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IN VITRO MICROPROPAGATION FROM SHOOT MERISTEMS OF TURKISH COWPEA (*VIGNA UNGUICULATA* L.) CV. AKKIZ

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Abstract

Multiple shoots from shoot meristems of three - five-day-old *in vitro* grown seedlings of Turkish cowpea (*Vigna unguiculata* L.) cv. Akkiz was obtained in MS supplemented with 0.50 mg/l BAP - 0, 0.10, 0.30 and 0.50 mg/l NAA. Callus induction was recorded on all cultures. Callusing was recorded on all cultures containing 0.5 mg/l BAP with and without NAA. However, increased diameter of calli was recorded on MS medium containing 0.5 mg/l BAP - 0.1, 0.3 and 0.5 mg/l NAA. The highest frequency (%) of shoot regeneration and mean number of shoots per explant was recorded on MS medium containing 0.5 mg/l BAP with out NAA. Addition of any concentration of NAA resulted in significant decrease in the frequency (%) of shoot regeneration and mean number of shoots per explant. Maximum mean number of 2.60 shoots per explant was obtained on MS without NAA. Regenerated shoots were rooted on MS containing 0.50 mg/l IBA where up to seven adventitous secondary shoots arose from the base of mother shoot were also recorded. These shoots could also be rooted easily on the same rooting medium. Rooted plants were adapted at room temprature in soil mix in pots. All plants flowered and set seeds in the growth room after three months.

Introduction

Cowpea (*Vigna unguiculata* L.) an important food grain legume crop, is popularly used as vegetable in the form of dry seeds, green seeds, green pods and tender green leaves in many parts of the world. It is also utilised for fodder and as a quick-growing cover crop. It grows easily in wide range of environments on poor and dry soils (Rachie 1985).

In vitro techniques such as micropropagation has proved a useful tool for propagation of number of food legume crops (Pierik 1993, Brar *et al.* 1997). Shoot Mertistem multiplication is generally used for producing virus free material and maintining germplasm via cryopreservation (Nehra and Kartha 1994). Reports regarding *in vitro* regeneration of cowpea by tissue culture describes using primary leaves (Muthukumar *et al.* 1995, Prem Anand *et al.* 2000, Ramakrishna *et al.* 2005), cotyledonary node (Van Le *et al.* 2002, Chaudhury *et al.* 2006), mature cotyledon (Muthukumar *et al.* 1995, Brar *et al.* 1999), embryonic axis (Popelka *et al.* 2006), mature embryo (Odutayo *et al.* 2005, Popelka *et al.* 2006) and epicotyl (Pellegrineschi 1997). However, very few reports (Kartha *et al.* 1981, Brar *et al.* 1997, Mao *et al.* 2006) describe the use of shoot meristem/apices/tip as explant of choice.

Our objective was to develop a reliable micropropagation system for Turkish cowpea cv. Akkiz using shoot meristem for future use in the mutiplication of *in vitro* genetically transformed cowpea plants. No previous report describes the micropropagation system in any of the Turkish cowpea cultivars.

Material and Methods

Seeds of Turkish cowpea cv. Akkiz obtained from the Department of Field Crops, Faculty of Agriculture, Ege University, Izmir, Turkey were surface sterilised with 70% commercial bleach (Ace- Turkey containing 5 - 6% NaOCl) for 5 minutes. Thereafter, they were rinsed 3 - 5 min with double distilled sterilized water and cultured for germination on MS basal medium (Murashige

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and Skoog 1962) supplemented with 3.0 % sucrose. Agar (0.65% - Duchefa Germany) was added to the culture medium after adjusting pH 5.6 - 5.8 before autoclaving at 121°C for 20 minutes. Initial experiments in our laboratory showed that it was not possible to sterilize the seeds against endogenic latent bacterial contamination. Therefore, the bacterial contamination was eliminated by adding 500 mg/l Augmentin (Smith-Klein-Beecham) in the seed germination media after autoclaving, before pouring the media into Petri dishes or Magenta GA7 vessels.

Shoot meristem explants were excised from three - four-day-old *in vitro* grown seedlings and cultured on MS containing 0.5 mg/l BAP 0, 0.10, 0.30 - 0.50 mg/l NAA supplemented with 2 mg/l yeast extract and 3.0 % sucrose gelled with 0.65% Agar. All regeneration media also contained 500 mg/l Augmentin added after autoclaving before pouring the media into Petri dishes or Magenta vessels to eliminate bacterial contaminations due to latent bacteria.

Initial experiments showed blackening of explants to a varying degree due to the presence of phenolics that affected regeneration from explants (data not shown). Therefore, to overcome this 5 g/l activated charcoal was also added to the culture medium.

The pH of all medium was adjusted to 5.6 - 5.8 using 0.1 N KOH or 0.1 N HC1 before sterilization and solidified by 0.65% agar. All cultures were incubated in growth chamber at 24 ± 2°C with 16 h photoperiod.

Regenerated shoots obtained from MS containing 0.5 mg/l BAP were excised aseptically and rooted on MS containing 0.5 mg/l IBA. After two weeks of culture, 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). 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 and pots containing *in vitro* regenerated plants were left in the growth room at room temperature with 70 % relative humidity reduced gradually to 40% in ten days time.

All treatments of regeneration experiments had three replicates containing five explants and all experiments were repeated twice. Data for frequency (%) of callus induction, callus diameter, frequency (%) of shoot regeneration, mean number of shoots per explant, shoot length and frequency of rooting were recorded and analyzed using one way ANOVA. The post hoc tests were performed using DMRT with the help of statistical software SPSS 12.00 for windows. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran 1967) before statistical analysis.

Results and Discussion

Shoot regeneration started within a week from the respective explants. Clear shoot regeneration from explants was observed after second week of culture with callus formation at the basal end of explants (Fig. 1A). Results of analysis of variance indicated no significant effects of plant growth regulators on the frequency of callus induction. The results showed a clear bearing of plant growth regulators on the callus diameter, frequency (%) of shoot regeneration, mean number of shoots per explant and mean shoot length ($p < 0.05$, Table 1). Callus induction was recorded on all regeneration media (Table 1). However, the intensity of callus formation increased with the increase of concentration of NAA. Minimum callus diameter of 1.66 cm was recorded on MS containing 0.50 mg/l BAP without NAA (Fig.1B). Maximum increase in the callus diameter (2.70 cm) was observed on MS containing 0.5 mg/l BAP.

Addition of NAA to the media had inhibitory effect on frequency (%) of shoot regeneration. Maximum shoot regeneration frequency of 66.67% was obtained on MS with 0.50 mg/l BAP.

Addition of NAA in any concentration resulted in dramatic decrease in the frequency (%) of shoot regeneration.

Maximum mean number of 2.60 shoots per explant was obtained on MS containing 0.5 mg/l BAP. Increase in the concentration of NAA resulted in corresponding decrease in the mean number of shoots per explant, such that least number of 1.08 shoots per explant were recorded on MS containing 0.50 mg/l BAP.

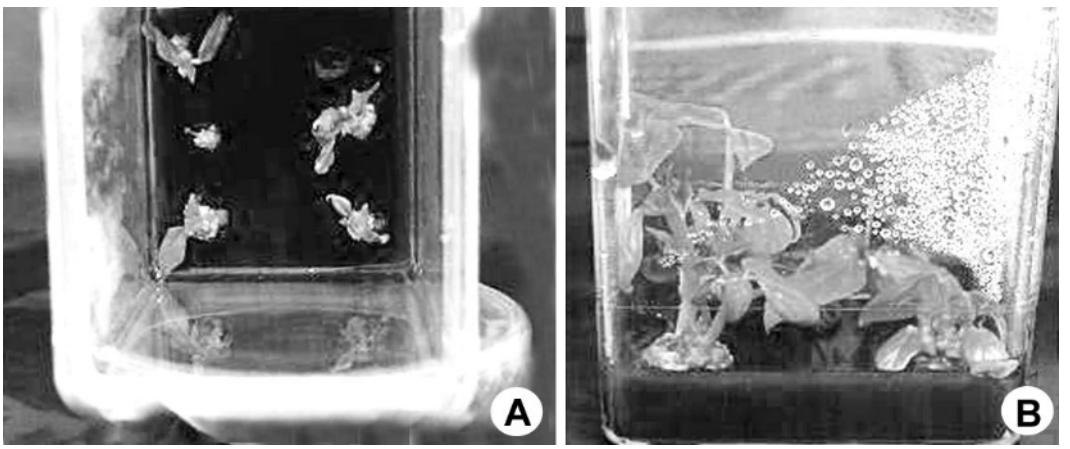

Fig. 1. Effects of various concentrations of BAP-NAA on shoot regeneration from shoot meristems. (A) initiation of shoot regeneration (B) expression of minimum callus diameter on MS medium containing 0.5 mg/l BAP without NAA.

Length of regenerated shoots ranged from 1.32 - 3.56 cm. The minimum shoot length of 1.32 cm was recorded on MS containing 0.5 mg/l BAP. The maximum shoot length of 3.56 cm was recorded on MS containing 0.5 mg/l BAP - 0.10 mg/l NAA.

Table 1. Effects of various concentrations of BAP-NAA on shoot regeneration behvior from shoot meristem explants of Turkish cowpea (*Vigna unguiculata* L.) cv. Akkiz.

Medium		Frequency (%) of callus induction	Callus diameter (cm)	Frequency (%) of shoot regeneration	Mean number of shoots per explant	Shoot length (cm)
BAP	NAA					
0.5	0.0	100.00	1.66 [b]	66.67 [a]	2.60 [a]	1.32 [b]
0.5	0.1	100.00	2.34 [ab]	55.33 [b]	2.00 [ab]	3.56 [a]
0.5	0.3	100.00	2.53 [a]	55.33 [b]	1.28 [ab]	3.03 [a]
0.5	0.5	100.00	2.70 [a]	55.33 [b]	1.08 [b]	2.50 [ab]

[1]Values within a column followed by different letters are significantly different at 0.05 level of significance using DMRT. [2] Each value is the mean of 6 × 2 replicates with 5 explants.

All regenerated shoots on MS containing 0.5mg/l BAP rooted easily on MS containing 0.5 mg/l IBA. Most of the shoots in rooting medium besides rooting produced secondary shoots with maximum of 7 shoots developing at the base of single shoots without callus formation (Fig. 2A). All the secondary shoots were more vigorous compared to mother shoot and rooted easily on MS containing 0.5 mg/l IBA. The plants were grown in the growth room at room temperature to maturity where they produced viable seeds (Fig. 2B).

Shoot meristem explant has proved useful for the propagation of other legumes and also for cowpea reported by several researchers in the past. The results clearly showed the effects of the concentration of BAP on the frequency (%) of callus induction, callus diameter, frequency (%) of shoot regeneration, mean number of shoots per explant and shoot length.

Fig.2. Rooting and adaptation of micropropagated plants. (A) rooted shoots on 0.5 mg/l IBA with multiple adventitous shoots arising from the base. (B) pod setting and flowering of *in vitro* regenerated plantlets after adaptation in the growth room.

Shoots with callus at the basal end of explants after second week of culture showed that the explant has high potential to regenerate callus even in the absence of NAA. However, addition of NAA resulted in more callus induction, which increased with the increase of concentration of NAA.

Increased concentration of NAA also increased the callus diameter which conformed the findings of Brar *et al.* (1997). However, increase in callus diameter had negative effects on shoot regeneration frequency and mean number of shoots per explant.

Addition of NAA to the MS containing 0.5 mg/l BAP inhibited the frequency (%) of shoot regeneration and mean number of shoots per explant was in agreement with Brar *et al.* (1997) with similar observation from shoot apices of cowpea.

Lesser number of shoots per explant might be due to heavy callusing because of NAA which ultimately, suppressed the frequency (%) of shoot induction from explants. Inhibitory effect of NAA to BAP medium on cowpea shoot regeneration and number of shoots per explant are also reported by Brar *et al.* (1997). They reported that increasing the concentration of 2,4-D or NAA inhibited cowpea shoot multiplication. However, maximum shoot regeneration was recorded on MS containing 0.5 mg/l BAP without NAA. This is contrary to the findings of Brar *et al.* (1997) who reported a fewer shoots on medium containing BAP only compared to medium containing both 2,4-D or NAA and BAP or Kn.

Addition of any concentration of NAA to the MS containing 0.5 mg/l BAP had promotory effect on shoot length. However, maximum shoot length was recorded on MS medium containing containing 0.5 mg/l BAP with 0.1 mg/l NAA. Further addition of NAA tended to inhibit shoot

elongation is in agreement with Brar *et al.* (1997). They found that increasing the concentration of auxins above 0.1 mg/l resulted in inhibition of shoot elongation.

Mao *et al.* (1996) reported that presence of IBA in the rooting medium had no effect on regenerated shoots from shoot apices on root induction. These results are in contradiction to the present results where IBA had positive effects not only on root induction but also on secondary shoot formation in the rooting medium.

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References

Brar, M.S., J.M. Al-Khayri, C.E. Shamblin, R.W. McNew, T.E. Morelock and E.J. Anderson. 1997. *In vitro* shoot tip multiplication of cowpea *Vigna unguiculata* (L.) Walp. In Vitro Cell. Dev. Biol. **33**: 111-118.

Brar, M.S., J.M. Al-Khayri, T.E. Morelock and E.J. Anderson. 1999. Genotypic response of cowpea *Vigna unguiculata* (L.) to *in vitro* regeneration from cotyledon explants. In Vitro Cell. Dev. Biol. **35**: 8-12.

Chaudhury, D., S. Madanpotra, R. Jaiwal, R. Saini, P.A. Kumar and P.K. Jaiwal. 2007. *Agrobacterium tumefaciens*-mediated high frequency genetic transformation of an Indian cowpea (*Vigna unguiculata* (L.) Walp.) cultivar and transmission of transgenes into progeny. Plant Sci. **172**: 692-700.

Gulati, A. and R.K. Jaiwal. 1992. *In vitro* induction of multiple shoots and plant regeneration from shoot tips of mung bean *(Vigna radiata* (L.) Wilezek). Plant Cell Tissue Organ Cult. **29**: 199-205.

Kartha, K.K., K. Pahl, N.L. Leung and L.A. Mroginski. 1981. Plant regeneration from meristems of grain legumes: soybean, cowpea, peanut, chickpea, and bean. Can. J. Bot. **59**: 1671-1679.

Mao, J.Q., M.A. Zaidi, J.T. Aranson and I. Altosaar. 2006. *In vitro* regeneration of *Vigna unguiculata* (L.) Walp. cv. Black eye cowpea via shoot organogenesis. Plant Cell Tissue Organ Cult. **87**: 121-125.

Murashige, T. and E.A. Skoog. 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Muthukumar, B., M. Mariamma and A. Gnanam. 1995. Regeneration of plants from primary leaves of cowpea. Plant Cell Tissue and Organ Cult. **42**: 153-155.

Muthukumar, B., M. Mariamma. K. Valuthambi and A. Gnanam. 1996. Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* (L.) Walp.) using *Agrobacterium tumefaciens*. Plant Cell Rep. **15**: 980-985.

Nehra, S.A. and K.K. Kartha. 1994. Meristem and shoot tip culture: Requirements and applications. *In:* Plant cell and tissue culture. (Vasil, I. and Thorpe, T.A. Eds.) Dordrecht, Netherlands: Kluwer Academic Publishers. pp. 37-70.

Odutayo, O.I., F.B. Akinrimisi, I. Ogunbosoye and R.T. Oso. 2005. Multiple shoot induction from embryo derived callus cultures of cowpea (*Vigna unguiculata* (L.) Walp. African J. Biotech. **4**: 1214-1216.

Pellegrineschi, A. 1997. *In vitro* plant regeneration via organogenesis of cowpea (*Vigna unguiculata* (L.) Walp.). Plant Cell Reports **17**: 89-95.

Pierik, R.L.M. 1993. *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrecht, The Netherlands pp. 183-195.

Popelka, J.C., S. Gollasch, A. Moore, L. Molvig and T.J.V. Huggins. 2006. Genetic transformation of cowpea and stable transmission of the transgenes to progeny. Plant Cell Rep. **25**: 304-312.

Prem Anand, R., A. Ganapathi, A. Ramesh, G. Vengadesan and N. Selvaraj. 2000. High frequency plant regeneration via somatic embryogenesis in cell suspension cultures of cowpea (*Vigna unguiculata* (L.) Walp). In Vitro Cell Dev. Biol. Plant **36**: 475-480.

Rachie, K.O. 1985. Introduction. *In:* Cowpea research production and utilization. (Singh, S.R. and Rachie K.O. Eds). John Wiley and Sons, New York pp. 22-27.

Ramakrishnan, K., R. Gnanam, P. Sivakumar and A. Manickam. 2005. *In vitro* somatic embryogenesis from cell suspension cultures of cowpea (*Vigna unguiculata* (L.) Walp.). Plant Cell Rep. **24**: 449-461

Sebastian, K.T. 1983. Shoot-tip culture and subsequent regeneration in cowpea. Sci. Hort. **20**: 315-317.

Snedecor, G.W. and Cochran, W.G. 1967. Statistical Methods. The Iowa State Univ Press, Iowa, USA. pp. 327-329.

Van Le, B.U.I., M.H.C. De Carvalho, Y. Zuily-Fodil, A.T.P. Thi and K.T.T. Van. 2002. Direct whole plant regeneration of cowpea (*Vigna unguiculata* (L.) Walp.] from cotyledonary node thin cell layer explants. J. Plant Physiol. **159**: 1255-1258.

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