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IN VITRO SHOOT MULTIPLICATION AND ROOTING OF ST. JOHN'S WORT (*HYPERICUM PERFORATUM* L.)

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Abstract

St. John's wort (*Hypericum perfor*atum L.) is one of the economically important plant of the 400 plant species from the genus Hypericum. Hypericum species contain a number of biological compounds and it is used as a medicinal herb due to its anti-inflammatory, antiviral, antimicrobial, antifungal and antidepressant activity. This study was aimed to study *in vitro* propagation and rooting of *H. perforatum.* Sterilized seeds were germinated in MS medium without plant growth regulator and after one month, shoot tips were separated from aseptically grown seedlings and used further as an explant. Explants were cultured in MS medium containing BAP or Kin at different concentrations (0, 0.25, 0.50, 1.0 and 2.0 mg L^{-1}) for shoot multiplication. After eight weeks, the highest number of shoots (63) was obtained from culture in MS with 0.50 mg L^{-1} BAP. Shoots successfully rooted in MS with IAA or IBA and without plant growth regulators. Rooted plantlets were acclimatized in pots containing 1:1 mixture of peat and perlite. It can be concluded that this study is an efficient system for *in vitro* shoot multiplication and rooting of St. John's Wort (*Hypericum perforatum* L.)

Keywords: BAP; *Hypericum perforatum*; *In vitro*; Kin; Shoot

Introduction

Hypericum perforatum L. is a perennial medicinal plant commonly known in Turkey as kantaron, binbir delik otu, kan otu, kılıç otu, yara otu or kuzukıran (Baytop, 1999). There are nearly 400 species of *Hypericum* that are present throughout the temperate regions of the world. The natural flora of Turkey is an important reserve that hosts 89 species from *Hypericum* genus, of which 43 are endemic (Davis, 1988; Guner *et al.,* 2000). The most common among them is *Hypericum perforatum* L.

Hypericum contains bioactive constituents like naphthodiantrones, phloroglucinols, flavonoids, xanthones and few other water-soluble compounds (Greeson *et al.,* 2001; Pasqua *et al.,* 2003). Naphthodianthrones, namely hypericin and pseudohypericin, are highly important constituents (Gadzovska *et al.,* 2005). *H. perforatum*, containing these phytochemicals with medicinal value (Treneva *et al*., 2014), possesses wound healing, bactericidal, diuretic, anti-inflammatory and tranquillizer activity. In addition, it has antiviral, anticancer, and antidepressant properties as well (Öztürk, 1997; Çırak, 2006).

H. perforatum is propagated through seeds and rhizome (Banerjee *et al.,* 2012). However, its cultivation is difficult due to development of dormancy in seeds (Çırak *et al.,* 2004 a, b), difficulties of planting seedlings, and its sensitivity towards anthracnose (*Colletotrichum gloeosporioides*) (Çırak, 2006). In addition, environmental conditions (Murthy *et al.,* 2014), diseases and pests may also affect the secondary metabolite content of the plant (Zobayed *et al.,* 2004) This has prompted to search for alternative methods for *Hypericum* production such as *in vitro* techniques involving plant cell and tissue cultures (Murch *et al.,* 2000; Gadzovska Simic *et al*., 2014; Murthy *et al.,* 2014). These techniques protect, enhance propagation and enable genetic improvement of medicinal plants to meet pharmaceutical needs (Shilpashree and Ravishankar, 2009).

In vitro techniques are procedures that are highly suitable for the fast and effective propagation of *Hypericum* species and also for the production of various secondary metabolites that these species contain (Pasqua *et al.,* 2003; Karakaş *et al.,* 2015). Studies conducted on the *in vitro* propagation of different *Hypericum* species were also associated to obtaining secondary metabolites in cell and tissue culture. It is possible to produce secondary metabolites through *in vitro* procedures and to increase the standard quality in a simple way. For this reason, *in vitro* micropropagation is accepted as an auxiliary technique for providing standard herbal material to the pharmaceutical/chemical industry (Gadzovska *et al.,* 2005; Motallebi-Azar and Kazemiani, 2011). Plants that are obtained through micropropagation are produced in culture rooms where environmental conditions such as temperature, light and humidity can be controlled and in this way it is possible to obtain a more homogenous product in terms of active substance content (Zobayed and Saxena 2004; Couceiro *et al.,* 2006).

It is reported by several researchers that plant regeneration in *H. perforatum* is obtained through the use of different types of explants such as seedling, hypocotyl and leaf (Cherry *et al.,* 2000; Zobayed *et al.,* 2004; Banerjii *et al.,* 2012; Akhtar *et al.,* 2013).

In the present study, the shoot tips of *H. perforatum* were used as explant and *in vitro* shoot propagation of this plant was studied.

Materials and Methods

Hypericum perforatum L. seeds were obtained the Medicinal and Aromatic Plants Research and Application Farm of Selcuk University Faculty of Agriculture and were used as a sterile explant source in this study. The seeds were surface-sterilized for 10 min using 20% diluted commercial bleach (Yetiş, Turkish trademark, containing 50% NaOCl) by stirring with a magnetic stirrer and rinsed three times with sterile deionized water. For germination, surface-sterilized seeds were then cultured in sterile magenta vessels containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without any plant growth regulators (PGRs).

MS medium containing 3% sucrose and solidified with 8% agar was used in all experiments. Deionized water was used in the preparation of the nutrient medium. The pH of medium was adjusted to 5.8 before being autoclaved at 121°C and 1.4 kg cm^{-1} pressure for 20 min. All the cultures were maintained at 24 °C with 4 LS fluorescent light a plant growth chamber (SANYO: MLR-351H, Japan) with 16 hours light and 8 hours dark cycles.

The shoot tips removed from 30 day old seedlings grown on MS medium were used as explant. The shoot tips were transferred to the MS medium containing benzyl amino purine (BAP) or kinetin (Kin) at different concentrations (0, 0.25, 0.50, 1.0, and 2.0 mg L^{-1}) for shoot proliferation. Each experiment was performed in three replicates using five explants per replicate. After 8 weeks of culture, the percentage of shoot forming explants and the number of shoots per explant were determined.

The regenerated shoots were transferred into MS media containing different concentrations of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (1.0, 2.0 and 3.0 mg L^{-1}) and without growth regulators for rooting. After four weeks of culture, rooting percentage and root lengths were determined. The seedlings after transferring to peat: perlite mixture (ratio 1:1) was kept in humidity chamber for acclimatization.

The statistical analysis was performed by using MSTAT-C statistical software. The data were compared using LSD test (Michigan State University, 1980).

Results and Discussion

After eight weeks of culture, there was no difference in the percentage of shoot forming explants cultured in either BAP or Kin at all concentrations used. All (100%) explants developed shoots indicating that neither BAP nor Kin influences shoot formation.

Table 1. The effect of BAP and Kin on shoot propagation of *Hypericum perforatum* L.

Plant Growth Regulator (mg L^{-1})	Number of shoot per explant		Mean
	BAP	Kin	
0	2.40 c	2.40 c	2.40 d
0.25	35.27 b	1.13 c	18.20 bc
0.50	62.93 a	1.20 c	32.07 a
1.0	55.27 a	1.20 c	28.23 ab
2.0	14.73c	1.00 c	7.87 cd
Mean	**34.12 A**	**1.39 B**	

$LSD_{0.01Cons}$: 10.49 $LSD_{0.01PGR \times Cons}$: 14.84

The effect of plant growth regulators (PGRs) on the number of shoots per explant was statistically significant ($p<0.01$). The effect of BAP on the number of shoots per explant was higher compared to that of Kin; as the mean number of shoots per explant was 34.12 on MS containing BAP and 1.39 on MS containing Kin (Table 1). According to study of Mohebalipour *et al.* (2012), BAP is the most reliable and effective cytokinin source in various medicinal plants. In addition, BAP is reported to be the most effective growth regulator in regeneration of *H. perforatum* L. plant from leaf (Pretto and Santarem, 2000) and seedling parts (Cellarova *et al.,* 1992; Karakaş *et al.,* 2009). Similarly, the results obtained in this study showed that BAP was more effective compared with Kin in shoot formations from tip explants.

Furthermore, there was a significant difference among different concentrations of PGRs ($p<0.01$). The highest number of shoots (32) was obtained at 0.50 mg L^{-1} concentration of PGRs (Table 1). Shoot propagation was achieved at all concentrations of BAP; the best result (63 shoots) was obtained in MS containing 0.50 mg L^{-1} BAP (Table 1). The increase in BAP concentration caused vitrification and deformation in regenerated shoots and thus decreased the number of healthy shoots per explant. The number of healthy shoots obtained in the 2 mg L^{-1} BAP containing medium (15 shoots) was lower than that obtained in the other media (Table 1). This was in line with findings of Jafari *et al.* (2011), which showed that high concentrations of BAP decrease the number of shoots and increase the formation of abnormal shoots.

The results obtained in MS containing Kin were similar to the control (Table 1). Cherry *et al.* (2000) have shown the use of Kin for shoot propagation in *H. androsaemum*, *H. patulum* and *H. grandiflorum* species. In the present study, the similar reaction of *H. perforatum* on media with Kin and without growth regulators may be due to genotype differences.

The simultaneous effect of PGRs and concentration on the number of shoots per explants was found to be statistically significant ($p<0.01$). In terms of the number of shoots per explant, the highest value of 63 was obtained with the 0.50 mg L^{-1} BAP containing medium, whereas the lowest value (1 shoot) was obtained with the medium containing 2.0 mg L^{-1} Kin (Table 1; Figure 1a; 1b). Akhtar *et al.* (2013) have shown that the leaf explants of *H. perforatum* obtained highest number of shoots (42) in 1.0 mg L^{-1} BAP containing medium. In another study, 25 shoots per hypocotyl explants of *H. perforatum* were obtained by the application of 1.0 mg L^{-1} BAP (Banerjii *et al.* 2012). The difference in the type of explant can explain the reason for obtaining the highest result at 0.50 mg L^{-1} BAP.

The regenerated shoots were then transferred to MS media without PGRs and with different concentrations of IAA or IBA (1.0, 2.0 and 3.0 mg L^{-1}) for rooting, and after four weeks, rooting rates and root lengths were evaluated.

The variance analysis performed on the data showed that PGRs, its concentration and combined effect of both PGRs and concentrations affected the rooting rate and root length.

Table 2. The effect of IBA and IAA on rooting in *Hypericum perforatum* L.

Plant growth regulator (mg l^{-1})	Percentage of rooting (%)		Mean	Root length (cm)		Mean
	IBA	IAA		IBA	IAA	
0	100 a	100 a	100 a	3.37 a	3.37 a	3.37 a
1.0	93.33 ab	60 d	76.67 b	2.86 abc	2.96 abc	2.93 b
2.0	80 bc	73.33 cd	76.67 b	3.07 ab	2.48 bc	2.77 b
3.0	86.67 abc	60 d	73.34 b	3.28 a	2.44 c	2.86 b
MEAN	**90 a**	**73.33 b**		**3.15 a**	**2.81 b**	
	$LSD_{0.01\ cons}$: 16.86			$LSD_{0.05\ cons}$: 0.42		
	$LSD_{0.05\ PGR\ x\ cons}$: 17.31			$LSD_{0.05\ PGR\ x\ cons}$: 0.59		

The effect of PGRs on rooting rate was found to be statistically significant ($p<0.01$). The higher percentage of rooting was recorded in media containing IBA (90%) than media containing IAA (73.33%) (Table 2). This suggests that IBA was more effective in rooting compared with IAA. It is known that auxins like IBA, IAA and naphthalene acetic acid (NAA) promote rooting in various *Hypericum* species (Shilpashree and Ravishankar, 2009;

Akhtar *et al.,* 2013). IBA is the most commonly used growth regulator for rooting in tissue culture and has been shown to increase rooting in variety of plants (Nissen *et al.,* 1990; Landi and Mezzetti, 2006; Kara, 2011). In addition, the rooting rate was higher (100%) in MS medium without PGRs compared with the medium containing PGRs. Gadzovska *et al.* (2005) found that *H. perforatum* shoots can be successfully rooted in media with or without auxin. Pretto and Santarem (2000) reported that the highest rooting rate is obtained in ½MS medium regardless of the presence of IBA. Similarly, Çırak (2006) found that there is no significant effect of PGRs on rooting. Murch *et al.* (2000) pointed out that the ability of *Hypericum* hypocotyl explants to form roots on media without exogenous auxins might be due to presence of high endogenous auxin level.

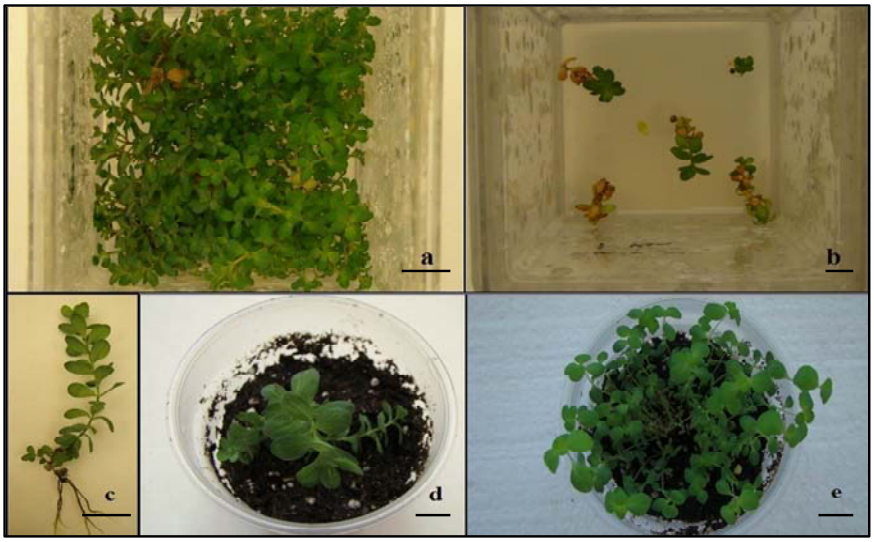

Figure 1. *In vitro* propagation of *Hypericum perforatum* L. Propagated shoots on MS medium with 0.50 mg L^{-1} BAP (a) and 1.0 mg L^{-1} Kin (b) Root development of regenerated shoots after 4 week on MS without PGR, (c). Acclimatized 1-4 week old plantlets after transfer to plastic pots (d-e) (bar: 1 cm)

The effect of concentrations on the percentage of rooting was found to be statistically significant ($p<0.01$). It was observed that rooting was 100% at the control medium, which did not contain any PGRs (Figure 1c). Other concentrations showed similar results (Table 2).

An *in vitro* study evaluating effect of different auxin and cytokinin levels on *H. perforatum* seedlings reported that IAA and IBA are effective in rooting based on their concentration in medium (Cellarova and Kimakova, 1999).

The combined effect of PGRs and concentrations on percentage of rooting were found to be statistically significant ($p<0.05$). The highest rooting rate (100%) was obtained on MS

medium without PGRs, followed by closest value of 93.33% that was obtained on the medium containing 1.0 mg L^{-1} IBA. The lowest rooting rate was 60% in medium containing 1.0 mg L^{-1} and 3.0 mg L^{-1} IAA. Gadzovska *et al.* (2005) reported that rooting decreases with the increase in IBA and IAA concentrations.

The effect of PGRs, concentrations and interaction between PGRs and concentration on root length was found to be statistically significant ($p<0.05$). In terms of growth regulator means, the root length of 3.15 cm was obtained in MS containing IBA and 2.81 cm in MS containing IAA (Table 2).

The best root length (3.37 cm) was obtained in MS medium without PGRs. In MS containing IBA, the highest value in terms of root length was 3.28 cm that was obtained at 3.0 mg L^{-1} IBA. The lower concentration of IBA caused the shortening of the roots. Franklin and Dias (2006) showed that the growth regulators are effective in root development and an increase in concentration of IBA causes decrease in root length but increase in number and thinness of newly formed roots. These variations could be attributed to genetic differences.

The effect of IAA was highest (i.e., root length 2.96 cm) at concentration 1.0 mg L^{-1}. As per Akhtar *et al.* (2013), auxin type and concentration significantly affect the root development and Coste *et al.* (2012) showed that an increase of the auxin level in the culture medium shortens the root length. Similarly, in this study, the concentration of IAA and root length was found to be inversely proportional.

Well-rooted shoots were rinsed with sterile water to remove residual rooting media and transferred to pots containing 1:1 mixture of peat and perlite and kept in a growth chamber at 24°C, 16 h photoperiod at 90% humidity maintaining the light/dark cycle. There were no morphological variations observed compared with the seed derived plants.

Conclusion

Micropropagation is a modern vegetative propagation technique that enables the propagation of genetically uniform and pathogen-free vegetative material regardless of time and place. The plants obtained through micropropagation are produced on culture media under controlled environmental conditions such as temperature, light and humidity. Therefore, the products obtained are more homogeneous in terms of active substance they contain. In addition, micropropagation also plays a significant role in the protection, propagation and genetic improvement of medicinal plants to meet pharmaceutical needs.

In this study, BAP was found to be more successful growth regulator in an *in vitro* propagation of *Hypericum perforatum* using shoot tips as explant. The most favorable concentration of BAP for number of shoots per explants was 0.5 mg L^{-1} (63 number of shoots). The regenerated shoots were able to form roots on MS with and without auxin. The MS without growth regulator provided the highest rooting value and hence can be used for rooting.

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