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Original Article

In vitro shoot Proliferation of *Hypericum perforatum* L. through Indirect and Direct Plant Regeneration

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Abstract

Hypericum perforatum L. (St. Johns' wort) is the most commercially important species of the genus *Hypericum* and contains a wide range of components including naphthodianthrones, phloroglucinols, tannins, xanthones, phenolic acids and essential oil. In order to establish an efficient protocol for regeneration, the effects of explant type and plant growth regulators on direct and indirect shoot regeneration in *H. perforatum* were evaluated. According to obtained results the media supplemented with 0.1 mg l^{-1} Benzyl Adenine (BA) was effective for shoot proliferation from shoot tip explants of *H. perforatum* that showed the highest shoots number (15.5 shoots per explant) and shoot height (2.07 cm). In second experiment a method for rapid micro propagation of *H. perforatum* through indirect plant regeneration from calli has been developed. The results demonstrated that a combination of auxin and cytokinin was needed for optimum callus induction and leaf segments were suitable explant for callus induction in *H. perforatum*. Callus induction was observed in most studied treatments but the highest callus volume (1.43 cm^3) was obtained by leaf segments in media supplemented with 0.25 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D)+1 mg l^{-1} Kinetin. Successful shoot regeneration from callus was observed in MS medium containing 0.5 mg l^{-1} BA, which resulted the highest shoot proliferation rate (61.75%) and maximum number of induced shoots (9 shoots per explant). All of shoots formed root in media with 0.5 mg l^{-1} Indole-3-Butyric Acid (IBA) on which 100% of the regenerated shoots developed roots with an average number of 5 roots per shoot. The plantlets were acclimatized and transferred to the greenhouse with 80% survival. This *in vitro* propagation protocol should be useful for conservation as well as mass propagation of *H. perforatum* plant.

Keywords: *Hypericum perforatum* L., Shoot formation, Callus induction, Micro propagation

Introduction

Hypericum perforatum L. commonly called St. John's wort, is an important perennial herb belonging to the family Hypericaceae [1]. This plant is a natural source of active compounds including naphthodianthrones (hypericin and pseudo hypericin), phloroglucinols, tannins, xanthones, flavonoids, phenolic acids and essential oil [2,3]. St. John's wort is stated to possess sedative and astringent properties, and has been used traditionally for the treatment of excitability, neuralgia, sciatica, menopausal neurosis, anxiety and depression [4]. Nowadays *H. perforatum* is the most economically important natural anti depression source [2,3,5]. The steadily increasing market interest in antidepressants effect of *H. perforatum* is one of the factors that have contributed to the economic value of this plant in herbal industry. This economic value of *H. perforatum* has stimulated the development of programs aimed to improve high-speed propagation, breeding and selection of this plant [6].

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Propagation of *H. perforatum* occurs by means of runners or from seeds. *In vitro* regeneration has been successfully achieved for many *Hypericum* L. species from a range of explants sources with different growth regulators. Recent studies have demonstrated that *in vitro* culture is an option for multiplication of different *Hypericum* species such *as H. foliosum* [7], *H. brasiliense* [8], *H. canariense* [9] and *H. perforatum* [10]. With the exception of *H. canariense* and *H. foliosum*, using whole seedlings or their excised parts as initial explants resulted in the formation of multiple shoots. Aseptically germinated seeds of *H. perforatum* and *H. maculatum* have been used as initial material for establishing cell cultures, which have been analyzed for the presence of hypericin, flavonoids, procyanidines and tannins [11].

With consideration this notice that tissue culture can provide an affordable alternative method for propagation with high speed to production of intensive plant material as well as suitable materials for breeding programs of *H. perforatum*, the objective of this study was to determine optimum explant and plant growth regulators for direct and indirect regeneration of *H. perforatum* for developing a high frequency of the plant multiplication system of this plant.

Material and Methods

The seeds of St. John's Wort were collected from Iranian native population in Azadshahr at Golestan province (37° 08′ latitude, 55° 17′ longitude and 143 m altitude). Voucher from this plant have been deposited at the herbarium of the Tehran University.

Seed Germination

In first experiment due to low germination rate of *H. perforatum* seeds, the effect of pretreatments to improve seed germination of this species was investigated. The seed pretreatments concluded: A) washing seeds with tap water for three days, B) soaking seeds in distilled water with some drops of detergent at 4 °C in darkness for three days, and C) soaking seeds in solution of hypochlorite sodium 60% for 20 minutes. The pretreated seeds were washed by water and set in petri-dishes (9 cm) with top paper method (25 seeds put in each petri-dishes). Then 4 ml distilled water was added in petri-dishes. Finally, the germination percentage was calculated for determination of best pretreatments. This experiment was done in the base of completely randomized design with three replications.

Shoot Proliferation

For preparation of sterile plantlets, the seeds of St. John's Wort were pretreated according to results of first experiment and then culture in 1.2 MS [12] growth regulator free-media. For shoot induction, when plantlets attained to height of 5-10 cm, aseptically the shoot tips of each plantlet were excised and cultured on MS medium with different concentrations of Benzyl Adenine (BA) included 0, 0.1, 0.25, 0.35 and 0.45 mg l^{-1} as well as high concentration of BA (0.5, 1 and 1.5 mg l^{-1}). Cultures were maintained at temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes. After 30 days the shoots number per explant, fresh weight and height of shoots were recorded. This experiment had four replications with three explants in each replication and was done in the base of completely randomized design. According to results of this experiment, the application of 0.1 mg l^{-1} BA resulted the highest shoot induction so was choose for next experiment.

In the experiment three, the effect of cytokine and auxin combination on shoots induction of *H. perforatum* was investigated. For this purpose the shoot tips of plantlets were cultured on supplemented media with 0.1 and 0.05 mg l^{-1} BA in combination with three concentrations (0, 0.01 and 0.05 mg l^{-1}) of IBA (Indole-3-Butyric Acid). Cultures were maintained at temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes. After 30 days, shoots number per explant, fresh weight of shoot and black glands number on leave were recorded. This experiment had four replications with four explants in each replication and was done in the base of completely randomized design.

Callus Induction

For callus induction the young leaves of 8-10 mm long and stem segments with 1-3 node of obtained *in vitro H. perforatum* plantlets from previous experiments were excised and cultured on media with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5 and 1 mg l^{-1}), BA (0.2, 0.5 and 1 mg l^{-1}), Kinetin (Kin) (0.2, 0.5 and 1 mg l^{-1}) and 1 mg l^{-1} Naphthalene Acetic Acid (NAA). All the cultures were then maintained at 25±1 °C in the dark. The callus induction, days to callus induction and callus

volume were recorded after 27 days. This experiment was done as a factorial in the base of completely randomized design with 4 replication and 4 explant in each replication. According to obtained results, leaves were the best explants for callus formation; therefore, the next experiment was done using leaves derived calli as initial explants.

Shoot Regeneration from Callus

For shoot regeneration, the leaves derived calli were transferred to supplemented medium with 0.5 and 1 mg l^{-1} of BA and Kin as well as the combination of 0.5 mg l^{-1} of BA and Kin with 0.1 mg l^{-1} IBA. The media contained macro and micro elements of MS and vitamins of B5 [13] medium. Cultures were maintained at temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes. After 6 weeks the shoots number, mean growth of shoots and black glands on leaves were recorded. This experiment had four replications with 10 explants in each replication and was done in the base of completely randomized design.

Root Induction

The shoots when attained to height of 1.5-3 cm were transferred to MS medium contained 0.5 and 1.5 mg l^{-1} of IBA and NAA. Cultures were maintained at temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes. Days to root induction, root induction, roots number, and root height were evaluated 8 weeks after culture. This experiment had four replications with four explants in each replication and was done in the base of completely randomized design.

Basal Medium and Culture Conditions

All media contained MS mineral salts and vitamins [12], 3% (w v^{-1}) sucrose and solidified with 0.8% agar. The pH of the media was adjusted to 5.7 before autoclaving for 20 min at 121 °C. 2,4-D, NAA, BA, Kin and IBA were added to media before autoclaving,.

Data Analysis

Results were analyzed statistically using the Statistical Analysis Program (SPSS ver.16.0). The mean values were calculated and compared by Duncan's multiple range tests ($P < 0.05$).

Results and Discussion

Seed Germination

The seeds of *Hypericum perforatum* due to inhibitor compounds that secrete from seed's capsule have dormancy and without any pretreatment show rarely or low germination rate [14]. It seems that the St. John's wort seeds have dormant embryo that depended on origin and seed age. New harvested seeds mostly have 1-3 months of post ripening period [15]. In present study, all pretreatments had positive effect on seed germination percentage that showed significant difference in comparison with control. In control treatment the seed germination percentage was low and irregular (Table 1).

The factors to increase of endogenous gibberellinic complex that leads to break of seed dormancy are as follow: seed storage, preliminary moisturizing and stratification, as well as washing, low temperature and darkness during germination [16].

Table 1 Effect of different pretreatments on seed germination of *Hypericum perforatum* L. and it's significant

Mean square for effect of different pretreatments on seed germination of *H. perforatum* L.		
Source of variation	df (degree of freedom)	Germination percentage
Pretreatment	3	1.54**
Error	8	0.033

** is significant at 1 percent probability level.

Treatments	Germination percentage
Control	80.75 b*
Washing with tap water (3 days)	97.87 a
Soaking in distilled water (3 days in fridge and darkness)	95.87 a
Socking in sodium hypochlorite 60 % (20 minutes)	96.87 a

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

In this study washing, soaking in solution of hypochlorite sodium, low temperature and darkness during germination was useful for access the highest seed germination of *H. perforatum*. Variability in germination ability of different plants seeds can be due to environmental or genetic differences [17,18]. Indeed, seed dormancy variability can serve as an adaptive strategy in unpredictable environments [19].

Shoot Proliferation

To induce and shoot proliferation, the *in vitro* shoot tips were transferred to shoot induction medium containing different concentration of BA. There was no significant differences for shoot proliferation in high concentration of BA (0.5, 1 and 1.5 mg l^{-1}) (data not shown), but there was significant differences in treatments with low BA concentrations (Fig. 1). Medium supplemented with 0.1 mg l^{-1} BA showed the highest organogenesis frequency level and by increasing the concentration of BA, shoot weight increased while shoot length decreased. According to obtained results, 0.1 mg l^{-1} BA was effective for proliferation of *H. perforatum* that showed the highest shoots number (15.5 shoots per explant) and shoot height (2.07 cm) (Table 2).

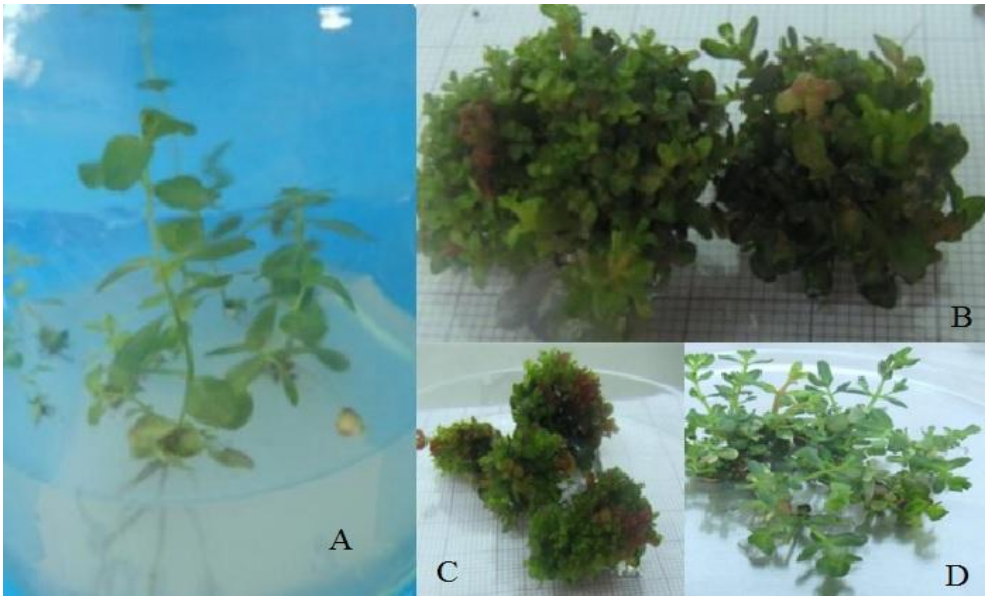

Fig. 1 (a) Two months old seedling of *Hypericum perforatum* L. on growth regulator free half MS medium. Multiplication of shoots on MS medium supplemented with 0.25 mg l^{-1} BA (b), 0.45 mg l^{-1} BA (c) and 0.1 mg l^{-1} BA (d).

Table 2 Effect of different concentrations of benzyl adenine on proliferation, weight and length of *Hypericum perforatum* L. shoots.

Mean squares for effect of different concentrations of benzyl adenine on proliferation, weight and length of *H. perforatum* shoots.

Source of variation	df	Number of shoots /explant	Shoot weight	Shoot length
BA concentration	4	39.125**	4.43**	0.61**
Error	15	0.84	0.15	0.005

** is significant at 1 percent probability level.

Benzyl adenine (mg l^{-1})	Number of shoots /explant	Shoot weight (g)	Shoot length (cm)
0	6.25 d*	1.8 b	2.61 a
0.1	14.5 a	2.12 b	2.07 b
0.25	12.75 a	3.65 a	0.086 c
0.35	10.25 c	3.83 a	0.69 c
0.45	10 c	4.17 a	0.49 b

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

Table 3 Effect of different concentration and combination of plant growth regulators on proliferation, weight and length of shoots as well as the number of black glands in leaves of *Hypericum Perforatum* L..

Mean squares for effect of different concentration and combination of plant growth regulators on proliferation, weight and length of shoots as well as the number of black glands in leaves of *H. perforatum*

Source of variation	df	Number of shoot /explant	Shoot weight	Shoot length	Number of black gland in leaves
Growth regulators	5	3.8**	0.73**	1.74**	15.9**
Error	18	0.04	0.005	0.04	1.25

** is significant at 1 percent probability level.

Plant growth regulators (mg l^{-1})	Number of shoot /explant	Shoot weight (g)	Shoot length (cm)	Number of black gland in leaves
0.1 BA	14.75 b*	1.62 b	1.99 a	12 b
0.05 BA	4.5 a	0.95 c	0.75 cb	10.5 cd
0.1 BA+0.05 IBA	16.5 a	1.97 a	2 a	14.75 a
0.1 BA+0.01IBA	9.25 c	0.9 c	0.58 c	11 cb
0.05 BA+0.05 IBA	6.5 d	0.77 cd	1.007 b	14 a
0.05 BA+0.01 IBA	4 e	0.61 d	0.64 c	9.75 c

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

As shown in figure 1, the BA concentrations higher than 0.1 mg l^{-1} induced the green mass of shoots with short internodes that the accounting of shoots number is difficult, so if the *in vitro* purpose was biomass production these concentrations are advisable. These results are agree with Ayan *et al*. [20]; Murch *et al*. [21] that reported the compact shoots induction in supplemented media with high concentrations of BA. Gadzovska *et al*. [22] reported the shoot induction of *H. perforatum* in medium with 0.1-2 mg l^{-1} BAP. According to their results, the high concentrations of BAP (2-5 mg l^{-1}) led to callus induction. Bezo and Stephonova [23] showed the highest shoot induction of *H. perforatum* in media with combination of BA and IBA. Therefore, in next experiment the effect of cytokinin (BA) and auxin (IBA) combination on shoot induction of *H. perforatum* was investigated.

The organogenesis frequency, number of black oil glands, as well as the number of induced shoots, varied according to used growth regulators. Similarly to Santarem and Astarita [24] reports, in this study the combination of cytokinin and auxin promoted the shoot formation of *H. perforatum*. The highest shoot induction was obtained in supplemented medium with 0.1 mg l^{-1} BA and 0.05 mg l^{-1} IBA that showed the highest shoots number (16.5 per explant), shoot weight (1.97 g) and shoot height (2 cm) as well as the highest number of black glands in leaves (14.5) (Table 3). The effects of cytokinins and auxins on shoot multiplication have been reported for *Hypericum* species. In *H. foliosum*, the highest number of shoots was obtained on media supplemented with BA and NAA [7]. Similar results were also reported for *H. canariensis* [9]. According to Briskin [25] the hypericin and pseudo hypericin, the important anti depression compounds in *H. perforatum* are in black glands of leave edges, so survey the effects of plant growth regulators on number of these glands is important.

Callus Induction

Callus cultures could be used for cell suspension initiation, studying of their morphogenetic potential and screening of secondary metabolite profile. In different species of *Hypericum* have successfully reported the production of hypericin, pseudohypericin and flavonoids in cell cultures that revealed the optimizing value of callus induction procedure in different species of *Hyperium* [11,26,27]. In present study the response of two *H. perforatum* explants (leaf and stem) to different levels and combinations of auxins and cytokinins were tested. Callus initiation was observed from the leaf and stem explants about 10 to 14 days after culture. The highest rate of callus induction (100%) were observed in most treatments (Table 4) except in the presence of 1 mg l^{-1} 2,4-D+1 mg l^{-1} NAA in both explants that showed very low callus induction (12-18%). The highest callus volume (1.43 cm^3) was obtained by leaf segments in supplemented media with 0.25 mg l^{-1} 2,4-D+1 mg l^{-1} Kin, also in stem explant the highest callus

volume was observes in 0.25 mg l^{-1} 2,4-D+1 mg l^{-1} Kin and 0.25 mg l^{-1} 2,4-D+1 mg l^{-1} BA treatments. Of the various concentrations and combination of growth regulators, the minimum response about callus induction was observed in the presence of 1 mg l^{-1} 2, 4-D in combination with 1 mg l^{-1} NAA (Table 4). These results demonstrated that a combination of auxin and cytokinin was needed for optimum callus induction and leaf segments were suitable explant for callus induction in *H. perforatum*. The achieved calli was compact, but they were brown shortly. Few species within the genus *Hypericum* L. have been used to produce callus. In *H. perforatum* seedling, different explants such as shoot apical meristem [22], stem segments [28] and leaves [29][30] were used for callus induction. According to Pretto and Santarem [31] the callus growth of *Hypericum* severity influenced by plant growth regulators of media culture. In *H. erectum* callus induction was obtained by culturing seedlings in the presence of IAA and BA under darkness [26]. Pretto and Santarem [31] reported that in *H. perforatum* 2,4-D did not successfully induce callus and combination of auxin and cytokinin was necessary for callus initiation. However in nodal explants of *H. brasiliense*, the combination of cytokinins and auxins did not support callus growth and callus was only obtained in the presence of 2,4-D or NAA using either MS or B5 medium [13]. These differences can be due to different cultivars, culture conditions, explant type and medium composition [29].

Shoot Regeneration from Callus

The regeneration frequency and number of induced shoots, varied according to used growth regulators. Shoot regeneration from *H. Perforatum* callus was induced in medium containing cytokinins alone or in combination with NAA.

Table 4 Effects of explant type and plant growth regulators on callus induction of *Hypericum Perforatum* L..

Mean squares for effects of explant type and plant growth regulators on callus induction of *H. perforatum*				
Source of variation	df	Days to callus induction	Callus induction	Callus volume
Growth regulators (G)	7	130.86**	14553.57**	1.48**
Explant (E)	1	4**	39.06**	0.21**
G×E	7	32**	16.74**	0.2**
Error	48	0.17	32.55	0.01

** is significant at 1 percent probability level.

Plant growth regulator (mg l^{-1})	Explant type	Days to callus induction	Callus induction (%)	Callus volume (cm^3)
Growth regulator-free media	0	0	0	0
	0	0	0	0
0.5 2,4-D+ 0.5 Kin	Stem	10 c*	100 a	0.40 ef
	Leaf	10 c	100 a	0.90 c
0.25 2,4-D+ 1 Kin	Stem	10 c	93.75 a	0.99 cb
	Leaf	10 c	100 a	1.43 a
1 2,4-D+ 0.2 Kin+ 0.1 NAA	Stem	10 c	100 a	0.34 f
	Leaf	10 c	100 a	0.65 d
0.5 2,4-D+ 0.5 BA	Stem	10 c	100 a	0.83 c
	Leaf	17 a	100 a	1.10 b
0.25 2,4-D+ 1 BA	Stem	10 c	100 a	0.99 cb
	Leaf	10 c	100 a	0.59 d
1 2,4-D+ 0.2 BA+ 0.1 NAA	Stem	10 c	100 a	0.53 ed
	Leaf	14 b	100 a	0.33 f
1 2,4-D+ 1NAA	Stem	14 b	12.5 b	0
	Leaf	7 d	18.7 b	0

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

The highest organogenesis frequency was obtained from cultured calli on medium supplemented with BA alone and no regeneration response from callus was observed in growth regulator free medium.

Successful shoot regeneration was obtained in MS medium containing 0.5 mg l^{-1} BA (Fig. 2a), which resulted the highest shoot proliferation rate (61.75%) and highest number of induced shoots (9 shoots per explant) (Table 5). In addition, black glands was visible at the first stage of leaf formation (Fig. 2b). In overall BA showed best shoot regeneration response than Kin, also the combination of BA and Kin did not show the significant difference. Supplemented media with BA alone or in combination with NAA, has previously been reported as being efficient in promoting shoot differentiation in several species [32]. In *H. perforatum*, BA was found to be most efficient in shoot formation [10] and for *H. canariense* and *H. foliosum*, the best results for shoot induction were obtained directly from apical or axillary buds cultured in media supplemented with BA and NAA [7][9]. In *H. erectum*, shoot primordia were induced in medium containing BA and IAA [26]. Callus obtained from anthers of *H. perforatum* was induced to shoot formation with using BA and NAA [33].

Table 5 Effect of different concentrations and combination of plant growth regulators on shoot regeneration from *Hypericum perforatum* L. callus.

Mean squares for effect of different concentrations and combination of plant growth regulators on shoot regeneration from *H. perforatum* callus.

Source of variation	df	Shoot induction	Number of shoot/explant	Number of black gland in leaves
Growth regulators	6	20.39**	41.14**	30.46**
Error	21	0.75	0.57	1.48

** is significant at 1 percent probability level.

Plant growth regulator (mg l^{-1})	Shoot induction	Number of shoot/explant	Number of black gland in leaves
Growth regulator-free media	0	0	0
1 BA	48.63 b*	6 b	12.5 a
0.5 BA	61.75 a	9 a	14 a
0.5 BA+0.1 IBA	20.5 c	4 c	9 b
1 Kin	10.5 dc	1.25 d	8 b
0.5 Kin	8 d	1.25 d	7.75 b
0.5 Kin+0.1 IBA	7.75 d	1.25 d	7.75 b

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

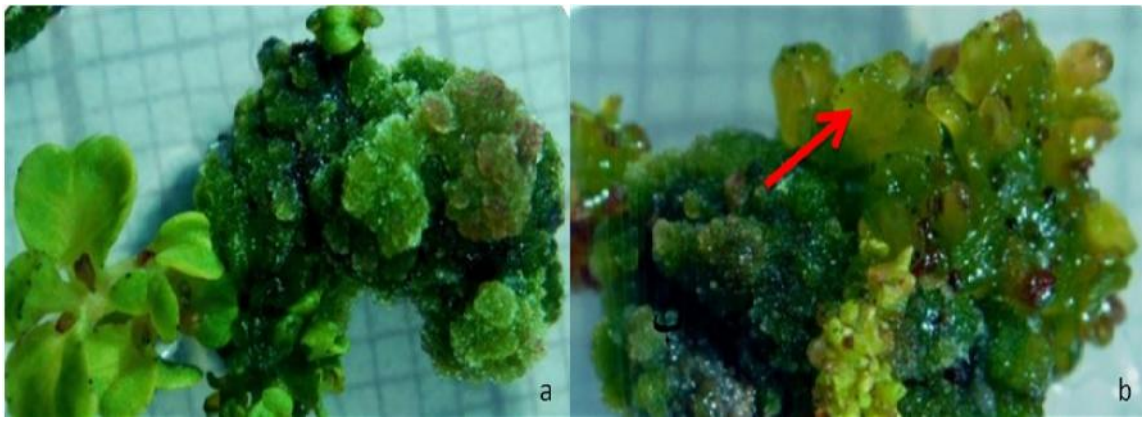

Fig. 2 (a) Adventitious shoot induction from callus on MS medium supplemented with BA (0. 5 mg l^{-1}) in *Hypericum Perforatum* L. (b) Black glands in *Hypericum perforatum* L. leaf at the first stage of formation (red arrow indicate the black gland in leaf).

Table 6 Effect of different concentrations of plant growth regulators on root formation of regenerated root primordia *perforatum* L. shoots.

Mean squares for effect of different concentrations of plant growth regulators on root formation of regenerated *H. perforatum* shoots.

Source of variation	df	Days to rooting	Root induction	Root length	Number of roots/explant
Growth regulators	4	602.05**	5792.5**	0.78**	114.175**
Error	15	1.78	176.25	0.013	0.7

** is significant at 1 percent probability level.

Plant growth regulators (mg l^{-1})	Days to rooting	Root induction	Root length (cm)	Number of main roots/explant	Number lateral roots/explant
Growth regulator-free media	17.75 a*	75 c	3.86 a	3 b	3 a
0.5 IBA	12 c	100 a	2.62 b	5 a	0.25 b
1.5 IBA	14 b	87.5 b	1.59 c	4.5 a	0
0.5 NAA	14 b	25 d	0.55 c	1 c	0
1.5 NAA	14 b	25 d	0.52 d	1 c	0

*Values followed by different letters are significantly different according to Duncan test at $P \leq 0.05$.

Rooting

The shoots when attained to about 1.5–3 cm in length were transferred to media supplemented with various concentrations of IBA and NAA for rooting. All of shoots formed root in media supplemented with 0.5 mg l^{-1} IBA on which 100% of the regenerated shoots developed roots with an average number of 5 roots per shoot. In addition, root induction was quick in this concentration and the root primordia was observed in 12 days after culture. In this experiment, NAA was not efficient in promoting rooting in shoots. The induced roots by high concentration of NAA were thicker and shorter without branches; thus, the survival rate of transplanted plantlets was lower. In addition, the longest root and the lowest number of roots per shoot were observed in plant growth regulator free medium and NAA treatments respectively (Table 6). Various medium and growth regulators to improve rooting in *Hypericum* species have been tested [7,10]. Among the growth regulators, auxins such as IBA, IAA and NAA have been used to induce root formation in several species of *Hypericum* L.. In *H. canariense*, rooting was obtained in the presence of either IBA or NAA [9]. Finally the rooted plantlets were hardened for 4 weeks and were transferred to a greenhouse with 80% survival.

In summary, we concluded that the pretreatment of seeds is necessary for high seed germination of *Hypericum perforatum.* In *H. perforatum* supplemented media with 0.1 mg l^{-1} BA is suitable for achievement the highest direct plant regeneration from shoot tips and callus induction was improved by selecting leave segments as initial explants when cultured on medium with 0.25 mg l^{-1} 2,4-D+1 mg l^{-1} Kin. Moreover, the highest shoot regeneration rate was achieved on medium supplemented with 0.5 mg l^{-1} BA that all formed root in media with 0.5 mg l^{-1} IBA. The described protocol for optimized the callus and shoot regeneration from *H. perforatum* provides a successful *in vitro*propagation technique for this species and can provide an alternative route to speed up the propagation and breeding program of *H. perforatum.*

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