Direct and Indirect Regeneration of Plants from Internodal and Leaf Explants of *Hypericum bupleuroides* Gris.

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Species of the genus *Hypericum* are of considerable interest worldwide because of their medicinal properties. *In-vitro* culture is a useful tool for both multiplication of the genus and studying its economically important secondary metabolites. Here, we present an effective *in-vitro* propagation method for *H. bupleuroides*. Leaf and internodal explants excised from 9-week-old, *in vitro*-germinated seedlings were cultured on a Murashige and Skoog (MS) medium supplemented with benzyladenine (BA; 1.0 or 0.1 mg L^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0 or 0.1 mg L^{-1}). Depending on the BA and 2,4-D combination used, these cultures produced adventitious shoot buds directly on the surfaces of both types of explants as well as excessive calli. Numerous shoots were obtained when the calli from both explant types were cultured on an MS medium supplemented with 2 mg L^{-1} BA. Internodal explants were more responsive than leaf tissues to direct and indirect plant regeneration. After shoots that regenerated from either the calli or the explant surface were excised, rooting was best on an MS medium lacking any growth hormones. These rooted plants were then acclimatized under greenhouse conditions, and 90% of regenerants had survived. Ours is the first report of *in-vitro* plant regeneration from *H. bupleuroides*.

Keywords: callus induction, *Hypericum bupleuroides*, plant growth regulators, plant regeneration, rooting, shoot induction

Hypericum is a genus comprising about 400 species of flowering plants in the family Guttiferae (formerly treated as the separate family of Hypericaceae). Members in this genus are used as traditional medicinal plants because of their wound-healing (Yazaki and Okuda, 1990), bactericide (Ishiguro et al., 1998), anti-inflammatory (Dias et al., 1998), diuretic, and sedative properties (Holz and Ostrowiski, 1987). In addition, numerous hybrids and cultivars have been developed as ornamental garden plants, such as *Hypericum* x *moserianum* (a hybrid of *Hypericum patulum* and *Hypericum calycinum*), *H. calycinum*, *H. patulum*, *Hypericum androsaemum*, *Hypericum frondosum*, and *Hypericum kalmianum* (AHS, 1980). The genus *Hypericum* now receives much publicity, not necessarily for its horticultural merit, but as an important component in the treatment of depression (Patocka, 2003). The genus has a nearly world-wide distribution, except in tropical lowlands, deserts, and arctic regions (Campbell and Delfosse, 1984). In Turkey, it is represented by 89 species, of which 43 are endemic (Davis, 1988). Of all the *Hypericum* sp., only *Hypericum perforatum* L. has been domesticated and well studied, while little research has been done with other members (Ayan et al., 2006).

Hypericum bupleuroides Gris. is a perennial, herbaceous medicinal plant that grows wild in damp, forested sites at high elevations in northeastern Turkey. It has traditionally been used in Turkish folk medicine to treat skin burns and intestinal disorders (Baytop, 1999). Although not yet domesticated in Turkey, this species has great pharmaceutical potential because of its well-documented phenolic (Özen et al., 2005) and hypericin (Çırak, 2005) contents.

In-vitro culture has been demonstrated as a viable option for multiplying such species as *Hypericum foliosum* (Moura, 1998), *Hypericum brasiliense* (Cardoso and Oliveira, 1996; Abreu et al., 2003), *Hypericum canariense* (Mederos, 1991), *Hypericum maculatum* (Kartnig and Brantner, 1990), and *Hypericum perforatum* (Cellarova et al., 1992; Murch et al., 2000; Pretto and Santarem, 2000; Bezo and Stefunova, 2001; Santarem and Astarita, 2003; Zobayed et al., 2004). That culturing system is a very useful tool for both studying and producing economically important secondary metabolites (Yazaki and Okuda, 1990; Dias et al., 1998; Kirakosyan et al., 2000, 2001; Bais et al., 2002; Sirvent and Gibson, 2002; Walker et al., 2002; Zobayed and Saxena, 2003; Hwang, 2006). Here, we present the first report of a procedure for callus production that yields cell masses for phytomedicinal studies. In addition, we describe an efficient protocol for direct and indirect plant regeneration from *H. bupleuroides*.

MATERIALS AND METHODS

Plant Material

All plant materials from *H. bupleuroides* were collected in Trabzon province of Turkey, and were identified by Dr. Hasan Korkmaz, Department of Biology, University of 19 Mayis, Samsun-Turkey. Voucher specimens were deposited in the herbarium of the Ondokuz Mayis University Agricultural Faculty (OMUZF # 62).

Explant Source

Seeds of *H. bupleuroides* were surface-sterilized in 75% ethanol for 2 min, then in a 15% (v/v) sodium hypochlorite solution (commercial sodium hypochlorite with 53% active constituent) for 25 min. This was followed by three rinses in sterile distilled water. The seeds were then germinated in magenta boxes containing an MS (Murashige and Skoog,

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1962) medium that was adjusted to pH 5.8 prior to autoclaving at 120°C for 20 min. The resultant cultures were incubated for 9 weeks at 20°C under an 18-h photoperiod. First and second pairs of leaves and the internodal segments of *in vitro*-grown seedlings (10 to 13 cm tall) were used as explant sources.

Callus Initiation

The explants were cultured in magenta boxes containing media supplemented with 0.1 or 1.0 mg L^{-1} each of BA and 2,4-D. Our callus initiation medium consisted of MS salts and vitamins, with the pH being adjusted to 5.8 before autoclaving at 121°C for 20 min. We tested the effect of growth regulators by combining BA with 2,4-D. Their selection and particular concentrations were based on results from previous studies of different *Hypericum* species (Pretto and Santarem, 2000; Bezo and Stefunova, 2001; Ayan and Çırak, 2005, 2006). A medium without plant growth regulators served as the control. All chemicals were purchased from Sigma (USA). Cultures were held, without sub-culturing, for 8 weeks at 26 ± 2°C, and under a 16-h photoperiod. Five explants were cultured in each magenta box (30 mL media) per treatment, with 4 replicates representing a total of 20 observations per treatment. The frequency of callus induction, callus fresh weights, and shoot numbers per explant were determined 8 weeks after culture initiation.

Shoot Induction and Multiplication

For shoot induction, calli were transferred to an MS medium supplemented with 2 mg L^{-1} BA (pH 5.8), and kept under the same conditions employed in our callus initiation. After 5 to 7 weeks, the number of shoots per callus was recorded.

Rooting and Acclimatization

To induce roots, shoots that had regenerated from either calli or the explant surfaces were excised and transferred into a full-strength MS medium (pH 5.8) supplemented with/without 1 or 2 mg L^{-1} indolebutyric acid (IBA). Five shoots were placed in one magenta box (30 mL media) per treatment, and data were recorded after 6 weeks of culture. After being removed from the boxes, the rooted plantlets were washed with tap water to remove rooting medium debris. These young plants were transplanted into boxes containing a 2:1 soil:sand mixture (autoclaved at 120°C for 20 min) under non-sterile conditions, and gradually exposed to ambient humidity. After 10 d, the acclimated plants were transferred to a partially shaded greenhouse and irrigated daily. Percent survival was recorded at 2-week intervals.

Statistical Analyses

Data obtained from this study were analyzed by ANOVA. Our experimental design was a factorial randomized-block arrangement with four replicates. Statistically significant averages were compared using Duncan's Multiple Range tests and, when necessary, the data were normalized using $x' = \sqrt{x} + 1$ transformation (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Callus Induction

When the goal is species conservation, callus induction and proliferation are effective tools for the micropropagation of plants, especially in cases of endemism or seed dormancy (Ayan and Cirak, 2006). However, cell suspension culturing is considered one of the best approaches for studying the biosynthesis of natural products, and calli are the richest source of cell mass when establishing such cultures (Walker et al., 2002). So far, the only *Hypericum* species reported to induce calli have been *H. erectum* (Yazaki and Okuda, 1990), *H. brasiliense* (Cardoso and Oliveira, 1996), *H. foliosum* (Maciel and Moura, 2000), *H. perforatum* (Dias et al., 1998; Pretto and Santarem, 2000; Bezo and Stefunova, 2001; Walker et al., 2002; Santarem and Astarita, 2003; Ayan et al., 2005), *H. linarioides*, *H. lydium*, *H. origanifolium*, *H. venustum*, and *H. scabrum* (Ayan and Çırak, 2005).

In the present study, the culturing of leaf and internodal explants on MS media containing various combinations of BA and 2,4-D resulted in both excessive callus induction and the formation of adventitious shoots directly on the explant surfaces. Success depended on the concentrations of growth regulators tested. Callus was optimally induced (100%) from both explant types within 20 to 25 d of inoculation when placed on an MS medium supplemented with either 1.0 mg L^{-1} BA plus 0.1 mg L^{-1} 2,4-D or 1.0 mg L^{-1} BA plus 1.0 mg L^{-1} 2,4-D. Our ANOVA results showed that BA and 2,4-D concentrations had a significant effect on the frequency of callus induction, callus fresh weights, and the number of shoot per callus/explant (Table 1). Callusing was initiated at the cut ends of either explant type, and eventually covered the entire explant surface. These calli were greenish and compact (Fig. 1a). Callus fresh weights ranged from 27.4 to 32.7 mg, and no significant difference was found among growth regulators and explant types. These results agree with those from previous studies where BA and 2,4-D were used as callus-inducing agents in *H. erectum* (Yazaki and Okuda, 1990), *H. brasiliense* (Cardoso and Oliveira, 1996), *H. perforatum* (Pretto and Santarem, 2000; Bezo and Stefunova, 2001; Santarem and Astarita, 2003; Ayan et al., 2005), *H. linarioides*, *H. lydium*, *H. origanifolium*, *H. venustum*, *H. scabrum* (Ayan and Çýrak, 2005), and *H. heterophyllum* (Ayan and Çırak, 2006).

In contrast, when both types of explants were cultured on MS media containing the opposite combination, i.e., 0.1 mg L^{-1} BA plus 1.0 mg L^{-1} 2,4-D, adventitious shoot buds were produced directly on the explant surfaces, without the intervening formation of calli. This adventitious formation was observed over the entire explant surface, and shoots elongated up to 3 cm under primary culture alone, without the usual need for sub-culturing. The number of shoots per explant averaged 27.3 and 20.3 for internodal and leaf explants, respectively. This callus induction and direct shoot regeneration in response to *in-vitro* culturing on media with BA and 2,4-D also has been observed with other horticultural crops. For example, Nehra et al. (1990) has shown that immature leaf explants taken from either *in-vitro* shoots or greenhouse-grown plants of the strawberry (*Fragaria x ananassa*; cv. Redcoat) can form calli and multiple shoots when

Table 1. Effect of BA and 2,4-D concentrations on callus initiation, callus fresh weights, and average number of shoots in *H. bupleuroides* Gris. from different explants.

Growth regulators (mg L^{-1})	Frequency of callus initiation (%)[a]		Callus fresh weight (mg FW per callus)		Number of shoots per callus or explant	
	Leaf	Internode	Leaf	Internode	Leaf	Internode
0.0 (control)	0b[b]	0b	0b	0b	0bC	0bC
1.0 BA + 0.1 2,4-D	100a	100a	30.8a	32.7a	16.7aB	33.6aA
1.0 BA + 1.0 2,4-D	100a	100a	27.4ab	30.4a	9.4abB	13.2bB
0.1 BA + 1.0 2,4-D	*	*	*	*	20.3aB	27.3aA

[a]Percentage of explants forming calli. [b]Values not followed by the same small letter within a column, or by the same capital letter within a row, are significantly different ($P < 0.01$) according to Duncan's Multiple Range test. *Culturing of leaf and internodal explants on MS medium supplemented with 0.1 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D resulted in direct shoot regeneration without intervening callus induction.

treated with various concentrations of BA and 2,4-D. Similarly, leaf explants of *Dianthus chinensis* cultured on an MS medium with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} 2,4-D produce adventitious shoot buds along with excessive calli (Kantia and Kothari, 2002).

Shoot Formation and Multiplication

When calli were cultured on the MS medium supplemented with 2 mg L^{-1} BA, shoot induction was very intensive (Fig. 1b). At various concentrations, BA can be an effective shoot-inducing agent in *Platycerium bifurcatum* (Camloha et al., 1994), *Chrysanthemum coronarium* (Lee et al., 1997), *Morus indica* (Sahoo et al., 1997), *Gardenia jasminoides* (Al-Juboory et al., 1998), and *Garcinia mangostana* (Te-chato and Lim, 2000). For *H. perforatum* in particular, BA is the most efficient in promoting shoot regeneration when leaves (Pretto and Santarem, 2000) or excised seedling portions (Cellarova et al., 1992) are the explant sources. Similarly, BA can be used together with NAA in shoot induction from anther-originated calli in that species (Kirakosyan et al., 2000).

The success of *in-vitro* shoot formation may depend upon the type of explant used (Zobayed and Saxena, 2003; Leng et al., 2004; Hong et al., 2004). For example, we observed that, depending on the callus induction media selected, the average number of shoots was significantly higher in calli obtained from internodal explants than from leaf explants. When derived from the former, the calli cultured on an MS medium with 1.0 mg L^{-1} BA plus 0.1 mg L^{-1} 2,4-D had the highest rate of shoot formation (33.6 shoots per callus), followed by internodes cultured on the MS medium with 0.1 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D (27.3 shoots per explant). Differences were significant among the different explant types and callus induction media with respect to the number of shoots produced per callus/explant (Table 1). We also noted that the culture response of internodal segments was superior to that of leaf explants in terms of both direct and indirect shoot regeneration. Such fluctuations in media response by different explant types also have been reported for *Lonicera japonica* (Georges et al., 1993), *Morus indica* (Sahoo et al., 1997), and *Gladiolus* sp. (Nhut et al., 2004).

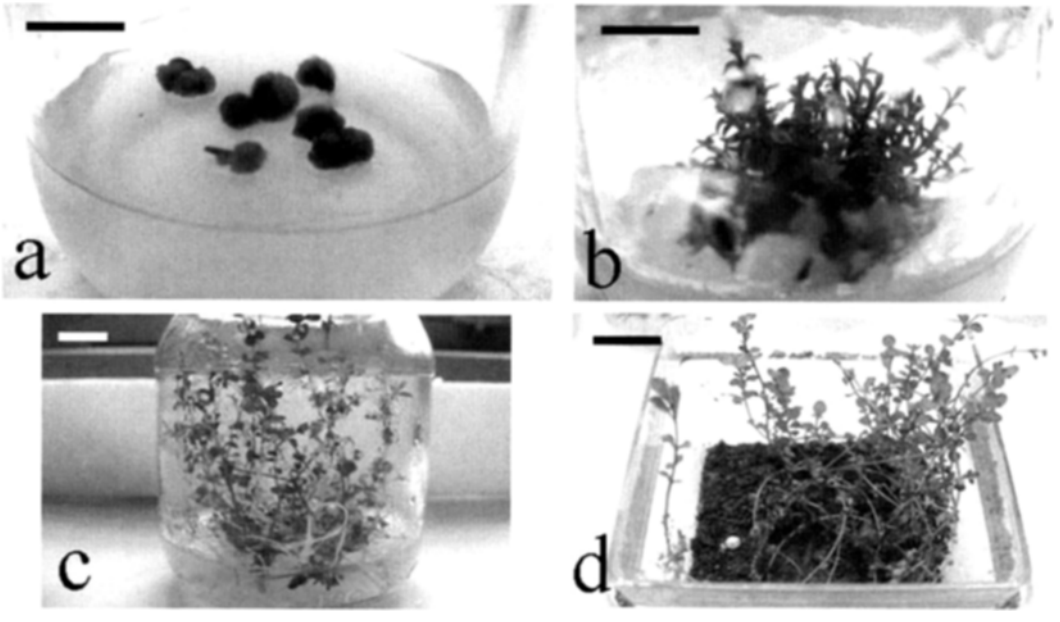

Figure 1. a. Callus formation from internodal explants cultured on MS medium supplemented with 1.0 mg L^{-1} BA plus 0.1 mg L^{-1} 2,4-D. **b.** Shoot induction in MS medium containing 2 mg L^{-1} BA from internodal explant-derived calli that had been induced on MS medium supplemented with 1.0 mg L^{-1} BA plus 0.1 mg L^{-1} 2,4-D. **c.** Rooting of shoots cultured on growth regulator-free medium. **d.** Regenerated plant under greenhouse conditions. Bars represent 1 cm.

Table 2. Effect of MS medium and the presence of IBA at different concentrations on root formation from shoots of *H. bupleuroides* Gris.

	Rooting media		
	MS	MS+IBA (1 mg L^{-1})	MS+IBA (2 mg L^{-1})
Percentage rooting (%)	90a*	40b	0c
Number of roots per shoot	7.1a	1.2b	0c

*Values not followed by the same small letter within a row are significantly different ($P < 0.01$) according to Duncan's Multiple Range test.

Rooting and Acclimatization

Indolebutyric acid, indoleacetic acid, and naphthaleneacetic acid are commonly used to improve rooting in different *Hypericum* species (Pretto and Santarem, 2000). Whereas in *H. canariense,* rooting is accomplished in the presence of either IBA or NAA (Mederos, 1991), these two growth regulators are entirely ineffective for inducing root formation from the shoots of *H. foliosum* (Moura, 1998). IBA and IAA are the most effective for rooting in *H. perforatum* (Cellarova and Kimakova, 1999).

In the present study, elongated shoots from both calli and explants were rooted successfully on a full-strength MS medium that lacked growth regulators (Fig. 1c). Interestingly, rooting capability deteriorated in the presence of IBA. A pronounced reduction in root formation was observed on an MS medium with 1 mg L^{-1} IBA, and no rooting was induced in the presence of 2 mg L^{-1} IBA (Table 2). The highest rooting percentage (90%) and root number (7.1) per shoot were obtained from a hormone-free medium. Moreover, the roots induced on an IBA-containing medium were thicker, shorter and less branched than those induced without IBA. Rooted plants from IBA-free media had longer nodes and greater vigor. Ibrahim and Debergh (2001) also have reported that elongated shoots of *Rosa hybrida* will root best on a growth regulator-free, half-strength MS medium.

Regenerated plants were transferred to non-sterile conditions for acclimatization and to conditions of steadily decreasing levels of humidity. After 10 d, 90% of regenerants had survived (Fig. 1d).

To conclude, a callus-mediated plant regeneration protocol is a critical requirement because it allows researchers to exploit techniques for *in-vitro* selection, somaclonal variation, and genetic engineering in efforts to improve plants (Sahoo et al., 1997). However, genetic mutations are more frequent in shoots regenerated from calli than from other explant types, particularly when the sub-culturing period is prolonged, because of the non-uniform nature of callus tissue (Kantia and Kothari, 2002). Therefore, adventitious shoot formation is a more reliable approach for clonal propagation because it prevents somaclonal variation in the cultures. We are now the first to describe a simple and effective system that is suitable for both adventitious shoot formation and cell mass proliferation from the leaves and internodes of *H. bupleuroides*. Our data show that internodal segments are superior to leaf explants for both purposes. If multiplication of selected clones is required, internodal explants can be cultured on an MS medium supplemented with 0.1 mg L^{-1} BA plus 1.0 mg L^{-1} 2,4-D. When elongated, the shoots are then transferred to a full-strength MS medium for rooting, making it possible to recover plants in four months. Likewise, if cell masses are required, calli can be obtained from internodal segments cultured on an MS medium containing 1.0 mg L^{-1} BA and 0.1 mg L^{-1} 2,4-D. Further analyses of cell suspension cultures and their secondary constituents are still needed.

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