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Short Communication

EFFECTS OF PRECONDITIONING, PLANT GROWTH REGULATORS AND KCL ON SHOOT REGENERATION OF PEANUT (*ARACHIS HYPOGEA*)

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ABSTRACT

Peanut (*Arachis hypogeae*) is an important legume used as green manure and cover crop also. In this study, preconditioned plumular apex explants with 20 mg L^{-1} BAP or unconditioned explants were cultured for 8 weeks on MS medium having 0.25-2.0 mg/l BAP with or without 0.25 mg L^{-1} NAA. 100 % shoot regeneration was recorded from both conditioned and unconditioned explants. However, conditioning of explants resulted in 1.5-2.0 fold more number of shoots per explants with relatively shorter shoots compared to unconditioned explants. Shoots per explant from unconditioned explants ranged 1.33-3.93 with 1.86-5.03 cm shoot length. Whereas, shoots per explant and shoot length of preconditioned explants ranged 2.73-5.87 and 0.77-1.75 cm respectively. In second experiment, 10, 15 and 20 µS/cm of KCl in combination with BA-NAA concentrations also resulted in 100 % shoot regeneration. Higher concentration of KCl imposed necrosis on the explants and generated shoots with low leaf area. Increased KCl concentration inhibited the shoots per explant but also enhanced the shoot length. In vitro regenerated shoots from both experiments were rooted successfully using IBA and acclimatized in the pots. Results revealed the superiority of preconditioning on shoot regeneration and KCl for shoot elongation.

Key words: Preconditioning, *in vitro*, Necrosis, Peanut, Plumular apex.

INTRODUCTION

Peanut (*Arachis hypogeae*) is an annual edible oil seed (Hassan *et al*., 2013) legume crops cultivated all over the world (Venkatachalam and Jayabalan, 1997) due to high oil and fatty acid contents. It contains 46.8% oleic acid as olein, 33.4% Linoleic acid as linolein and 10.0% palmitic acid as palmitin. Besides that, it also contains lignoceric acid, arachidonic acid, arachidic acid, behenic acid, tearic acid, and other fatty acids (Venkatachalam and Kavipriya, 2012). Peanut is also suitable for green manure, cover crop (Balkcom *et al*. 2007), hay (Hill, 2002) and an intercrop (Langat *et al*. 2006). It also provides raw material of household commodities to industrial byproducts. Peanut is used in different forms by humans due to the presence of niacin (Morris *et al*. 2004) and polyphenols (p-coumaric acid), resveratrol (Sanders *et al*. 2000) and antioxidant activities (Blomhoff *et al*. 2008).

High consumption of peanuts and commercial value of crop make this plant important for improving the varieties that can tolerate biotic and abiotic stresses. Furthermore, increasing nutritional value of seeds and adaptation to different environmental conditions (Venkatachalam and Kavipriya, 2012) are other prerequisites for conventional breeding program or for modern biotechnological techniques for improving peanut. Plant tissue culture protocols can be employed for genetic transformation studies. However, peanut is highly recalcitrant plant (Heatley and Smith, 1996) like other legumes and there is need to develop new protocols or to modify the present regeneration systems. There is need to use new explants or modifying the regeneration protocols.

KCl is an important ingridient of basal mediums used for in vitro micropropagation or shoot regeneration. High concentrations of KCl are usually used for screening of species/cultivars for salt stress. However, higher KCl concentration in the culture media lead to salt stress and may affect in vitro plant regeneration behavior like callus induction (Santos *et al*. 2001; Boyko *et al*. 2010a), necrosis (Santos *et al*. (2001), shoot regeneration frequency (Zahid *et al*. 2014), shoots per explant (Sotiropoulos *et al*., 2006**)** and shoot length (Ahire *et al*. 2013). Besides that, KCl also effects genetic transformation in plants. Boyko *et al*. (2009) reported decreased homologous recombination frequency due to absence of potassium ion in the MS medium. Whereas, positive effects of chloride ions on homologous recombination rates (RRs) has been reported by Boyko *et al*. (2010a) in Arabidopsis.

In this study, we checked the efficacy of initial preconditiong with BA on shoot regeneration potential of plumular apex followed by possible effects of different

concentrations of KCl in the presence of BA or BA+NAA.

MATERIALS AND METHODS

Seeds of cultivated peanut cv. Osmaniye were collected from the Department of Field Crops, Cukurova University, Adana, Turkey. The seeds were subjected to surface sterilization using 100 % commercial bleach (5 % NaOCl) for 10 min followed by 3 x 5 min rinsing with distilled sterilized water. After sterilization, embryos were isolated carefully under sterile conditions without any mechanical damaging. Thereafter, mature embryoes were preconditioned on MS medium (Murashige and Skoog, 1962) containing 20 mg/l BA for 10 days. At the same time, embryos were also inoculated on BA free medium for 10 days.

After 10 days of initial preconditioning, plumular apex (both preconitioned and unconditioned) were excised from embryos and subsequently incubated on MS medium provided with 0.25, 0.50, 1.0 and 2.0 mg L^{-1} BAP with or without 0.25 mg L^{-1} NAA for 8 weeks. In another experiment, the same culture media were used along with KCl to bring the EC of the medium to 10, 15 and 20 µS/cm with the help of WTW 3.15 conductivity meter. Thereafter, preconditioned plumular explants were cultured on these mediums for 8 weeks to check the response of explant to regeneration. From both experiments, data regarding callus and shoot regeneration frequency, shoots per explant and shoot length were taken and statistical analysis was performed.

Regenerated shoots of ≈1-1.25 cm long from both experiments were separated from explants and cultured for adventitious rooting using 0.25, 0.50, 1.0 and 2.0 mg L^{-1} IBA in the culture media. Ten shoots were cultured in each treatment and replicated thrice. Similarly, 10 shoots from each KCl medium were also cultured for rooting. After 4 weeks, data regarding rooting response were taken into account for statistical analysis. *In vitro* rooted plants from both regeneration and KCl were removed from medium, washed with water and awaited in the water for 10-15 min before transfer to pots containing peat moss. Thereafter, the pots were tightly covered with polythene bags in a way to create vaccume inside and kept in the growth room for 7-10 days to maintaining 80-90 % humidity. However, humidity was gradually decreased by making holes followed by complete opening of bags after 15 days.

All culture media used in the study were MS medium solidified with 0.65 % agar and provided with 3.0 % sucrose. Culture media were also enriched with 1.0 % PVP (Polyvinylpyrrolidone) to control necrosis. All chemicals (agar, MS, sucrose, PVP, KCl,) used in this study were purchased from Duchefa Biochemie B.V. (Haarlem, the Netherlands) and Sigma-Aldrich Co. (St. Louis, MO, USA). The pH was adjusted between 5.6-5.8 prior to autoclave. Media were autoclaved at 104 kPa atmospheric pressure and 120 °C for 20 min. The cultures were incubated at 24±2°C with 16 h light (35µmpl photons m^{-2} s^{-1}) photoperiod provided by Philips® cool white fluorescent tubes. All experiments were replicated thrice and experiments were repeated twice.

Data regarding regeneration, salt stress and rooting were subjected to One Way analysis of variance (ANOVA: SPSS17 for Windows, SPSS Inc., Chicago, IL,USA). Means were compared using Duncan's multiple range test (DMRT) at 0.05 level of significance. All treatments were arranged in a completely randomized design. Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cocharan, 1967) prior to statistical analysis.

RESULTS

In this study, plumular apex explants isolated from unconditioned mature embryos or preconditioned with 20 mg/l BA for 10 days were cultured on MS medium supplemented with 0.25-2.0 mg/l BA singly or supplemented with 0.25 mg/l NAA for 8 weeks. Shoot initiation started within one week (Figure 1a) followed by multiple shoot induction (Figure 1b) within 2 weeks. Whereas, multiple shoot regeneration with callus induction started from basal end of explant after four weeks of culture. After 8 weeks of culture, data from unconditioned (Figure 1c) and preconditioned explants (Figure 1d) were taken. Results showed 100 % callus induction and shoot regeneration irrespective of initial conditioning. However, shoots per explant and shoot length showed statistical difference of preconditioning compared to unconditioned plumular apex.

Initial preconditioning with 20 mg/l BA resulted in 1.5-2 fold more number of shoots per explant on all culture mediums compared to unconditioned explants. Unconditioned explants generated 1.33-3.93 shoots per explant compared to preconditioned explants, which scored 2.73-5.87. Contrarily, negative impact of preconditioning was evident on shoot length that ranged 0.77-1.75 cm compared to unconditioned explants with range of 1.86-5.03 cm (Table 1). Results further revealed the importance of growth variants especially the presence or absence of NAA in the culture medium. Higher concentration of BA (2.0 mg/l) with or without NAA was optimized for higher shoots from unconditioned explants. On the other hand, BA+NAA in the culture medium enhanced the shoot induction of preconditioned explants compared to mediums with only BA. Similarly, results on mean shoot length also showed clear impact of preconditioning and NAA in the culture medium. Presence of NAA in the culture mediums did not affect the shoot length of unconditioned explants and almost statistically similar lengths were obtained. Shoot length of unconditioned explants ranged 1.86-5.03 with longer

shoots obtained from 0.50 mg/l BA+0.25 mg/l NAA (Table 1). Contrarily, preconditioning significantly hindered the shoot elongation and relatively shorter shoots were regenerated in the presence of NAA with BA compared to BA used singly. Shoot length ranged 0.77-1.76 cm for preconditioned explants (Table 1).

In another experiment, plumular apex explants preconditioned with 20 mg/l BA were cultured on same culture mediums given in Table 1 with addition of 10, 15 and 20 µS/cm of KCl in order to check the effects of BA-NAA+KCl concentrations on shoot regeneration. Results on callus induction and shoot regeneration showed no negative/positive effect of KCl concentration in the culture medium and recorded 100%. However, KCl concentration exerted statistically significant but variable effects on shoots per explants and shoot length with necrosis and relatively smaller leaves on all KCl containing mediums. There was clear relationship between KCl and BA or BA+NAA on shoots per explant as maximum shoots per explant were achieved on medium provided with 2.0 mg/l BA+15 µS/cm KCl. Shoots per explants ranged 2.60-5.40, 2.60-6.00 and 2.47-5.40 respectively for 10, 15 and 20 µS/cm KCl (Table 2). In general, shoots per explants decreased with increased KCl concentration with 0.25 and 0.50 mg/l BA with or without NAA. Whereas, static results were achieved on different KCl concentrations with 1.0 and 2.0 mg/l BAP with 0.25 mg/l NAA and all KCl concentrations.

Results on shoot length clearly showed the positive impact of KCl concentration. Relatively longer shoots above 1.0 cm long were achieved on the medium supplemented with 20 µS/cm KCl on all BA-NAA concentrations except of medium containing 2.0 mg/l BAP+0.25 mg/l NAA. Shoot length at 20 µS/cm KCl ranged 0.81-1.54 cm. Whereas, shoot length of 15 and 10 µS/cm KCl respectively ranged 0.63-1.37 cm and 0.80-1.46 cm (Table 3). In general, inclusion of KCl exerted positive effects on mean shoot length irrespective of BA-NAA concentration.

Results on rooting revealed the insignificant effects of IBA on rooting frequency and recorded 100% from regeneration experiment and experiment with different concentrations of KCl in the culture medium. Regenerated shoots from both experiments were also successfully acclimatised in the pots.

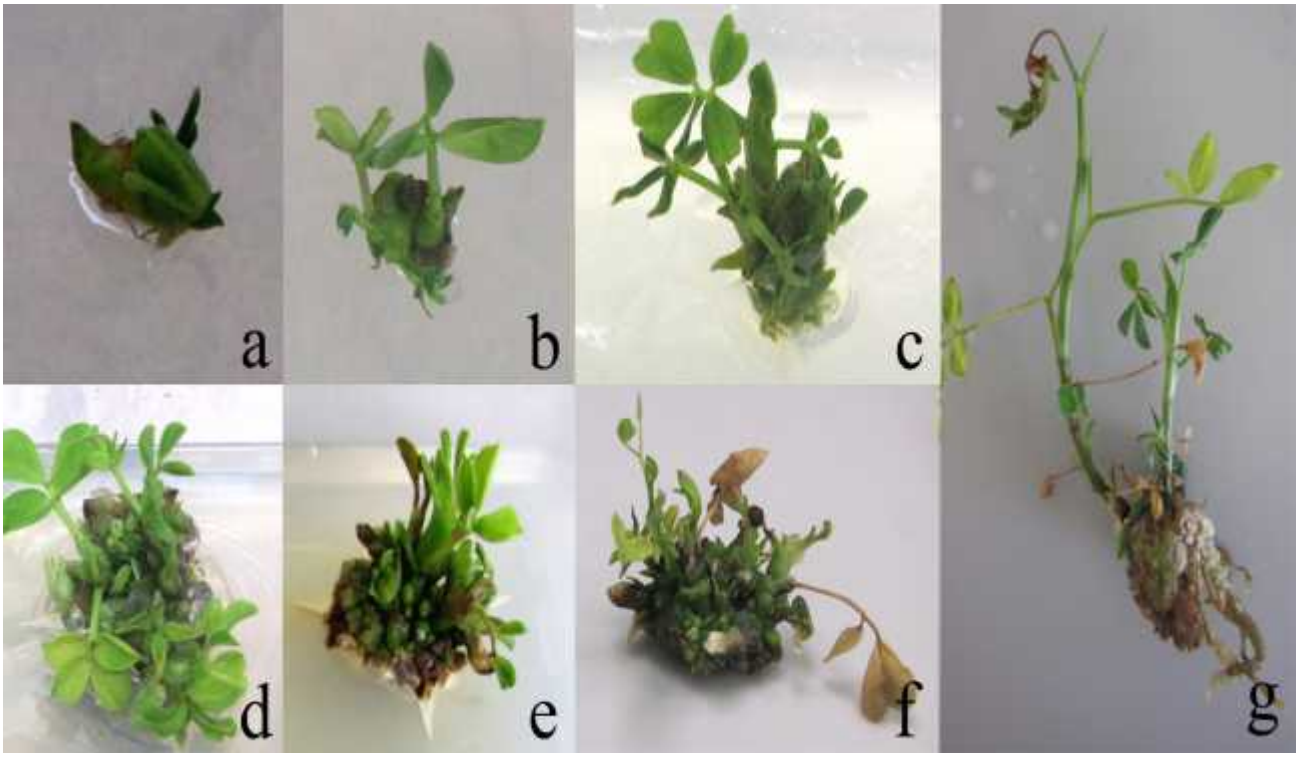

Figure 1. In vitro shoot regeneration from preconditioned plumular apex explants of peanut (a) shoot initiation from plumular apex explant within 1 week, (b) multiple shoot induction after 2 weeks (c) shoot induction from unconditioned and (d) preconditioned plumular apex explant (e) necrosis on explants with (f) low leaf area (g) rooted plantlets

Table 1. Effects of Preconditioning with BA on shoot regeneration of plumular apex explant of peanut (*Arachis hypogeae*).

BA (mg/l)	NAA (mg/l)	ShootS per Explant		Shoot Length (cm)	
		Unconditioned	Preconditioned with 20 mg/l	Unconditioned	Preconditioned with 20 mg/l
0.25	-	1.33f	4.33cd	3.97b	1.75f
0.50	-	1.93f	4.73bc	4.04b	1.76f
1.00	--	2.93e	4.73bc	2.49cd	0.94g
2.00	-	3.00e	2.73e	1.86ef	1.18g
0.25	0.25	1.93f	5.40ab	4.11b	0.93g
0.50	0.25	1.40f	4.87bc	5.03a	0.96g
1.00	0.25	1.93f	5.20ab	2.83c	0.87g
2.00	0.25	3.93d	5.87a	2.23de	0.77g

Values within a column followed by different letters are significantly different at 0.01 level of significance using DMRT

Table 2. Effects of KCL dosage on shoots per explant of preconditioned plumular apex explant of peanut (*Arachis hypogeae*).

BA (mg/l)	NAA (mg/l)	Control	10 µS/cm	15 µS/cm	20 µS/cm
0.25	-	4.33c	3.20cd	3.27bc	3.07cd
0.50	-	4.73bc	4.00bc	2.73c	2.47d
1.00	-	4.73bc	5.33a	4.60abc	2.73cd
2.00	-	2.73d	2.60d	6.53a	3.87bc
0.25	0.25	5.40ab	2.87d	2.60c	2.67cd
0.50	0.25	4.87bc	5.40a	2.93bc	2.47d
1.00	0.25	5.20abc	4.53ab	5.27ab	4.80ab
2.00	0.25	5.87a	5.33a	6.00a	5.40a

Values within a column followed by different letters are significantly different at 0.01 level of significance using DMRT

Table 3. Effects of KCL dosage on shoot length of preconditioned plumular apex explant of peanut (*Arachis hypogeae*).

BA (mg/l)	NAA (mg/l)	Control	10 µS/cm	15 µS/cm	20 µS/cm
0.25	-	1.76a	1.42abc	1.02abcd	1.49a
0.50	-	1.75a	1.51a	1.19ab	1.50a
1.00	--	0.94b	1.26abcd	0.83bcd	1.42ab
2.00	-	1.18b	1.46ab	0.63d	1.17bc
0.25	0.25	0.93b	1.15abcd	1.13abc	1.16bc
0.50	0.25	0.96c	0.80d	1.37a	1.54a
1.00	0.25	0.87b	0.90bcd	1.14abc	1.03cd
2.00	0.25	0.77b	0.83cd	0.72cd	0.81d

Values within a column followed by different letters are significantly different at 0.01 level of significance using DMRT

DISCUSSION

Plumule or plumular apex are very potent explants for in vitro shoot regeneration and are successfully demonstrated for *in vitro* shoot regeneration of other legumes like pea (Molnar *et al*. 1999), pigeon pea (Surekha *et al.* 2005), cowpea (Aasim *et al*. 2009), lentil (Aasim. 2012); chickpea (Aasim *et al.* 2013) and grasspea (Barpete *et al*. 2014). However, use of plumular apex explants is limited in the literature due to difficulties in isolating explant properly from embryo. Therefore, best procedure adapted was to either culture of embryos on MS medium free of plant growth regulators or preconditioned with higher concentrations of cytokinin for few days as suggested by Aasim *et al*. (2013). 100 % callus induction and shoot regeneration were scored from plumular apex explants in line with Aasim *et al*. (2013) and Barpete *et al*. (2014). However, variable callus and shoot induction from pulse treated longitudinally sliced cotyledonary node of dwarf chickling were reported by Saglam (2012). In contrast, low shoot regeneration frequency from different explants has been reported in

peanut (Sharma and Anjaiah, 2000; Shan *et al*. 2009; Burns *et al*. 2012; Venkatachalam and Kavipriya, 2012).

Results further illustrated the positive effects of preconditiong which yielded 1.5-2 fold more shoots per explants compared to unconditioned explants. This might be due to actively division of cells at initial stage (Von Arnold and Tillberg 1987; Brar *et al*. 1999; Madhulatha *et al*. 2004; Aasim *et al*. 2009; Saglam 2012; Barpete *et al*. 2014) which also induced callus on the explants (Aasim *et al*. 2009, 2013). Results further emphasized on the importance of growth variants after preconditioning on shoot regeneration behaviour. Unconditioned explants showed higher requirement of BA or BA+NAA for achieving maximum shoots per explant. However, shoots per explants of preconditioned explants were greater on all culture mediums compared to unconditioned explants in line with the findings of Aasim *et al*. (2011, 2013). Contrarily, initial preconditioning inhibited the shoot regeneration in line with Aasim *et al*. (2011, 2013) in chickpea, Aasim (2012) in lentil and in grass pea by Barpete *et al*. (2014). Similarly, presence of NAA in the culture emdium also exerted variable efefcts on shoots per explant and shoot length. Although, shoots per explant incraesed due to the availibility of NAA in the culture medium but with relatively shorted shoots. Similar effects of preconditioning with BA and NAA or auxin in the postconditioned medium on shoots per explant and shoot length has been reported in lentil (Aasim 2012) and chickpea (Aasim *et al*. 2013).

In second experiment, we checked the regeneration potential and behaviour of plumular apex explant by culturing on each BA-NAA concentration with three different KCl levels (10, 15 and 20 µS/cm) and compared it with control experiment. Results showed no positive or negative bearings of KCl on callus induction. Contrarily, Boyko *et al*. (2010b) stated positive effects of 50 mM KCl on calli of Arabidopsis. Whereas, Santos *et al*. (2001) reported decreased callus induction with increased KCl concentration of sunflower. Likewise callus induction, KCl had no negative effect on shoot regeneration frequency compared to control. Zahid *et al*. (2014) reported negative effects of KCl concentration on shoot regeneration frequency of rice cultivars. Besides that, necrosis on the callus (Figure 1e) and leaves with relatively low leaf area (Figure 1f) were clearly observed due to KCl proved the findings of Santos *et al*. (2001), who also observed necrosis at higher KCl concentration in sunflower with low leaf area.

Although, callus induction and shoot regeneration were not affected by KCl with each BA-NAA concentration, shoots per explant and shoot length showed positive bearings of KCl. Increased KCl concentration significantly enhanced the shoots per explant and maximum shoots were scored at medium with 2.0 mg/l BA+15 µS/cm KCl. Contrarily, Ahire *et al*. (2013) stated the negative effects of KCl on shoots per explant. Whereas, Sotiropoulos *et al*. (2006**)** reported insignificant effects of 0-40 mM KCl on shoots per explants of GF677 and Nemared prunus rootstocks. Our results also revealed the positive effefcts of KCl on shoot length of preconditioned explants which resulted in longer shoots of more than 1 cm. However, these results are contradictory to the findings of Ahire *et al*. (2013) who gained shorter shoots in response to KCl.

In vitro rooting and acclimatization is the prerequisite for successful plant tissue culture protocol. Peanuts are somewhat recalcitrant to rooting when exposed to different auxins in the culture medium (Hassan *et al*. 2013). However, we achieved 100% rooting from both experiments (Figure 1g). Aasim *et a*l. (2009, 2010) and Barpete *et al*. (2014) also reported 100% rooting from preconditioned explants in cowpea and grasspea respectively. Whereas, low rooting in chickpea (Aasim *et al*. 2011) and lentil (Aasim 2012) has also been reported. After successful rooting, rooted plantlets were acclimatized under growthroom conditions also.

This study presents the efficacy and supermacy of initial preconditiong with BA and compared it with unconditioned plumular apex explant followed by postconditioning on different BA-NAA concentartions. Besides that, shoot regenration behaviour of preconditioned plumular apex explant was also tested on different concentrations of KCl with BA-NAA which resulted in greater shoot length without any negative effects on shoot regenertaion behaviour.

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