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Full Length Research Paper

High efficiency indirect shoot regeneration and hypericin content in embryogenic callus of *Hypericum triquetrifolium* Turra

Esin Akçam Oluk[1]*, Serpil Orhan[2], Özgür Karakaş[3], Ali Çakır[1], and Ahmet Gönüz[4]

[1]Ege University, Science Faculty, Biology Department, Bornova, 35100 İzmir, Turkey.
[2]Ege University, Engineering Faculty, Bioengineering Department, Bornova, 35100 İzmir, Turkey.
[3]Dicle University, Science and Arts Faculty, Biology Department, Diyarbakir, Turkey.
[4]Çanakkale 18 Mart University Science and Arts Faculty, Biology Department, Turkey.

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A method to induce indirect regeneration from *Hypericum triquetrifolium* Turra was described in the present study. Callus was induced from cotyledon explants of 35 days old aseptic seedlings on semi-solid MS supplemented with IAA (0.5 mg.L^{-1}) combined with BAP (2 mg.L^{-1}). Meristemoids developed on the surface of callus by decreasing of the cytokinins and plantlet regeneration with 100% frequency through these embryogenic calli occured in semi-solid medium when the PGRs were removed completely. Embryogenic calli obtained during the experiments were analyzed for their hypericin content. And they were found to produce hypericin as 48 µg/g DM. Regenerated plantlets were rooted in MS containing 1 mg/L IAA. The highest percentages (94%) of survival of transferred plantlets to free-living circumstances were limited when they were acclimatized in sand : peat : perlite (1:1:1; v/v/v) mixture.

Key words: *Hypericum triquetrifolium*, indirect regeneration, hormone-free MS.

INTRODUCTION

Hypericum sp. have been notified to have a complex mixture of bioactive compounds mainly naphtodianthrons with antidepressive and antiviral activities (Bombardelli and Morazzoni, 1995), flavanoids preventing cardio-vascular diseases (Chu et al., 2000) and hypericin and hyperforin with their wide array of pharmacological effects (Patocka, 2003). A diverse tissue culture systems as a viable option for multiplying of *Hypericum* sp. have been developed. Wojcik and Podstolski (2007) have criticized leaf explant response of *Hypericum perforatum* while Çirak et al. (2007) have reported direct and indirect regeneration from *Hypericum bupleuroides* Gris. Additionally, the bioactive productivity of tissue cultures of *Hypericum maculatum* (Kartnig and Brantner, 1990), *Hypericum erectum* (Yazaki and Okuda, 1990), *H. perforatum* (Bais et al., 2002) and *H. bupleuroides* (Çirak, 2005) have been investigated.

Hypericum triquetrifolium Turra is a perennial herbaceous, distributed wildly in Eastern Europe and Mediterranean region. The plant is reported as a sedative and antispasmodic for intestine and bile disorders (Saya et al., 2001), besides its antiinflammatorial and antioxidative effects (Couladis et al., 2002) in traditional use. This species has not been subjected to *in vitro* studies as much as the other Hypericum species, especially *H. perforatum* have been. However, Karakas et al. (2008) have examined hypericine content of micropropagated plants and Akçam Oluk and Orhan (2009) have reported thidiazuron induced micropropagation of the plant.

In vitro plantlet production through the embryogenic callus phase can be scaled up several fold more than with

*Corresponding author. E-mail: esak_ol@yahoo.co.uk. Tel:_+9 0232 3884000. Fax: +9 0232 3881036.

Abbreviations: **IAA,** Indole-3-acetic acid; **BAP,** 6-benzylaminopurine; **NAA,** naphtelene aceticacid; **BA,** benzyl adenine; **MS,** Murashige-Skoog; **PGR,** plant growth regulator; **DM,** dry mass; **2,4-D,** 2,4-dichloro-phenoxyacetic acid.

direct regeneration. This process also involves somaclonal variation presenting an oppurtunity to select plants with desirable features (Cuenca et al., 2000), e.g. increased secondary metabolite content in medicinal plants. Many secondary metabolites such as phenolics are biosynthesized mainly during cell differentiation (Matsuki, 1996). Embryogenic calli obtained *in vitro* have been regarded as much productive in comparison to non-embryogenic calli for the many secondary metabolites (Kovacevic et al., 2000; Akçam Oluk, 2006; Niham et al., 2009). Therefore, in the present study, indirect regeneration capacity and hypericin content of the cotyledon explant derived embryogenic calli of *H. triquetrifolium* Turra was undertaken. Investigation of bioactive compounds in regenerated plantlets through this species is however intended in the future.

MATERIALS AND METHODS

Plant materials

H. triquetrifolium Turra seeds collected from Kaz Mountains in Turkey, seasonally. They were surface sterilized by immersing in a 70% (v/v) ethanol for 30 s, and then in a 20% (v/v) commercial bleach (5% NaOCl) plus 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) for 25 min. Subsequently, they were washed 4 times with sterilized distilled water. Sterilized seeds were sawn on 0.8% (w/v) water-agar, in a growth chamber at 24 ± 4°C under darkness.

Callus ınitiation, shoot regeneration and multiplication

Cotyledon explants of 35 days old seedlings (0.2 cm) were excised and placed horizontally on semi-solid MS (Murashige-Skoog, 1962) medium supplemented with 0.5 mgL^{-1} IAA + 2 mgL^{-1} BAP for callus induction. MS medium devoids of PGRs was referred as the control. The explants were incubated in 100 ml erlenmeyer flasks in a growth room that had 16/8 photoperiod and 25 ± 2°C temperature. Each erlenmeyer was containing five explant and each experimental variant had 16 replicates. The frequency of callus induction was recorded after 1 month of culture beginning. The induced calli were transferred into semi-solid medium supplemented with 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} BAP for shoot induction. Approximately 5 pieces of callus (1 g each) were placed in culture vessels and the cultures were subcultured twice with 15 days of intervals under the same environmental conditions as employed for callus cultures. Then, a part of appeared embryogenic calli was used for hypericin analysis and the rest was transferred to hormone-free MS. Regeneration was determined at the end of 2 weeks. Primary somatic embryos were subcultured onto fresh medium for further production of secondary embryos, and thus followingly the secondary to tertiary, etc.

Rooting and acclimatization

Regenerated shoots were sepparated from the calli and rooted and acclimatized due to our previous report on micropropagation of *H. triquetrifolium* Turra (Akçam Oluk and Orhan, 2009).

Sigma chemicals and vessels were utilized in all experiments. pH of the all media were adjusted to 5.8 with 1 M KOH before autoclaving at 121°C, 1.1 kg/cm^2 for 15 min. All the experiments repeated three times.

Hypericin determination

Freshly harvested embryogenic calli were immediately washed with distilled water. The calli were dried in air for three days and then of 200 mg were placed in vials containing chloroform (10 ml) and extracted in a sonicator to remove their chlorophyll contents. This process was repeated twice. Following that, the chloroform was removed in a vacuo. After removing the chloroform, the samples were re-extracted with methanol (10 ml) in the sonicator. With methanol, the extraction process was repeated three times and then, the methanol was removed from the vacuole. The final samples were dissolved in methanol 1 ml of supernatant was placed in a test tube. Absorbance was measured at 589 nm on a spectrometer (Shimadzu UV-160). The spectrometer analysis was performed by using an eight point calibration curve generated with pure hypericin (Figure 2). The hypericin was obtained from Sigma.

RESULTS

Callus initiation began with the swelling of cultured cotyledons at a frequency of 98.2% within one month in 0.5 mg/l IAA and 2 mg/L BAP. They continued to grow in this medium as green, friable clusters of undifferentiated cells (Figure 1A). The growth rate of the callus was relatively constant and doubling time was about two weeks. After two subcultures, meristemoids appeared on the entire surface of callus upon transferring onto medium containing lower BAP (0.5 mg/L) (Figure 1B). And differentiation of these meristemoids into somatic embryos occured only when they were subcultured in the medium devoids growth regulators (Figure 1C). A moiety of these embryogenic callus were analyzed for their hypericin content. They were found to produce this compound at a level of 48 µg/g DM. The rest of the cultures, kept in hormone-free MS, continued to produce new embryoids for several subcultures. The embryoids germinated and formed noumerous shoots (more than 50 shoots per g callus) usually without roots (Figure 1D). Rooting (Figures 1E and F) and acclimatization (Figure 1H) of the regenerants were performed according to our previous report (Akçam Oluk and Orhan, 2009). No plants have yet grown large enough to flowering nor to employ for their hypericin content.

DISCUSSION

2,4-D and NAA did not result callus from cotyledon explants in this study, however 2,4-D in *H. perforatum* (Pretto and Santarem, 2000) and *H. bupleuroides* (Çirak et al., 2007), and NAA in *H. perforatum* (Karppinen et al., 2006) are suggested. Regeneration via somatic embryogenesis has been reported for several species of *Hypericum*. The researchers have suggested that the shoot production may be induced from anther derived callus in the presence of BA and NAA (Kirakosyan et al., 2000), while BA alone may be used in leaf origined callus (Pretto and Santarem, 2000) of *H. perforatum.* BA alone is also reported as very intensive shoot inducing agent

Figure 1. A: Intensive callus growth from cotyledon explants of *Hypericum triquetrifolium* Turra in 0.5 mg/l IAA+2 mg/l BAP. **B:** Meristemoids (arrows) appearing on the entire surface of callus. **C:** Embryoids developing from meristemoids (arrows indicate special glands on the margin of the leaves). **D:** De novo shoot formation on the surface of the calli. **E, F:** Root development in the presence of 1 mg/l IAA. **G:** Red pigmentation (arrows) in the calli indicating hypericin accumulation. **H:** Acclimatized plantlets.

from callus of *H. bupleuroides* (Çirak et al., 2007). We transferred calli into the medium containing lower level of BAP for shoot inducion, as Gadzovska et al. (2005) referenced. Meristemoids appeared on the callus but, remained meristematic and did not undergo any additional differentiation in the presence of BA (0.5 mg/l) and IAA (0.5 mg/l). Differentiation of meristemoids to somatic embryos depended on the remove of the growth regulators from the medium, as Nessler (1982) described detaily in opium poppy. Support of hormone-free medium on shoot regeneration from callus has also been underlined for sugarbeet (Doley and Saunders, 1989). In our study, obtained regenerants acclimatized readily, alived (94%) and are still continuing to grow in the greenhouse of our department.

Cell or callus cultures of the medicinal plants are reported less or non-productive for the complicated secondary metabolites. Differentiation is emphasized as fundamental for the biosynthesis of such compounds (Verpoorte et al., 1993; Hsu and Pack, 1989). Hypericin is an aromatic policyclic anthrone, a class of colored or pigmented chemical substance. Accumulation of hypericins in cell cultures of *H. perforatum* has been shown to be dependent on cellular and tissue differentiation (Kirakosyan et al.,

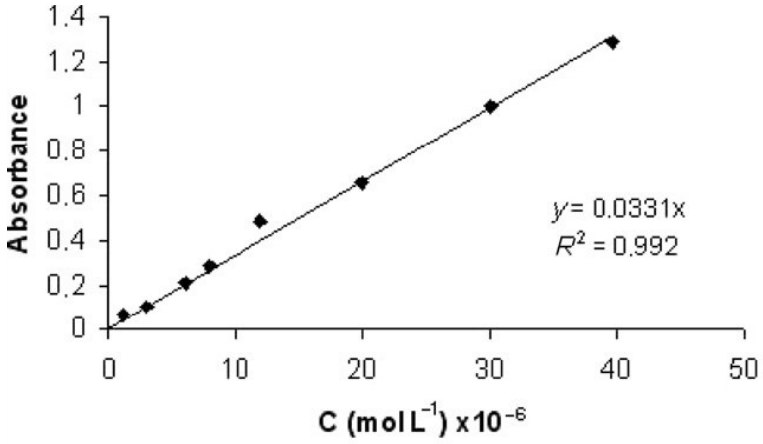

Figure 2. Calibration curve of pure hypericine.

2000) since they accumulated in special glands on the margin of the leaves (Cellarova et al., 1995). These structures were highly visible in our cultures (Figure 1C). Red pigmented hypericin dots were also observed after germination of embryoids (Figure 1G). Gadzovska et al. (2005) have studied hypericin content of calli, regenerated shoots and micropropagated plantlets of *H. perforatum*. They found these cultures to produce hypericin at a level of 15 - 20, 25 - 50 and 30 - 100 µg/g DM, respectively. Karppinen et al. (2006) have similarly shown the concentrations of hypericins, pseudohypericins and phloroglucinols increased with tissue differentiation, in the same species.

Total aerial parts of wild *H. triquetrifolium* are reported to contain 0.43% w/w of hypericin (Alali et al., 2004). Karakas et al. (2008) have studied this compound of the micropropagated plants obtained in the presence of 1 and/or 2 mg/l BAP and found the compound at a level of 0.013 and 0.012% w/w, respectively, in this species. Thus, we can interpretate that our embryogenic calli are productive for hypericin (48 µg/g DM) being as 0.05% higher than native plant (Alali et al., 2004).

To our knowledge, there is no report on the callus induction nor the indirect regeneration and hypericin content of callus tissues in *H. triquetrifolium*. In conclusion, the regeneration protocol first described here could be used for multiplying of the plant and the embryogenic cultures could be used in the large-scale production of hypericin from *H. triquetrifolium*. The bioactive compound analysis of the plants those were obtained by rgeneration through this embryogenic and hypericin productive callus cultures will also be performed in the future study.

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