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Full Length Research Paper

TDZ x IBA induced shoot regeneration from cotyledonary leaves and *in vitro* multiplication in safflower (*Carthamus tinctorius* L.)

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A high frequency adventitious shoot regeneration protocol for safflower (*Carthamus tinctorius* L.) cv. Dinçer with high yield (Turkish cultivar) using cotyledonary leaves of 10 day-old seedlings were optimized by studying the influence of different combinations of thidiazuron (TDZ) and indole-3-butyric acid (IBA). Cotyledonary leaves were cultured on Murashige and Skoog medium (MS) supplemented with different concentrations and combinations of TDZ and IBA. The highest percentage of regenerated shoots (33.33%) and the highest number of shoots per explant (6.5) occurred on a MS medium containing 0.5 mg/l TDZ and 0.25 mg/l IBA. Furthermore, cotyledonary nodes and meristem tips of 14 day-old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of N^6-benzylamino-purine (BAP) alone or combination of α-naphthalene acetic acid (NAA). Direct multiple shoots from cotyledonary nodes and meristem tips developed within 18 - 21 days in most media tested. 100% shoot multiplication was achieved from cotyledonary node and meristem tip on a range of MS media supplemented with different concentrations of BAP and NAA.

Key words: *Carthamus tinctorius*, regeneration, cotyledonary leaves.

INTRODUCTION

Safflower, (*Carthamus tinctorius* L.), is a member of the family *Compositae* or *Asteraceae*, important oilseed crop of semi-arid regions and cultivated mainly for its seed. The crop is cultivated in India, Mexico Argentina, Australia, Canada, China, Spain, Italy, Turkey, Iraq, Iran, Egypt, and Ethiopia and is an alternative oil crop for the dry lands of these countries. The crop has high oil content (35%) and its oil is golden yellow in color and contains high amount of linoleic acid. It occupies a unique position among oil seed crops due to the high linoleic content of its seed oil which has therapeutic value (Nikam and Shitole, 1999). The crop has been also traditionally grown for its flowers, used for colouring, flavouring foods and making dyes exclusively as a source of red dye carthamin extracted from its florets (Dajue and Mündel, 1996). It has resistance to cold, drought and salinity stress. Therefore, it could be grown successfully on dry lands and surrounding regions which have insufficient precipitation.

Successful utilization of plant biotechnology for plant improvement requires the development of an efficient shoot regeneration system from cultured cells or tissues. The development of an efficient micropropagation protocol can highly support breeding of this potential and adaptive oil crop and the establishment of cell culture has considerable potential in the future as an alternative for the production of new secondary metabolites (Stockigt et al., 1995). Gene transfer manipulations are also used for genetic modification of important yield characters and composition of fatty acids in oil seed yielding crop. An efficient and reliable *in vitro* plant regeneration system is

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Abbreviations: TDZ, thidiazuron; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; BAP, N^6-benzylamino-purine; and NAA, α-naphthaleneacetic acid.

a principal need for genetic transformation studies. Plant regeneration from hypocotyls (George and Rao, 1982; Nikam and Shitole, 1999), immature embryos, seedling leaves (Orlikowska and Dyer, 1993; Nikam and Shitole, 1999) and root (Nikam and Shitole, 1999) was achieved via organogenesis and somatic embryogenesis in this important crop. In addition to these, callus induction and *in vitro* plantlet regeneration systems were optimized using cotyledonary explants by direct organogenesis (George and Rao, 1982; Orlikowska and Dyer, 1993; Tejovathi and Das, 1997; Nikam and Shitole 1999; Mandal and Gupta 2001; Neetika et al., 2005) and direct somatic embryogenesis (Mandal et al., 1995; Mandal et al., 2001; Mandal and Gupta, 2003). It is very important to increase shop multiplication frequency for genetic transformation studies of safflower. However, regeneration frequency and rooting capacity of safflower were low (Nikam and Shitole, 1999). The interaction of explant and growth regulator was found to be significant in the earlier investigations on safflower tissue culture. (George and Rao, 1982; Sujatha and Suganya, 1996; Orlikowska and Dyer, 1993). The differences among different parts of the same plant may be attributed to the various levels of endogenous plant growth regulators of explants from different positions (Özgen et al., 1998; Uranbey et al., 2005). Though there are many reports on the regeneration of safflower, there are still no data concerning regeneration using thidiazuron (TDZ) and indole-3-butyric acid (IBA) combinations as a cytokinin-auxin source for cotyledonary leaves of safflower. Use of IBA with TDZ might be best treatment to eliminate the secretion of phenolic substances. The aim of the current study was therefore to determine the role of the combinations of TDZ and IBA for cotyledonary leaves and to increase direct shoot regeneration frequency using cotyledonary node and meristen tip of safflower cv. Dinçer (a high yielding Turkish cultivar).

MATERIAL AND METHODS

Plant material

Seeds of safflower cultivar Dinçer were obtained from Anatolia Agricultural Research Institute Eskişehir, Turkey. Dinçer cv. is a high yielding genotype and adapted to the semi arid conditions of Central Anatolian and Transitional Zones in Turkey.

Surface sterilization, isolation of explant sources and culture conditions

The seeds were surface-sterilized in 70% ethanol for 2 min and then in 30% commercial bleach (Axion) containing 6% sodium hypochlorite for 30 min. Surface sterilized seeds were germinated in Magenta (GA-7) vessels containing 40 ml of MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. Cotyledonary explants were excised from germinating seeds after 10 days. The explants were cut across discarding the petiole and the lower 1 - 2 mm of cotyledonary base. Edges of cotyledonary leaves were also trimmed off. The callus and shoot induction medium was composed of MS basal medium containing 3% sucrose and 0.8% agar. The cotyledonary leaves were cultured on the callus and shoot induction media supplemented with different concentrations of thidiazuron (TDZ; 0.05, 0.1 and 0.5 mg/l) and indole-3-butyric acid (IBA; 0.25 and 0.5 mg/l) for adventitious soot regeneration studies in Petri dishes (100 x 10 mm). Cotyledonary nodes and meristem tips were also excised from germinating seeds after 14 days for direct shoot organogenesis. Cotyledonary nodes (2 - 3 cm length) and meristem tips (1 - 2 mm length) were cultured on MS basal medium supplemented with various concentrations of N^6-benzylamino-purine (BAP; 0.5 and 1.0 mg/l) alone or combination of α-naphthaleneacetic acid (NAA; 0.02 and 0.2 mg/l) in Magenta (GA-7) vessels to determine their influence on shoot multiplication. Stock solution of 1 mg/ml TDZ was prepared either by using dimethyl sulphoxide (DMSO; Sigma Technical Information bulletin 1996) or 50% ethanol as solvent. Ethanol diluted TDZ was incorporated into the medium and retained its high activity even after autoclaving as described by Khawar et al. (2004).

Scoring for adventitious soot regeneration and microprogation was done after 8 - 10 weeks of culture. The medium was adjusted to pH 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 121ºC, 1.4 kg/cm^2 for 20 min. The cultures were kept at 24 ± 2ºC under cool white fluorescent light (35 µmolm^{-2}s^{-1}) in 16 h photoperiod. Samples of all explants were subcultured onto fresh optimum callus and shoot induction media every 30 days in all experiments. In order to induce rooting, the shoots (3 - 4 cm long) regenerated from different explants were excised and were transferred to half strength MS medium supplemented in Magenta vessels containing 50 ml of medium. Rooted plantlets were acclimatized to ambient conditions and later established under greenhouse conditions.

Experimental design

Each treatment had three replicates consisting of Petri dishes containing 10 explants for adventitious soot regeneration study. Each treatment had four replicates consisting of Magenta vessels each containing 3 explants for micropropagation. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range test using MSTAT-C computer programme (Michigan State University). Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis. All the experiments described here were repeated at least two times and all results were pooled.

RESULTS AND DISCCUSION

Most of cotyledonary leaves elongated, enlarged and formed green-yellow colored compact callus about 22 - 25 days after culture initiation (Figure 1a). Although induction of callus was observed in all media, there was no statistically difference among the concentrations of TDZ and IBA (Table 1). Most of explants formed callus in all concentrations of TDZ and IBA. Shoot proliferation from cotyledonary leaves was visible after 25 - 30 days in all media tested (Figure 1b). These shoot primordia subsequently developed into normal shoots 45 - 50 days after culture initiation in all concentrations of TDZ and IBA (Figure 1c). TDZ is very soluble in DMSO with slight solubility in water (Khawar et al., 2004). Although most published reports describe the use of DMSO as a solvent

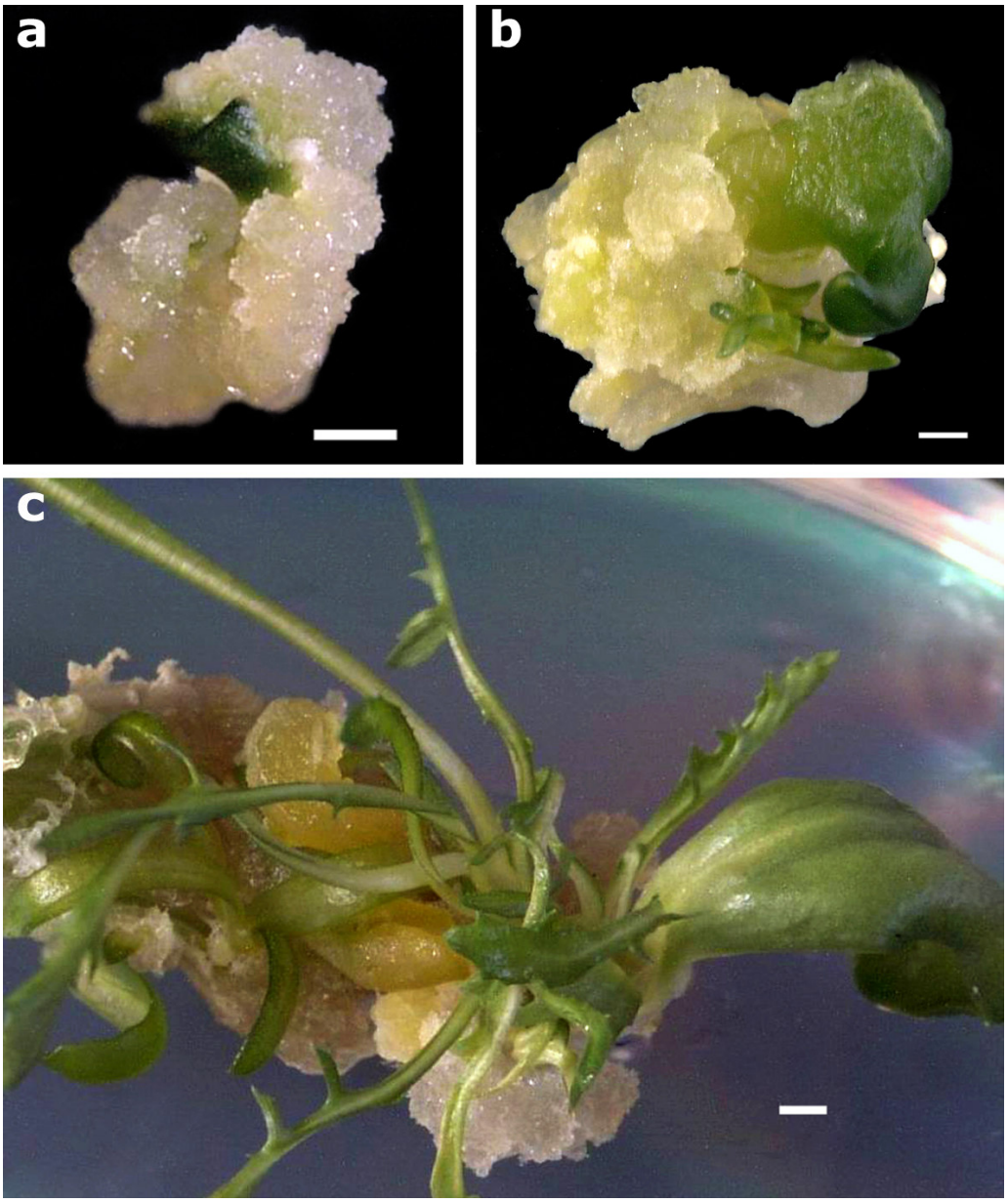

Figure 1. Adventitious shoots regeneration cotyledonary leaves of safflower on a medium supplemented with 0.5 mg/l TDZ and 0.25 mg/l IBA. (a) Compact callus formation after 22 - 25 days of culture. (b) Appearance of shoot initials on cotyledonary leaves after 25 - 30 days of culture. (c) Developed adventitious shoots from cotyledonary leaves after 45 - 50 days of culture. Bar = 0.5 cm for a, c and 0.75 cm for b

for TDZ, we observed that the use of DMSO as a solvent resulted in necrosis on explants with no callus formation at the preliminary studies as reported in lentil by Khawar et al. (2004). Ethanol diluted TDZ was used in the experiment. Perbal (1988) also stated that DMSO may have a detachment or killing effect on sensitive cells.

The percentage of explants producing shoots and the number of shoots per explant were influenced by concentrations of TDZ and IBA tested ($p<0.01$). The percentage of regenerated shoots fluctuated between 0.00 - 33.33%. The highest percentage of regenerated shoots (33.33%) and the highest number of shoots per explant (6.5) occurred with 0.5 mg/l TDZ and 0.25 mg/l IBA. Considering both percentage of explants producing shoots and the number of shoots per explant, the best shoot multiplication was achieved on a range of media supplemented with 0.5 mg/l TDZ and 0.25 mg/l IBA. Drastic reductions in shoot regeneration were also observed when decreasing of concentrations of TDZ. Higher TDZ concentrations increased shoot regeneration

Table 1. Effect of various concentrations of TDZ and IBA on adventitious shoot regeneration from cotyledonary leaves of safflower.

Growth regulator (mg/l)		Callus ratio (%)	Explants roducing shoots (%)	Mean number of shoots per explant
TDZ	IBA			
0.5	0.50	93.33^{ns}	6.70*bc	2.8*b
0.5	0.25	96.66	33.33a	6.5a
0.1	0.50	96.66	20.00b	3.1b
0.1	0.25	90.00	10.00b	1.3c
0.05	0.5	90.00	16.70b	1.1c
0.05	0.25	100.00	0.00c	0.0d

*Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.
^{ns}Not significant.

and resulted in stunted shoots, as has been reported for pea (Malik and Saxena, 1992) and lentil (Khawar et al., 2004). In most cases, callus formation and number of shoots originated from responding region were either low or negligible in safflower. Maximum of 7.5 shoots from entire cotyledonary explants of safflower have been reported up to date (Nikam and Shitole, 1999). These results show the importance of TDZ and suggest that a higher dose of TDZ induces high frequency of shoot regeneration from cotyledonary leaves. The shoot organagogenesis of some crops in tissue culture have been recently achieved using thidiazuron (TDZ), a substituted phenylurea compound with cytokinin activity (Malik and Saxena, 1992; Kanyand et al., 1994; Kim et al., 1997; Jain and Rashid, 2001, Singh et al., 2002; Hosseini and Rashid, 2003; Thomas, 2003; Uranbey, 2005). Gill and Saxena (1992) suggested a crucial role of TDZ in the interaction with endogenous hormones in reprogramming the mode of morphogenesis from organogenesis to somatic embryogenesis possibly by releasing, synthesising, protecting or even inhibiting auxins *in situ* in combination with other sub-cellular metabolic changes, particularly in key regulatory enzyme and related proteins. Frequency of shoot organogenesis may be increased with combinations of TDZ and IBA. Combinations of TDZ-IBA in the media revealed an efficient pathway for shoot proliferation in cotyledonary leaves of safflower. The use of IBA with TDZ might be best treatment to eliminate the secretion of phenolic substances and this effect might be also due to the oxidation of phenols by auxin oxidase. Çöçü et al. (2004) stated that combination of TDZ and IBA induced shoot regeneration from cotyledonary nodes in *Calendula officinalis*. Our results partially support these reports and clearly underline the importance of combination of TDZ and IBA in efficient morphogenesis from cultured cotyledonary or other explant types. No abnormality, necrosis or chlorosis was observed during the culture. Adventitious shoot buds were induced directly on the adaxial surface of the cotyledons of eight safflower cultivars after 14 d of culture initiation on MS medium supplemented with various levels BA (Mandal and Gupta, 2001). Tejovathi and Das (1997) obtained about 20 - 73% plant regeneration when cotyledonary leaves from 4 - 6 day-old seedlings of safflower were cultured on modified Gamborg's medium (B5) supplemented with different concentrations of BA. The standardized protocol involves culture of cotyledonary explants that are 2 cm long, 2 to 6-day-old seedlings on MS supplemented with 1.0 mg/l BAP and 0.1 mg/l in safflower (Neetika et al., 2005). Whereas, we firstly cultured entire cotyledonary leaves 10 day-old on MS supplemented with different concentrations and combinations of TDZ and IBA as a cytokinin-auxin source. Thus, the TDZ and IBA combinations tested for cotyledonary leaves at the present investigation as the frequency of shoot induction was high.

In the present study, basal regions of cotyledons isolated from 10 day-old seedlings were used. Similarly, regeneration frequency was found to be slightly more in entire cotyledons than pieces of cotyledonary and more shoots were produced on explants cut from the most basal region of cotyledons from 5 to 7 day-old seedlings than from older seedlings (Nikam and Shitole, 1999).

Cotyledon nodes and meristem tips were also excised from germinating seeds for direct shoot organogenesis studies. Prolific shoot regeneration from cotyledonary node and meristem tips were observed after 18 - 21 days of culture (Figure 2ab). Differential response of the two explants was observed on the different media for shoot multiplication. The effects of combinations of BAP x NAA and explants on shoot multiplication were statistically significant. Also, explant types and plant growth regulators interaction was significant ($p<0.05$) (Table 2). Most of explants produced shoots and green shoot initials were seen on a range of media containing BAP and NAA within four weeks. The highest percentage of regenerated shoots (100.00%) was achieved on a range of media supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA or 1.0 mg/l BAP + 0.2 mg/l NAA in cotyledonary node explants. Whereas, the highest shoot multiplication capacity (100%) was obtained on a medium containing 0.5 mg/l BAP alone, 0.5 mg/l BAP + 0.02 mg/l NAA or 1.0 mg/l

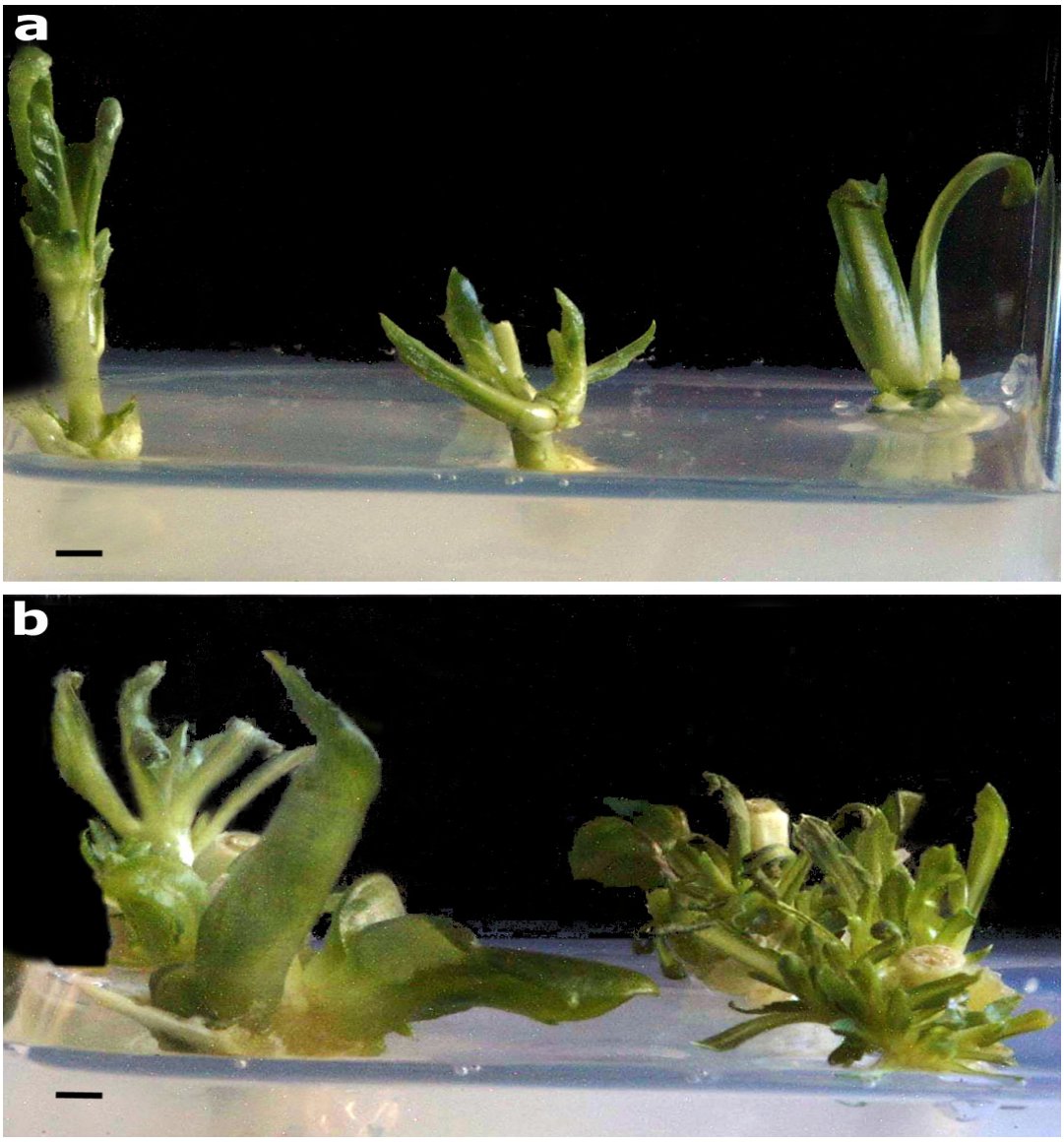

Figure 2. *In vitro* micropropagation of safflower. (a) Prolific shoot formation from meristem tips on a media supplemented with 1.0 mg/l BAP + 0.2 mg/l NAA after 18-21 days of culture. B) Formation of multiple shoots on cotyledonary node on a media supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA after 18-21 days of culture. Bar = 0.1 cm for a and b.

Table 2. Effects of BAP and NAA combinations on shoot multiplication from cotyledonary node and meristem tip of safflower.

Growth regulator (mg/l)		Explants producing shoots (%)		Mean number of shoots per explant	
BAP	**NAA**	**Cotyledonary node**	**Meristem tip**	**Cotyledonary node**	**Meristem tip**
0.5	0.0	77.77*ab	100.00*a	2.33*b	1.33*b
0.5	0.2	100.00a	88.88ab	4.72a	5.67a
0.5	0.02	66.66bc	100.00a	2.49b	2.67b
1.0	0.0	88.88ab	33.33c	2.77b	4.41ab
1.0	0.2	100.00a	100.00a	3.21ab	4.23ab

*Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.

BAP + 0.2 mg/l NAA in meristem tips. Considerable decreases in shoot multiplication were also observed on a medium containing 1.00 mg/l BAP alone in meristem tips. The highest number of shoots per explant was also obtained from a medium supplemented with 0.5 mg/l BAP + 0.2 mg/l NAA in both explants. Both explant types gave high multiple shoots and the results further showed that cotyledon node and meristem tips explants were very favorable explants with a high multiplication ratio (100%) at any concentration of BAP and NAA. Selection of a suitable explant at correct developmental stage plays a key role in the successful establishment of culture under *in vitro* conditions. Morphological integrity of an explant along with the proper choice of plant grow regulators strongly influence induction of optimal callus and shoot regeneration (Khawar et al., 2005). The multiple shoot induction rate and morphogenetic response significantly varied to a greater extent according to the explant type and plant growth regulators concentrations (Özgen et al., 1998; Uranbey, 2005). Type of explant and culture medium with specific growth regulator concentrations influenced the organogenesis in the present study and cotyledonary node and meristem tips could be used for rapid clonal propagation with optimized culture medium.

All regenerated shoot tips (20 - 35 mm length) were excised and rooted readily in half strength MS medium supplemented. Rooting was observed from the cut ends of the shoots within 30 days in most media tested. All of the developing roots were physically vigorous and healthy.

Conclusion

In conclusion, the results recorded during the present investigation clearly suggest that entire cotyledon leaves obtained from 10 day-old seedlings of safflower are very important explant type for efficient shoot regeneration. Furthermore, the present study underlines the importance of combinations of TDZ and IBA for high shoot regeneration from cotyledonary leaves by organogenesis. Furthermore, cotyledonary node and meristem tips have a great direct shoot multiplication potential and may be used easily transformation studies. We have been trying to obtain transgenic shoots from cotyledonary leave explants of various safflower genotypes using disarmed GV 2260, EHA 105 and LBA 4404 *Agrobacterium tumefaciens* strains that will potentially lead to large scale production of transgenic safflower.

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