cereals by the incorporation of proline is a usual phenomenon (Kothari and Varshney, 1998). The salt resistance embryoids showed a poor germination as compared to the control. A significant increase in the germination of embryoids (both control and salt resistant) was observed on half strength LS medium supplemented with 5% sucrose and 8 mM proline. Stimulation of somatic embryogenesis and regeneration in rice by exogeneously provided proline have been reported by Kavikishore *et.al.* (1999). Similar observations on the effect of proline on rice have also been made by Raval and Chattoo (1993). Proline has been shown to benefit the adaptation of cultured cells by adjusting osmotic potential in response to changing external water potential. It has been assumed that proline act as active solute and as an enzyme protectent (Kim and Janick, 1991). Proline also act as a regulator of fatty acid composition in both zygotic and somatic embryos as has been shown in soybean (Shoemaker and Hammod, 1988), *Brassica* (Avjioglu and Knox, 1989), *Jojoba* (Wang and Creelman, 1986).

In the present study, better germination of somatic embryoids and well developed plantlets have been observed in liquid medium compared to solid medium. Though the growth of the embryogenic cultures was normal in liquid medium, the embryoids showed poor germination on transfer to semisolid medium. Jana *et. al.* (1994) reported that mango somatic embryos developed better in liquid medium than in semisolid medium and better germination was observed in semi-solid medium than in liquid medium. Choi *et al.* (1998) reported that small plantlets could be obtained within three weeks, when cotyledonary stage embryos were transferred to half strength MS liquid medium with 3×10^{-5} M GA_3. An

exogenous supply of BA in MS basal medium found to improve somatic embryo development and germination in banana and oil palm (Bertossi *et. al.*, 1999).

The occurrence of somaclonal variation in plants regenerated via *in vitro* culture is reported in many plant species and can be considered as a novel source for crop improvement. The production of albino plants is one of the most frequent and conspicuous manifestation of somaclonal variation. The production of albinos may be due to the excess amount of aminoacids, growth regulator treatments or different stress factors. In the present study a very low percentage (1-2%) of albino plants was observed only in adapted embryoids. Tsukahara *et. al.* (1996) reported regeneration of albinoplants in rice both in solid and liquid medium.

The use of artificial seeds for obtaining plants has been reported for several crops of agronomic interest (Gray and Purohit, 1991). Encapsulated embryoids of *Eucalyptus citrioda* have an *in vitro* germination rate of 40% (Muralidharan and Mascarenhas, 1995) but the germination rate was only 10% in sandal wood embryoids encapsulated in 3% sodium alginate (Bapat and Rao, 1988). Storage of encapsulated embryos significantly reduced the survival and plant recovery after two months storage at 23 ± 2°C but at lower temperatures (4-6°C) the recovery of plantlets was significantly increased. In the present study well developed embryoids encapsulated in 3% alginate have been stored at different temperatures (2°C, 4°C, 6°C) for varying durations (10, 20, 30 days). Among various temperatures tried for storage 4°C was found to be the optimum. Highest percentage of germination was observed in embryoids stored at 4°C for a duration of 10 days. Increasing the duration of storage at 4°C resulted in progressive reduction in the percentage germination of embryoids. The decline in germination of stored encapsulated embryos may be due to the oxygen deficiency in gel beads

and its rapid drying (Redenbaugh *et al.*, 1991). In *Eleusine coracana* Gaertn, the storage of the capsules for more than 14 days resulted in the loss of tissue viability (George and Eapen, 1995).

Plant tissue culture serves as an excellent tool to understand the basic physiology and biochemistry associated with growth and differentiation of plants. Salinity decreases protein synthesis and increases its hydrolysis in many plants. The total protein content of both embryogenic and non-embryogenic calli increased sharply during the culture periods. NaCl showed a reduction in protein content in salinized embryogenic cells more than that of non-embryogenic ones. When stress is imposed plants will undergo metabolic changes that enable them to tolerate stress (Leone *et. al.*, 1994). Santos *et. al.* (1996) studied the effect of exogeneous (6mM) proline on embryogenic and organogenic maize callus subjected to salt stress (0.4-1.2% NaCl w/v). It was observed that total protein content of embryogenic callus was higher in the presence of proline as compared to the control. However the protein content of organogenic callus was significantly reduced by proline and salt. Total soluble protein profile in rice callus cultures revealed two polypeptides of molecular weight 26 and 41 kDa under NaCl and mannitol stress (Basu *et. al.* 1997). Physiological changes of salt adaptation process are accompanied by increase and decrease in relatively small sets of cellular proteins. These stress induced proteins may play a role in osmotic stress tolerance.

Proline is one of the naturally occurring compound which functions as compatible or counteracting solutes. Salinity causes the accumulation of free aminoacids, especially proline which usually attains ten times more than that in the control. Proline status of plant organs and cell cultures still continue to be an active

area of research in stress physiology (Jain *et. al.*, 1991b). Present study indicates an increase in the level of free proline from 10th day and reached a maximum level at the end of fourty days. Salanized cells of both embryogenic and non-embryogenic calli showed a high content of proline. Li (1990) and Reddy and Vaidyanath, (1988) showed an increase in proline content in salt treated cells of *Cajanus cajan*. The higher level of proline observed in embryogenic cells may be an indication of their stress tolerance. *In vitro* studies indicate that proline is much less inhibiting than equivalent concentration of NaCl to enzyme and protein synthetic machinery (Brady *et. al.*, 1984). Proline also protects cells against heat denaturation (Paleg *et al.*, 1981; Santoro *et. al.*, 1992). Proline also function as hydroxyl radical scavenger and may stabilize membranes by interacting with phospholipids (Rudolph *et. al.*, 1986). Proline also acts as a cryoprotectant in plant cells (Santarius, 1992) and help in osmotic adjustment (Ketchum *et. al.*, 1991; Voetberg and Sharp 1991). Gangopadhyay *et. al.* (1997) reported that tobacco tissues adapted to a low concentration of NaCl (85 mM) showed low growth with high proline content compared to tissues adapted to a low concentration of mannitol (165 mM). In the present study also in all cases the adapted cultures exhibited more level of proline. In maize, it was observed that organogenetic callus exhibited an increased level of proline in presence of 0.4-1.2% w/v NaCl while embryogenic callus showed a reduced level of proline under the influence of same concentration of NaCl (Santos *et. al.* 1996). The increased level of proline biosynthesis in transgenic tobacco plants confers increased tolerance to hyper osmotic stress (Kishor *et. al.*, 1995). In *Oryza sativa* L. a concomitant increase in proline content with increase in concentration of NaCl was observed (Reddy and Vaidyanath, 1986). To counteract the effect of increased accumulation of salt ions in the

vacoules, proline has been reported to increase in the cytoplasm to act as an osmoticum.

The activity of many enzymes have been reported either to increase or decrease as a result of salanization of the medium. Growth regulators have also been reported to protect plants from salt injury. In wheat seeds IAA helped to overcome Na_2SO_4 induced depression of root growth (Levitt, 1980). In the present study, IAA-oxidase activity was stimulated under the influence of salt in embryogenic callus and in the non-embryogenic control. This may be because of the exogeneous application of IAA in the embryogenic medium. Jasrai *et. al.* (1988) demonstrated an inverse relationship between endogeneous IAA levels and IAA-oxidase activity in *Kalanchoe mortagei*. Chauhan and Prathapasenan (1998, 1999) reported a low activity of IAA-oxidase in callus cultures of two rice cultivars under the influence of hydroxyproline. Vincent *et. al.* (1992) reported a higher activity of IAA-oxidase in differentiating callus of *Kaempferia galanga* L.

Carbon metabolism is one of the important factors determining the salt tolerant phenotype (Cushman *et.al.*, 1990). The accumulation of sugars has been shown to act as an osmoticum under NaCl stress (Sacher and Staples, 1985). Amylase is a key enzyme for breaking down starch into sugars. Under the influence of salinity embryogenic callus exhibited a higher reduction in amylase activity compared to that of the non-embryogenic calli. Kavikishor and Mehta, (1989) reported a higher activity of amylase in organogenic callus cultures of tobacco.