Adventitious shoot regeneration from stem internode explants of *Verbena officinalis* L., a medicinal plant

Arzu UÇAR TÜRKER, Buhara YÜCESAN, Ekrem GÜREL

Abant İzzet Baysal University, Department of Biology, Bolu - TURKEY

Abstract: *Verbena officinalis* L. (vervain) is a medicinal plant that shows several biological activities, including antimicrobial, antiinflammatory, analgesic, neuroprotective, hypnotic/sedative, gastroprotective, hepatoprotective, anticancer, cicatrizant, and antioxidant. This report describes an efficient plant regeneration system for *V. officinalis* via adventitious shoot development from stem internode and petiole explants. Stem internode explants were more effective than petiole explants in terms of both the number of shoots per explant and the percentages of explants forming shoots. Increasing benzyladenine (BA) concentrations steadily increased shoot formation up to 13.32 μM, but 22.22 μM BA caused a sharp decrease. The highest number of shoots (17.1 shoots per explant at 100% frequency) was obtained when 13.32 μM BA was combined with 5.71 μM indole-3-acetic acid (IAA). Rooting of the regenerated shoots was readily achieved when multiple shoots were singled out and cultured on medium containing varying concentrations of different auxins [IAA, indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), or naphthalene acetic acid (NAA)]. IBA was more effective in terms of the number of roots developed, producing a mean of 7.1 roots per shoot at 4.92 μM, whereas IAA was more effective for the frequency of shoots developing roots: 100% of the shoots rooted at 5.71 μM IAA. More than 90% of the regenerants survived through the hardening off process, when they were transferred to foam cups and kept under growth room conditions for 2 weeks before finally being moved to room conditions at low humidity. Approximately 3 months after the transfer to room conditions, the flowering of the regenerated plants could be observed.

Key words: *Verbena officinalis*, in vitro culture, stem internode explant, micropropagation

Tıbbi bir bitki olan *Verbena officinalis* L.'in gövde internodal eksplantlarından adventif sürgün rejenerasyonu

Özet: *Verbena officinalis* L. (mine çiçeği) tıbbi bir bitki olup, anti-mikrobiyel, anti-inflamatuar, analjezik, nöroprotektif, hipnotik/sedatif, gastroprotektif, hepatoprotektif, anti-kanser, sikatrizan ve antioksidant gibi çeşitli biyolojik aktiviteler göstermektedir. Gövde internodal ve petiyol eksplantlarından adventif sürgün gelişimi yoluyla başarılı bir bitki rejenerasyon sistemi, bu türde ilk defa bu çalışmayla ortaya konulmaktadır. Eksplant başına sürgün sayısı ve sürgün oluşturan eksplantların yüzdesi bakımından, gövde internodal eksplantları petiyol eksplantlarından daha fazla etkili olmuştur. 13,32 μM'a kadar artan benziladenin (BA) konsantrasyonu sürgün oluşumunu düzenli olarak artırırken 22,22 μM BA ani bir düşüşe sebep olmuştur. En yüksek sürgün verimi (eksplant başına ortalama 17,1 sürgün ve eksplantların % 100'nün sürgün verdiği), 13,32 μM BA ile 5,71 μM indol-3-asetik asit (IAA)'nın kombinasyonunda elde edilmiştir. Çoklu sürgünler tek tek ayrılarak çeşitli konsantrasyonlarda farklı oksin içeren ortamlarda [IAA, indole-3-butirik asit (IBA), 2,4-diklorofenoksi asetik asit (2,4-D), veya naftalen asetik asit (NAA)] kültüre alındığında, rejenere olmuş sürgünler kolaylıkla köklendirilmiştir. Gelişen kök sayısı bakımından, 4,92 μM IBA, sürgün başına ortalama 7,1 kök üreterek daha etkili olurken, kök oluşturan sürgünlerin yüzdesi bakımından ise 5,71 μM IAA % 100 sürgün köklenme oranıyla daha başarılı olmuştur. Oluşan rejenerantlar saksılara aktarıldıklarında, % 90'dan fazlası alıştırma sürecini başarıyla tamamlamış ve düşük nemli bir ortama aktarılmadan önce iki hafta süreyle büyüme odası koşullarında tutulmuştur. Oda şartlarına aktarıldıktan yaklaşık üç ay sonra rejenere olmuş bitkilerin çiçeklendiği gözlenmiştir.

Anahtar sözcükler: *Verbena officinalis*, in vitro kültür, gövde internodal eksplant, mikropropagasyon

Introduction

Verbena officinalis L. (vervain), belonging to the family Verbenaceae, is a perennial herb growing by roadsides and in sunny pastures. It grows wild throughout most of Europe and North Africa, as well as in China and Japan. In the 1st century AD, Dioscorides called vervain the "sacred herb," and for many centuries it was taken as a cure-all (1). The leaves and flowering stems are used in folk medicine as analgesics, nervines, antibacterials, anticoagulants, antispasmodics, astringents, depuratives, diaphoretics, mild diuretics, emmenagogues, galactogogues, stimulants, expectorants, antirheumatics, tonics, and vulneraries (1-4). The antimicrobial (5), antiinflammatory (6-9), analgesic (8), neuroprotective (10,11), hypnotic/sedative (12), gastroprotective (9), hepatoprotective (13), anticancer (14), cicatrizant, and antioxidant (9) activities of *V. officinalis* have already been reported. *V. officinalis* contains iridoids (15-21), flavonoids (19,21-23), triterpenoids (7,20), verbascosides (7,24), phenylethanoids (21), phenolic acid derivatives (19), and volatile oils (1). As to its geographic distribution and survival, it was reported that climatic fluctuations account for the seasonal dormancy of vervain seeds that is critical for their geographical distribution (25,26). Plant tissue culture is an alternative method of commercial propagation and has been widely used for the commercial propagation of a large number of plant species, especially medicinal plants that are vulnerable to climatic changes (27,28).

The present study describes, to our knowledge for the first time, an efficient in vitro plant regeneration protocol for *V. officinalis* via adventitious shoot development from stem internode and petiole explants cultured on media containing different concentrations and combinations of various plant growth regulators.

Materials and methods

Seeds of *V. officinalis* were collected from Abant İzzet Baysal University's Gölköy Campus (Bolu, Turkey) in 2006. Identification of the species was made according to the method of Davis (29), and voucher specimens (AUT-2005) were deposited at the Abant İzzet Baysal University Herbarium (Bolu,

Turkey). To break the dormancy, seeds were cold stratified by being kept at 0 and -12 °C for 1 week each, respectively, before finally being kept at 0 °C for another week. The treated seeds were then sterilized by immersion in 50% H_2SO_4 (sulfuric acid) for 20 min, followed by several rinses with sterile distilled water. Seeds were then kept in 70% ethanol for 1 min before being soaked in a 1.5% $HgCl_2$ (mercuric chloride) for 4 min.

Sterilized seeds were germinated in plastic petri dishes containing Murashige and Skoog Minimal Organics (MSMO) medium (4.43 g L^{-1}, Sigma Chemical Co., St. Louis, MO, USA) (30) supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} Difco-Bacto agar (pH 5.7), and then autoclaved for 20 min at 121 °C and 105 kPa. After 1 week of incubation on this medium, seedlings were transferred to Magenta containers (GA-7 vessel, Sigma Chemical Co.) containing the same medium for an additional 4 weeks of incubation. For shoot regeneration, leaf lamina (5 × 5 mm pieces), petiole (5 mm segments), and stem explants (10 mm of internode segments) were excised from 5-week-old seedlings and placed in plastic petri dishes containing 20 mL of MSMO medium supplemented with different combinations and concentrations of plant growth regulators: benzyladenine (BA; 0.44, 2.22, 4.44, 13.32, or 22.22 μM) and indole-3-acetic acid (IAA; 0.57, 2.86, 5.71, or 17.12 μM), or thidiazuron (TDZ; 0.23, 0.45, or 2.27 μM) and IAA (0.57, 2.86, or 5.71 μM). Following 3 weeks of culturing on these media, regenerated shoots were transferred to Magenta containers containing the same MSMO media for shoot elongation for 2 weeks. Well-developed shoots (3-4 cm in length) were then singled out and placed on a rooting medium containing MSMO medium supplemented with varying concentrations of different auxins, IAA (2.86, 5.71, or 17.12 μM), IBA (2.46, 4.92, or 14.76 μM), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.45, 2.26, or 4.52 μM), or naphthalene acetic acid (NAA; 0.54, 2.69, or 5.37 μM), for 3 weeks. All cultures were incubated at 23 °C in a growth room with a 16 h photoperiod under cool white light at 22-28 μmol $m^{-2} s^{-1}$ and 55-60% relative humidity. For hardening off, the plantlets (5-6 cm length) were washed with sterile distilled water to remove traces of agar medium, and were then transferred to foam cups (6 cm in diameter) containing sterile potting soil. The plantlets were kept

in a growth chamber for 2 weeks before being transferred to room conditions at 20-22 °C with low humidity (25%-35%). For shoot regeneration, the mean number of shoots per petiole or stem explants and the percentage of explants producing shoots were calculated. For rooting, the number of developing roots per shoot and the percentage of shoots producing roots were determined. Eight replicates for shoot regeneration and 10 replicates for rooting experiments were used for each treatment, and both experiments were repeated 3 times. All data were analyzed by analysis of variance (ANOVA), and mean values were compared using Duncan's multiple range test using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

Results

Effects of Combinations of BA or TDZ with IAA on Shoot Regeneration. Three different types of explants (leaf lamina, petiole, and stem internode) excised from 4-week-old sterile seedlings were cultured on MSMO medium containing BA or TDZ in combination with IAA. The regeneration efficiency was found to be higher in stem internode explants than petiole explants with regard to both the mean number of shoots per explant and the percentages of explants forming shoots in all responsive combinations and concentrations of plant growth regulators tested (Table 1). For petiole and stem internode explants, microshoots started to appear after 13 days with no visual evidence of an intervening callus phase for all combinations of plant growth regulators, thus suggesting a direct shoot organogenesis (Figure 1). On the other hand, leaf lamina explants produced no shoots at all in any of the treatments listed in Table 1; thus, no data is provided. Control treatments involving no plant growth regulators, as well as those treatments that did not use the auxin IAA in the combination (i.e. when BA or TDZ was used alone), again produced no shoots at all in either the petiole or the stem internode explants.

Taking the data on shoot production from stem internode explants into account, increasing concentrations of BA from 0.44 to 13.32 μM steadily increased shoot regeneration, while 22.22 μM BA caused a dramatic decrease in terms of both the mean

number of shoots per explant and the percentage of explants producing shoots. The highest number of shoots (17.1 shoots per explant at 100% frequency) was obtained when 13.32 μM BA was combined with 5.71 μM IAA (Table 1 and Figure 1). This combination was also the most effective for petiole explants, producing 5.1 shoots per explant with a 79% frequency.

When cultured on media containing combinations of different TDZ (0.23, 0.45, or 2.27 μM) and IAA (0.57, 2.86, or 5.71 μM) concentrations, petiole explants did not produce any shoots (Table 1). For stem internode explants, although combinations of TDZ with higher IAA concentrations seemed to be slightly more effective, the differences between the treatment means were not statistically significant.

Using the same explants, we also tested several combinations of other plant growth regulators for shoot regeneration of *V. officinalis*, including TDZ (0.45, 2.27, or 4.54 μM) and 2,4-D (0.45, 2.26, or 4.52 μM); TDZ (0.45, 2.27, 4.54, or 13.62 μM) and IBA (0.49, 2.46, 4.92, or 14.76 μM); kinetin (2.32, 4.65, 13.94, or 23.23 μM) and IAA (0.28, 0.57, 2.86, or 17.12 μM); kinetin (2.32, 4.65, 13.94, or 23.23 μM) and 2,4-D (0.45, 2.26, or 4.52 μM); and BA (2.22, 4.44, or 13.32 μM) and NAA (5.37, 16.11, or 26.85 μM); however, these combinations produced very few, if any, shoots (data not provided).

Effects of Different Auxins on Rooting of the Regenerated Shoots. Regenerated shoots were singled out and cultured on MSMO medium containing different auxins: IAA, IBA, 2,4-D, or NAA. Although the highest number of roots per shoot was observed with 4.92 μM IBA (7.1 roots per shoot at 80% frequency), the number of responsive shoots was higher when the rooting medium contained IAA, especially at the 5.71 μM concentration, which induced 100% rooting (Table 2 and Figure 1). The control treatment involving no auxin did not produce any roots at all. The other auxins, 2,4-D and NAA, were less effective for root induction, yet higher concentrations of 2,4-D (2.26 or 4.52 μM) produced no roots at all. It was also observed that 2,4-D promoted a moderate amount of callus development at the basal part of the shoots, which were in contact with the medium. The rooted shoots were transferred to foam cups containing potting soil and kept under

Table 1. Shoot regeneration from petiole and stem internode explants cultured on MSMO medium containing different combinations of BA or TDZ with IAA after 3 weeks in culture. Data presented as mean number of shoots per explant ± standard error (SE). Mean values with the same letters within columns are not significantly different at P > 0.05.

| Treatments (μM)	Petiole Explants		Stem Internode Explants	
	Mean no. of shoots per explant ± SE	% of explants forming shoots	Mean no. of shoots per explant ± SE	% of explants forming shoots
Control	0	0	0	0
0.44 BA	0	0	0	0
0.44 BA + 0.57 IAA	0	0	0	0
0.44 BA + 2.86 IAA	0	0	1.0 ± 0.5 [gh]	42
0.44 BA + 5.71 IAA	0	0	0	0
0.44 BA + 17.12 IAA	0	0	0	0
2.22 BA	0	0	0	0
2.22 BA + 0.57 IAA	0	0	0	0
2.22 BA + 2.86 IAA	0.6 ± 0.3 [cd]	50	2.5 ± 0.6 [f]	75
2.22 BA + 5.71 IAA	0	0	2.9 ± 0.5 [f]	79
2.22 BA + 17.12 IAA	0	0	0	0
4.44 BA	0	0	0	0
4.44 BA + 0.57 IAA	0	0	0	0
4.44 BA + 2.86 IAA	1.8 ± 0.4 [b]	75	4.5 ± 0.8 [e]	88
4.44 BA + 5.71 IAA	0.3 ± 0.3 [d]	17	4.5 ± 0.4 [e]	100
4.44 BA + 17.12 IAA	0	0	0	0
13.32 BA	0	0	0	0
13.32 BA + 0.57 IAA	0	0	2.0 ± 0.1 [fg]	42
13.32 BA + 2.86 IAA	1.5 ± 0.7 [bc]	50	5.6 ± 1.3 [cde]	79
13.32 BA + 5.71 IAA	5.1 ± 1.3 [a]	79	17.1 ± 0.7 [a]	100
13.32 BA + 17.12 IAA	0	0	0	0
22.22 BA	0	0	0	0
22.22 BA + 0.57 IAA	0	0	0	0
22.22 BA + 2.86 IAA	0	0	2.1 ± 0.3 [fg]	33
22.22 BA + 5.71 IAA	1.3 ± 0.5 [bc]	33	4.3 ± 0.5 [e]	46
22.22 BA + 17.12 IAA	0	0	0	0
0.23 TDZ	0	0	0	0
0.23 TDZ + 0.57 IAA	0	0	7.0 ± 0.3 [bc]	100
0.23 TDZ + 2.86 IAA	0	0	2.0 ± 0.5 [fg]	75
0.23 TDZ + 5.71 IAA	0	0	5.3 ± 0.9 [de]	92
0.45 TDZ	0	0	0	0
0.45 TDZ + 0.57 IAA	0	0	2.8 ± 1.0 [f]	50
0.45 TDZ + 2.86 IAA	0	0	6.0 ± 0.3 [bcde]	100
0.45 TDZ + 5.71 IAA	0	0	7.5 ± 0.4 [b]	100
2.27 TDZ	0	0	0	0
2.27 TDZ + 0.57 IAA	0	0	0	0
2.27 TDZ + 2.86 IAA	0	0	6.0 ± 0.3 [bcde]	100
2.27 TDZ + 5.71 IAA	0	0	6.3 ± 0.4 [bcd]	100
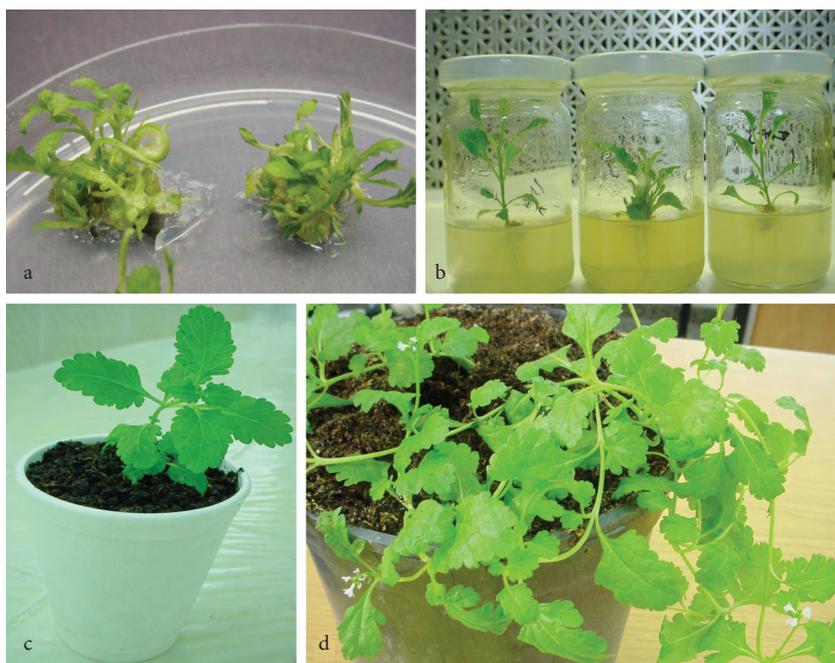
Figure 1. In vitro regeneration of vervain (*Verbena officinalis* L.): **a**) Shoot development from stem internode explants cultured on medium containing 13.32 μM BA and 5.71 μM IAA after 3 weeks in culture, **b**) Rooting of the regenerated shoots on medium containing 4.92 μM IBA after 3 weeks in culture, **c**) Regenerated plants transferred to foam cups containing sterile potting soil, **d**) Flowering of a regenerated plant after transferring to room conditions.

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

Auxins (μM)	Mean number of roots per shoot ± SE	% of shoots that rooted
Control	0	0
2.86 IAA	4.5 ± 0.6 [bc]	93
5.71 IAA	5.6 ± 0.7 [abc]	100
17.12 IAA	5.9 ± 1.1 [ab]	83
2.46 IBA	4.4 ± 1.1 [bc]	67
4.92 IBA	7.1 ± 1.2 [a]	80
14.76 IBA	3.6 ± 1.0 [cd]	57
0.45 2,4-D	1.7 ± 0.5 [de]	70
2.26 2,4-D	0	0
4.52 2,4-D	0	0
0.54 NAA	1.0 ± 0.3 [e]	63
2.69 NAA	0.6 ± 0.2 [e]	50
5.37 NAA	0.4 ± 0.2 [e]	37

growth room conditions (Figure 1). Approximately 90% of the regenerated plants survived the hardening off process. Approximately 3 months after transfer to room conditions, the flowering of regenerated plants could be observed (Figure 1).

Discussion

Although *Verbena officinalis* is a valuable medicinal plant showing several biological activities, there has been, to our knowledge, no published work on the in vitro propagation of this species. In this report, we describe an efficient in vitro plant regeneration protocol via adventitious shoot development from stem internode explants cultured on media containing different concentrations of BA or TDZ in combination with different concentrations of IAA. In this report, we mainly present the results of the effects of combinations of BA and TDZ with the auxin IAA. However, we also tested a wide range of other combinations of several plant growth regulators, including TDZ and 2,4-D, TDZ and IBA, KIN and IAA, KIN and 2,4-D, and BA and NAA at varying concentrations for shoot regeneration efficiencies, but presented no data for these experiments since they produced too little success, producing either very few shoots or, as in many cases, no shoots at all.

A serious dormancy problem of the vervain seeds was encountered at the beginning of our study, which resulted in very low rates of, or in many cases, no germination. To overcome this problem, a sequential treatment of cold stratification was employed by keeping the seeds at 0 °C and then -10 °C for 1 week each, and finally keeping them at 0 °C for another week. These treatments improved the germination ability of the seeds considerably, resulting in an average of 50% germination. Seed germination could be a severe problem in many species due to dormancy; however, cold pretreatment and/or weakening the seed coat by physical or chemical means before surface sterilization have been proved to be effective ways of promoting germination (26,31-33), as was the case in our study.

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 the endogenous content of the hormones (34). 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 of certain plant species (35). When we compared leaf, petiole, and stem internode explants, it was clear that stem internode explants were more productive for shoot formation than leaf or petiole explants. This result is consistent with the results of Tamura et al. (36), who reported a higher regeneration frequency with internodal stem segments than leaf lamina discs of different hybrid cultivars of *Glandularia peruviana* (formerly known as *Verbena peruviana) × Glandularia tenera* (formerly known as *Verbena tenera*) cultivars when cultured on Murashige and Skoog basal medium supplemented with 2.27 µM TDZ and 1.43 µM IAA. Stem internode explants have been previously shown to be the most productive explant source for several species, including carnation (37), gypsophila (38), and *Adenophora triphylla* (39). This effect might be partly attributed to the intense vascular nature of the stem tissue since, in some cases, parenchyma cells surrounding the vascular bundle or cambial cells per se could be actively involved in the initiation of primordia development, which then leads to an organogenic process (40). Hosoki and Katahira (41) reported the micropropagation of *Verbena tenera* by subculturing one-node explants on Murashige and Skoog (MS) medium supplemented with BA at concentrations of 0, 0.44, and 4.4 µM. Shoot length was the longest in the BA-free basal medium, and increasing BA concentrations slightly suppressed the shoot elongation. However, we observed no shoot formation from leaf lamina, petiole, or stem internode explants at 0, 0.44, 2.22, 4.44, 13.32, or 22.22 µM BA in our study (Table 1).

Our data indicated that cultured explants certainly required the presence of auxin in addition to cytokinin in the culture medium in order to achieve shoot organogenesis, as no shoot development could be obtained when BA or TDZ was used alone. Even in the cases where auxin was supplied at relatively low concentrations (i.e. 0.57 µM; see Table 1), shoot development was not observed, suggesting that cells needed to be exposed to a certain concentration of auxin in order to become competent enough to

induce cell proliferation and dedifferentiation. Auxin is generally considered necessary for the acquisition of the meristematic competence of the responsive cells. Once this competence is established, further embryonic (42) or organogenic (40) development due to excessive auxin concentrations have often been found to be inhibitory.

Rooting of the regenerated shoots was readily achieved when multiple shoots were singled out and cultured on media containing different auxins (Table 2 and Figure 1). In terms of the mean number of roots per shoot explant, the greatest number of roots was obtained when the culture medium was supplemented with 4.92 µM IBA. However, the auxin IAA was more effective in terms of the frequency of the shoots that rooted, with 2.86, 5.71, and 17.12 µM IAA resulting in 93%, 100%, and 83%, respectively. The other auxins tested for root induction (2,4-D and NAA) were much less effective for root formation, suggesting a phenomenon of auxin-type-specific rooting in this species. Hosoki and Katahira (41) showed that basal MS medium was suitable for the rooting of *V. tenera* among the tested IBA concentrations (0, 0.49, or 4.9 µM). Although root number increased with IBA treatment, root length was extremely short and all shoots formed calli at the base with 4.9 µM IBA. However, in our study, no root formation was observed with auxin-free basal medium (control) (Table 2), and callus formation was not observed with 4.92 µM IBA in the rooting of *V. officinalis*. Although 4.9 µM IBA caused 13.6 roots per shoot with *V. tenera* (41), 7.1 roots per shoot were observed with *V. officinalis* in our study (Table 2). On the other hand, the percentages of shoots that rooted were similar in both studies for 4.9 µM IBA (79% for *V. tenera* and 80% for *V. officinalis*) (41) (Table 2).

In conclusion, an effective in vitro propagation protocol via adventitious shoot development was achieved for *V. officinalis*. Thus, the protocol described in this report is hoped to contribute to future studies on large-scale production of certain plant metabolites with important biological activities from in vitro propagated elite materials year round, as well as to possible studies on the improvement of *V. officinalis* via genetic transformation technologies. Although *V. officinalis* and some other medicinal plants can be found in the wild easily, they are generally subjected to some herbicides and attacked by some insects and pathogens. In vitro micropropagation of medicinal plants provides pesticide- or disease-free plants and produces a large quantity of vegetative planting stock easily. In addition, with an in vitro propagation method, unlimited plant material can consistently be obtained throughout the whole year, and genetically uniform plant materials (less genetic diversity) can be produced easily with higher seed germination. Additional studies are, however, recommended for the further improvement of in vitro regeneration efficiency and for the establishment of suspension cultures for a large-scale metabolite production.

Corresponding author:
Ekrem GÜREL
Abant İzzet Baysal University,
Faculty of Arts and Sciences,
Department of Biology,
14280 Bolu - TURKEY
E-mail: gurel_e@ibu.edu.tr

References

1. Chevallier A. The Encyclopedia of Medicinal Plants. Dorling Kindersley Limited. London; 1996.

2. Grieve M. A Modern Herbal, vol. 2. Dover Publications. New York; 1982.

3. Baytop T. Türkiye'de Bitkiler ile Tedavi. Nobel Tıp Kitabevleri. Istanbul; 1999.

4. Guarrera PM, Forti G, Marignoli S. Ethnobotanical and ethnomedicinal uses of plants in the district of Acquapendente (Latium, Central Italy). J Ethnopharmacol 96: 429-44, 2005.

5. Hernández NE, Tereschuk ML, Abdala LR. Antimicrobial activity of flavonoids in medicinal plants from Tafí del Valle (Tucumán, Argentina). J Ethnopharmacol 73: 317-22, 2000.

6. Calvo MI, Vilalta N, San Julian A et al. Anti-inflammatory activity of leaf extract of *Verbena officinalis* L. Phytomedicine 5: 465-67, 1998.

7. Deepak M. Handa SS. Antiinflammatory activity and chemical composition of extracts of *Verbena officinalis*. Phytotherapy Res 14: 463-65, 2000.

8. Calvo MI. Anti-inflammatory and analgesic activity of the topical preparation of *Verbena officinalis* L. J Ethnopharmacol 107: 380-82, 2006.

9. Speroni E, Cervellati R, Costa S et al. Effects of differential extraction of *Verbena officinalis* on rat models of inflammation, cicatrization and gastric damage. Planta Med 73: 227-35, 2007.

10. Lai SW, Yu MS, Yuen WH et al. Novel neuroprotective effects of the aqueous extracts from *Verbena officinalis* L. Neuropharmacology 50: 641-50, 2006.

11. Lai SW, Yu MS, Yuen WH et al. Potential neuroprotective agent from botanical extract: an experience of using *Verbena officinalis* against beta-amyloid peptide neurotoxicity. Neurosignals 15: 146-47, 2006.

12. Carlini EA. Plants and the central nervous system. Pharmacol Biochem Behav 75: 501-12, 2003.

13. Singh B, Saxena A, Chandan BK et al. Hepatoprotective activity of verbenalin on experimental liver damage in rodents. Fitoterapia 69: 135-40, 1998.

14. Dudai N, Weinstein Y, Krup M et al. Citral is a new inducer of caspase-3 in tumor cell lines. Planta Med 71: 484-88, 2005.

15. Rimpler H, Schafer B. Hastatoside, new iridoid from *Verbena officinalis* and *Verbena hastata* (Verbenaceae). Tetrahedron Lett 17: 1463-64, 1973.

16. Rimpler H, Schafer B. Hastatoside, a new iridoid from *Verbena hastata* L. and *Verbena officinalis* L. Z Naturforsch C 34: 311-18, 1979.

17. Damtoft S, Godthjælpsen L, Jensen SR et al. Age-dependent variations of the efficiency of iridoid biosynthesis in *Verbena officinalis*. Phytochemistry 22: 2614-15, 1983.

18. Makboul AM. Chemical constituents of *Verbena officinalis*. Fitoterapia 57: 50-51, 1986.

19. Calvo MI, San Julian A, Fernandez M. Identification of the major compounds in extracts of *Verbena officinalis* L. (Verbenaceae) by HPLC with post-column derivatization. Chromatographia 46: 241-44, 1997.

20. Deepak M, Handa SS. 3 alpha,24-dihydroxy-urs-12-en-28-oic acid from *Verbena officinalis*. Phytochemistry 49: 269-71, 1998.

21. Muller A, Ganzera M, Stuppner H. Analysis of the aerial parts of *Verbena officinalis* L by micellar electrokinetic capillary chromatography. Chromatographia 60: 193-97, 2004.

22. Reynaud J, Couble A, Raynaud J. Flavonoids of *Verbena officinalis* L. J Plant Physiol 135: 380-81, 1989.

23. Carnat A, Carnat AP, Chavignon O et al. Luteolin 7-diglucuronide, the major flavonoid compound from *Aloysia triphylla* and *Verbena officinalis*. Planta Med 61: 490, 1995.

24. Hansel R, Kallmann S. Verbascoside, a main constituent of *Verbena officinalis*. Arch Pharm 319: 227-30, 1986.

25. Woodword FI. Life at the edge: a 14-year study of a *Verbena officinalis* population's interactions with climate. J Ecol 85: 899-906, 1997.

26. Brandel M, Schütz W. Seasonal dormancy patterns and stratification requirements in seeds of *Verbena officinalis* L. Basic and App Eco 4: 329-37, 2003.

27. Rout GR, Samantaray S, Das P. In vitro manipulation and propagation of medicinal plants. Biotechnol Adv 18: 91-120, 2000.

28. Naika HR, Krishna V. Plant regeneration from callus culture of *Clematis gouriana* Roxb. – a rare medicinal plant. Turk J Biol 32: 99-103, 2008.

29. Davis PH. Flora of Turkey and the East Aegean Islands, vol. 7. Edinburgh University Press. Edinburgh; 1982.

30. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-97, 1962.

31. Eisvand HR, Arefi HM, Tavakol-Afshari R. Effects of various treatments on breaking seed dormancy of *Astragalus siliquosus*. Seed Sci Technol 34: 747-52, 2006.

32. Olmez Z, Temel F, Gokturk A et al. Effect of cold stratification treatments on germination of drought tolerant shrubs seeds. J Environ Biol 28: 447-53, 2007.

33. Vandelook F, Bolle N, Van Assche JA. Seed dormancy and germination of the European *Chaerophyllum temulum* (Apiaceae), a member of a trans-Atlantic genus. Ann Bot 100: 233-39, 2007.

34. Trigiano RN, Gray DJ. Plant Tissue Culture: Concepts and Laboratory Exercises, 2nd edition. CRC Press. Boca Raton; 2000.

35. Yucesan B, Turker AU, Gurel E. TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). Plant Cell Tiss Org Cult 91: 243-50, 2007.

36. Tamura M, Togami J, Ishiguro K et al. Regeneration of transformed verbena (*Verbena × hybrida*) by *Agrobacterium tumefaciens*. Plant Cell Rep 21: 459-66, 2003.

37. Watad AA, Ahroni A, Zuker A et al. Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Sci Hortic 65: 313-20, 1996.

38. Ahroni A, Zuker A, Rozen Y et al. An efficient method for adventitious shoot regeneration from stem-segment explants of gypsophila. Plant Cell Tissue Organ Cult 49: 101-106, 1997.

39. Chen CC, Chen SJ, Sagare AP et al. Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC. (Campanulaceae) - an important medicinal herb. Bot Bull Acad Sinica 42: 1-7, 2001.

40. Gurel E, Wren MJ. In vitro development from leaf explants of sugar beet (*Beta vulgaris* L.): rhizogenesis and the effect of sequential exposure to auxin and cytokinin. Ann Bot 75: 31-38, 1995.

41. Hosoki T, Katahira S. Micropropagation of *Verbena tenera* by node culture. Plant Cell Tissue Organ Cult 36: 373-375, 1994.

42. Charrière F, Sotta B, Miginiac E et al. Induction of adventitious shoots or somatic embryos on in vitro cultured zygotic embryos of *Helianthus annuus*: variation of endogenous hormone levels. Plant Physiol Biochem 37: 751-57, 1999.