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Full Length Research Paper

Direct plant regeneration from *in vitro*-derived leaf explants of *Hypericum spectabile*, a medicinal plant

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An efficient and reproducible procedure is described for direct plant regeneration using *in vitro* regenerated leaf explants of *Hypericum spectabile*. The leaf explants were cultured on MS media supplemented with six different concentrations (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg/L) of BAP and kinetin seperately. All of the BAP concentration, shoot regeneration occurred directly without callus formation, but the number of shoots changed, depending on the different concentrations of BAP. The highest and the lowest number of shoots were obtained on the medium supplemented with 1.0 and 2.5 mg/L of BAP (90 to 50%, respectively). In the present study, the medium containing 0.25, 0.5 and 2.5 mg/L Kin did not promote adventitious shoots formation. Among concentrations of Kin, the best results in terms of both shoots number and morphogenic properties were obtained from MS medium supplemented with 1.0 mg/L Kin (60%). *In vitro* regenerated plants induced roots on half-strength MS medium containing all the concentrations (0.25, 0.5, 1.0, 2.0 mg/L) of IAA. However, the medium supplemented with 0.5 mg/L IAA (100%) was found to be optimum for inducing root. Rooted 5- to 6-week-old *in vitro* regenerated plants were transferred into a greenhouse for acclimatization.

Key words: *Hypericum spectabile*, direct regeneration, leaf explant, *in vitro*, PGRs.

INTRODUCTION

Hypericum (Hypericaceae) is one of the plants used traditionally in medicine, crop protection, and flavoring, as well as fragrance in food (Isman et al., 2001; Daferera et al., 2003). The *Hypericum* genus, a member of the Clusiaceae family, is represented in Turkey by 77 species, 30 of which are endemic (Kaya and Aksakal, 2005). Hypericin, a major bioactive component of *Hypericum* species, is widely used in neurological disorders and depression (Okpanyi et al., 1990). Recently, hypericin has been shown to have antitumor activity (Vandenbogaerde and Witte, 1995; Kamuhabwa et al., 2000), highly active against HIV (Takahashi et al., 1989; Meruelo et al., 1988), and exhibits a number of pharmacological effects. Pharmacological experiments have shown that this species has antiulcerogenic, anticiceptive, anti-inflammatory, antitumor, antimicrobial and antioxidant activities (Apaydin et al., 1999; Sokmen et al., 1999; Ozturk et al., 2002; Conforti et al., 2007; Toker et al., 2006; Hakimoğlu et al., 2007; Kizil et al., 2008). However, this species have been known for their antidepressant, analgesic, spasmolytic, antiviral and wound healing effects for many years (Pasqua and Avota, 2003; Wojcik and Podstolski, 2007).

The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine (Murch et. al., 2000a). Micropropagation, as an advanced vegetative propagation technique, can serve in the production of a great number of genetically uniform plants and pathogen-free transplants in a limited time and space (Canter and Thomas, 2005; Zobayed and Saxena, 2003; Santarem and Astarita, 2003). Murch et al. (2003) consider *in vitro* propagation of medicinal plants a successful strategy that addresses the problems associated with supply and variability in product quality. Furthermore, plant tissue and cell cultures are also

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Abbreviations: BAP, N^6-benzylaminopurine; **IAA,** Indole-3-acetic acid; **Kin,** kinetin; **MS,** Murashige and S koog (1962) medium; **PGRs,** Plant growth regulators.

important tools which allow extensive manipulation of the biosynthesis of secondary compounds and yield a higher productivity compared to that of intact plants (Kirakosyan et al., 2001). Plant regeneration of the *Hypericum* species has been achieved by using as explants the whole seedling or their excised parts (Ćellárová et al., 1992; Bernardi et al., 2007; Ayan and Çirak, 2008; Namli et al., 2009; Namli et al., 2010), hypocotyl sections (Murch et al., 2002; Zobayed et al., 2004), leaves (Pretto and Santarem, 2000) and leaf discs and stem segments (Ayan et al., 2005), adventitious roots (Goel et al., 2009) using various types and concentrations of cytokinins and auxins.

To our knowledge, this is the first report on direct plant regeneration from leaf explants of *Hypericum spectabile*, which will provide an alternative source for *H. spectabile* genetic manipulation in the future. Therefore, the aim of this research was to develop an efficient protocol for inducing direct plant regeneration from *in vitro* leaf explants. Also, this study will be useful for exploitation of somaclonal variation of *H. spectabile*.

MATERIALS AND METHODS

Plant material and preparation of explants

H. spectabile were collected from Adiyaman province of Turkey (Figure 1A). Voucher specimens are kept at the Herbarium of Dicle University, Faculty of Science. The seeds of *H. spectabile* were used as explants to initiate *in vitro* cultures. They were surface-sterilized by immersion in a 5% (w/v) commercial bleach solution (NaOCl) for 10 min. after pre-sterilization processes, which included washing with tap water for 5 to 10 min followed by rinsing with 70% (w/v) ethanol for 30 s. followed by repeated rinsing with sterile distilled water.

In the first stage of our study, the seeds were germinated on the hormone-free MS (Murashige and Skoog, 1962) medium supplemented with 30 g/L sucrose and 5.45 g/L agar (w/v). After 10 days of culture, the shoots (2 to 3 cm long) obtained from seeds were cultured on MS medium supplemented with 0.25 mg/L BAP. After that, new shoots to on fresh culture medium every 3 to 4 weeks were subcultured.

Culture media and conditions

In the second stage of our study, for direct shoots regeneration experiments, the leaves were used as explant sources. This explants were cultured on MS medium containing six different concentrations of BAP and kinetin (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg/L) seperately.

All media were adjusted to pH 5.8 prior to autoclaving (120°C for 20 min), and *in vitro* cultures were maintained at 25 ± 2°C with light at 40 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) ("cool white" fluorescent lamps, 75 W) a photoperiod of 16 h. Adventitious shoots formation from leaf explants response was examined after 5 weeks of culture.

Rooting and acclimatization

For rooting induction, elongated shoots (2.5 to 3.5 cm long) were isolated and transferred on half strength MS medium containing 20 g/L sucrose and four different concentrations of IAA (0.25, 0.5, 1.0, 2.0 mg/L) seperately. All the media were adjusted to pH 5.8 prior to autoclaving (120°C for 20 min) and the cultures were maintained under dark conditions for one week. Then, the cultures were maintained at 25±2°C with 16 h photo period (40 µmol m^{-2} s^{-1}) provided mercury fluorescent lamps.

For acclimatization, rooted plantlets were removed from culture vessels, washed carefully with water to remove adhering medium, The plantlets were covered with a Pyrex beaker after being potted in plastic cups containing a sterile 1:1:1 mixture of sand, soil and peat. To maintain 90 ± 5% relative humidity for at least 2 weeks, and then, the humidity was gradually reduced to 65 ± 5% before transfer into greenhouse conditions.

RESULTS AND DISCUSSION

Induction of adventitious shoots directly via organogenesis from leaf explants

Hypericum genus is commonly used as a medicinal plant for its anti-depressant properties. Harvesting such medicinal plants from nature is causing a loss of genetic diversity. Plants with very small seeds like *Hypericum* cannot be cultured by traditional methods, easily. The plant has many active compounds (for example, hypericine) and is traditionally used for its sedative, antihelminthic, anti-inflammatory and antiseptic effects. It is also reported to be effective in the treatment of burns and in the treatment of gastrointestinal diseases (Conforti et al., 2002). Directly adventitious shoots formation without callus phase is very important because plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus (Lee and Phillips, 1988; Karam and Al-Majathoub, 2000).

The effects of auxins and cytokinins on shoot multiplication have been reported earlier for *Hypericum* species (Cellárová et al., 1992; Moura, 1998). This study describes the basic procedures for adventitious shoots induction from leaves of *H. spectabile.* The adventitious shoots formation was observed in 9 different treatments, though the efficacy varied greatly among types and concentrations of plant growth regulators.

In the first stage of our research, surface sterilized seeds were germinated (75% germination rate) on the hormone-free MS medium about within 7 to 10 days (Figure 1B). After germination of seeds, *in vitro*-grown seedlings produced 4 to 6 nodes. The shoot apices with two to four leaves were isolated as primary explants and cultured on MS medium supplemented with 0.25 mg/L BAP (Figure 1C) for the establishment of aseptic cultures. After 3 to 4 weeks of culture, leaves of *in vitro* grown seedling were used as sources of explants.

In the second stage of our research, the leaf explants were cultured on MS medium supplemented with BAP and Kin at various concentrations (Figure 1D). After one week, the size of the explants had increased, and cluster primordial formation was seen at the cut ends or on surface of leaves, particularly in concentrations of BAP

Figure 1. Direct plant regeneration from *in vitro*-derived leaf explants of *H. spectabile*.(A) *H. spectabile* plants in the field. (B) Seeds were germinated on the hormone-free MS basal medium. (C) Multiple shoots grown on MS medium supplemented with 0.25 mg/L BAP. (D) Aspect of leaf explants cultured on MS medium. (E) *In vitro* culture response and shoot induction from leaf explants. (F) Direct shoot formation from leaf explants on MS medium supplemented with 1.0 mg/L BAP. (G) Adventitious shoots formation on MS medium supplemented with 0.5 mg/L BAP and (H) 0.25 mg/L BAP. (I) Aspect of shoot formation from leaf explants on MS medium supplemented with 2.5 mg/L Kin and (J) 1.0 mg/L Kin. K) Rooting microshoots on half strength MS medium supplemented with 0.5 mg/L IAA. (L) Direct regenerated plantlets originated from microshoots following establishment in soil.

(Figure 1E). Among tested concentrations, 1.0 mg/L BAP (Figure 1F) was found to be most effective as it produced maximum number of adventitious shoots (29.6 per explant) and shoot length (3 cm per explant). In this study, increasing concentrations of BAP (from 1.5 to 2.5 mg/L) reduced adventitious shoots formation (Figure 2a). Similar responses were observed in low BAP concentrations (0.25 and 0.5 mg/L) for adventitious shoots formation (Figure 1G and H).

In our research, the adventitious shoots were successfully propagated after two subcultures in the presence of BAP. Our findings are compatible with those of Pretto and Santarem (2000), who reported that in *H. perforatum*, BAP was found to be the most efficient in promoting shoot regeneration when leaves were used as the explant. Among the various concentrations of Kin

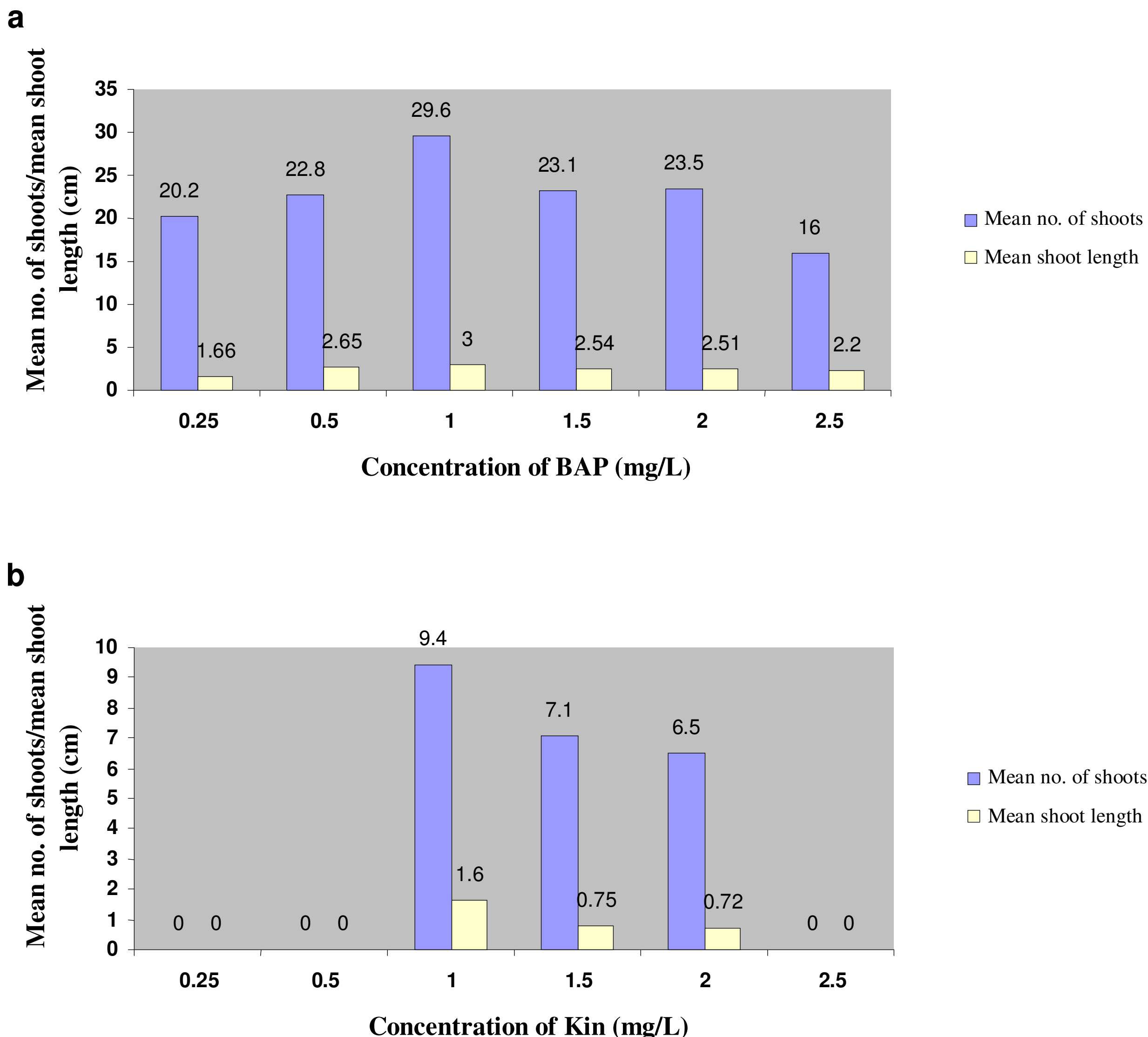

Figure 2. Effect of BAP and Kinetin (a, BAP and b, Kin) on direct shoot regeneration and shoot elongation from leaf explants of *H. spectabile*.

tested, 1.0 mg/L Kin proved to be the most effective for inducing of adventitious shoots (9.4 per explant) and shoot length (1.6 cm per explant). Low concentrations (0.25 and 0.5 mg/L) have not been effective on shoots formation (Figure 2b). Moreover, no shoots were induced when the highest level of Kin (2.5 mg/L) were added to the culture medium (Figure 1I), and browning observed on the surface of the leaves in the 3 weeks of cultures. The medium containing Kin (0.25, 0.5 and 2.5 mg/L) as a supplement did not promote shoot-bud regeneration even after subculturing in the same media. However, the increase in Kin concentration caused adventitious shoots formation, but very slow development of new shoots were observed. Among concentrations of Kin, the best results in terms of both shoot number and morphogenic properties were obtained from MS medium containing 1.0 mg/L Kin (Figure 1J). But, here Kin-supplemented MS medium had a lower shoots formation percentage as compared to BAP.

The average frequency of direct plant regeneration per explant on MS medium supplemented with 1.0 mg/L BAP (Figure 3) in case of leaf explants was found to be 90%. However, 1.5, 2.0 and 2.5 mg/L BAP showed 75, 60 and 50% response, respectively. Similarly, the average frequency of direct plant regeneration on MS medium supplemented with 1.0 mg/L Kin was found to be 60%, as shown in Figure 3. As a result, the present investigation showed that adventitious shoots could be regenerated directly from leaf explants of *H. spectabile* and in this investigation, we induced shoots directly via organogenesis from leaf explants. The concentrations of BAP were more suitable than concentrations of Kin for

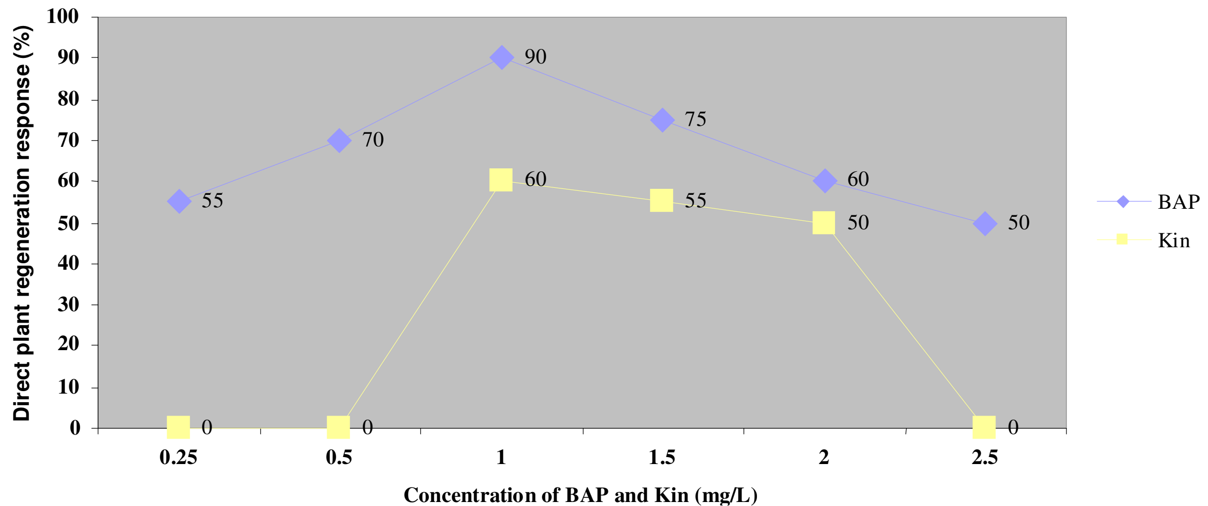

Figure 3. Influence of BAP and Kin concentrations (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/L) on direct plant regeneration frequency of leaf explants of *H. spectabile.*

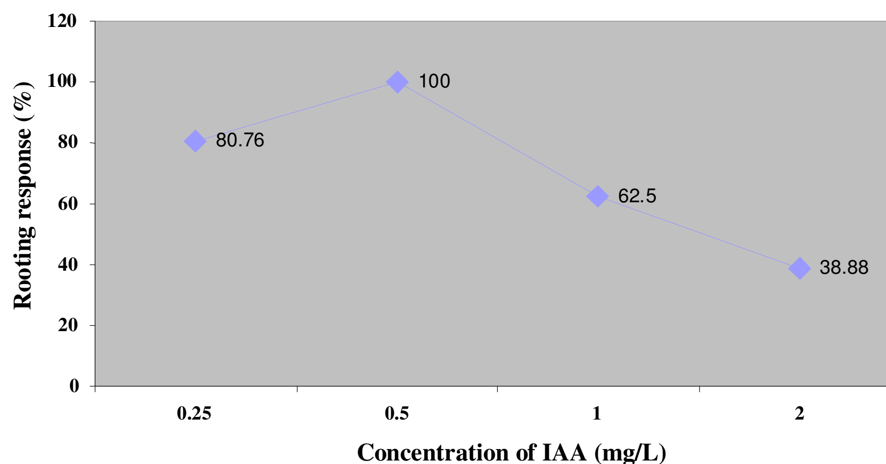

Figure 4. Effect of IAA concentrations (0.25, 0.50, 1.00 and 2.00 mg/L) on rooting in direct regenerated shoots from leaf explants of *H. spectabile.*

direct shoots regeneration. In this research, the highest direct plant regeneration rate occured on the medium containing 1.0 mg/L BAP.

Induction of roots and acclimatization of *in vitro* rejenerated plants

Regenerated shoots (2.5 to 3.5 cm in length) were excised and transferred individually to a rooting medium (half strength MS, 20 g/L sucrose) supplemented with various concentrations (0.25, 0.5, 1.0 and 2.0 mg/L) of IAA. In the rooting stage, the root initiation occurred within 15 days; an efficient root system was present after 6 weeks of culture. The roots induction was observed in all concentrations of IAA (Figure 1K). However, the addition of 0.5 mg/L IAA to the medium was most effective at inducing root formation (100% rooting). The increase in IAA concentration (1.0 and 2.0 mg/L) resulted in significant reduction in the root induction rate (62.50 to 38.88%, respectively). Nevertheless, adding lower concentrations of IAA significantly increased number of roots per explant (Figure 4). Well developed root system and high-quality rooted plantlets ensure acclimatization. Plants were normal in appearance and morphologically (Figure 1L).

IAA and IBA are the most effective for rooting in *H. perforatum* (Cellárová and Kimakova, 1999). In our research, elongated shoots were rooted on half strength MS medium supplemented with different concentrations of IAA and, the root formation rate was changed depending on the concentration of IAA. However, our

data did not match those of Ayan et al. (2005). They reported that shoots of *H. perforatum* were rooted very intensively on MS medium supplemented with 1 mg/L of IAA. In our study, the best result was obtained on half strength MS medium supplemented with 0.5 mg/L of IAA and 20 g/L sucrose.

Conclusion

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors. This paper reviews the achievements and advances in the application of tissue culture and genetic engineering for the *in-vitro* regeneration of medicinal plants from various explants and enhanced production of secondary metabolites.

A simple, efficient, high frequency protocol on direct regeneration from leaf explants of *H. spectabile* has been demonstrated here for the first time. This protocol offers a potential system for micropropagation and genetic improvement of *H. spectabile* in the future. Additionally, this method could be useful for large scale multiplication as well as *in vitro* conservation of germplasm of this medicinal species.

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