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In Vitro Shoot Regeneration of NAA-Pulse Treated Plumular Leaf Explants of Cowpea

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Abstract

Cowpea (*Vigna unguiculata L.*) is an economically important grain legume crop and is an important source of dietary protein in many of the developing countries. The present study reports the effect of pulse treatment duration, concentration of NAA and presence of NAA in the culture medium on shoot regeneration from plumular leaf explant of Turkish cowpea cv. 'Akkiz' and 'Karagoz'. Pulse treatment of mature embryos with 20 mg l^{-1} NAA for 1 and 3 weeks followed by culturing of plumular leaf explant on MS medium containing 0.25, 0.50 and 1.0 BAP with 1.0, 2.0 and 4.0 mg l^{-1} NAA promoted somatic embryogenesis in both cultivars. Longer duration of pulse treatment was deleterious resulting in browning and consequently death of the embryos on explants. Pulse treatment with 20 mg l^{-1} NAA for one week was less deleterious and developed two plantlets after the explants were transferred to MS0 medium after 6 weeks through somatic embryogenesis in cv. 'Akkiz'. Pulse treatment with 10 mg l^{-1} NAA for 1 week showed 33.33-50.00 % and 25.00-50.00% shoot regeneration frequency in cv. 'Akkiz' and 'Karagoz' respectively on MS medium containing 0.25-1.00 mg l^{-1} BAP. Maximum number of 2.50 shoots each per explant were recorded in cv. 'Akkiz' and 'Karagoz' on MS medium containing 1.00 and 0.50 mg l^{-1} BAP respectively. Contrarily, maximum shoot length of 8.98 cm of cv. 'Akkiz' and 9.42 cm of cv. 'Karagoz' was recorded on MS medium containing 0.50 mg l^{-1} BAP and 1.00 mg l^{-1} BAP respectively. Regenerated shoots were rooted on MS medium containing 0.5 mg l^{-1} IBA and and acclimatized in growth room at room temprature where they produced viable seeds.

Keywords: cowpea, *in vitro*, plumular leaf, pulse treatment, shoot regeneration

Introduction

Cowpea (*Vigna unguiculata* L.) is a tropical herbaceous leguminous plant used as vegetable in most parts of the world and it is cultivated under diverse soils and climatic conditions. Cowpea is one of the essential crops for rural population diet and less costly source of protein (25% content) for rural people in West Africa (Cisse, 1996).

Auxins in combination with cytokinins are generally used for *in vitro* shoot regeneration in many plants. Previous studies suggest use of auxins at initial stage like 2,4,5-trichlorophenoxyacetic acid, (Muthukumar *et al.*, 1995); 2,4-D (Ganapathi and Anand, 1998; Prem Anand *et al.*, 2000 and Ramakrishnan *et al.*, 2005) followed by culturing on cytokinin-auxin combinations for induction of callus and somatic embryogenesis in cowpea. Pulse treatment with cytokinins for shoot induction in cowpea using BAP at higher dose has also been reported by Brar *et al.* (1999) and Aasim *et al.* (2009a). This study aimed to investigate the effects of duration and concentration of NAA pulse treatment on *in vitro* shoot regeneration from plumular leaf explants of Turkish cowpea cv. 'Akkiz' and 'Karagoz'.

Material and methods

Seeds of Turkish cowpea cv. 'Akkiz' and 'Karagoz' were obtained from the Department of Field Crops, Faculty of Agriculture, Ege University, Izmir, Turkey. Seeds were surface sterilized with 70% commercial bleach (Ace-Turkey containing 5-6% NaOCl) for 15 and 20 minutes for cv. 'Akkiz' and 'Karagoz' respectively followed by 3x5 min rinsing with bidistilled sterilized water. Mature embryos were taken aseptically and pulse treated with different concentrations of NAA in different time periods followed by culturing of plumular leaf explants on MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of BAP with or without NAA in three different ways.

Experiment 1: Mature embryos were pulse treated with 20 mg l^{-1} NAA for 3 weeks followed by culture of plumular leaf explants on MS medium containing 0.25, 0.50 and 1.0 BAP with 1.0, 2.0 and 4.0 mg l^{-1} NAA.

Experiment 2: Mature embryos were were pulse treated with 20 mg l^{-1} NAA for 1 week followed by culturing of plumular leaf explants on MS medium containing 0.25, 0.50 and 1.0 BAP with 1.0, 2.0 and 4.0 mg l^{-1} NAA.

Experiment 3: Mature embryos were pulse treated with 10 mg l^{-1} NAA for 1 week followed by culturing of plumular leaf explants on MS medium containing 0.25, 0.5, 0.75 and 1.00 mg l^{-1} BAP.

Regenerated shoots were excised aseptically and rooted on MS medium containing 0.5 mg l^{-1} IBA (Aasim *et al.*, 2008; Aasim *et al.*, 2009a, b). The pH of all media was adjusted to 5.6-5.8 using 0.1 N KOH or 0.1 N HC1 after adding 3.0% sucrose. The agar (0.65%-Duchefa Germany) was added before autoclaving at 118 kPa and 121°C for 20

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minutes. All cultures were incubated in growth chamber at 24±2°C with 16 h light photoperiod.

After two weeks of culture on rooting media, agar was carefully removed from the roots and the plants were kept submerged in water for 15 min before transferring them to pots containing clay, sand and organic matter (1:1:2). The pots were covered with low density transparent polythene bags (160 Gauge-40 microns) to maintain the internal humidity and placed in growth room at room temperature. After one week, polythene bags were removed gradually reducing 70% relative humidity in growth room to 40% in ten days time.

All treatments of regeneration experiments had three replicates containing four explants and all experiments were repeated twice. Data for frequency (%) of shoot regeneration, mean number of shoots per explant, shoot length and frequency of rooting was recorded and analyzed using one way ANOVA. The post hoc tests were performed using DMRT with statistical software SPSS 16.00 for windows. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

Results

In the first experiment, callus induction started on all explants after 2 weeks followed by regeneration of somatic embryos after 6 weeks, irrespective of the concentration of growth regulators in the regeneration media. After initiation of somatic embryogenesis, the explants were transferred to MS0 medium for maturing of somatic embryos and obtaining plants. After 4 week of culture on MS0, the embryos started to develop roots on the mini plantlets developed from somatic embryos with shoot initials in both cultivars. The developing embryos were sticky, difficult to separate and could not mature to develop in to plants.

The results of the second experiment also showed induction of callus followed by somatic embryogenesis on all explants after transferring the explants to MS0 medium for maturing of embryos after 6 weeks. There after, 4 weeks, majority of the embryos died due to browning of explants. Only two somatic embryos could be matured to get two plantlets with healthy roots and shoots (Fig. 1 ab) from the explants previously cultured on MS medium containing 0.5 mg l^{-1} BAP- 1.0 mg l^{-1} NAA from cv. 'Akkiz'. In 3rd experiment, callus induction started from petiole end of plumular leaf explant (Fig. 2a) on all culture medium followed by initiation of shoot primordia which developed into shoot (Fig. 2ab). These shoots attained height of 3-4 cm in 4 weeks. Analysis of variance showed statistically significant but variable effect of pulse treatment with NAA on plumular leaves on shoot regeneration, number of shoots per explant and mean shoot length of both cultivars on MS medium containing 0.25-1.00 mg l^{-1} BAP.

Shoot regeneration frequency of cv. 'Akkiz' and cv. 'Karagoz' ranged 33.33-50.00% and 25.00-50.00%, respectively (Tab. 1). However, maximum shoot regeneration of cv. 'Akkiz' and cv. Karagöz was recorded on MS medium containing 1.00 and 0.75 mg l^{-1} BAP. BAP concentrations in the culture medium showed variable effect on number of shoots per explant in both cultivars. Number of shoots per explant ranged 1.17-2.50 and 1.20-2.50 in cv. 'Akkiz' and 'Karagoz' respectively (Tab. 1). Shoots per explant increased proportionally with increase in BAP concentration with maximum of 2.50 shoots per explant on MS medium containing 1.00 mg l^{-1} BAP in cv. 'Akkiz'. Contrarily, maximum number of 2.50 shoots per explant in cv. 'Karagoz' was recorded on MS medium containing 0.50 mg l^{-1} BAP. Mean shoot length of cv. 'Akkiz' and cv. 'Karagoz' ranged 2.40-8.98 cm and 4.17-9.42 cm respectively (Tab. 1). Maximum shoot length (8.98 cm) in cv. 'Akkiz' was recorded on MS medium containing 0.50 mg l^{-1} BAP. Contrarily, maximum shoot length in cv. 'Karagoz' was recorded on 1.00 mg l^{-1} BAP.

Regenerated plantlets from experiment 2 were directly transferred to pots and placed in growth room at room temperature where they failed to acclimate. All regenerated shoots from third experiment were not difficult to root and they were easily acclimatised under growth room conditions where they flowered and set viable seeds.

Tab. 1. Effect of 10 mg l^{-1} NAA pulse treatment for 1 week on shoot regeneration frequency, shoots per explant and mean shoot length of plumular explant of cowpea cv. 'Akkiz' and 'Karagoz'

BAP	Shoot regeneration frequency (%)		Shoots per explant		Shoot length (cm)	
	'Akkiz'	'Karagoz'	'Akkiz'	'Karagoz'	'Akkiz'	'Karagoz'
0.25	41.67^{b}	33.33^{b}	1.17^{c}	1.20^{c}	6.49^{b}	4.17^{c}
0.50	33.33^{c}	25.00^{c}	1.33^{bc}	2.50^{a}	8.98^{a}	5.67^{bc}
0.75	41.67^{b}	50.00^{a}	1.67^{b}	1.67^{b}	3.56^{c}	4.48^{c}
1.00	50.00^{a}	33.33^{b}	2.50^{a}	2.00^{ab}	2.40^{d}	9.42^{a}

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test

Fig. 1. Plantlet regeneration on plumular leaf of cv. 'Akkiz' pulse treated with 20 mg l^{-1} NAA for 1 week; (a) plantlet and roots on explant on MS medium containing 0.50 mg l^{-1} BAP with 1.0 mg l^{-1} NAA; (b) plantlet with root

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Fig. 2. Plantlet regeneration on plumular leaf of cv. 'Akkiz' pulse treated with 10 mg l^{-1} NAA for 1 week; (a) shoot regeneration initiation; (b) explants with and without shoot regeneration; (c) acclimatised plants in growth room

Discussion

The study presented establishment of *in vitro* regeneration protocol of plumular leaf explants from Turkish cowpea cv. 'Akkiz' and 'Karagoz' after pulse treatment of mature embryos with 10 and 20 mg l^{-1} NAA for different durations. The results showed that pulse treatment of explants with 20 mg l^{-1} NAA for 1 and 3 week induced high somatic embryogenesis in both cultivars. However, 3 weeks pulse treated explants failed to induce shoot regeneration due to browning and partially due to over dose of NAA for a longer period of time. The results are contradictory to the findings of Bhatti (2001) who reported 3.33-30.00% shoot regeneration with 1.66-2.16 shoots per explant from 20 mg l^{-1} NAA for 10 days pulse treated immature embryonic axis explant in lentil.

The results further showed that decreasing pulse treatment duration had positive effect on shoot regeneration and 2 shoots were recorded in cv. 'Akkiz', which is in line with Bhatti (2001), who obtained shoot regeneration from embryonic axis in lentil cv. Emre after 10 days pulse treatment with 20 mg l^{-1} NAA followed by culture on MS medium containing 4.0 mg l^{-1} BAP and 0.25 mg l^{-1} NAA.

Plumular leaves induced callus initiating at petiole ends of plumular leaf explants after pulse treatment with 10 mg l^{-1} NAA for 1 week. The results are in line with Muthukumar *et al.* (1995). They reported induction of callus on primary leaf explants that initiated from petiole ends.

The results further, showed that shoot growth in 2nd experiment was very low that might be due to (i) duration of pulse treatment, (ii) high concentration of NAA for pulse treatment or (iii) presence of NAA in the culture medium in combination with BAP. Results of 1st and 2nd experiment showed that decreasing pulse duration induced low shoot regeneration. Results further showed that decreasing NAA concentration of pulse treatment and culturing of explants on MS medium devoid of NAA (containing only different concentrations of BAP) positively increased the shoot regeneration and number of shoots per explant in both cultivars. Similarly, Muthukumar *et al.* (1995) obtained callus by culturing primary leaf explants on Gamborg's B5 medium containing $8x10^{-7}$ M 2,4,5-trichlorophenoxyacetic acid, $1x10^{-2}$ M L-glutamine and $1x10^{-4}$ M adenine sulphate and shoot regeneration occurred on transferring callus to B5 basal medium containing BAP. Similarly, Brar *et al.* (1997) and Aasim *et al.* (2008) also reported addition of NAA in the culture medium inhibited shoot regeneration frequency and mean number of shoots per explant on shoot meristem of cowpea. Results further showed that shoot length of both cultivars was dependant on BAP concentration and number of shoots per explant. Maximum shoot length of cv 'Akkiz' and minimum shoot length of cv. 'Karagoz' was recorded on MS medium containing 1.00 mg l^{-1} BAP. Similarly, explants with more shoots had poor shoot length compared to explants with low number of shoots, with longer shoots.

Regenerated plantlets from 2nd experiment failed to establish in the growth room. Contrarily, regenerated shoots from 3rd experiment rooted easily on MS medium containing 0.5 mg l^{-1} IBA (Aasim *et al.,* 2008; Aasim *et al.,* 2009a, b) and adapted well in growth room at room temperature and produced viable seeds at maturity (Fig. 2c). Aasim *et al.,* 2008; Aasim *et al.,* 2009a,b also reported successful adaptation of *in vitro* regenerated plants of cowpea cv. 'Akkiz' under growth room conditions.

Conclusions

Pulse treatment of 20 mg l^{-1} NAA for 3 weeks increased the somatic embryogenesis of plumular leaf explant but failed to regenerate plantlets. Whereas, decreasing pulse treatment time from 3 to 1 week had similar effect on somatic embryogenesis and induced very low number of plantlets. On the other hand, decreasing NAA concentration from 20 mg l^{-1} to 10 mg l^{-1} and culture media devoid of NAA promoted shoot regeneration frequency and mean number of shoots per explant but decreased the somatic embryogenesis in both cultivars. Regenerated shoots from experiment three were acclimatized easily under growth room conditions.

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