African Journal of Biotechnology Vol. 8 (15), pp. 3506-3510, 4 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Thidiazuron induced micropropagation of *Hypericum triquetrifolium* Turra

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Accepted 15 May, 2009

Hypericum **genus is commonly used as a medicinal plant for its anti-depressant properties. Harvesting such medicinal plants from the nature is causing a loss of genetic diversity. Plants with very small seeds like** ***Hypericum triquetrifolium*** **Turra cannot be cultured by traditional methods, easily. In the present study, a rapid micropropagation protocol was developed for** ***Hypericum triquetrifolium*** **Turra. Native seeds, collected from Kaz Mountains in Egean region of Turkey were used as starting material. Water-agar (89%) germinated six weeks old seedlings were placed in ten different medium. The most intensive shoot multiplication rate per explant was recorded at 5.95 on the MS medium supplemented with 1.25 mg/L Thidiazuron (TDZ) plus 0.5 mg/LIndole-3-acetic acid (IAA). Root development (5-6 per shoot) was obtained on Murashige and Skoog (MS) medium supplemented with 1 mg/L IAA. After rooting** ***in vitro*****, the plantlets morphologically similar to mother plants were easily acclimatized within a month and survived (93%) upon transfer to greenhouse conditions.**

Key words: *Hypericum triquetrifolium*, micropropagation, medicinal plant, thidiazuron.

INTRODUCTION

Hypericum species have been known for their anti-depressant, analgesic, spasmolytic, antiviral and wound healing effects for many years (Pasqua and Avota 2003; Wojcik and Podstolski, 2007). One of these species, *Hypericum triquetrifolium* Turra is native to Eastern Europe and Mediterranean area. Information about the plant specimen records for Turkey exists in detail (Karakas et al., 2008). 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). Stability of active compounds, in the plant material, is important for the pharmaceutical applications and requires standardized growth conditions (Wojcik and Podstolski, 2007). On the other hand, *H. triquetrifolium* Turra has very small seeds giving difficulties to its sexual propagation. Existing limited traditional cultivation of medicinal plants, in general, does not meet increasing commercial demand. Micropropagation, as an advanced vegetative propagation technique, can serve in the production of a great number of genetically uniform plants in a limited time and space (Canter and Thomas, 2005).

Although the presence of successful *in vitro* micropropagation protocols for several *Hypericum* species have been reported (Pretto and Santarem, 2000; Murch et al., 2000; Bernardi et al., 2007), only one study on *H. triquetrifolium* appears in the literature (Karakaş et al., 2008). Also, the efficiency of TDZ on *H. triquetrifolium in vitro* micropropagation has not been demonstrated, yet TDZ is a plant growth regulator which is reported to be more effective than other cytokinines (example, BAP, a frequently used one) on shoot multiplication (Kim et al., 2006). In this attempt to increase the efficiency of propagation, an *in vitro* multiplication system using different concentrations of thidiazuron in combination with IAA, comparing with BAP on shoot proliferation rates in seedlings as initial explants from *in vitro* germinated seeds of *H. triquetrifolium* Turra, which is wildly distribu-

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Abbreviations: TDZ, Diphenyl urea derived *N*-phenyl-*N'*-1, 2, 3-thiadiazol-5-ylurea (thidiazuron); **IAA**, Indole-3-acetic acid; **BAP**, 6-benzylaminopurine; **MS**, Murashige-Skoog.

ted in Turkey, was investigated.

MATERIALS AND METHODS

Establishment of *in vitro* seedlings

The seeds of *Hypericum triquetrifolium* Turra, native to Turkey, were collected seasonally from Kaz Mountains in the Egean region of Turkey and were incubated in 70% (v/v) ethanol for 30 s followed by surface-decontamination in 20% commercial bleach (containing 5% NaOCl) solution containing two drops Tween 20 for 25 min.

Afterwards, the seeds were washed four times (5 min) with sterile distilled water prior to placing onto water-agar (0.8% [w/v], A7921-Sigma-Aldrich) or ½ strength MS medium (Murashige and Skoog, 1962) solidified with 0.8 % (w/v) agar. One seed placed in one test tube (5 cm x 9.5 cm) containing 25 ml of medium. Cultures were maintained at 27 ± 4°C under darkness. And they were monitored every day for germination for a period of 8 weeks.

Culture induction and multiplication of plantlets

After germination, plant material obtained through six weeks old seedlings, discarded of two/third of their cotyledons and roots (app.1 cm in height), was introduced *in vitro* in test tubes containing 25 ml MS medium supplemented with 3% sucrose and solidified with 0.8% agar. TDZ (0.5; 0.75; 1 and 1.25 mg/l) and BAP (0.5; 0.75; 1 and 1.25 mg/l), in combination with IAA (0.5 mg/l) as growth regulators were examined for shoot multiplication. Hormone free medium was used as control. The cultures were kept at 27 ± 4°C under cool white fluorescent light (4000 lux) with a 16 h photoperiod and they were monitored for shoot production regularly (everyday). Multiplication rate was evaluated during 30 d, by counting the appeared adventitious shoots per explant. The MS medium was supplemented with 1.25 mg/L TDZ and 0.5 mg/L IAA was then regarded as the maintenance medium. Shoot tips excised from the multiplicated shoots were used for all subsequent subculture cycles at three weeks of intervals, in test tubes containing 25 ml of maintenance medium. The pH of the media was adjusted to 5.8 with 1 M KOH before autoclaving at 121°C, 1.4 kg/cm^2 for 20 min.

Each experiment consisted of one explant per culture vessel and fifteen replicate vessels per treatment. The experiments were repeated at least three times. Data was analyzed using ANOVA and the data means ± SE of at least three different experiments were conducted and compared using Duncan's multiple range test at the rate of 5% of significance set.

Rooting and acclimatization

1 mg/L IAA added or hormone free MS was used for rooting. Plantlets, rooted in 1 mg/L IAA in MS, were observed for uniformity and vigor and transplanted to pots containing either perlit or sand : peat : perlite (1:1:1, v/v/v) mixture in a growth chamber with 100% humidity. Rooting experiments were subjected to independent t-test to see whether the observed differences were significant or not at 5% significance level. The pots were sealed with Parafilm® (P6543, Sigma) in order to avoid dehydration for the first few days. Benomyl, ® 50 Wp Valles (50% benomyl active gradient; Du Pont) (0.5 ml/l) as fungicide, was used on the first day after transplanting *in vivo* to prevent fungal growth. After ten days, the lids were opened and the humidity remained at 100% for one month. Acclimatized plantlets were then transferred to pots containing compost enriched greenhouse soil. The greenhouse, into which acclimatized plantlets were transferred, had 96% humidity and 75 µmol m^{-2} s^{-1} light intensity. The temperature fluctuated between 16 and 28°C.

RESULTS AND DISCUSSION

Germination on ½ MS was lower (67%) and the seedlings grown from germinated seeds had some growth disorders as necrotic callus-like structures which never developed any further (data not shown). The seeds in water-agar germinated successfully within 3 - 6 weeks (Figure 1A). However, the germination followed a non-synchronous course as some seeds germinated within three weeks whereas few started to germinate at the end of 6 weeks. Finally, 89% of the seeds gave healthy seedlings after 45 days. According to the study of Karakas et al. (2008), full strength MS resulted in 69.2% of germination rate that decreased to 21.7% when BAP was used in the medium for *H. triquetrifolium*.

Plant regeneration of *Hypericum* species has been achieved using whole seedlings or their excised parts (Cellárová et al., 1992), hypocotyls sections (Murch et al., 2000) and leaves (Pretto and Santarém, 2000) as explants, and using various types and concentrations of cytokinins and auxins (Santarem and Astarita, 2003; Wójcik and Podstolski, 2007, Bernardi et al., 2007; Karakas et al., 2008) as growth regulators, indicating that organogenic response might be controlled by diverse factors. We used several concentrations of TDZ or BAP in combination with 0.5 mg/L IAA in the guidance of above mentioned well-documented studies.

First initial explants started to multiplicate within three weeks in all treatments. They tended to be accompanied by callus, produced at the shoot base (Figure 1B, C). The best response appeared on the medium containing 1.25 mg/L TDZ plus 0.5 mg/L IAA with 5.95 multiplication rate per explant, at the end of 30 d (Table 1; Figure 1B). Decrease in the concentration of thidiazuron in the medium reduced the multiplication rates ranging from 4.20 - 4.88. Multiplication rate (3.19-3.23) also decreased when TDZ was replaced by BAP (Table 1; Figure 1C). Although shoot number was influenced significantly by the type of cytokinine, elongation appeared to be not dependent on it (Figure 1D, E; Table 1). TDZ, nevertheless, seemed more effective than BAP, although no significant differences were detected with respect to shoot elongation potential (Table 1). Furthermore, when the medium contained TDZ and IAA at a ratio of 1.25:0.5 or 1:0.5 (mg/L), the former PGR combination was utilized for continuous culture of *H. triquetrifolium* Turra since the shoot elongation appeared so close in both. Finally, after one month, the shoot tips of the multiplicated shoots were excised and transferred into maintenance medium. Consequently, proliferated shoots grew vigorously (Figure 1F) giving healthy uniform clones, and no further losses were recorded thereafter.

Data from studies conducted by Pretto and Santarem (2000) and Santarem and Astarita (2003) in *H. perforatum* L., support the use of auxins for rooting. However, the reports from other laboratories indicated that PGRs were not necessarily needed for rooting in *H. erecta* (Kim

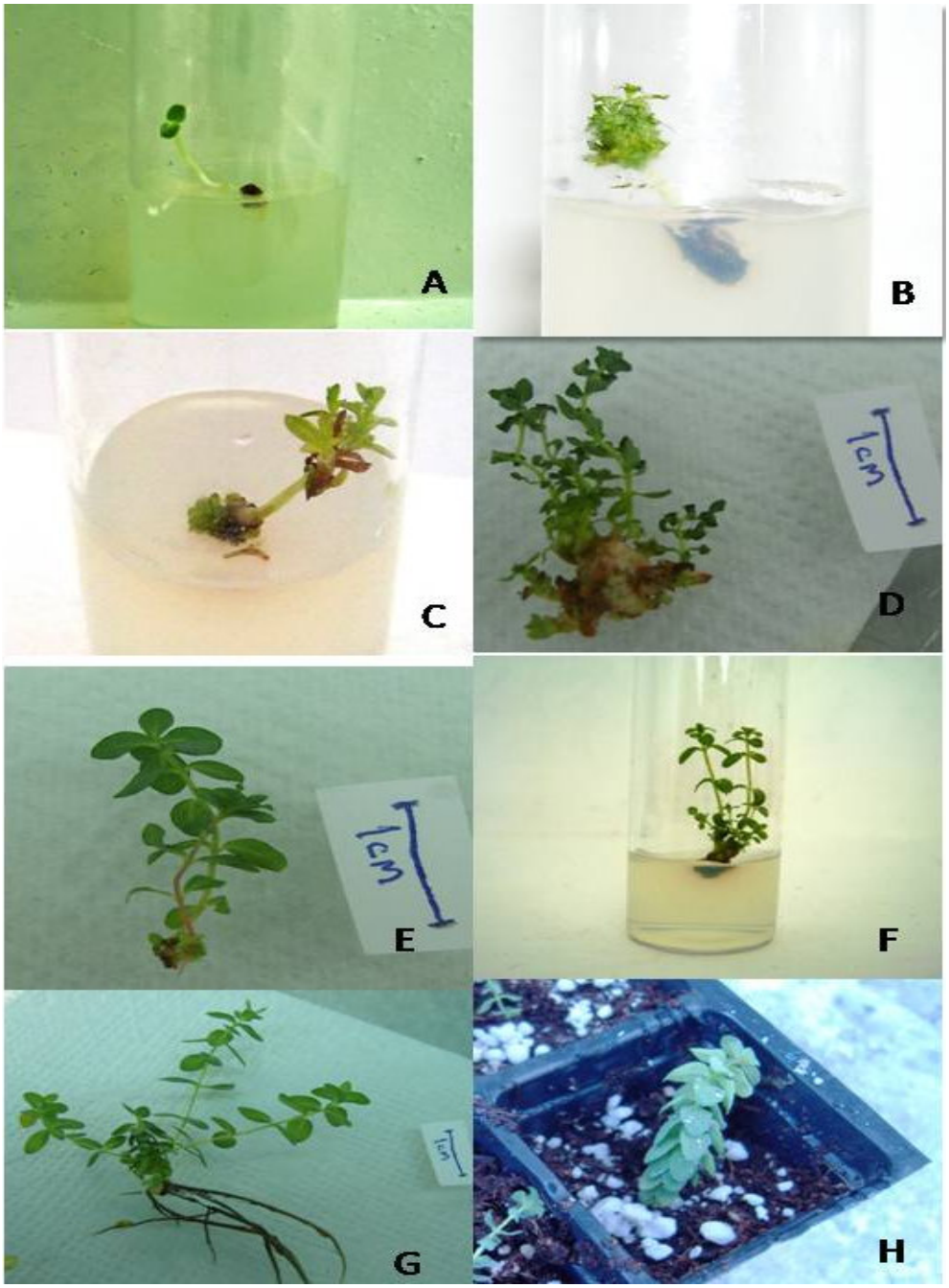

Figure 1. A. A germinating seedling (4 weeks in culture). **B.** Direct shoot proliferation on the medium with 1.25 mg/L TDZ and 0.5 mg/L IAA. **C.** Decrease in shoot proliferation when the BAP (1.25 mg/L combined with 0.5 mg/L IAA) was used instead of TDZ. **D.** Shoot lenght was appeared approximately similar either in the presence of TDZ (1.25 mg/L combined with 0.5 mg/L IAA, (**E.**) or in the presence of BAP (1.25 mg/L combined with 0.5 mg/L IAA). **F.** Vigorously grown (3 weeks old) shoot tips of multiplicated shoots in maintenance medium (1.25 mg/L TDZ + 0.5 mg/L IAA). **G.** Rooting of plantlets in the medium with 1 mg/L IAA. **H.** Acclimated plantlet on the sand : peat : perlite (1:1:1; v/v) substrate.

et. al, 2006), *H. perforatum* cv. 'Anthos' (Murch et al., 2000); or rooting might be induced with or without auxins in *H. perforatum* (Wojcik and Podstolski, 2007). No rooting values were reported for *H. triquetrifolium* Turra by Karakas et al. (2008). After *in vitro* multiplication in the maintenance medium, obtained adventitious shoots (three weeks old) were transferred into rooting medium. *H. triquetrifolium* was found to be able to produce five or six adventitious roots (Figure 1G) (Table 2) in MS under the presence of 1 mg/L IAA (rooting medium), whereas

Table 1. Seed germination and multiplication and elongation of shoots for *Hypericum triquetrifolium* Turra in different media.

Medium	Seed germination (%)	MS medium (BAP and IAA as mg/L)	Multiplication rate (n=45)	Shoot length (cm)
½ MS	67.8	+ 0	1.2f	0.40h (± 0.03)
Water-gar	89.9	+ 0.5 BAP + 0.5 IAA	3.12e	1.78g (± 0.08)
		+ 0.75 BAP + 0.5 IAA	3.20d	2.81f (± 0,11)
		+ 1 BAP + 0.5 IAA	3.22d	3.32e (± 0.07)
		+ 1.25 BAP + 0.5 IAA	3.23d	3.22d (± 0.04)
		+ 0.5 TDZ + 0.5 IAA	4.83b	2.98c ± 0.14)
		+ 0.75 TDZ + 0.5 IAA	4.86c	3.45b (± 0.06)
		+ 1 TDZ + 0.5 IAA	4.76b	3.61a (± 0.06)
		+ 1.25 TDZ + 0.5 IAA	5.96a	3.59a (± 0.09)

Values within columns followed by different letters are significantly different at $p \leq 0.05$, according to Duncan's multiple range tests.

Table 2. Rooting of *H. triquetrifolium* Turra plantlets on different media (in 3 weeks) and the survival rate of the plantlets upon transplantation to different substrates (in 4th week).

Medium	Mean no. of root per plantlet*	Root length* (cm)	Transplantation Substrate	% of survival rate
MS(Hormone-free)	1.8 (± 0.10)	1.4 (± 0.13)	Perlit	47
MS+1 mg/L IAA	5.7 (± 0.03)	4.2 (± 0.18)	Sand:Peat:Perlite (1:1:1; v/v)	93

*$p \leq 0.05$ according to "t" test.

one or two weak roots were recorded on hormone-free MS, in this study. The roots in rooting medium, reaching an average length of 4.2 cm, were significantly stronger (Figure 1G) (Table 2), as well.

Micropropagated plantlets through existing organized meristems, such as shoot tips or axillary buds, exhibit normally no signs of visible morphological variation and are genetically identical with donor plants (Hu and Wang, 1983; Bajaj et al., 1988). Confirming the fact, after three weeks in rooting medium, rooted plantlets were morphologically uniform with longer nodes and greater vigor besides uniform coloring and leaf shape (Figure 1G), in our trials. The performance of the microplants was observed to be highly dependent on the transplantation substrate as best survival rate (93%) was obtained upon their transfer to sand: peat: perlite substrate under 100% humidity (Table 2). Perlite alone resulted in lower survivability (47%).

Approximately six weeks later, acclimatized plantlets were transferred to greenhouse successfully (Figure 1H), and further on (app. within two months) these *in vitro* derived plants developed into mature, fully formed individuals similar to their nature-grown congenerics.

From the literature concerning this work, it is understood that, investigations have been performed previously on *H. perforatum*. The present paper reported for the first time TDZ induced micropropagation of *H. triquetrifolium* Turra. In conclusion, we described an efficient *in vitro* multiplication method for *H. triquetrifolium* Turra. Through this method, important genotypes can be micropropagated in large numbers within a short period of time, and the clones induced can be conserved. Moreover, explants of these *in vitro* plants may be used to establish a bioreactor system to produce important compounds, in the future studies.

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