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EFFICIENT *IN VITRO* PROPAGATION FROM PRECONDITIONED EMBRYONIC AXES OF TURKISH COWPEA *(VIGNA UNGUICULATA* L.) CULTIVAR AKKIZ

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Abstract - Cowpea is an important grain legume crop. The study reports an efficient *in vitro* multiplication and shoots regeneration protocol from preconditioned embryonic axes of the Turkish cowpea cultivar Akkiz. The embryonic axes were preconditioned with 10 mg/l BA on agar solidified MS medium for 5 days. Thereafter they were cultured on MS medium containing 0.25, 0.50, 0.75 and 1.00 mg/l BA with or without 0.10 mg/l NAA. Mean frequency (%) of shoot regeneration, number of shoots per explant and shoot length decreased with each increase in BA concentration used singly. However, a positive increase was recorded in all parameters in the presence of 0.10 mg/l NAA in the regeneration medium. A maximum mean number of 10.33 shoots per explant was recorded on an MS medium containing 1.00 mg/l BA -0.1 mg/l NAA. Regenerated shoots were rooted on an MS medium containing 0.50 mg/l IBA. Rooted plants were acclimatized at room temperature in soil mix contained in pots where they were subjected to **an** intermittent mist-water spray for 24 h that maintained 90% relative humidity during the first few days which was gradually reduced to 40% for 10 days. All plants flowered and set seeds in a greenhouse after 3 months.

Key words: Cowpea, micropropagation, embryonic axes, preconditioning

Abbreviations: NAA- α Naphtalene Acetic acid, BA- 6 Benzyladenine, IBA- Indole 3 Butyric acid.

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INTRODUCTION

The cowpea (*Vigna unguiculata* L.) is a highly drought-tolerant summer annual, short duration legume crop of savanna regions in the tropics and subtropics of Africa, Asia, and South America. It is grown on a limited area in the Marmara region of Turkey (Peksen 2004) and can play an important role in supplementing human diet due to its high protein content, calories, minerals and vitamins (Phillips et al. 2003). It is mainly used as a vegetable in the form of dry seeds, green seeds, green pods and tender green leaves, or as fodder, cover crop and green manure.

By introducing new varieties many improvements have been observed in the cowpea but conventional breeding methods may not provide ultimate solutions against several biotic and abiotic stresses. These can be easily solved by the judicious application of biotechnological methods and genetic transformation techniques. Therefore, there is an urgent need to develop and optimize reliable, efficient, and reproducible methods of regeneration and genetic transformation for the cowpea. Previous tissue culture studies report the use of primary leaves (Muthukumar et al. 1995; Prem Anand et al. 2000; Ramakrishna et al. 2005), the cotyledon node (Van Le et al. 2002; Chaudhury et al. 2006), the mature cotyledon (Muthukumar et al. 1996; Brar et al. 1999, Popelka et al. 2006), the mature embryo (Odutayo et al. 2005; Popelka et al. 2006), the hypocotyl (Pellegrineschi 1997) and shoot meristem/apices/tip (Kartha et al. 1981; Brar et al. 1997; Mao et al. 2006, Aasim et al. 2008, Aasim et al. 2009a), immature cotyledon (Prem Anand et al. 2001; Choi et al. 2003) and plumular apices (Aasim et al. 2009b).

This study was aimed at improving previous regeneration methods by developing a shoot rege-

neration system from an embryonic axis explant of the Turkish cowpea cultivar Akkiz for effective use in *Agrobacterium* mediated genetic transformation.

MATERIAL AND METHODS

Seeds of the Turkish cowpea cv. Akkiz were obtained from the Department of Field Crops, Aegean University, Izmir, Turkey. They were surface-sterilized with 70% commercial bleach (Ace-Turkey containing 5% NaOCl) for 5 min and rinsed for 3x5 min with bi-distilled sterilized water. Mature embryos were separated aseptically from cotyledons and preconditioned on an MS medium (Murashige and Skoog 1962) containing 10 mg/l 6 benzyladenine (BA- Cat No. B3408 Sigma Aldrich Chemical Co. St. Lo. Mo.), solidified with 0.65% agar (Cat No. P1001.1000, Duchefa RV Haarlem, the Netherlands) and 3% sucrose for 5 days. The agar was added after adjusting the pH of the media to 5.6 - 5.8 with 0.1 N KOH or 0.1 N HCl before autoclaving at 121°C, under pressure (118 kPa) for 20 min. Preconditioned embryos cultured on an MS medium served as control.

Thereafter, the embryonic axes were excised from the preconditioned embryos under aseptic conditions and cultured on 35 ml MS basal medium containing 0.25 0.50, 0.75 and 1.00 mg/l BA - with or without 0.10mg/l α naphthalene acetic acid (Sigma Aldrich Chemical Co.) in Petri dishes. The media was also supplemented with 3% sucrose and 0.65% plant Agar (Cat No. P1001.1000, Duchefa RV Haarlem, the Netherlands). The agar was added after adjusting the pH of the media to 5.6 - 5.8 with 0.1 N KOH or 0.1 N HCl before autoclaving for 20 min. All cultures were maintained in Sanyo MLR Plant Growth Chambers at 24±2°C at 42 μ Mol photon m^{-2} s^{-1} and 16 h light photoperiod. Initial experiments showed that the cowpea exhibited severe blackening of explants due to phenolic compounds that affected their regeneration capacity. To overcome this problem, 1 mg/l Polyvinylpyrrolidone (Sigma Aldrich) was also added to all regeneration media. Initial experiments also showed the severe problem of endogenic latent bacterial contaminations (data not shown). Therefore, 500 mg/l of the antibiotic Augmentin (Glaxo Smith Kline) was also added to the culture media before pouring into Petri dishes after autoclaving and cooling to 45°C. Augmentin is an antibiotic agent with a notably broad spectrum of activity against the commonly occurring bacterial pathogens.

Approximately 2-3 cm-long regenerated shoots were excised aseptically after six weeks of regeneration and rooted on 35 ml of MS medium containing 0.5 mg/l IBA Indole 3 butyric acid (Sigma Aldrich Chemical Co.) in Magenta GA7 vessels for two weeks. Thereafter, the agar was carefully removed from the roots and the plants were kept submerged in water for 15 min before transferring them to pots containing vermiculite, organic matter and sand (1:1:1). The pots were transferred to the greenhouse at room temperature, where they were subjected to intermittent mist-water spray with HR-15 Cool Mist humidifier with humidistat turned on for 24 h and maintaining a relative humidity of 90% during the first few days. The duration and frequency of misting assisted in maintaining a film of water on the leaves of the plants that prevented wilting. It was gradually reduced to 40% that lasted 10 days.

All treatments of the regeneration experiments had three replicates comprising of 6 explants and were repeated twice (3 replications x 6 explants x 2 repeats = 36 explants). The data was analyzed using one-way analysis of variance (ANOVA) and post hoc tests were performed using Duncan's Multiple Range Test with the help of statistical software SPSS 16.00 for Windows. The data presented in percentages were subjected to arcsine transformation (Snedecor and Cochran 1967) before statistical analysis.

RESULTS

The present investigation showed that preconditioned embryonic axes could be regenerated di

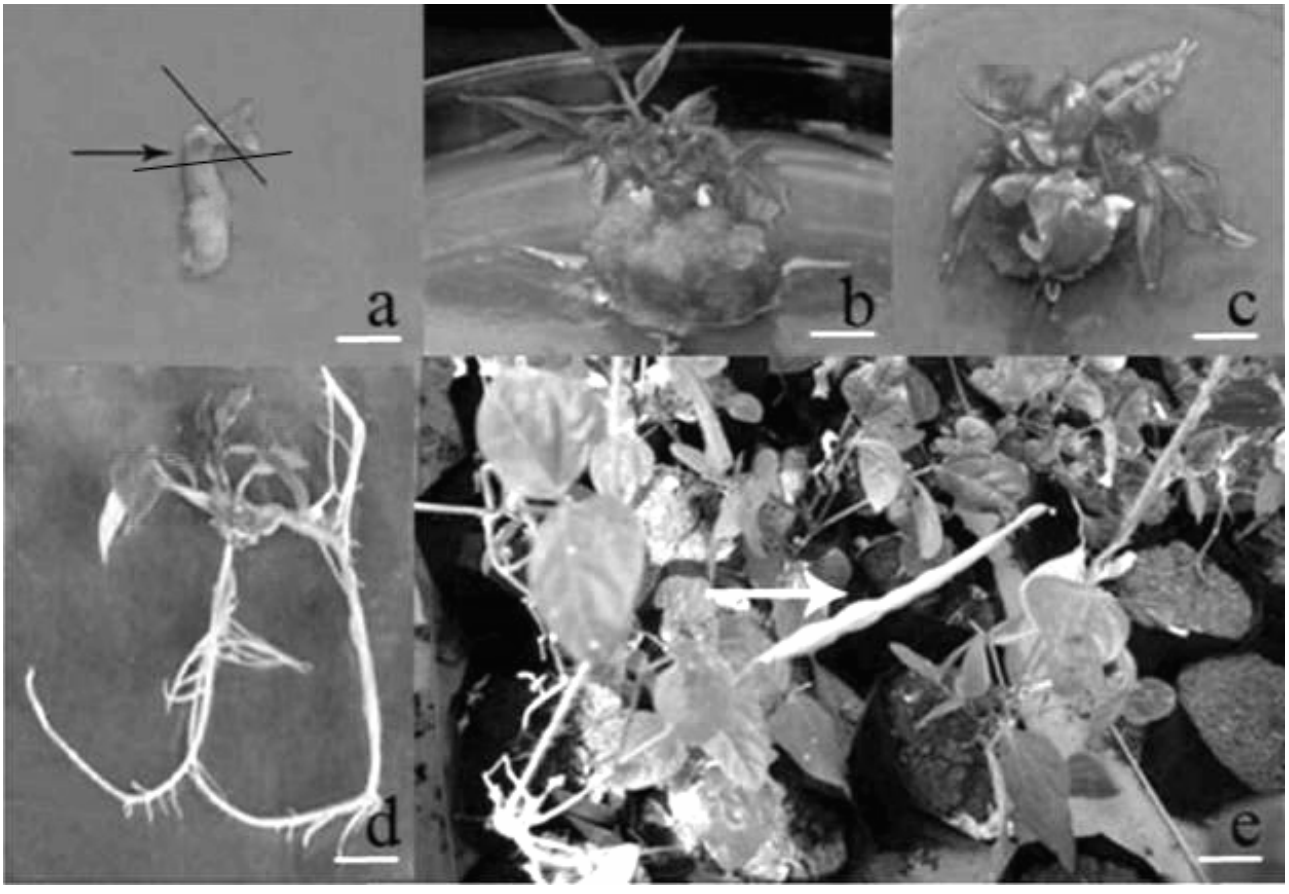

Fig. 1. Shoot regeneration from embryonic axes explants of cowpea (a) preconditioned embryo with swelled embryonic stem (b,c) conversion of swelling to callusing and shoot regeneration (d) rooting on an MS medium containing 0.5 mg/l IB A and induction of multiple secondary shoot on rooting media (e) acclimatization, flowering and seed setting.

rectly on an MS medium containing different combinations of BA-NAA. Regeneration of preconditioned embryos started with a swelling of the embryonic stem (hypocotyl) (Fig 1a). Both embryonic stem and plumules were removed aseptically to obtain the embryonic axes. These explants swelled further and this was followed by the induction of callus and shoot primordia within 3-4 days on an MS medium containing any concentration of BA with or without NAA (Fig 1b). The primordia differentiated into dark green shoot buds and clear shoots in 2 weeks. Analysis of variance results revealed the significant effects ($p<0.05$) of plant growth regulators on the explant in terms of callus diameter (cm), mean frequency (%) of shoot regeneration, number of shoots per explant and shoot length (Table 1). It was noted that BA promoted more callusing in the presence of NAA compared to BA used singly. The callus diameter ranged from 1.11 (on an MS medium containing 0.25 mg/l BA) to 1.71 cm (on an MS medium containing 0.75 mg/l BA-0.1 mg/l NAA). Each increase in BA concentration without NAA had an inhibitory effect on the frequency (%) of shoot regeneration, mean number of shoots per explant and mean shoot length. The minimum shoot regeneration frequency of 8.33%, 0.75 shoots per explant and shoot length of 0.38 cm was recorded on the MS medium containing 1.00 mg/l BA.

A general comparison of regeneration showed that the presence of 0.1 mg/l NAA in the

Table 1. Effects of various concentrations of BA-NAA on shoot regeneration behavior from shoot meristem explants of Turkish Cowpea *(Vigna unguiculata* L.) cultivar Akkiz.

Culture medium		Frequency (%) of Callus induction	Callus diameter (cm)	Frequency (%) of shoot regeneration	Mean number of Shoots per explant	Shoot length (cm)
BA (mg/l)	NAA (mg/l)					
0.25	0.00	100.00	1.11^{d}	41.67^{c}	1.78^{bc}	1.29bc
0.50	0.00	100.00	1.13^{d}	33.33^{cd}	2.67^{bc}	1.32bc
0.75	0.00	100.00	1.21^{cd}	16.67^{cd}	1.50^{bc}	0.83cd
1.00	0.00	100.00	1.22^{cd}	8.33^{d}	0.75c	0.38d
0.25	0.10	100.00	1.40^{bc}	44 44	2.67^{bc}	2.13a
0.50	0.10	100.00	1.48^{b}	55.56^{b}	2.75^{bc}	1.70ab
0.75	0.10	100.00	1.71^{a}	66.67^{b}	3.89^{b}	1.59ab
1.00	0.10	100.00	1.38^{bc}	100.00^{a}	10.33^{a}	1.31bc

Values within a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

regeneration medium overcame the inhibitory effects of BA used singly and induced more favorable conditions for shoot regeneration and elongation. The maximum number of 10.33 shoots per explant with 100% shoot regeneration was recorded on the MS medium containing 1 mg/l BA-0.1 mg/l NAA (Fig 1c). No callusing and development of single shoots were recorded on the preconditioned embryonic axes on MS medium (control). However, the longest shoots of 2.13 cm were recorded on the MS medium containing 0.25 mg/l BA - 0.1 mg/l NAA, which showed a corresponding decrease at higher concentrations of BA -0.1 mg/l NAA.

All regenerated shoots on MS medium containing 0.5mg/l BA rooted easily on MS medium containing 0.5 mg/l IBA. The primary shoots also regenerated secondary shoots at the base (Fig. 1d). All secondary shoots also rooted easily on MS medium containing 0.5 mg/l IBA. Morphologically no difference could be recorded between plantlets obtained through the rooting of primary or secondary shoots. They were acclimatized and grown to maturity at room temperature in the greenhouse. Ninety percent of the rooted plantlets survived acclimatization and produced viable seeds in the greenhouse (Fig. 1e).

DISCUSSION

The powerful combination of conventional and genetic modification breeding has the potential of greatly enhancing the productivity of cowpeas by increasing its resistance to pests, diseases, *Striga*, and abiotic stress, as well as improving seed quality and other traits that impact on cowpea utilization for fodder and grain (Machuka et al. 2000). The cowpea is of major economic importance to many tropical African, Asian, and Latin American countries with poor technical expertise and weak scientific and biotechnological infrastructure. This has made it very difficult for the scientists of these countries to improve the plant using local facilities, and has made it of immense importance for these studies to be carried out elsewhere in order to improve the plant for higher yield and better nutrition of the population.

The results showed that preconditioned embryonic axes of cowpea could easily be used to regenerate shoots on various concentrations of BA with or without NAA with variable callusing. It is assumed that callus induction at the basal end of all explants was due to the preconditioning with BA, which resulted in the fast multiplication of cells leading to callusing even in the absence of NAA. Each increase in the concentration of BA used singly was inhibitory for any of the factors studied. The addition of NAA positively increased the mean shoot length on the culture media. The results appear to show the presence of NAA as an important inducing factor not only for organogenesis but also for shoot growth. The maximum mean number of 10.33 shoots per explant was obtained on an MS medium containing 1 mg/l BA with 0.1 mg/l NAA. The results are in agreement with Aasim et al. (2009b), who recorded multiple shoots on plumular apices excised from the mature embryos of cowpea cv. Akkiz after preconditioning with 10mg/l BA for 5 days followed by culture on an MS medium containing different concentrations of BA with or without NAA. They found that the inclusion of 0.1mg/l NAA had a positive effect on callusing and shoot length. Contrarily, they obtained a maximum mean number of 7.11 shoots per explant on an MS medium containing 1.00mg/l BA. Aasim et al. (2009a) also found multiple shoots from shoot meristems on *in vitro* grown seedlings of Turkish cowpea cv. Akkiz obtained on an MS supplemented with 0.50 mg/l BA - 0, 0.10, 0.30 and 0.50 mg/l NAA. They recorded shoot regeneration on all cultures containing 0.5 mg/l BA with and without NAA. The addition of any concentration of NAA resulted in a significant decrease in the frequency (%) of shoot regeneration and the mean number of shoots per explant. The maximum mean number of 2.60 shoots per explant was obtained on MS without NAA.

The results suggest that the presence of NAA is an important inducing factor not only for organogenesis but also for shoot growth, as the regeneration potential of the embryonic axes was enhanced due to synergized BA-NAA action, which could not be achieved when only BA was used. This also helped to ultimately increase the frequency (%) of shoot induction from the explants. The results are in agreement with Brar et al. (1997). Similar results were reported by Hammatt (1996), who cultured the distal and proximal portions of transversely cut cotyledons of *Fraxinus excelsior* L. They also found that the inclusion of NAA increased the callus diameter of the explants.

Regenerated shoots rooted and acclimatized easily in the external environment. The promotional effect of IBA on secondary shoot regeneration in the rooting medium is also an important observation. The results are in agreement with Aasim et al. (2008), who had very similar observations of secondary shoot regeneration on rooting medium due to the presence of 0.5 mg/l IBA in cowpea. Additional research is needed to understand the mechanism and the nature of the interaction between the explant and IBA that resulted in the induction of secondary shoots in the rooting medium. This would be greatly beneficial to describe the mechanism of IBA action and behavior of plant more exactly.

CONCLUSION

The present work provides a new window of opportunity for shoot organogenesis on pre conditioned embryonic axes and indicates that this protocol can be used as a viable approach for regeneration in the cowpea. This study also provides an opportunity to meet our objective to develop a reliable micropropagation system for future use in the multiplication of *in vitro* genetically transformed cowpea plants.

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