Effects of Explant Types and Different Concentrations of Sucrose and Phytoharmones on Plant Regeneration and Hypericin Content in *Hypericum perforatum* L.

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Abstract: *Hypericum perforatum* L. is a medicinal perennial known as "St. John's wort" in Western Europe and used in the treatment of mild to moderate depression. Hypericin, a dianthrone red pigment, is considered responsible for reversing the depression symptoms. The goal of this study was to produce this pigment via micropropagation and thereby describe a suitable micropropagation method for this plant. To achieve this objective, leaf discs and stem segments were cultured on Murashige and Skoog (MS) medium supplemented with kinetin and 2,4-dichlorophenoxyacetic acid (0.5, 1 and 1.5 mg l^{-1}) and sucrose concentrations (30, 40 and 50 g l^{-1}) in darkness at 26 ± 2 °C. The highest value was obtained from MS medium supplemented with 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin in terms of callus initiation frequency. In addition, callus initiation was observed in all media supplemented with different constituents. When obtained, calli were cultured on MS basal medium supplemented with 1 mg l^{-1} of benzyladenine for shoot induction, while the same basal medium supplemented with 1 mg l^{-1} of indolacetic acid was employed for rooting. The effects of factors tested on both regeneration and hypericin content were various. Number of shoots per callus and hypericin percentage were investigated and found to be higher in leaf discs originated from regenerants (19 shoots per callus and 0.048% hypericin); being easily acclimated in greenhouse conditions.

Key Words: *Hypericum perforatum* L., micropropagation, hypericin.

Abbreviations: BA-6-benzyladenine; 2,4-D-2,4-dichlorophenoxyacetic acid; IAA-indolacetic acid; IBA-indolebutyric acid; NAA-α-naphthaleneacetic acid.

Farklı Sukroz İçerikleri, Bitki Büyüme Düzenleyicileri ve Eksplant Tiplerinin *Hypericum perforatum* L.'de Rejenerasyon Yeteneği ve Hypericin İçeriğine Etkileri

Özet: Dünya literatüründe St. John's wort olarak bilinen kantaron (*Hypericum perforatum* L.) günümüzde depresyon tedavisinde de yaygın olarak kullanılan çok yıllık bir tıbbi bitkidir. Bitkinin antidepresan etkisinin bir dianthrone pigmenti olan hypericinden kaynaklandığı düşünülmektedir. Bu çalışmada hypericini doku kültürü yöntemiyle üretmek ve kantaron için etkili bir mikro çoğaltım sistemi tanımlamak amaçlanmıştır. Bu amaçla yaprak diskleri ve gövde segmentleri kinetin, 2,4-dichlorophenoxyacetic acid (0.5, 1 ve 1.5 mg l^{-1}) ve sukroz (30, 40 ve 50 g l^{-1}) içeren Murashige & Skoog ortamlarında, karanlık şartlarda ve 26 ± 2 °C de sekiz hafta boyunca kültüre alınmışlardır. Bitki büyüme düzenleyicileri içeren bütün ortamlarda kallus oluşumu gözlenmiştir. Ancak, kallus oluşum sıklığı bakımından en yüksek değerler 30 g l^{-1} sukroz, 0.5 mg l^{-1} 2,4-D ve 0.5 mg l^{-1} kinetin içeren MS ortamından elde edilmiştir. Elde edilen kalluslar sürgün oluşumu için 1 mg l^{-1} benzyladenine ; kök oluşumu için ise 1 mg l^{-1} indolacetic acid içeren MS ortamlarında alt kültüre alınmışlardır. Kallus başına sürgün sayısı ve hypericin içeriği bu sürgünlerde araştırılmıştır. Yaprak disklerinden gelişen kallusların oluşturduğu rejenerantlarda bu değerler daha yüksek olup sırasıyla 19 sürgün/kallus ve % 0.048 olarak tespit edilmiştir. Elde edilen rejenerantlar başarılı bir şekilde sera şartlarına adapte olmuşlardır.

Anahtar Sözcükler: Kantaron, mikroüretim, hypericin.

Introduction

Hypericum perforatum L. (St. John's wort) is a well-known traditional medicinal plant that has been used for centuries for the treatment of several diseases, such as skin lesions, eczema, burns and microbial, inflammatory and psychological disorders (Dias et al., 1998, Ishiguro et al., 1998, Barnes et al., 2001; Sanchez-Mateo et al., 2002). This feature is obviously attributed to the

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presence of a broad spectrum of secondary metabolites, mainly naphthodianthrons, phloroglucinols, (Greeson et al., 2001) and xanthones (Hostettmann and Hostettmann, 1989). In particular, aromatic polycyclic diones, e.g., hypericin and pseudohypericin, are of great interest as their antiviral, anticancer and antidepressant activities have been documented previously (Meruelo et al., 1988; Lavie et al., 1989; Agostinis et al., 2002; Sanchez-Mateo et al., 2002). These naturally occurring red pigments are only available in *Hypericum* species (Gerassim and Kitanov, 2001). Therefore, all these features manifest the economic importance of the genus *Hypericum* and in particular of *Hypericum perforatum* L. This phenomenon is further evidenced by the fact that the market for *Hypericum perforatum* L. has exceeded $210 million in the USA and $570 million worldwide annually (Sirvent and Walker, 2002).

In vitro systems have been reported as an effective tool for obtaining genetically uniform plants, which can be a source of variable pharmaceutical preparations (Santarem and Astarita, 2003). Plant regeneration of *H. perforatum* L. has been achieved using as explants whole seedlings or their excised parts (Cellarova et al., 1992; Brutovska et al., 1994), hypocotyl sections (Murch et al., 2000a; Santarem and Astarita, 2003; Zobayed et al., 2004) and leaves (Pretto and Santarem, 2000; Bezo and Stefunova, 2001) using various types and concentrations of auxins and cytokinins.

Production of secondary metabolites via plant cell and tissue cultures yields various advantages, including standardisation and quality. These criteria are also valid for the main economically important chemicals in St. John's wort, namely hypericin, pseudohypericin and hyperforin (Zobayed et al., 2004). *Hypericum perforatum* L. is a unique species that undergoes multiple forms of reproduction in the wild. Several reproductive processes have been identified in wild harvested *Hypericum perforatum* L. (Matzk et al., 2001). This reproductive flexibility has led to high chemical variability in field grown plants. Therefore, the application of in vitro techniques provides an approach for the production of standardised plant material (Murch et al., 2000b). Hypericin is generally extracted from flowers of wild or cultivated *Hypericum perforatum* L. plants. Both the limited area of occurrence of this plant and seasonal harvesting necessitate a search for alternative methods for production of these compounds. In addition, plant

tissue and cell cultures are important tools which allow extensive manipulation of the biosynthesis of the secondary compounds and yield a higher productivity compared to that of intact plants (Kirakosyan et al., 2001).

The aim of the study was to evaluate the effects of some tissue cultures on plant regeneration and thereby to investigate the changes in the hypericin content of tissue cultures established from *Hypericum perforatum* L.

Materials and Methods

Plant material

Two initial explants, leaf discs (7 mm in diameter) and stem segments (10 mm in height), were obtained from young shoots 8 weeks old and 20-25 cm in height of *Hypericum perforatum* L. plants growing wild in the 19 Mayıs University campus area, Samsun, Turkey, at the beginning of the summer of 2002. Leaf discs and stem segments were surface sterilised in a 20% (v/v) commercial sodium hypochlorite solution containing 0.05% (v/v) Tween 20 for 20 min, followed by rinsing in sterile distilled water.

Callus initiation

The explants were cultured in media supplemented with different culture constituents in magenta boxes. The callus initiation medium contained Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962). The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Different sucrose concentrations (30, 40 and 50 g l^{-1}), plant growth regulators (kinetin and 2,4-D) and their concentrations (0.5, 1 and 1.5 mg l^{-1}) were tested (Table 1). All chemicals were purchased from Sigma. Medium without sucrose or plant growth regulators was used as the control. Cultures were kept in darkness at 26 ± 2 °C for 8 weeks without sub-culturing. Six explants were cultured per 25 ml magenta box, in 4 replicates, representing a total of 24 observations per treatment. The frequency of callus induction and callus fresh weight was determined 8 weeks after culture initiation.

Shoot induction and multiplication

For shoot induction, calli were transferred to shoot induction medium containing MS salt and vitamins, 1 mg l^{-1} of BA and 30 g l^{-1} of sucrose, pH 5.8, and kept under the same conditions employed in callus initiation for 6 weeks (Pretto and Santarem, 2000). Then they were

Table 1. The media used for callus initiation.

Code	Media
1	MS (control)
2	MS + 30 g l^{-1} of sucrose
3	MS + 40 g l^{-1} of sucrose
4	MS + 50 g l^{-1} of sucrose
5	MS + 30 g l^{-1} of sucrose + 0.5 mg l^{-1} of 2,4-D + 0.5 mg l^{-1} of kinetin
6	MS + 30 g l^{-1} of sucrose + 1 mg l^{-1} of 2,4-D + 1 mg l^{-1} of kinetin
7	MS + 30 g l^{-1} of sucrose + 1.5 mg l^{-1} of 2,4-D + 1.5 mg l^{-1} of kinetin
8	MS + 40 g l^{-1} of sucrose + 0.5 mg l^{-1} of 2,4-D + 0.5 mg l^{-1} of kinetin
9	MS + 40 g l^{-1} of sucrose + 1 mg l^{-1} of 2,4-D + 1 mg l^{-1} of kinetin
10	MS + 40 g l^{-1} of sucrose + 1.5 mg l^{-1} of 2,4-D + 1.5 mg l^{-1} of kinetin
11	MS + 50 g l^{-1} of sucrose + 0.5 mg l^{-1} of 2,4-D + 0.5 mg l^{-1} of kinetin
12	MS + 50 g l^{-1} of sucrose + 1 mg l^{-1} of 2,4-D + 1 mg l^{-1} of kinetin
13	MS + 50 g l^{-1} of sucrose + 1.5 mg l^{-1} of 2,4-D + 1.5 mg l^{-1} of kinetin

transferred to the photoperiod (8/16 light/dark, 1200 lux). After 5-7 weeks, the number of shoots per treatment was recorded.

Hypericin Determination

Seven-week-old plantlets exposed to shoot induction medium under light conditions were used for hypericin determination. Freshly harvested plantlets were immediately washed with distilled water and kept in a deep-freeze (-80 °C) for a week. The frozen material was then lyophilised using a Modulyo freeze-drier (Edwards, UK). Dry samples (50 mg) were placed in vials containing chloroform (10 ml) and then homogenised using a Ultra Turrax T25 (Jankel & Kunke, IKA Laborteknik, Germany). This procedure was repeated until chlorophyll contents of samples were fully removed. The final samples were then centrifuged at 13000 x g for 10 min. The colourless supernatant was pipetted off and replaced by methanol. The samples were re-homogenised and kept in a water bath (40 °C) for 18 h. After centrifugation, 1 ml of supernatant was placed in a test tube. Absorbance was measured at 590 nm. The hypericin content was calculated using the formula,

$$\% \text{ hypericin (mg)} = \frac{A.590}{718} \times \frac{1}{\text{g sample/100 ml}} \times 100$$

where A_{590} is the absorbance of the sample, expressed as percentage of hypericin content in a given dry material (Kaya, 1998).

Rooting

To induce roots, elongated shoots were excised and transferred into MS medium supplemented with 30 g l^{-1} of sucrose and 1 mg l^{-1} of IAA, pH 5.8. Four shoots were placed in a magenta box (30 ml media) per treatment. Data were recorded after 4 weeks of culture.

After removal from magenta boxes, the rooted plantlets were washed with tap water to remove rooting medium debris. The young plants were transplanted into boxes containing a soil and sand mixture (2:1) autoclaved at 120 °C for 20 min under nonsterile conditions and gradually exposed to ambient humidity. After 10 days, the acclimated plants were transferred to a greenhouse, maintained under partial shade and irrigated daily. The percentage of survival was recorded at 2-week intervals.

Statistical analyses

The study was performed as a whole one time and data obtained from this study (means of callus formation, callus fresh weight, number of induced shoots, hypericin content of shoots and frequency of rooting) were analysed using ANOVA. Experimental design was a factorial randomised block arrangement with 4 replicates. Statistically significant averages were compared using Duncan's multiple range test, and when necessary data were normalised using x' = √x+1 transformation (Gülümser et al., 2002). The statistical analysis was performed using MSTAT.

Results and Discussion

Callus Initiation

Callus formation was observed in all media studied except for those supplemented with different sucrose concentrations but without plant growth regulators. The effect of different media on callus induction was insignificant (Table 2). Hypericin is the only pigment supplying the colour red in response to methanol extraction (Cellarova et al., 1994) and the majority of calli were green and friable exhibiting dense red-pigmented areas that indicate the presence of hypericin (Figure 1a). It is noteworthy that shooting was also observed in some media without being sub-cultured (data not shown).

There are few reports concerning callus formation in *Hypericum* species such as *H. erectum* (Yazaki and Okuda, 1990), *H. brasiliense* (Cardoso and Oliveira, 1996) and *H. foliosum* (Maciel and Moura, 2000). In our findings, the highest frequency of callus induction was obtained from the leaf discs cultivated on the medium supplemented with 30 g l^{-1} of sucrose, 0.5 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin (Table 2, medium code 5). According to previous reports, 2,4-D and kinetin proved to be effective plant growth regulators for callus

induction and cell suspension cultures from *H. perforatum* L. (Bais et al., 2002; Travis et al., 2002).

Callus induction and proliferation are useful tools for the study of biosynthesis of natural products and the factors affecting it (Pretto and Santarem, 2000) and plant cell suspension cultures are often an effective system in which to study the biological significance of bioactive metabolites under in vitro conditions, as well as for producing natural products for bioprocessing applications (Travis et al., 2002). The in vitro production of hypericin (Bais et al., 2002), pseudohypericin (Kirakosyan et al., 2001), hyperforin (Sirvent and Gibson, 2002) and flavonoids (Kartnig and Brantner, 1990) has been reported previously. Callus growth of *H. perforatum* L. seemed to be significantly affected by the factors investigated in our study. Fresh cell mass was higher in those obtained from stem segments (Table 2). The highest mass (88 mg, based on callus fresh weight) was obtained from stem segments cultured in the medium containing 50 g l^{-1} of sucrose, 1 mg l^{-1} of 2,4-D and 1 mg l^{-1} of kinetin (medium code 12). This was followed by leaf discs cultured in the same medium with 86 mg.

The increasing effect of 2,4-D and kinetin on callus growth was only observed in the medium containing

Table 2. Effects of media consisting of different cytokinin, auxin and sucrose doses on callus initiation, callus fresh weight, average number of shoots, hypericin percentage of shoots and rooting rate of shoots in *Hypericum perforatum* L. from different explants.

Media	Frequency of callus initiation (%)*		Callus fresh weight (mg/magenta)		Number of shoots per callus		Hypericin content of shoots (%)		Rooting rate (%)	
	Leaf disc	Stem segment	Leaf disc	Stem segment	Leaf disc	Stem segment	Leaf disc	Stem segment	Leaf disc	Stem segment
1	-	-	-	-	-	-	-	-	-	-
5	35	34	5 n**	7.4 m	31 a	19 c	0.052	0.061	76 h	67 ı
6	24	28	8.3 l	10.2 j	16 d	14 ef	0.052	0.017	78 h	95 a
7	31	26	4.1 n	2.3 o	23 b	21 c	0.023	0.047	91 bc	94 ab
8	31	24	8.3 kl	9.1 k	24 b	19 c	0.037	0.022	80 gh	94 ab
9	27	26	12.1 i	16.3 g	14 de	13 f	0.015	0.023	91 cd	88 ef
10	31	30	17.2 g	14.1h	18 def	17 def	0.063	0.061	-	-
11	24	23	45 f	67 c	8 g	6 h	0.075	0.005	87 fg	95 a
12	26	24	86 b	88 a	17 d	19 c	0.044	0.023	92 ab	87 g
13	27	24	51 e	69 d	16 d	12 ef	0.025	0.020	89 de	92 ab
Mean	25.6	23.9	26.33 B***	31.44 A	19 A	15.5 B	0.048 A	0.031 B	76.0	79.1

*Percentage of explants forming callus; **Values followed by different small letters in columns and ***capital letters in bottom row are significantly different (P < 0.01) according to Duncan's multiple range test; - no response.
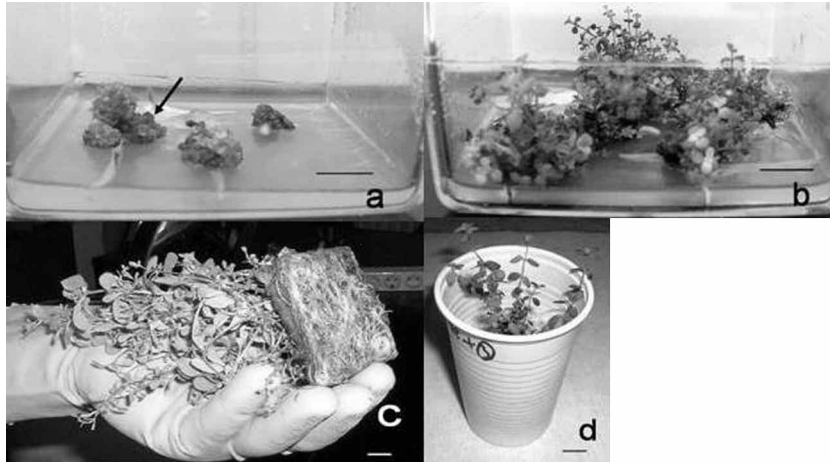
Figure 1. a) Callus formation and red pigmentation from leaf disc cultivated on 30 g l^{-1} of sucrose, 0.5 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin. Arrow indicates dense red-pigmented areas.
b) Shoot induction in MS medium supplemented with 1 mg l^{-1} of BA from callus induced on MS supplemented with 30 g l^{-1} of sucrose, 0.5 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin
c) Rooting of shoots cultured on the medium containing 1 mg l^{-1} of IAA
d) A regenerated plant in greenhouse conditions. Bars represent 1 cm.

sucrose (50 g l^{-1}), yielding the highest mass as fresh weight (1 mg l^{-1} of 2,4-D and kinetin), whereas they did not seem to have the same effect in media containing 30 or 40 g l^{-1} of sucrose. In addition, other 2,4-D and kinetin concentrations (0.5 and 1.5 mg l^{-1}) yielded a higher callus mass than those of the remaining treatments in the presence of 50 g l^{-1} of sucrose. These results are similar to those in a previous report that indicated the highest callus fresh weight was obtained from stem segment cultured in the medium supplemented with 1 mg l^{-1} of 2,4-D and 1 mg l^{-1} of kinetin in *H. perforatum* (Bezo and Stefunova, 2001).

Shoot Induction

When calli were cultured on MS medium supplemented with 1 mg l^{-1} of BA, very intensive shoot induction was observed (Figure 1b). BA, alone or in combination with NAA, is known to be an effective shoot inducing agent in different species (Blakesley and Constantine, 1992). For *H. perforatum* L., BA was found to be the most efficient in promoting shoot regeneration when leaf (Pretto and Santarem, 2000) or excised seedling parts (Cellarova et al., 1992) were used as the explant. Similarly, BA can be used together with NAA in shoot induction from anther-originated callus (Kirakosyan et al., 2000a).

In vitro shoot formation may be subjected to change depending upon the explant types used (Zobayed and Saxena, 2003). Likewise, in this study the average number of shoots was significantly higher in calli obtained from leaf discs than in those from stem segments (19 and 16 shoot per callus, respectively, Table 2). The calli obtained from leaf discs and cultured on the medium supplemented with 0.5 mg l^{-1} of 2,4-D, 0.5 mg l^{-1} of kinetin and 30 g l^{-1} of sucrose had the highest shoot formation (31 shoots per callus), followed by the calli obtained from leaf discs and cultured on the medium supplemented with 0.5 mg l^{-1} of 2,4-D, 0.5 mg l^{-1} of kinetin and 40 g l^{-1} of sucrose or 1.5 mg l^{-1} of 2,4-D, 1.5 mg l^{-1} of kinetin and 30 g l^{-1} of sucrose (24 and 23 shoots per callus, respectively). Similarly, a changeable response to shoot induction media from different explants was reported by Gupta and Conger (1998) in *Panicum virgatum*.

No correlation was observed between the organogenic capacity and fresh weight of calli obtained, as the highest number of shoots, 31 and 24, were observed on calli showing average fresh weights of 5.0 and 8.3 mg, respectively (Table 2). The result is similar to that of Pretto and Santarem (2000), who reported no correlation between the organogenic capacity and fresh weight of calli in *H. perforatum* L.

Studies concerning shoot induction and multiplication in *H. perforatum* L. have reported an average 20 to 22 shoots, depending on the factors evaluated (Bezo and Stefunova, 2001; Pretto and Santarem, 2000). We reported a procedure allowing the production of up to 30 shoots per callus. However, it is important to note that induction of up to 50 shoots per callus for *H. perforatum* L. was reported by Santarem and Astarita (2003) employing thidiazuron and NAA in addition to BA and kinetin as callus inducing agent. The marked difference between the 2 reports may derive from the plant growth regulators used.

Hypericin contents of shoots

Only a few studies have reported the presence of hypericin in organ and cell cultures established from *H. perforatum* L. (Kirakosyan et al., 2001; Bais et al., 2002; Sirvent and Gibson, 2002; Travis et al., 2002). Biosynthesis of hypericin and its derivatives is correlated with the extent of cell differentiation, attaining its highest level during leaf morphonogenesis (Kirakosyan et al., 2000b). Thus, the shoots obtained from calli cultured on the medium containing 1 mg l^{-1} of BA were used for hypericin determination. According to the variance analysis, explant types was the only factor affecting the hypericin content of regenerants. As a mean of other factors tested, the hypericin percentage of shoots obtained from leaf disc-originated calli was higher than that of stem segment-originated ones (0.048% and 0.031% respectively, Table 2). Explant types is one of the main factors regulating and directing secondary metabolite synthesis (Murch et al., 2000a). In addition, hypericin was most intensively found in oil glands of leaves (Cicracelli et al., 2001). Therefore, leaves are the preferred organs for hypericin extraction (De Smet and Nolen, 1996). Consequently, the marked difference observed between the hypericin percentages of shoots obtained from either leaf disc or stem segment originated calli may be related to the above facts.

Rooting

IBA, IAA and NAA have commonly been used to improve rooting in different *Hypericum* species (Pretto and Santarem, 2000). IAA and IBA are the most effective for rooting in *H. perforatum* (Cellarova and Kimakova, 1999).

In this study, elongated shoots were rooted very intensively in MS medium supplemented with 1 mg l^{-1} of IAA (Figure 1c) except for the shoots obtained from calli cultured on the medium supplemented with 40 g l^{-1} of sucrose, 1.5 mg l^{-1} of 2,4-D and 1.5 mg l^{-1} of kinetin.

A pronounced difference was determined in rooting percentages of shoots (Table 2). The difference may be related to the media used for callus induction. The highest rooting percentage was formed by the shoots obtained from stem segment-originated calli cultured on media supplemented with 30 g l^{-1} of sucrose, 1 mg l^{-1} of 2,4-D and 1mg l^{-1} of kinetin or 50 g l^{-1} of sucrose, 0.5 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin (95%). Explant types had no effect on rooting rate.

Our findings concerning the rooting percentages are much higher than those of earlier reports which indicate IBA was the best growth regulator for the promotion of root induction with a range of rooting percentages from 23.3 to 47.6 (Pretto and Santarem, 2000). The main difference might be attributed to the use of IAA, instead of IBA.

Regenerated plants were transferred to non-sterile conditions for acclimatisation and to conditions supplying progressively lower humidity levels. After 10 days, 90% of regenerants recorded up to 100% survival (Figure 1d).

Conclusion

We describe an effective model concerning the regeneration and hypericin content of *H. perforatum* L. If the multiplication of selected clones is required, leaf discs or stem segments may be appropriate explants. MS medium supplemented with 30 g l^{-1} of sucrose, 0.5 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin was most suitable for callus initiation. Once induced, calli are transferred to 1 mg l^{-1} of BA containing medium for shoot induction and shoots can be rooted intensively in MS medium supplemented with 1 mg l^{-1} of IAA. Thus, regenerated plants can be obtained after 5 months. As far as the in vitro production of hypericin is concerned, leaf disc could be preferred as explant, instead of stem segment.

Acknowledgements

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