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Regeneration and Acclimatization of Salt-Tolerant *Arachis hypogaea* Plants Through Tissue Culture

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Abstract. Excised embryos of *Arachis hypogaea* were cultured on Murashige and Skoog's medium (MS medium) supplemented with different combinations of growth hormones. The highest frequency of callus proliferation (80%) was recorded on MS medium mixed with 1.0 mg/l of 2,4-D and 0.5 mg/l of BAP. These cultures were treated with 0.65 mg/l of trans-4-hydroxy-L-proline (HyP) and various concentrations (0.1-0.5%) of NaCl. In all cases the presence of salt reduced the fresh mass of callus. Shoot regeneration in the cultures took place when transferred to MS medium supplemented with 1.0 mg/l of kinetin (Kin) and 0.5 mg/l of 6-benzylaminopurine (BAP). Percentage of shoot regeneration decreased with the increase of NaCl (0.1- 0.5%) in the shoot regeneration medium. Root formation in these cultures took place when the cultures were nurtured on MS medium free of growth hormones. Regeneration, hardening and acclimatization of the salt-tolerant plants was conducted.

Keywords: tissue culture, *Arachis hypogaea*, salt-tolerant plant, *in vitro* regeneration, salt-tolerant peanuts

Introduction

The peanut plant (*Arachis hypogaea*) is an annual legume. It belongs to the family Leguminoseae and genus *Arachis*. The genus *Arachis* is native to a region encompassing Central Brazil and the neighbouring countries (Moretzsohn *et al.*, 2004). The peanut seeds contain approximately 50% oil on dry weight basis (Isleib *et al.*, 2004). It is a rich source of fat, proteins and vitamins B_1, B_2, nicotine and vitamin E (Chopra *et al.*, 1958).

Soil salinity has long been on the list of global catastrophes. Areas affected by soil salinity are rapidly increasing in the Middle East and North Africa. The total area affected by mineral toxicity is about 25% of the World's potentially arable land (Raghava and Nabors, 1985). In the South and Southeast Asia, an area of 50 million hectares is adversely affected by salinity (Gamborg *et al.*, 1988). Salinity has also created an alarming situation in Pakistan. Owing to salinization, Pakistan loses 40,000 hectares per year (Jones and Gorham, 1986).

Application of tissue and cell culture techniques to enhance the adaptation of plants to stresses is relatively recent. Excess salt, usually NaCl, is the most widespread chemical condition inhibiting plant growth in nature (Casey, 1972; Epstein, 1976). The advantages of tissue culture technique for selecting salt-resistant mutants have been discussed by Melchers (1972). This approach was applied successfully by Zenk (1974), who selected a cell line that was able to grow on medium containing 0.17 M NaCl from the haploid culture of *Nicotiana sylvestris*. Another successful selection was reported by Dix and Street (1975), who selected a number of cell lines of *Nicotiana sylvestris* and *Capsicum annuum* capable of growing in liquid medium containing upto 0.34 M NaCl. Plants were regenerated from resistant cell lines obtained after two to six passages in media containing 0.34 M NaCl (Dix, 1980). Croughan *et al*. (1978) isolated NaCl-resistant cells, which could grow on a medium containing 0.17 M NaCl, from cell culture of alfalfa (*Medicago sativa*). Plants have been regenerated from salt-tolerant cell lines using the procedure of Staverek *et al*. (1980). Fitch and Moore (1981) have reported the regeneration of salt tolerant plants of sugarcane on medium furnished with 0.20-0.34 M NaCl. Similar results were documented by Rains *et al*. (1980) with rice on cells selected in the presence of 0.26 M NaCl. Screening of salt-resistant varieties of rice was also conducted through International Rice Research Institute (IRRI) tissue culture breeding programme (Yamada and Loh, 1984). Callus has been induced from seeds placed on high salt medium, and plants have been regenerated from salt-resistant callus. Kochba *et al*. (1980) reported the isolation of 'shamouti' orange (*Citrus sinensis*) callus lines with increased resistance to NaCl.

Mukesh- Jain *et al*. (1993) successfully employed *in vitro* methods to isolate glyphosate-tolerant lines of peanuts from mutagenized and non-mutagenized callus cultures. Moreover, Jain *et al*. (2002) selected cell lines of *Arachis hypogaea* in the presence of high salt (NaCl), and a herbicide (glyphosate), which yielded 4.2-4.5x and 3.9-4.6x elevated glyoxylase-I activity, respectively.

In vitro production of salt-tolerant callus lines and multiple shoot regeneration on 0.1% NaCl-stressed callus of peanuts from mature excised embryos has been reported by Harun (1997). However, no salt-tolerant plant has been reported to be produced *in vitro*. The present research was undertaken to produce salt-tolerant plants of peanut through tissue culture.

Materials and Methods

Plant material, treatments and culture. In the present study, peanut seeds were used. The peanuts (*Arachis hypogaea*), variety VarBard-479 were supplied by the National Oil Seed Development Project, NARC, Islamabad, Pakistan. The peanuts were first peeled off and then the seeds alongwith their membranous coat were surface sterilized with 1% mercuric chloride solution for 3-4 min, followed by a dip in 75% alcohol containing 10% Tween 80 for 3 min. The seeds were rinsed three times with autoclaved, distilled water (Compton and Veillenx, 1991).

The embryos were excised from the seeds and used for tissue culture studies. The embryos were inoculated onto Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962). In a 100 ml Erlenmeyer flask, 50 ml of the medium was inoculated with two embryos. Ten flasks were used for each treatment. The MS medium was always used at ½ strength and supplemented with vitamins at full strength and 5% (w/v) sucrose (Ullah, 1986).

The growth hormones kinetin and 6-benzylaminopurine (Kin and BAP, respectively) were added to the medium. Moreover, prior to the treatment of the cultures with salt stresses, the callus was administered with 0.65 mg/l of trans-4-hydroxy-L-proline (HyP) alongwith MS medium containing 1.0 mg/l of 2, 4-D and 0.5 mg/l of BAP. The pH of the medium was adjusted to 5.8 with 1.0 N NaOH. The medium was solidified with 0.8% (w/v) Difco-bacto agar and autoclaved at 15 psi. The cultures were placed at 25±2 °C under illumination of fluorescent lamps of 2500 lux for 16 h per day cycle (Compton and Veillenx, 1991).

Controlled room acclimatization. Cotton plugs of the culture flasks were removed prior to the hardening of the plantlets. The salt-tolerant plantlets, alongwith the controls, were transferred to the pots containing a mixture of sandy-loam and decomposed leaf manure in equal quantities. Half strength Knop's solution was administered to the plantlets (Steward, 1963). The pots were covered with polyethylene covers. The regenerated plants were placed for hardening in controlled temperature culture room (25±2 °C) with 16 h photoperiod. These potted plants were allowed to stay in the controlled temperature room for four weeks. The plants were then placed in varying temperature according to the prevailing season, i.e., 30 °C day and 25 °C at night (on an average) for four weeks, watering with ½ strength Knop's solution was done, alternative with tap water. Polyethylene covers were continuously changed with larger ones consequent upon the growth of the plants. In laboratory, the plants were exposed to rays of morning sun daily for half to one hour, or they were placed near glass windows to receive diffused daylight. Polyethylene covers were removed gradually during the last two weeks (Akram *et al*., 1988).

Field acclimatization. Field atmospheric temperature during these studies was at the average maximum of 32 °C and 38 °C during May and June-August, while the minimum average temperature during these months was 25 °C and 28 °C, respectively. At this stage, the plants were transferred to the open environment in a field nursery, but protected from direct exposure to sun by keeping them under shade of larger trees for one week. However, they were placed in such a position as to allow them exposure to the afternoon sun. Canal water was administered to the plants. The plants were then transferred to the sandy-loam soil. Nitrophos manure was broadcast to the soil before the transplantation of plants to the open field and irrigated with canal water.

Results and Discussion

Callus induction. Excised embryos nurtured on MS medium, without hormones, died after a culture period of four weeks. The addition of various combinations of growth hormones proved to be effective in inducing growth in the embryos. Auxin and cytokinin, used alone, had no effect on callus induction. However, the highest frequency of callus (80%) was recorded on MS medium containing 2,4-D (1.0 mg/l) and BAP (0.5 mg/l), as may be noted from Table 1. White, nodulated type callus developed on this medium in eight weeks of culturing. Subculturing of the callus, four weeks after being placed in culture medium supplemented with 2,4-D (1.0 mg/l) and BAP (0.5 mg/l) led to the production of greenish-yellow nodular callus (Fig. 1).

trans*-4-Hydroxy-L-proline (HyP) *treatment. *trans*-4-Hydroxy-L-proline (HyP) is an amino acid which helps the cultures to physiologically tolerate stresses, such as drought, salinity, freezing, etc. Before treating the cultures with salt stresses, the callus was administered with 0.65 mg/l of HyP, alongwith the MS containing 1.0 mg/l of 2,4-D and 0.5 mg/l of BAP. Callus browning, with poor proliferation during the first four weeks of culturing, was noticed (Fig. 2). However, the callus regained its vigour. Reasonably good amount of greenish-yellow, compact and nodular callus was obtained during the next four weeks of culturing (Fig. 3).

Effect of salinity on callus. The fresh and dry weights of callus, in response to the salinity treatments, are given in Table 2. NaCl added to MS medium supplemented with 0.65 mg/l of HyP + 2,4-D (1.0 mg/l) + BAP (0.5 mg/l) enhanced the growth of embryo derived from callus. In all cases, the presence of salt in the medium reduced callus growth as compared to the growth in the control medium. Fresh weight of callus was reduced to 22%, when grown on medium containing 0.4% NaCl and at the same salt level, the dry mass dropped to almost 25% of the control (Fig. 4).

Shoot regeneration. The calli treated with 5 mg/l of HyP were excised and inoculated on the shoot regeneration medium, MS medium supplemented with various concentrations of BAP and Kin (0.5, 1.0, 1.5, 2.0 mg/l). About 90% of shoot regeneration took place on MS medium in eight weeks of culturing, when the medium was supplemented with 1.0 mg/l of Kin and 0.5 mg/l of BAP (Table 3). With the objective of salt stress induction, the calli were cultured on the same medium alongwith various concentrations of NaCl (0.1, 0.2, 0.3, 0.4 and 0.5%), and the observations so obtained are presented in Table 4.

Slight browning of callus tissue was noted within the first week of subculture on medium containing 0.1% NaCl. However, the callus soon revived its growth and a copious amount of creamy-white callus, together with multiple shoots developed after four weeks of culture period (Table 4). Shoot regeneration rate was 90% (Fig. 5). Creamy, luxuriant callus growth was also obtained on the medium containing 0.2% NaCl within four weeks, and about 80% of shoot regeneration took place. With an increase in the salinity level to 0.3%, callus growth was further slowed down. The callus was brownish-green in colour. Moreover, small green spots (buds) were observed after the third week of subculture, which ultimately grew into shoots. Approximately 30% of the total cultures regenerated shoots.The callus treated with 0.4% NaCl remained green. Moreover, a few green buds regenerated after fourth week of subculturing, which developed into shoots. About 20% of the total cultures developed into shoots. Furthermore, a complete negative response was observed in cultures, subcultured to a medium supplemented with 0.5% NaCl. Callus growth was very slow with no signs of regeneration. The tissue remained green for a culture period of one week after which it started turning brown, which ended in the death of the culture.

Root induction. Shoots regenerated in cultures treated with 0.1-0.4% NaCl alongwith control were placed in MS medium without the addition of any hormone at 25±2 °C in culture room with 16 h light period. A high rate of rooting was observed within the short period of two to three weeks.

The salt-tolerant regenerated plants, after hardening in the controlled laboratory conditions, were transferred to field conditions, where they were observed to survive well (Fig. 6).

When tissue is subjected to salt stress, a difference in osmotic potential (OP) develops between the tissue and the surrounding medium. This OP is greater within the tissue than its

Table 1. Callogenic responses of seed embryo explants, cultured during eight weeks on MS medium supplemented with various types and levels of growth regulators

Growth regulator composition		Callus frequency (%)*
2,4 – D (mg/l)	BAP (mg/)	
0.5	0.5	50
0.5	1.0	30
0.5	2.0	20
1.0	0.5	80
1.0	1.0	40
1.0	2.0	25
2.0	.5	40
2.0	1.0	35
2.0	2.0	30

*values are frequency (number of responding explants/total number of explants x 100); each value is the average of 10 replicates; BAP = 6-benzylaminopurine

Table 2. Fresh and dry weights (g) of callus in response to salinity treatments

Salt concentration (NaCl, %)	Fresh weight (g)	Dry weight (g)
0.0	2.520	0.085
0.1	1.432	0.065
0.2	0.953	0.050
0.3	0.741	0.042
0.4	0.563	0.034

Table 3. Shoot regeneration in calli treated with 0.65 mg/l of trans-4-hydroxy-L-proline and later inoculated on various concentrations of 6-benzylaminopurine (BAP) and kinetin (Kin)

Growth regulators		Culture time (weeks)	Shoot regeneration* (%)
BAP (mg/l)	Kin (mg/l)		
0.5	0.5	8	30
0.5	1.0	8	90
0.5	1.5	8	80
0.5	2.0	8	30

*number of responding callus cultures/total number of callus cultures x 100

Fig. 1. Rapidly growing greenish-yellow nodular callus on MS medium supplemented with 0.5 mg/l of 6-benzylaminopurine and 1.0 mg/l of 2, 4-D.

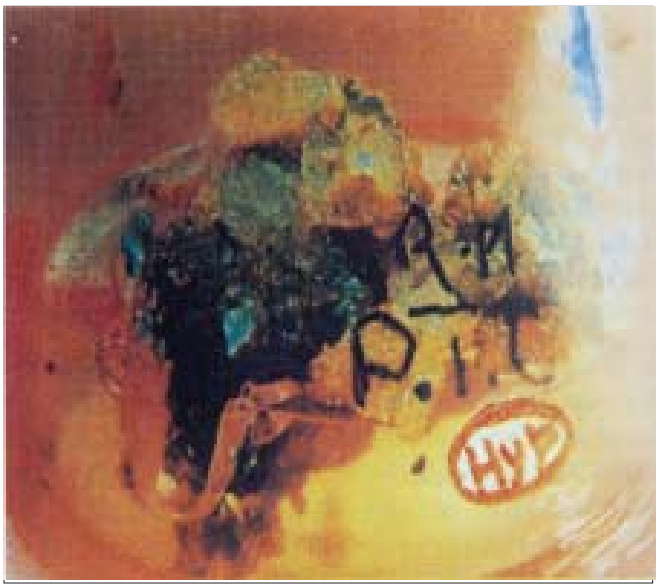

Fig. 3. Greenish-yellow, compact and nodular callus on maintenance medium supplemented with 0.65 mg/l of trans-4-hydroxy-L-proline during the next four weeks of culturing.

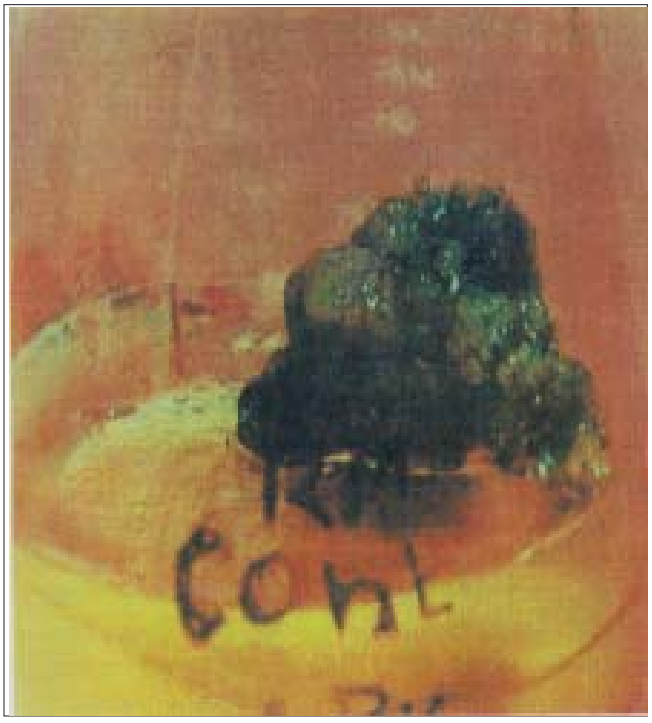

Fig. 2. Callus browning with poor proliferation during the first four weeks of culturing on MS medium supplied with 0.65 mg/l of trans-4-hydroxy-L-proline alongwith 1.0 mg/l of 2,4-D and 0.5 mg/l of 6-benzylaminopurine.

Fig. 4. Fresh mass of callus reduced (right) when grown on medium containing 0.4% NaCl, as compared to mass in the control (left).

surrounding medium. Therefore, more water is lost from tissue than is absorbed. As a result, adverse effect of dehydration starts to appear. As HyP is one of the osmotic regulating agents that helps the tissue to endure low water status and maintain turgor, it protects protein membrane, or other cellular components, from adverse effects of dehydration (Hanson and Ghitz, 1982; Hsia, 1973). HyP (0.65 mg/l) was administered to cultures prior to salt treatment in order to avoid the adverse effect of dehydration in protein membrane and other cellular components of the cultures. Gulati and Jaiwal (1993) have also reported that the HyP-resistant callus line of *Vigna radiata* showed greater tolerance to NaCl than the wild type. Gadallah (1999) reported that on the administration of HyP to salt-stressed bean plants, reduced membrane injuries and improved growth were noted. A group of researchers administered glutathione, instead of HyP, for the interaction of cellular processes with growth and development under salt (NaCl)-stressed conditions (Jain *et al.*, 2002).

In the present study, the callus was induced in excised embryo explants nurtured on MS medium along with various combinations of BAP and 2,4-D. Embryo as an explant has been also used for the initiation of culture in *Anethum graveolens*, *Ilex aquifolium*, *Corylus avellana* (Hu *et al.*, 1978; Stewart *et al*., 1970). Pronounced growth in callus took place on the medium containing 2,4-D (1.0 mg/l) and BAP (0.5 mg/l). Our results confirm the findings of Ilahi *et al*. (1988) on the induction of callus in corms of *Crocus sativus*, which are also in agreement with the results of Jabeen (1988) and Aziz (1994) in respect of the objective of inducing callus in ginger and carnation, respectively. However, Catapaw *et al*. (2001) induced high frequency of embryogenic callus on medium supplemented with 5.0 mg/l of 2, 4-D.

In another set of experiments, 0.5 mg/l of BAP and 1.0 mg/l of Kin was used for inducing shoot regeneration. Similar effects of BAP and Kin on the regeneration of shoots from callus of *Arachis hypogaea* have been reported by Ullah (1986). Chengalrayan *et al.* (1995) also reported that BAP and Kin were the best combination for achieving shoots from callogenic buds of peanuts. Our results, as well as the investigations reported in literature confirm that BAP and Kin are excellent for shoot regeneration.

The effect of different concentrations of NaCl added to MS medium containing 2,4-D and BAP on the growth of embryo derived calli, expressed as fresh and dry weight bases have been shown in Table 2. In all cases, the presence of salt in the culture media reduced callus growth as compared to the growth on control medium. These results confirm the findings of Youssef *et al*. (1998) on the effect of salt stress on calli of *Nigella sativa.* The reduction of callus growth in response to salinity has been explained by several workers. The toxic effects induced by specific ions caused damage and reduction in the growth of tobacco cells. The growth of *Datura innoxia* cells was inhibited severely by NaCl, especially at the concentration above 166.6 mM. It has been further shown that callus growth of a maize genotype was reduced to 68.6% of the control, on fresh weight basis, when grown on media containing 166.6 mM NaCl (Lusardi *et al.*, 1991).

Tissue culture techniques for screening and plant regeneration, with increased stress tolerance, are being applied in many species. Croughan *et al*. (1978) were successful in regenerating salt-resistant plants of alfalfa upto 1% NaCl level. Dix and Street (1975) selected salt-tolerant cell lines of *Nicotiana sylvestris* and *Capsicum annuum*, which were capable of growing in 0.34 M NaCl.

Increasing salt concentrations decreases hydration in the cultures. The decrease is smaller in tolerant varieties. The tolerance of *in vitro* grown cultures is due to their capacity to

Table 4. The effect of different salt concentrations on shoot regeneration from 0.65 mg/l of trans-4-hydroxy-L-proline (HyP) treated calli

Salinity level (%)	Growth regulators		Culture time	Shoot regeneration rate*	Callogenesis**	Texture/ appearance
	BAP (mg/l)	Kin (mg/l)	(weeks)	(%)		
Control	0.5	1	4	90	++++	greenish-yellow, nodular
0.1	0.5	1	4	90	++++	creamy-white
0.2	0.5	1	4	80	+++	creamy-white
0.3	0.5	1	4	30	++	brownish-green
0.4	0.5	1	4	20	+	brownish-green
0.5	0.5	1	4	-	+	dark-brown

*number of responding callus cultures/total number of callus cultures x 100; ** + = slight; ++ = good; +++ = very good; ++++ = excellent; BAP = 6-benzylaminopurine; Kin = kinetin

Fig. 5. Regeneration of plantlets/culture on MS medium treated with 1.0 mg/l of kinetin, 0.5 mg/l of 6-aminobenzylpurine and 0.1% of NaCl.

Fig. 6. Salt tolerant plants transferred to pot and placed under field conditions: 0.3% salt-tolerant regenerated plant.

accumulate salt (Transcosa *et al.*, 1999). NaCl stress decreases the rate of growth of cultures, i.e., root, shoot, etc., as compared with the control (Panneerselvam *et al*., 1998). Our results are, therefore, in agreement with the observations of these authors.

Conclusion

In the present studies, 0.1-0.4% salt tolerant plants were regenerated. These plants were then transferred to field conditions alongwith control where they were observed to complete their life cycle.

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