EFFICIENT PLANT REGENERATION OF BITTERSWEET (*SOLANUM DULCAMARA* L.), A MEDICINAL PLANT

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(Received: May 8, 2008. Accepted: September 10, 2008)

ABSTRACT

Solanum dulcamara L. (bittersweet) is a medicinal plant that has been used to treat skin diseases, warts, tumors, felons, arthritis, rheumatism, bronchial congestion, heart ailments, ulcerative colitis, eye inflammations, jaundice and pneumonia. A reliable in vitro culture protocol for bittersweet was established. Explants (leaf and petiole segments) were cultured on Murashige and Skoog minimal organics (MSMO) medium with various plant growth regulator combinations. Leaf explants formed more shoots than petiole explants. Plant regeneration was observed through indirect organogenesis with both explants. Best shoot proliferation was obtained from leaf explants with 3 mg/l BA (benzyladenine) and 0.5 mg/l IAA (indole-3-acetic acid). Regenerated shoots were transferred to rooting media containing different levels of IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), NAA (naphthalene acetic acid) or 2,4-D (2,4 dichlorophenoxyacetic acid). Most shoots developed roots on medium with 0.5 mg/l IBA. Rooted explants were transferred to vermiculate in Magenta containers for acclimatization and after 2 weeks, they were planted in plastic pots containing potting soil and maintained in the plant growth room.

KEY WORDS: *Solanum dulcamara*, bittersweet, common nightshade, in vitro culture, plant regeneration.

INTRODUCTION

Solanum dulcamara L. (bittersweet), a semi-woody herbaceous perennial vine, is a member of the Solanaceae family. It has a long history of use in the treatment of skin diseases, arthritis, rheumatism, bronchial congestion, heart ailments, ulcerative colitis, jaundice and pneumonia (Grieve 1982; Chevallier 1996; Baytop 1999). It is native to Europe, North Africa, and Northern Asia and occurs in a very wide range of habitats, from woodlands to scrubland, hedges and marshes (Grieve 1982; Dobelis 1990; Chevallier 1996).

Bittersweet is a rich source of glycosides (Rönsch and Schreiber 1966; Friedman and McDonald 1997) and used in the commercial production of steroidal hormones (Ehmke et al. 1995). Aqueous extract of *S. dulcamara* works as a platelet activating factor inhibitor so it thins the blood (Tunon et al. 1995). Alcoholic extract of *S. dulcamara* showed tumor-inhibitory activity against Sarcoma 180 in mice and systematic fractionation of the extract has led to isolation and characterization of β-solamarine as an active principle (Kupchan et al. 1965). A haploid clone of *S. dulcamara* was obtained by anther culture from isolated protoplasts of shoot tip (Binding and Mordhorst 1984). Pluronic F-68 has been used as growth-stimulating additives to plant culture media on the growth of transformed roots, callus and protoplasts of *S. dulcamara* (Kumar et al. 1992).

Bittersweet is a valuable medicinal herb, but there are no reports on an in vitro culture protocol of this species. The present study describes, to our knowledge for the first time, an efficient in vitro plant regeneration protocol through indirect organogenesis for *S. dulcamara* via adventitious shoot development from leaf and petiole explants cultured on medium containing different concentrations and combinations of various plant growth regulators.

MATERIAL AND METHODS

Seeds of *S. dulcamara* were collected from Abant Lake, Bolu/Turkey in September of 2006. Identification of the species was made by using "Flora of Turkey and The East Aegean Islands" (Davis 1978) and voucher specimens (AUT-1438) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey.

Seeds were washed with an anti-bacterial soap, rinsed with distilled water and surface sterilized by shaking in 20% ethanol for 20 minutes followed by rinsing well with

sterilized distilled water and then dipped into 20% Domestos® (5% sodium hypochloride) for 20 minutes. They were finally washed with sterile distilled water three times. After surface sterilization of the seeds, they were placed in sterile, disposable petri dishes containing Murashige and Skoog's minimal organics (MSMO) medium (4.43 g/l MSMO, Sigma Chemical Co., St. Louis, MO, USA; Murashige and Skoog 1962) with 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121˚C and 105 kPa).

After a one week incubation in this medium, seedlings were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing the same medium for an additional three weeks. For shoot regeneration, leaf lamina (36 mm^2) and petiole segments (5-6 mm) were excised from four weeks old sterile seedlings and placed in sterile disposable petri plates containing 4.43 g/l MSMO with different combinations and concentrations of plant growth regulators; kinetin (1, 3 and 5 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1 and 0.5 mg/l); benzyladenine (BA; 0.1, 0.5, 1 and 3 mg/l) and indole-3-acetic acid (IAA; 0.5 and 1 mg/l); BA (0.5, 1 and 3 mg/l) and naphthalene acetic acid (NAA; 1, 3 and 5 mg/l); thidiazuron (TDZ; 0.1, 0.5, 1 and 3 mg/l) and IAA (0.5 and 1 mg/l); TDZ (0.1, 0.5 and 1 mg/l) and 2,4--D (0.1, 0.5 and 1 mg/l); TDZ (0.1, 0.5, 1 and 3 mg/l) and indole-3-butyric acid (IBA; 0.1, 0.5, 1 and 3 mg/l).

After three weeks, regenerated shoots were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MSMO for shoot elongation for an additional two weeks. Shoots were then separated individually and placed in rooting medium containing MSMO and varying concentrations of different auxins; indole-3-acetic acid (IAA; 0.5, 1 and 3 mg/l); indole-3-butyric acid (IBA; 0.5, 1 and 3 mg/l); 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 0.1 and 0.5 mg/l) or naphthalene acetic acid (NAA; 0.5, 1 and 3 mg/l).

All cultures were incubated at 22˚C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$). After three weeks, rooted explants were transferred to vermiculate (Agrekal®) in Magenta containers for acclimatization and after 2 weeks, they were transferred to plastic pots containing potting soil.

Data analysis

Mean number of shoots per explant and the percentage of explants producing shoots were determined after 2 weeks incubation on elongation medium. Moreover, mean number of roots per shoot and percentage of shoots that rooted were determined after 3 weeks incubation on rooting medium. Both shoot regeneration and rooting experiments had 10 replications for each explant and were repeated three times. Results were expressed as means ± standard error of the mean. All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

S. dulcamara is a valuable medicinal herb, but there has been almost no interest in the in vitro propagation of this species. We therefore, aimed to develop a protocol for high frequency regeneration of bittersweet plants by adventitious organogenesis.

Two different explants (leaf and petiole) were used for the regeneration experiments. When leaf and petiole explants were cultured on MSMO medium containing BA or kinetin in combination with 2,4-D, IAA or NAA, generally all explants tested formed shoots with BA in combination with IAA, and kinetin in combination with 2,4-D.

Best shoot proliferation was obtained with leaf explants. Petiole explants formed fewer shoots than leaf explants (Table 1). Overall, number of shoots per explant was lower for all explants on media with BA plus NAA combinations when compared to BA plus IAA combinations (Table 1).

With leaf explants, the greatest number of shoots per explant was observed on media with 3 mg/l BA plus 0.5 mg/l IAA (40±7.07 shoots per explant; 100% explants formed shoots) (Table 1; Fig. 1a). Media containing 3 mg/l kinetin (33.88±1.95 shoots per explant; 100% explants formed shoots) and 3 mg/l BA (33.13±1.62 shoots per explant; 100% explants formed shoots) were also effective for shoot formation with leaf explants (Table 1).

With petiole explants, the greatest number of shoots per explant was observed on media containing 3 mg/l kinetin plus 0.1 mg/l 2,4-D (28±0.82 shoots per explant; 100% explants formed shoots) and 3 mg/l kinetin (10.38±0.61 shoots per explant; 100% explants formed shoots) (Table 1).

When leaf or petiole explants were cultured on media containing BA or kinetin in combination with 2,4-D, IAA or NAA, it is evident that the frequency of shoot formation was closely related to concentrations of the auxin supply (Table 1). Increasing concentrations of auxin severely inhibited shoot development. For example, among leaf explants investigated, 1 mg/l IAA in combination with 3 mg/l BA decreased mean number of shoots per explant to 1.38 and the number of the shoots producing explants to 62% while 0.5 mg/l IAA in combination with 3 mg/l BA induced shoots in all explants and mean number of shoots per explant was 40 (Table 1). Similarly, among petiole explants investigated, although 0.5 mg/l 2,4-D in combination with 3 mg/l kinetin did not form shoots, 0.1 mg/l 2,4--D in combination with 3 mg/l kinetin was the most effective combination for shoot formation.

In addition, some root formation was observed in the treatments which were used higher NAA (3 and 5 mg/l), 2,4--D (0.5 mg/l) or IAA (1 mg/l). Such effects of auxin on shoot and root developments are in agreement with the classical findings of Skoog and Miller (1957); a low ratio of auxin to cytokinin induces shoots, while the reverse situation induces roots.

Callus was observed for leaf and petiole explants before shoot formation (indirect organogenesis). In control experiments (plant growth regulator free medium) none of the explants formed shoots (Table 1).

Leaf and petiole explants did not form shoots on media containing TDZ in combination with IAA, IBA or 2,4--D (data not shown). Only 0.1 mg/l TDZ formed shoots on leaf and petiole explants (Table 1). TDZ was not an effective growth regulator for shoot induction of *S. dulcamara* explants.

Regenerated shoots were separated individually and put on rooting medium. They formed roots in 3-4 weeks (Fig. 1b). If we compare among auxins investigated for rooting, IAA and IBA were better than 2,4-D and NAA (Table 2).

TABLE 1. Shoot development from leaf and petiole explants incubated on media containing combinations of BA, kinetin or TDZ with 2,4-D, IAA or NAA after 2 weeks incubation on elongation medium. Data are presented as mean number of shoots per explant ± standard error (SE).

[a, b, c, d, e, f, g, h, i, j] Mean values with the same letters within vertical columns are not significantly different ($P>0.05$) (i.e., Comparison of hormon combinations for each explant).

[xy] Mean values with the same letters within horizontal columns are not significantly different ($P>0.05$) (i.e., Comparison of explants for each hormon combination).

Treatments		EXPLANTS			
		Leaf		Petiole	
MSMO medium without growth regulators		Mean # of shoots per explant	% explants forming shoots	Mean # of shoots per explant	% explants forming shoots
		–	–	–	–
Kinetin (mg/l)	**2,4-D (mg/l)**				
1.0	0.0	26.6±1 cd x	100	9.0±0.4 c y	100
1.0	0.1	23.8±2.7 de x	100	7.63±0.8 c y	100
1.0	0.5	–	–	–	–
3.0	0.0	33.9±2 b x	100	10.4±1.6 b y	100
3.0	0.1	13.9±0.6 fg y	100	28.0±0.8 a x	100
3.0	0.5	22.5±3.7 de	100	–	–
5.0	0.0	11.9±0.9 g	100	–	–
5.0	0.1	10.4±0.8 gh x	100	4.88±0.7 d y	100
5.0	0.5	–	–	–	–
BA (mg/l)	**IAA (mg/l)**				
0.1	0.0	–	–	–	–
0.1	0.5	21.50±3.4 de x	100	4.25±1.3 d y	75
0.1	1.0	4.88±0.6 hij x	100	3.5±0.2 d y	100
0.5	0.0	18.63±5 ef x	100	1.5±0.2 ef y	100
0.5	0.5	20.13±3.3 de x	100	8.38±2.1 e y	100
0.5	1.0	11.0±2.1 g x	100	9.0±1.4 bc x	100
1.0	0.0	23.25±6.1 de x	100	4.38±1.2 d y	100
1.0	0.5	19.0±3.1 ef x	100	1.75±0.4 ef y	87.5
1.0	1.0	2.25±0.9 ij x	75	1.0±0.4 ef x	50
3.0	0.0	30.0±6.6 bc x	100	8.5±0.6 c y	100
3.0	0.5	40.0±7.1 a x	100	4.88±1.2 d y	100
3.0	1.0	1.38±0.5 j	62.5	–	–
BA (mg/l)	**NAA (mg/l)**				
0.5	0.0	21.4±3 de x	62.5	2.0±0.6 e y	62.5
0.5	1.0	4.63±1.4 hij	62.5	–	–
0.5	3.0	–	–	–	–
0.5	5.0	–	–	–	–
1.0	0.0	21.3±3.3 de	100	–	–
1.0	1.0	8.13±0.7 ghi	100	–	–
1.0	3.0	–	–	–	–
1.0	5.0	–	–	–	–
3.0	0.0	33.1±1.6 b	100	–	–
3.0	1.0	22.5±4.9 de	100	–	–
3.0	3.0	4.0±1.1 ij	87.5	–	–
3.0	5.0	–	–	–	–
TDZ (mg/l)	**IAA (mg/l)**				
0.1	0.0	8.0±2.4 ghi x	100	2.0±1.2 e y	25
0.1	0.5	–	–	–	–
0.1	1.0	–	–	–	–
0.5	0.0	–	–	–	–
0.5	0.5	–	–	–	–
0.5	1.0	–	–	–	–
1.0	0.0	–	–	–	–
1.0	0.5	–	–	–	–
1.0	1.0	–	–	–	–
3.0	0.0	–	–	–	–
3.0	0.5	–	–	–	–
3.0	1.0	–	–	–	–

The greatest number of roots per explant was observed on media with 0.5 mg/l IBA (Table 2; Fig. 1b). Media containing 3 mg/l IAA (9.2±4.43 roots per explant; 80% explants formed roots) and 1 mg/l IBA (7.7±1.51 roots per explant; 90% explants formed roots) were also good for root formation (Table 2).

Fewer explants produced roots as IBA concentration increased, 100% of the explants formed roots at 0.5

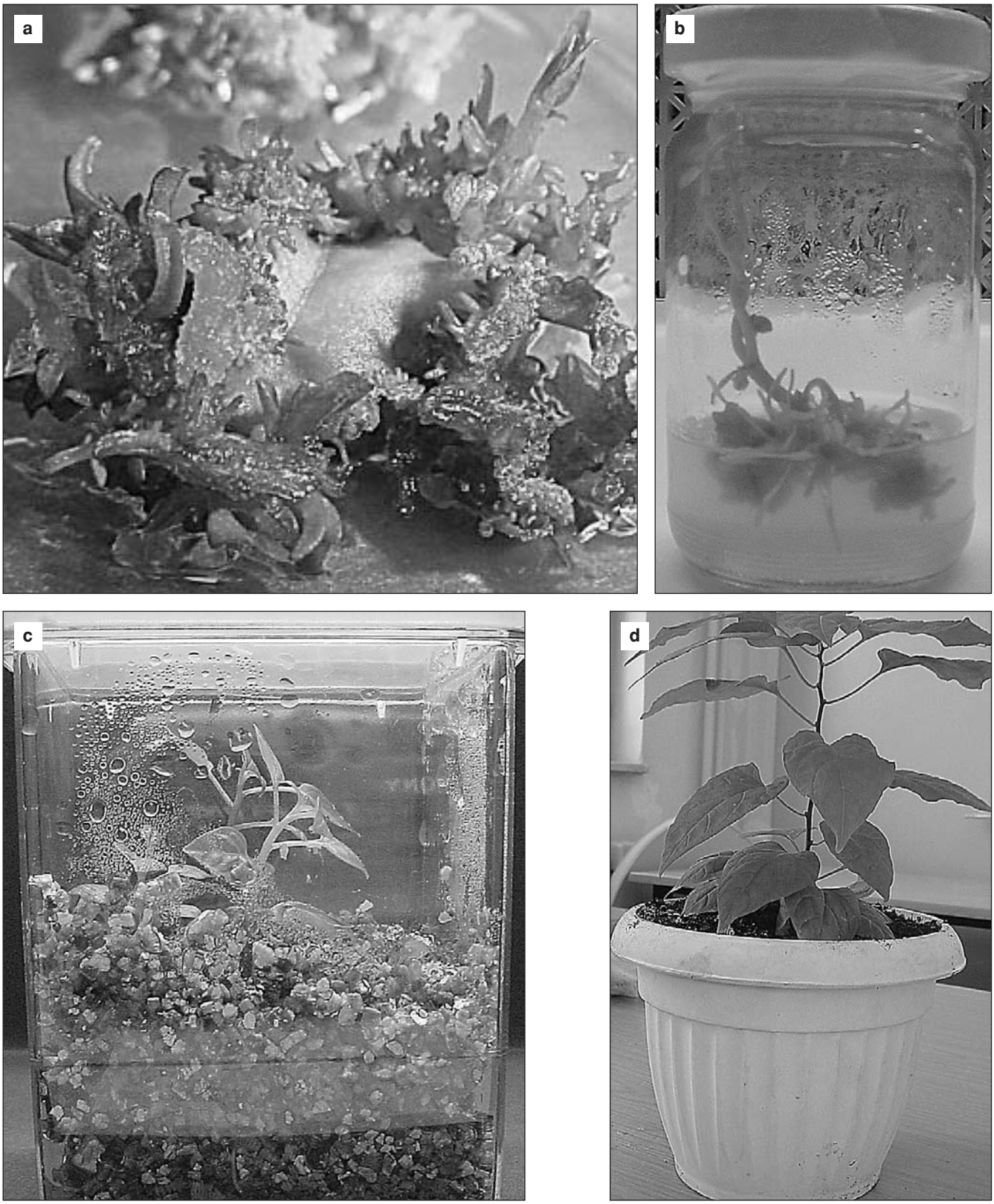

Fig. 1. In vitro regeneration of bittersweet (*S. dulcamara*): **a)** shoot regeneration through indirect organogenesis from leaf explant cultured on MSMO medium containing 3 mg/l BA+ 0.5 mg/l IAA after 3 weeks incubation on regeneration medium; **b)** rooting of the regenerated shoots in medium containing 0.5 mg/l IBA; **c)** regenerated plants in Magenta containers including vermiculate for acclimatization; **d)** regenerated plants transferred to plastic pots containing sterile potting soil under growth room conditions.

mg/l IBA compared to 10% at 3 mg/l IBA. Similar pattern was observed with NAA concentrations on rooting. Although 60% of the explants formed roots at 0.5 mg/l NAA, root formation was not observed with 3 mg/l NAA (Table 2). In control experiments (no auxin added to the media) none of the explants formed roots (Table 2).

Some of the regenerated shoots on media with 3 mg/l kinetin plus 0.5 mg/l 2,4-D, 0.5 mg/l BA plus 1 mg/l IAA

TABLE 2. Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots after 3 weeks incubation on rooting medium. Data are presented as mean number of roots per shoot ± standard error (SE). Means with the same letter within columns are not significantly different at $P>0.05$.

Treatments	Mean # of roots per explant	% explants forming roots
MSMO medium without growth regulators	–	–
IAA (mg/l)		
0.5	5.2±1.3 [bc]	90
1	4.1±1 [bc]	80
3	9.2±4.4 [ab]	80
IBA (mg/l)		
0.5	11.6±1.4 [a]	100
1	7.7±1.5 [ab]	90
3	0.5±0.5 [c]	10
2,4-D (mg/l)		
0.05	3.5±1.4 [bc]	50
0.1	4.5±1.3 [bc]	80
0.5	0.3±0.3 [c]	10
NAA (mg/l)		
0.5	6.4±3.3 [ab]	60
1	0.2±0.1 [c]	20
3	–	–

and 3 mg/l BA plus 3 mg/l NAA combinations formed roots and these regenerants directly transferred to vermiculate and then to pots without rooting procedure.

Rooted plants were transferred to Magenta containers including vermiculate for acclimatization (Fig. 1c). After 2 weeks, they were transferred to soil (Fig. 1d). More than 90% of the shoots survived through the hardening off process when the rooted shoots (regenerated plantlets) were transferred to pots and kept under growth room conditions for four weeks before finally moving to room conditions at low humidity.

Although there are some studies on *S. dulcamara*, there are no reports on an efficient protocol for *in vitro* regeneration of this plant. In some studies only callus initiation medium was used for callus production. For example, Emke and Eilert (1986) compared the steroidal alkaloid soladulcidine quantity in callus, regenerated plants and suspension cultures of *S. dulcamara*. They obtained callus on Eriksson medium with 1 mg/l NAA+0.02 mg/l kinetin or Murashige and Skoog (MS) Basal medium with 1 mg/l 2,4-D+0.4 mg/l kinetin. They reported that for plant regeneration, callus which was not subcultured and left growing for 2 months showed the tendency to regenerate roots or shoots only on MS basal medium with 1 mg/l 2,4-D+0.4 mg/l kinetin. Also in our study, kinetin plus 2,4-D combinations provided rapid clonal propagation of this species (Table 1). Similarly, Khanna et al. (1988) compared the solanine content of callus grown on revised Murashige and Skoog medium supplemented with 1 mg/l of 2,4-D after harvesting 2, 4, 6 and 8 weeks.

Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Schwarz and Beaty 1996). Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success. In our study, when leaf and petiole explants were compared, leaf explants were much more productive for shoot formation than petiole explants with all plant growth regulator combinations and concentrations (Table 1). Similarly, leaf explant was also reported as the responsive explant in terms of shoot organogenesis with some members of Solanaceae family; *Solanum surattense* Burm. f. (Baburaj and Thamizhchelvan 1991), *Solanum commersonii* Dun. (Iapichino et al. 1991), *Solanum tuberosum* L. (Yadav and Sticklen 1995; Nadolska-Orczyk et al. 1995; Hussain et al. 2005), *Solanum nigrum* L. (El-Ashaal et al. 1999), *Solanum laciniatum* Ait. (Okrslar et al. 2002) and *Lycopersicon* spp. (Lech et al. 1996; Jabeen et al. 2005). Four of the studies (Baburaj and Thamizhchelvan 1991; El-Ashaal et al. 1999; Okrslar et al. 2002; Hussain et al. 2005) showed that BA was the most succesful growth regulator for *Solanum* spp., like bittersweet.

Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout et al. 2000). In order to have standardized formulations, the chemical constituents from plants and their parts are required to be uniform both qualitatively and quantitatively. Furthermore, an ever increasing demand of uniform medicinal plants based medicines warrants their mass cloning through plant tissue culture strategy (Chaturvedi et al. 2007). This protocol can be helpful in the large scale production of certain secondary products of *S. dulcamara*. Micropropagation of *S. dulcamara* can provide a mass production of pesticide, herbicide and disease free plants on a commercial scale and unlimited plant materials can consistently be obtained throughout the whole year. Moreover, this protocol can provide plant material for future pharmacological, physiological and biochemical studies.

ACKNOWLEDGEMENTS

The authors are grateful to The Scientific and Technological Research Council of Turkey (TUBITAK) for financial support [TBAG-2278(103T024) and TBAG-HD/27 (105T040)].

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