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Comparison of Shoot Regeneration on Different Concentrations of Thidiazuron from Shoot Tip Explant of Cowpea on Gelrite and Agar Containing Medium

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Abstract

Cowpea (*Vigna unguiculata* L. Walp) is an important legume grown all over the world as grain crop, animal fodder, cover crop, gren manure and vegetable. The present study compares effects of agar and gelrite on micropropagation from shoot tip explant of two Turkish cowpea cultivars Akkiz and Karagoz using 0.15, 0.15, 0.35 mg/l Thidiazuron (TDZ), 3 g/l activated charcoal, 2 mg/l yeast extract with and without 1.25 mg/l Polyvinylpyrrolidone (PVP). To overcome problem of endogenic bacterial contaminations, all cultures contained 500 mg/l augmentin and incubated at 24 ± 2o C in 16 h light photoperiod for eight weeks. Therafter, all explants were transferred to MS medium for two weeks for shoot regeneration and elongation under same incubation and photoperiod conditions. The results showed that frequency of shoot regeneration increased with increase in TDZ concentrations in both cultivars on both agar and gelrite gelled medium. Both cultivars showed maximum mean number of shoots per explant in gelrite compared to agar gelled medium. Maximum number of 4.72 and 2.86 shoots per explant were recorded on MS medium containing 0.25 mg/l TDZ in cv. Akkiz and cv. Karagoz respectively. Hyperhydricity was recorded on some regenerated shoots, which was more prominant on agar. Agar gelled medium had greater shoot length compared to gelrite medium in both cultivars. Regenerated shoots were rooted easily on MS medium containing 0.50 mg/l IBA with regeneration of mean number of 4 secondary shoots on cv. Akkiz and 3 on cv. Karagoz. Rooted plantlets were successfully hardened in the growth chamber and subsequently established in the greenhouse; where they flowered and set seeds. The recorded survival rate of the plants was 100%. Plants looked healthy with no visible detecTab. phenotypic variations.

Key words: micropropagation, gelling agents, regeneration, in vitro, cowepea

Introductıon

Cowpea (*Vigna unguiculata* L. Walp.) is a summer annual herbaceous, drought tolerant legume, widely grown in Africa and many parts of the world as grain crop and animal fodder. Sometimes grown for cover crop, green manure but is more generally used as vegetable. Cowpea seed is a nutritious component in the human diet, as well as a livestock feed and is consumed by more than 200 million people on daily basis in many parts of Africa (Popelka *et al*., 2006).

Shoot tip multiplication is generally used for producing virus free material and maintining germplasm via cryopreservation (Nehra and Kartha, 1994). Micropropagation through shoot tip culture has considerable importance as a tool for clonal propagation in legumes (Gulati and Jaiwal , 1992; Pierik, 1993, Brar *et al*., 1997) and could help for easy multiplication of genetically transformed plants. Generally, solidification of regeration media is achieved by adding one or the other type of gelling. Every gelling agent has variable effect on regeneration as has been reported (Chevreau *et al*., 1997, Chauvin *et al*., 1999, Ozel *et al*., 2008).

Agar has been used as gelling agent in most of the plant regeneration studies on cowpea (Kartha *et al*., 1981; Odutayo *et al*., 2005; Popelka *et al*., 2006) with the exception of Ramakrishna *et al*. (2005) and Mao *et al*. (2006); who used phytagel. However, no report describes comparative effect of gelling agents on plant regeneration in cowpea.

The paper compares effects of agar and gelrite on micropropagation from shoot tip of two Turkish cowpea cultivars with aim to investigate the factors responsible for plant regeneration and possible use of the results in breeding and genetic transformation experiments.

Materials and methods

Seeds of two Turkish cowpea cultivars (Akkiz and Karagoz) were obtained from the Department of Field Crops, Faculty of Agriculture, Ege University, Izmir, Turkey. Surface sterilized seeds were germinated in growth chamber at 24 ± 2 oC in 16 h light photoperiod on MS10 basal medium containing 0.7% agar (Duchefa, Germany) and 3.0 % sucrose. Shoot tip explants were obtained from 3-4 days old *in vitro* grown seedlings.

They were cultured in upright position on MS medium (Murashige and Skoog, 1962) with 3.0 % sucrose gelled with 0.65% agar, and 0.21% Gelrite (Sigma) containing 0.15, 0.25 and 0.35 mg/l Thidiazuron-TDZ, 2 mg/l yeast extract with and without 1.25 mg/l PVP (PolyVinyl Proline) as an anti oxidant and 3 g/l active charcoal.

It may be mentioned that agar contain D-galactose, 3,6-anhydrogalactose and agaropectin containing 1,3-glycosidically linked D-galactose (Sigma-Aldrich, St. Louis, MO) and gelrite contain glucuronic acid, rhamnose and glucose (Phytotechnology lab. catalogue, 2006)]. Besides this, both have variable gelling strength (Ozel *et al*. 2008).

The pH of all cultures was adjusted at 5.6-5.8 before autoclaving. Initial experiments showed that the explants were infested with high endogenic bacterial contaminations; therefore, to overcome the problem, all cultures contained 500 mg/l of an anti biotic-Augmentin (Glaxo-Smith Kline, England). The explants were incubated in growth chamber at 24± 2 oC temprature with 16 h light photoperiod.

After 8 weeks of culture, the explants were transferred to MS medium to reduce stress of plant growth regulators on shoot regeneration and elongation. Thereafter, two weeks these shoots were excised from explants and transferred to MS medium containing 0.5 mg/l IBA for rootng.

After two weeks on rooting media, the rooted shoots (plantlets) were taken out from the Magenta vessels® and submerged in water for 30 minutes before transfer to pots containing sterilized peat moss. Pots were covered with transparent polythene bags and placed in growth chamber at room temprature. After seven days, the bags were removed and the plants were allowed to grow at room temprature with 50 % relative humidity.

Statistical analysis

Each treatment for both regeneration and rooting experiments had 8 replicates with 4 explants in each replication (8 x4=40 explants). Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Least Significant Difference Test or t test using "SPSS 16 for Windows" computer program. Data given in percentages were subjected to arcsine (√x) transformation (Snedecor and Cochran, 1967) before statistical analysis.

Results

After two to three days on culture media, the explants started bleeding reddish pink coloured phenolic compounds which were more visible in gelrite compared to agar gelled medium in cultures that did not contain PVP and active charcoal, irrespective of the TDZ concentration. The explants swelled after 9-10 days and showed development of variable number of shoot meristems but the phenolic compounds inhibited the growth of these shoot meristems in to full shoots (numerical results not shown). Therefore, these experiments were discarded at initial stages.

The problem was not observed when the media contained PVP and active charcoal. High frequency of callus induction was recorded in both cultivars, which started from the basal cut ends on both gelling media. (Tab. 1). The explants began to swell with in two to three days of culture with variable number of shoot meirstems after 9-10 days of culture. These developed in to well developed shoots after two weeks of culture.

Frequency (%) of shoot regeneration increased progressively with each increase in TDZ concentrations from 0.15-0.35 mg/l in both cultivars on agar and gelrite gelled MS medium. Hyperhydric shoots were observed on some explants of both cultivars in agar and gelrite containing media. Cv. Akkiz was more promising with negligible hyperdricity. Cv. Karagoz; showed hyperhydricity in both gels; however, it was very prominant in agar gelled medium at 0.25 and 0.35 mg/l TDZ.

The results indicated visible efect of TDZ on shoot rgeneration: the cultivars (genotypes) also played a significant role in the induction of number of shoots per explant. A sharp variation exiasted in the number of shoots regenerated on agar and gelrite based media. Mean number of 2 to 2.06 and 1.89 to 4.72 (Fig. 1) shoots per explant were recorded in cv. Akkız on agar and gelrite base media respectively. Similarly, mean number of 2.39 to 2.78 and 1.39 to 2.86 shoots per explant were recorded in cv. Karagöz on agar and gelrite vbased media respectively (Table1). Irre-

Tab.1. Effects of various concentrations of TDZ on shoot regeneration of cowpea cv. Akkiz and Karagoz on agar and gelrite gelled media

TDZ (mg/l)	Frequency (%) of callus induction				Frequency (%) of shoot regeneration			
	Akkiz		Karagoz		Akkiz		Karagoz	
	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite
0.15	100.00	89.00b	100.00	77.67b	44.33c	89.00b	89.00a	77.67c
0.25	100.00	100.00a	100.00	55.67c	66.67b	100.00a	89.00a	89.00b
0.35	100.00	100.00a	100.00	100.00a	100.00a	100.00a	78.00b	100.00a
TDZ (mg/l)	Mean number of shoots per explant				Shoot length (cm)			
	Akkiz		Karagoz		Akkiz		Karagoz	
	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite
0.15	2.00b	1.89bc	2.39ab	1.39b	2.27c	1.88a	4.53a	3.49a
0.25	2.84a	4.79a	2.78a	2.86a	2.62b	1.79a	3.20b	2.86b
0.35	2.66ab	2.44b	1.91b	2.11b	4.03a	1.45b	2.70c	1.39c

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

spective of the gelling media, maximum number of shoots per explant were recorded on MS media containing 0.25 mg/l TDZ.

Longer shoots were recorded on agar based media in both cultivars compared to shoot length on gelrite based media. Each increase in TDZ concentration from 0.15-

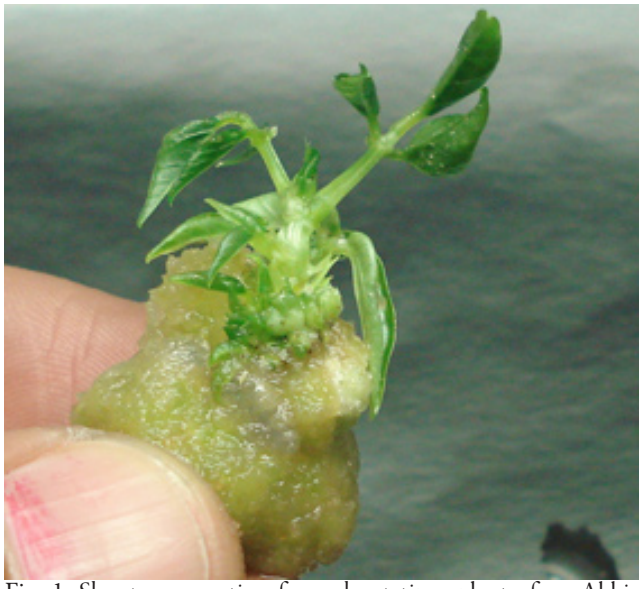

Fig. 1. Shoot regeneration from shoot tip explant of cv. Akkiz bar=0.5 cm.

0.35 mg/l also improved the mean shoot length from 2.27-4.03 cm on MS medium gelled with agar in cv. Akkiz,. However, gelrite resulted in inhibition in shoot length with each increase in TDZ concentration with mean shoot length of 1.79 to 1.45 cm. Cv. Karagoz behaved variably and showed reduction in shoot length with each increase in the concentration of TDZ on agar and gelrite with range of 4.53 to 2.70 cm and 3.49 to 1.39 cm respectively.

Rhizogenesis

All shoots of cv. Akkız regenerated on MS medium containing 0.25 mg/l TDZ gelled with agar and gelrite solidified medium were rooted easily on MS medium containing 0.5mg/l IBA. Contrarily, the shoots of cv. Karagoz regenerated on 0.25 mg/l TDZ in agar based medium were highly hyperhydric and did not root. However, this problem was not observed in gelrite regenerated shoots on 0.25 mg/l TDZ. Once the rooting started, each of the rooted shoots were transferred to single magenta for better growth and development of both roots and shoots (Fig. 2a). The results showed mean number of 2.95 roots in cv Akkiz and 3.44 roots per shoot in cv Karagoz. Besides rooting, IBA also promoted mean number of 4 secondary shoots per rooted shoot on cv. Akkiz and 3 in cv. Karagöz . Secondary shoots were also rooted on MS medium containing 0.5 mg/l IBA. All plants established successfully and set seeds at maturity in the greenhouse (Fig. 2b). Plants looked healthy with no visually detecTab. phenotypic variations.

Discussions

This experiment made use of agar and gelrite to know the best gelling media to be use as gelling agent in the tissue culture of cowpea. The genotypic changes in cowpea were not expected because cultures were grown with normal life regulatory factors such as same levels of plant growth regulators and nutrient medium, light source, and growth temperature, during the course of experiment. Different types of sugars in agar and gelrite and variable hardness of respective gels due to biochemical and structural differences affected the molecular diffusion of growth regulators and nutrients through the medium. It also resulted in quantitative and qualitative variations in the number of shoots of the cultured explants from both cultivars in agreement with Ozel *et al*. (2008).

The results showed clear influence of gelling agents and concentration of plant growth regulators (TDZ) and addition of active charcoal on the frequency (%) of callus induction, frequency (%) of shoot regeneration, mean number of shoots per explant and shoot length.

Cytokinins and auxins alone or in combinations are commonly used for plant regeneration in legumes. Thidiazuran (TDZ) is cytokinin like substance which facilitates efficient micropropagation and induce greater axillary proliferation than many other cytokinins at low concentrations (Huetteman 1993). Previous reports on successful plant regeneration in cowpea indicates mainly the use of BAP (Brar *et al*. 1997; Odutayo *et al*. 2005; Muthukumar *et al*. 1995; Brar *et al*. 1999; Chaudhury *et al*. 2007), 2,4 D (Ramakrishnan *et al*. 2005; Prem Anand *et al*. 2007); 2,4,5-T (Muthukumar 1995) and Zeatin (Prem Anand *et al*. 2000). However, Van Le (2002) reported plant regeneration from thin cell layer explants obtained from cotyledonary node using TDZ in combination with IBA in cowpea.

In vitro regeneration of cowpea by using primary leaves (Ramakrishnan *et al*. 2005; Muthukumar *et al*.1995; Prem Anand *et al*. 2000), cotyledonary node (Chaudhury *et al*. 2007; Van-Le 2002), mature cotyledon (Muthukumar *et al*. 1995; Brar *et al*. 1999), embryonic axis (Popelka *et al*. 2005), mature embryo (Odutayo *et al*. 2005), hypocotyl (Pellergrieschi 1997) and immature cotyledon (Prem Anand *et al*. 2000; Choi *et al*. 2003) has also been described previously. However, only Brar *et al*. (1997), Kartha *et al*. (1981), Mao *et al*. (2006) and Aasim *et al*. (2008) describe the use of shoot meristem/apices/tip as explant of choice. Shoot meristem explant has proved useful for the propagation of legumes including cowpea.

As expected, the shoot meristems cultured on different concentrations of TDZ showed variable behavior on agar and gelrite gelled media for induction of shoots and shoots cultured on MS medium containing different con-

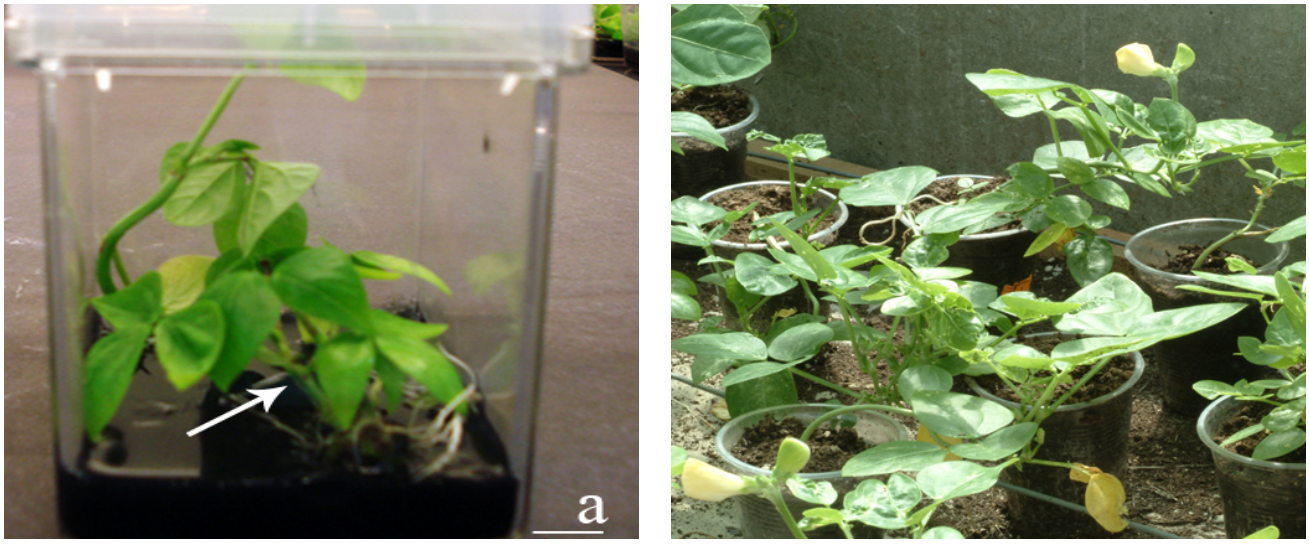

Fig. 2. Rooting and acclimatisation of tissue cultured plants (a) well developed rooted shoot of cv. Akkız (b) acclimatised and flowering plants in the greenhouse. bar Fig. 2a=1cm, Fig.2b=1.5 cm.

centrations of TDZ. Comparing growth parameters, the best adventitious shoot regeneration was obtained on gelrite gelled media showing that it provided better diffusion of media components to the plant tissues, allowing better *in vitro* development. This resulted in increased availability of plant growth regulators and other nutrients in the respective media and contributed to enhanced growth and regeneration from shoot meristems in agreement with Ozel *et al*. (2008).

Comparing agar and gelrite retardation in the shoot length at different concentrations of TDZ in gelrite medium might also be due to higher viscosity of gelrite offering inhibition due to lesser diffusion of nutrients through the medium. It was concluded that all variations in shoot regeneration were primarily due to the kind of gelling agent followed by the concentration of TDZ and the variations in genotypes. The total yield of tissues, shoot initiation and proliferation is dependent on the carbohydrate supply (Upper *et al*. 1970). It was found that agar made better supply of carbohydrate that resulted in the maximum elongation of shoots compared to gelrite.

Longer shoots were recorded on agar based medium compared to gelrite based medium in all treatments in both cultivars, which showed the positive effect of agar compared to gelrite in both cultivars.

Rhizogenesis

Hyperhydric shoots irrespective of the type of cultivar on agar gelled medium were transferred to MS medium to overcome the problem.Where, the shoots recovered but no rooting was observed on rooting medium containing 0.5 mg/l IBA. Ziv (1991) related hyperhydricity with ethylene accumulation, gelling agent concentration or type, nutrient compositionon of the medium. However, this problem was not noted on shoots regenerated on gelrite gelled media. All regenerated shoots rooted easily on MS medium containing 0.5mg/l IBA. Interestingly, the rooting media also promoted shoot regeneration and resulted in induction of secondary shoots developing at the base from a single shoot on agar gelled medium (Fig. 2a). No secondary shoot regeneration was observed on gelrite gelled medium. Secondary shoots were also rooted on the same rooting media. All the secondary shoots were more vigorous compared to mother shoots and rooted easily on MS medium 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). It was concluded that

- Shoot meristems behaved variabley on MS medium gelled with agar and gelrite in both cultivars. Shoot elongation behavior in both cultivars also varied depending upon the gelling agent and concentration of TDZ.
- MS medium containing 0.25 mg/l TDZ was the best for obtaining maximum number of shoots per explant from both cultivars. However, gelling with agar was associated with hyperhydricity.
- It was not difficult to root the regenerated shoots on MS medium containing 0.5 mg/l IBA, which besides inducing roots also induced secondary shoots on the explants.
- Additional research is needed to understand the mechanism of action of IBA for induction of adventitious shoots. Knowledge of this would be beneficial to describe the interaction of IBA and cowpea shoot meristem explants more appropriately.

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