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ADAPTATION TO POLYETHYLENE STRESS MAINTAINS TOTIPOTENCY OF CELL LINES OF *ORYZA SATIVA* L. CV. SWAT-1 FOR A LONGER PERIOD

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Abstract

Cell suspension cultures of rice cv Swat-1 were adapted to osmotic (polyethylene glycol, PEG) and ionic (lithium chloride) stresses to find components of stress having greater inhibitory effects on regeneration frequency of cells. In early stage suspension were in cell aggregates or in micro calli form and regeneration frequency of one month old suspensions was about 74%. One month old cultures were incrementally adapted to 20% PEG and 20 mM LiCl. After 50 repeated batch cultures the size of cell aggregates of control and LiCl adapted cell lines turned into fine suspensions, while the suspension of PEG adapted lines remained in small clusters with whitish pale coloration. The relative growth rates of adapted and unadapted cell lines were similar at respective media but the regeneration potential of unadapted and LiCl adapted lines dropped to 30% and 10 %, respectively. In contrast regeneration frequency of PEG adapted line increased to 80% with early recovery of plantlets. These data reveal that regeneration capacity of cell cultures decreases with age of cultures and with ionic stress, while osmotic stress not only maintain but enhance regeneration frequency of cell cultures over a longer time.

Introduction

Environmental stresses have been recognized as the most detrimental factors leading to decline in plants productivity around the world. Among the abiotic stresses, salinity is one of the major and ever-present threat to crop yields, in arid and semiarid regions of the world, while NaCl is the most abundant source of salinity in the soil (Flowers & Yeo, 1995). Rice is highly sensitive to salt stress (Bohnert *et al*., 1995; Flowers & Yeo, 1995; Tamura *et al*., 2003; Hmida-Sayari *et al*., 2005; Bashir *et al.*, 2010). Attempts to improve the salt tolerance through conventional breeding programmes have met with very limited success (Bohnert & Jensen, 1996).

The use of tissue culture techniques has the potential to increase the stress tolerance of plants because plant cells contain a complete species genome and are totipotent. One of the areas in which the *In vitro* selection approach has been used efficiently is plant breeding (Barkat & Abdel-Latif, 1996). Tissue culture techniques make possible the screening and selection or adaptation of a large population of cells and regeneration of plants in a small space and controlled environment than conventional field trails. The relatively higher plasticity of rice to *in vitro* techniques than other graminacious monocots has resulted in its utility as a model system for studying physiology and biochemistry of other plants as well (Christau, 1994). *In vitro* selection for cells having increased tolerance to salt stress has been reported in different crops (Bressan *et al*., 1981; Harms & Oretli, 1985; Barkat & Abdel-Latif, 1996; Jain, 2001) but NaCl stress caused a decrease in callus induction and regeneration capacity of the cell/calli

Since under salt stress plants/tissues experience both ion toxicity and osmotic components of stress simultaneously, particularly under prolonged stress duration (Munns & Tester, 2008). The adverse effects of NaCl on regeneration may be the consequences of osmotic and ion specific components. Therefore, the success of in vitro selection for tolerance to NaCl stress is dependent upon the development of efficient and reliable regeneration systems keeping in view the effects of individual components of salt stress (ionic or osmotic) on regeneration efficiency. For osmotic stress polyethylene glycol (PEG) of high molecular weight has long been used for plants and cells (Ruf *et al*., 1967; Kaufman & Eckard, 1971; Corchete and Guerra, 1986), because PEG having high molecular weight is a non penetrating osmoticum lowering the water potential of nutrient solutions without being taken up (Lawlor, 1970). On the other hand LiCl has been used as an alternative for ionic stress of NaCl because it has shown co-tolerance towards NaCl and caused reduction in growth at very low concentration that has no osmotic effect (Shah *et al*., 1993; 2002). The present study was undertaken by developing cell lines tolerant to osmotic and ion specific components of stress by adapting them to 20% PEG and 20 mM LiCl, with the objective to find which component of stress exerts greater inhibitory effect on the regeneration capability of rice cell lines (cv. Swat-1).

Materials and Methods

Calli were induced from mature seed of rice (*Oryza sativa* L.) cv Swat-1. Dehulled seeds were surface sterilized in 70% ethanol for 30 seconds followed by a 15 minutes washing with 70% bleach. After five washes with sterilized distilled water, seeds were incubated onto Murashige & Skoog (MS) medium (1962) supplemented with 2 mg/l 2, 4 -D, 0.25 mg^{-1} kinetin, 2 g^{-1} casein hydrolysate, 30 g^{-1} sucrose and pH was adjusted to 5.8 and solidified with 9 g^{-1} agar. All the cultures were incubated in the dark at 27 ± 2°C. Following fourth subculture of 28 days, rapidly growing friable calli were sub-cultured and used for suspension cultures. Suspension cultures were established by inoculation of calli into 50 ml liquid MS medium in 200 ml Erlenmeyer flasks. Cultures were incubated at 100 rpm in shaking incubator at 27 ± 2 °C. After two weeks dense suspension were sub-cultured into fresh medium by decanting to about 1/10 dilution every 12-15 days depending on growth of suspensions.

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After preliminary experiments with different concentrations of PEG (0, 5, 10, 15, 20, 25 and 30%) and LiCl (0, 5, 10, 15, 20, 25 and 30 mM), 20% PEG and 20 mM LiCl was selected for adaptation to osmotic and ionic components of stress, these concentrations substantially reduced the growth to about 95% but with out a total inhibition of growth.

Selection procedure: A multi-step procedure (Shah *et al*., 2002) was used to raise adapted lines. Cell lines were subjected to an incremental increase of PEG and LiCl stresses. The sequence of increasing PEG and LiCl concentrations were 5% PEG and 5 mM LiCl (5 Passages), 10% PEG and 10 mM LiCl (10 passages), 15% PEG and 15 mM LiCl (15 Passages) and 20% PEG and 20 mM LiCl for 20 passages. Concurrently, control lines were maintained in the absence of PEG and LiCl.

Measurement of growth: Growth of cell suspension was estimated as relative growth rate (RGR) of sedimented cell volume (SCV) by the method of Shah *et al*., (1993).

$$RGR\ (week)^{-1} = [\ln (SCV_{final}) - \ln (SCV_{initial})] / weeks$$

Regeneration of plantlets: Modified MS medium (Murashige & Skoog, 1962) was used to regenerate plantlets from suspension culture. The medium was modified by omitting 2, 4–D and kinetin and supplemented with NAA1mg^{-1} + BAP 1mg^{-1} + D sorbitol 30000 mg^{-1} + agar 9000 mg$^{-1.}$ Initially thirty days old unadapted suspension cultures were transferred to regeneration medium to record the regeneration percentage. The cultures were incubated at 27^{o}C ± 2^{o}C with a 16 hours photoperiod. After 28 days calli with embryiods or without embryiods were sub-cultured on to the fresh medium, a necessary step to promote conversion of embryiods into plants (Bingham *et al*., 1975). Later another subculture was made depending upon the conditions of the calli/plantlets. After adaptation, adapted and unadapted cell lines were inoculated on regeneration medium. Well developed plantlets were transferred to water culture following Mae (1993).

Results and Discussion

The data on callus induction frequency of Swat-1 on MS growth medium was recorded for 50 replicate (seeds). The quality of calli was assessed on visual observation as calli size, compactness and color. The callus induction frequency was found 90%, out of which 30% calli were of low quality (hard and dark brown in coloration), 40% were average in quality and 30% calli were of good quality eg., friable, large enough in size and bright yellowish in colour.

Further suspension cultures were developed from friable calli and 50 replicates of one month old suspension cultures were transferred to regeneration medium to asses the regeneration frequency of cells (Fig. 1). At the end of 2nd week top of the some proliferated calli were seen as green spots, with the passage of time numbers of green spots increased which later appeared in embryoid form. However, no proper plantlets were recorded during first culture of 28 days. After 28 days all calli were transferred to fresh medium, during this passage well developed plantlets in 12 cultures (24% regeneration) were observed. Rest of the differentiated and undifferentiated replicates were transferred to fresh medium for 3rd passage. During this passage 25 cultures (50% regeneration) produced plantlets. The cumulative regeneration percentage over three passages added up to 74% (Fig. 1). Rest of the calli became hard and brown, which lately turned dark. In many replicates only roots or shoot or deformed structures were observed, which were not considered as regenerants.

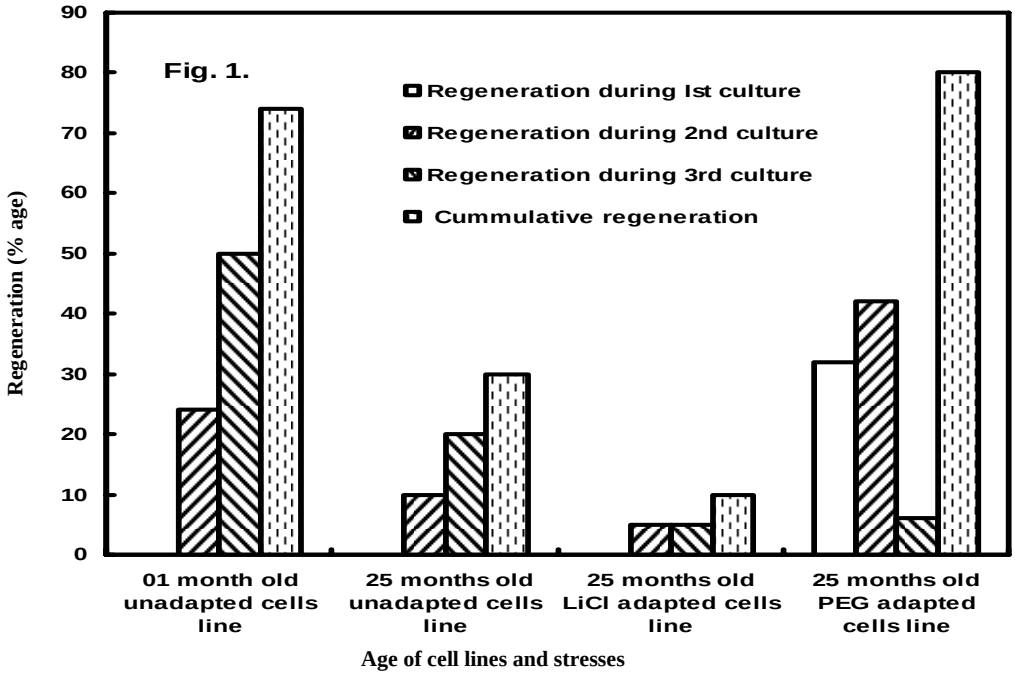

Fig. 1. The effects of age of cell suspensions and adaptation to osmotic and ionic stresses on regeneration frequencies of adapted and unadapted cell lines of rice cultivar Swat-1.

Following adaptation both the adapted and unadapted cell lines were compared for their relative growth rates and regeneration frequencies. The RGR values recorded were 0.99, 0.98, 0.97.5 for unadapted, LiCl and PEG adapted cell lines at their respective media i.e., control medium for unadapted, 20% PEG or 20 mM LiCl supplemented media for PEG and LiCl adapted lines, respectively. The similar RGRs values were indicative of the fact that 20% PEG and 20 mM LiCl was no more a stressful environment for PEG and LiCl adapted lines. Visual observation revealed that suspensions of unadapted and LiCl adapted lines turned into smaller cell aggregates with a large proportion of fine cell suspensions compared to suspensions of PEG adapted cells line.

When 25 months old adapted and unadapted cell lines were cultured on regeneration medium the regeneration frequency of unadapted and LiCl adapted cell lines decreased to 30% and 10%, respectively (Fig. 1). The decrease in regeneration potential of over time is consistent with the general trend that cultured cells loose their capacity for plant regeneration after few subcultures (Abe & Futuhara, 1986; Gobel *et al*., 1986; Reddy, 1986; Binh *et al*., 1992). Where as, more reduction in regeneration frequency of LiCl adapted line may be associated with toxic effects of Li ions. On the other hand regeneration percentage of PEG adapted line increased to 80%. The trend of regeneration were 32% during first culture on regeneration medium 42% and 6% during subsequent 2nd and 3rd cultures respectively (Fig. 1). Furthermore, it was observed that plantlets of PEG adapted line were more vigorous and healthier than plantlets from other cell lines (Fig. 2).

Fig. 2. Plantlets from 25 months old unadapted and polyethylene glycol adapted (a and b) cell lines of rice *Oryza sativa* L. cv. Swat 1 on regeneration medium and on water culture (c and d).

The development of fine cell suspensions or smaller cells aggregates after several subcultures in liquid medium and under LiCl stress with corresponding decrease in regeneration and maintenance of large cell aggregates in PEG adapted line with enhanced regeneration is consistent with the findings of Nagamori *et al*., (2001). They have reported that formation of cell clusters is the result of intercellular attachment due to pectin properties of cell wall and by strength of shear stress of medium. In our case it can be speculated that continuous culturing and ionic stress (LiCl) might have decreased the ratio of intercellular attachment and disturbed shear forces consequently fine suspensions developed with reduced regeneration capability. In contrast osmotic stress (PEG) might have increased intercellular attachment with balanced shear forces that have allowed the cells to remain in cluster form, a property associated with enhanced regeneration.

This pattern of regeneration response reveals that (i) prolonged culturing of cells reduce regeneration frequency of cells, (ii) adaptation to ionic stress aversely affects the regeneration efficiency of rice cells line, (iii) adaptation to osmotic stress (in this case 20% PEG) not only enhances regeneration capability of undifferentiated cells/tissues but also produce plants in a shorter period. Therefore, it is recommended that PEG might be added in culture medium for maintenance of totipotency of undifferentiated cells for a longer time and to shorten the regeneration time, a prominent improvement in tissue technology for crop improvement programs.

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