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Direct *in vitro* Regeneration of *Arachis Hypogaea* L.(Jl-24) and Study of Salt Tolerance

KEYWORDS: Peanut JL-24, embryo culture, salt tolerance, BAP, NAA

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ABSTRACT *An efficient protocol for complete in vitro plant regeneration of Arachis hypogea Linn (.JL-24) variety is devised in the present study. Immature embryos when cultured on MS1 regeneration media (MS + 2.5 mg/L BAP + 0.2mg/l NAA) has shown a higher frequency of regeneration. There are no such reports of direct regeneration using embryo culture. Efforts have been made to develop salt stress tolerant plants using this protocol by subjecting in vitro developed plant to different NaCl regimen (concentration) in MS2(MS1+ NaCl) medium. The regenerated plants survived and were found to be tolerant to a maximum concentration of 1.0mg/L NaCl*

INTRODUCTION

Groundnut or Peanut (Arachis hypogea Linn) has been an important cash crop in semi arid tropics, cultivated at large for edible oil seed and its protein value. In India, the production rate has been next to China (FAO, 1996). But, in recent years, huge losses in terms of quality and quantity (yield) has occurred due to biotic and abiotic stress. The loss estimated to be around US $520 million and is supposed to be due to low genetic diversity of peanut variant in Asian land races (Mrognisky et al., 1988) subjecting them to be susceptible to pest and disease (Sharma and Lavanya, 2002) resulting in decline in yield.

To meet the food demands of growing population, traditional approaches and classical breeding have several limitations (Sellar et al., 1991). Plant breeding for peanut essentially accounts for vegetative, reproductive and quantitative traits that are associated with yield and those with economic values (Vasil, 1994). These limitation can be overcome by tissue culture and genetic engineering techniques; supplementing the traditional method and thus enhance the yield (Durham And Parrot, 1992). Although the cultivated groundnut is recalcitrant in tissue culture (Cheng et al., 1992; Heady and Smith, 1996), plantlet regeneration has been possible through somatic embryogenesis, organogenesis and callus cultures (Chengalrayan et al., 1994).

Tissue culture response in groundnut is strongly influenced by plant genotypes (M C Kently et al., 1995). The success in obtaining regeneration in tissue culture of legumes has been mainly due to the shift in the emphasis from medium selection to genotype selection. Conventional breeding has lead to the improvement of few peanut traits like seed yield and drought tolerance (Moss and Stalker, 1987). However, due to limited applicability, many of important agronomic traits like tolerance to water stress, uniform fruit maturity and nutritional quality have yet to be improved. Plant growth regulators are known to enhance yield, oil and fatty acid content in peanut (Malik et al., 1993). Many efforts have been devoted to develop an efficient in vitro regeneration system, because of their calcitrance to tissue culture regeneration (Sellar et al., 1990, Baker and Wetzstein 1992). Some researchers have adopted embryo rescue technique to overcome the event of quiescence (Moss and stalker, 1987).In essence transformed embryos continue, repetitive embryo production under selection, resulting in a reduction in chimeric events and in a greater number of embryos available for conversion into plants (Parott et al., 1991)

Though more than a hundred varieties of peanut are released for cultivation in India, efforts are still underway to find out sources of tolerance to salinity for use in the genetic improvement of peanut. A few germplasm accessions of cultivated peanut have been identified as tolerant of salinity (Adkins et al, 1995: Alpa J. et al., 2008).

In the present investigation, an attempt is made to standardize protocol for complete in vitro regeneration of groundnut and evaluate the usefulness of embryo cultures towards salt tolerance in peanut genotypes in vitro.

MATERIALS AND METHODS

Plant material and explants preparation

A. hypogaea L. (JL-24) variety seeds were collected from farmers field in Visakhapatnam. Seeds were then stored in air tight container for a period of 4 weeks excluding moisture using dried silica gels. Immature heart shaped embryos were excised from surface sterilized seeds. The seeds were soaked overnight before being subjected to surface sterilization using 0.1% (w/v) mercuric chloride ($HgCl_2$) solution, for 5 minutes. Thorough rinsing in sterile double distilled water was repeated thrice for 2 minutes each time. Embryo are carefully removed by separating the cotyledons and inoculated them on MS medium MS1(MS + 0.2 mg/l BAP + 2mg/l NAA). MS medium (Murashige and Skoog, 1962) was reconstituted from stock solutions by supplementing B5 vitamins and different concentrations of BAP (6-benzylaminopurine) i.e 1.5, 2.0, 2.5, 3.0mg/l and NAA (0.1, 0.2, 0.3, 0.4 mg/L). The pH of the media was adjusted to 5.8 and autoclaved. All the cultures were maintained at 26 $\pm$ 2^0C under a16 hr photoperiod (30 $\mu mEm^{-2}{}_{s}{}^{-1}$) intensity. Each single culture tubes were inoculated with 2 explants each. A total number of twenty explants were tested in each batch. The experiment was repeated twice and observation was done on regular base for 4weeks and final data was gathered until 6 week.

In order to evaluate the salt tolerance potential of JL- 24 variety, in regeneration medium, the above said regeneration protocol was repeated with four different concentration of sodium chloride viz., 1.0, 1.5, 2.0, 2.5 mg/l. by incorporating into MS1 medium. This medium was designated as MS2. The explants were observed for the response after 1 week. Cultures are incubated at 26 $\pm$ 2^0C and 16 hrs photoperiod. The experiment was repeated twice and observed for visible response for 21 days.

Results and Discussion:

Effect of hormone combinations and in vitro regeneration

Groundnut (Arachis hypogea Linn..JL-24 variety used in this study was the material of choice. Since, this cultivar is sus-

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ceptible to both drought and salt and the best regeneration response was obtained with variety JL 24 (used as control variety) regardless of the sterilizing agent used (Maina et al., 2010). The response was rapid, with shoots arising in multiples in shooting media having only BAP within one week. When single shoots were transferred to rooting media with different concentration of NAA, root induction occurred within 7 days. The time required for regeneration was found to be fast with higher frequency of regeneration (Fig 1a,1b, 1c and 1d) and Table 1. This study reports regeneration of peanut using a protocol, which first of its reports in embryo culture technique. Such a technique is essential, as transformed embryos continue, repetitive embryo production under selection, resulting in a reduction in chimeric events and in a greater number of embryos available for conversion into plants (Parott et al., 1991). Previous reports have highlighted on regeneration via somatic embryogenesis or organogenesis (Chengalrayan et al., 1994). There is absence of a unifying mechanism for induction, development and expression of SE(Somatic Embryos) in the different species and genotypes in which studies have been conducted (Vıctor M. Jimenez, 2005). The present study describes the effect of plant growth regulators for inducing direct regeneration using embryo culture.

Induction of sodium chloride (Nacl) induced salinity tolerance: Different concentrations of sodium chloride(Nacl) 1.0, 1.5, 2.0, and 2.5 mg/l was incorporated into MS medium (MS2) to create salinity (high salt). The explants viz immature zygotic embryos are cultured in these 'saline' media to evaluate the difference in response to the level of 'salinity'. The rate of survival of the explants in these media was taken as a measure of their tolerance to 'salinity'. Four levels of sodium chloride viz., 1.0, 1.5, 2.0, 2.5 mg/l were used from the observation of salt tolerance.

The shoots with green leaves were considered as tolerant and those with brown leaves or without leaves as susceptible. The observed values were converted to percentage (%). The resulting percentage was used for interpreting the responses of the cultivars to the level of salinity stress. The rate of survival of the explants decreased drastically with the increased concentration of Nacl. This was evident from the browning and drying up of the explants (Fig 2 and table 2). In the lower concentrations of sodium chloride (1.0%) the embryos had good survival rate (85%). But, with the increase in the concentration to 1.5% the survival rate decreased with a maximum of 50%. However, when the concentration raised further to 2.5% the rate of survival has come down indicated a very high selection pressure which is above the threshold of the crop. In spite of the large amount of research conducted on peanut, since a decade , the mechanisms by which plant hormones involve in somatic embryogenesis is not clear(Vıctor M. Jimenez, 2005)

Table 1 : Effect of different concentrations of plant growth regulator BAP on regeneration from embryonated cotyledonary explants of local variety of Peanut

Conc.of hormone (mg/l)	No. of explants inoculated	No. of explants Showing response	Type of response	No. of shoots formed per explants (30 days)	No. of plantlet formed per 20 explant (60 days)	% Frequency of regeneration
BAP 1.5	20	10	Shoots along with rooting	1-2	8-10	50
BAP 2.0	20	14	Shoots along with rooting	2-3	10-14	70
BAP 2.5	20	18	Shoots along with rooting	5-6	15-20	90
BAP 3.0	20	12	Shoots along with rooting	2-4	11-15	60

Note: NAA at 0.2mg/l was incorporated separately in MS + BAP medium(MS2).

Tab1e 2 : Effect of different concentrations of sodium chloride (Nacl) on regeneration from embryonated cotyledonary explants of local variety of Peanut

Conc.of Nacl (mg/l)	No. of explants inoculated	No. of explants showing response	Type of response	No. of shoots formed per explants (30 days)	% Frequency of shoot formation
1.0	20	17	Multiple shoots with rooting	2-4	85
1.5	20	10	Multiple shoots with rooting	1-2	50
2.0	20	-	-	-	-
2.5	20	-	-	-	-

Fig 1: Direct In vitro regenerated plant of A. hypogea.L(JL-24) in MS1 at different stages.

Fig.1.1:3rd d- showing shoots.

Fig.1.2: Primary roots -10th d

Fig.1.3 Secondary roots & shoots -15th d

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Fig.1.4: complete plant-20 d (photographed later on 35th d)

Fig 2: Embryo explants in in vitro at different stages of development in MS2 (containing higher concentration of NaCl ie above 1mg/l)

Fig:2.1: Browning of plumule -5th d

Fig: 2.2: Unhealthy plant- 18th d.

CONCLUSION

The availability of an efficient regeneration system is an essential prerequisite for utilizing in genetic transformation. Usually embryos abort early in the reproductive cycle in classical breeding experiment; and hence in vitro techniques using embryo culture are necessary to recover many desirable traits in the genus (Stalker and Eureda, 1988).The use of embryo culture indicated that rescuing heart-shaped embryos of interspecific hybrids is possible. But, direct regeneration protocol adopted in this study, has not been reported with high degree of success. Such regeneration system can be made ideally suited for genetic transformation research when done on large scale. By imposing antibiotic selection on transformed tissues transgenic chimeras can be eliminated. In conclusion, the protocol developed here has proved to be an efficient approach for the micropropagation of groundnut through embryo culture technique, under salt stress conditions. By means of in vitro salt stress studies, it is indicative of peanut of local variety possess, salt stress tolerance property.

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