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Original Article

Effects of Methyl Jasmonate and Chitosanon Shoot and Callus Growth of Iranian *Hypericum perforatum* L. *in vitro* Cultures

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Abstract

The present study was investigated the effects of two elicitors methyl jasmonate (0, 125, 250 and 500 µM) and chitosan (0, 50, 150 and 250 mg L^{-1}) on shoot and callus growth of Iranian *Hypericum perforatum* L. *in vitro* cultures in separate experiments. The experiment was done in the base of completely randomized design with five replications and five explants in each replication. Methyl jasmonate significantly reduced the regeneration of cultured shoots and developed shoots necrosis as well as the growth of treated calluswith methyl jasmonate was ceased. In spite of methyl jasmonate, chitosanwas more effective in stimulating the growth of cultured explants. Chitosan significantly increase the regeneration of *H. perforatum* shoots and the highest regenerated shoots number (9.29 shoots per explant) and shoot weight (1.82 g) were obtained in 250 mg L^{-1}chitosan. The growth of callus in response to chitosan was higher than control treatment and the treated calli with 250 mg L^{-1} chitosan showed the highest diameter and weight. The essential oil content and chemical compositions of *in vitro* proliferated Iranian *H. perforatum* shoots were also investigated. The essential oil content was 0.02% (w w^{-1}). The characterization of essential oil by GC and GC/MS analyses, allowed the identification of 35 volatile constituents, accounting for 97.01% of the total oil composition and sesquiterpenes (51.72%) represented by γ–gurjunene (22.34%) were the main compounds.

Keywords: Chitosan, Elicitor, Essential oil, *Hypericum perforatum* L., Methyl jasmonate

Introduction

Hypericum perforatum L. (St. John's wort) belonging to *Hypericum* L. genus, is widely known herb due to its remarkable pharmaceutical properties [1]. In different organs of the related species to *Hypericum* genus (leaf, stemand flower tissues) were produced a broad spectrum of valuable compounds with antiviral, antitumor,neuroprotective, antioxidant and antidepressant properties included naphthodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), flavonoids (hyperoside, rutin or quercitrin), xanthones and essential oils [2,3].

Although the chemical variations among different *Hypericum* species and varieties have been reported but the chemical composition of some species has not been surveyed. *H. perforatum* is one of the less well-studied species that may synthesize moderate quantities of hypericins and hyperforin as well as appreciable amounts of essential oils [3]. Due to the commercial importance of *Hypericum* secondary metabolites, there is great interest in enhancing their production by means of biotechnology.

Invitro plant cultures proper the possibility of ensuring sustainable conservation, mass production and introductionof new biodiversity. The used of elicitation strategy at *in vitro* condition can be effective in stimulation of regeneration and

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bioactive compound production in cultivated *in vitro* explants [2,4].The elicitor such as jasmonic acid and salycilic acid significantly influenced the cells and shoots growth; also enhanced the hypericins and hyperforin content in both cell suspension and shoot cultures of different species as well as *H. perforatum* [4-7].The growth of the elicited cells of *H. perforatum* with jasmonic acid was always below or equal to the control values [5]. In elicited callus of *H.perforatum* L. compact callus with distinct necrotic regions of dead cells were formed [8]. In addition, the *H. perforatum* cell suspension cultures treated with the *Aspergillus* mycelia extract were negatively affected for growth and turned brown in coloration; however these cultures were accompanied with high production of secondary metabolites [9].

Bioactive compounds of *in vitro* plants are usually differed from those in natural plants about their quantity and quality. In *Hypericum* species contradictive reports are about this issue for different phytochemical compounds. Dias *et al*. [10] reported that the flavonoids contentin callus culture of *H. perforatum* and *H. androsaemm*were in trace or absent, however in the survey of Shilpashree and Ravishankar Rai [11] flavonoids was accumulated in higher content in *in vitro* cultures of *H. mysorense*. There are a few reports regards to essential oil content and quality of *Hypericum* species in *in vitro* conditions. Literature review shows that the variation of essential oil content and constituents of *in vitro* and in vivo *Hypericum* plants depends on species [12].

In the present study, the effect of methyl jasmonate (MeJA) and chitosan on shoot and callus growth of Iranian *H. perforatum* under *in vitro* cultures was investigated. In addition, the essential oil content and chemical compositions of *in vitro* proliferated Iranian *H. perforatum* shoots were also surveyed.

Material and Methods

Plant materials, treatments and growing conditions

Seeds of *Hypericum perforatum* were collected from Iranian native population in Azadshahr at Golestan province. Voucher from this plants been deposited at the herbarium of the Tehran University**.**

The seeds were sterilized by immersion in 70% (vv^{-1}) ethanol solution (60 s), followed by immersion in a 60% (vv^{-1}) sodium hypochlorite solution (20 min) and five rinses with sterile distilled water. The sterile seeds were germinated on half-strength Murashige and Skoog (MS) medium [13] free ofplant growth regulators. For shoot induction, aseptically the shoot tips of each plantlet were excised and cultured on MS medium containing0.1 mg L^{-1}benzyl adenine (BA) and 0.05 mg L^{-1}indole-3-butyric acid (IBA). Elongated shoots were used as experimental material for subsequent experiments.

For shoot elicitation with MeJA and chitosan, in two separate experiments the shoot segments 2-3 cm were transferred to MS medium with 0, 125, 250 and 500µM MeJA and 0, 50, 150 and 250 mg L^{-1} chitosan. The cultured shoots in these concentrations of MeJA lost their viability so the experiment was replicated with lower concentrations included 0, 25, 50, 75 and 100 µM of MeJA. Control cultures received 500 µl ethanol 95% and 50 µM acetic acid 0.1 M in MeJA and chitosan treatments, respectively. Cultures were maintained at temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes at 40 µmol m^{-2} s^{-1}. After 3 weeks, the formed shoots number per explants, shoot fresh weight and the number of necrosis shoots in each experiment were recorded. Each experiment was done based on completely randomized design with six replicates, and four explants in each replication.

For callus induction, the young leaves were excised and cultured on media with 0.25mg l^{-1}2,4-Dichlorophenoxyacetic acid (2,4-D) and 1 mg l^{-1} Kin (Kinetin). All the cultures were then maintained at 25±1 °C in the dark. The proliferation calli were elicited with MeJA and chitosan so the callus segments with determined weight and diameter were transferred to MS medium with 0, 125, 250 and 500 µM MeJA and 0, 50, 150 and 250 mg L^{-1} chitosan in two separate experiments. Control cultures received 500 µl95% ethanol and 50 µM acetic acid 0.1 M in MeJA and chitosan treatments, respectively. Cultures were maintained at temperature of 25±1 °C in the dark. After 3 weeks, the fresh weight and diameter of callus segments were recorded. Each experiment was done based on completely randomized design five replications and five explants.

Basal medium and culture conditions

All media contained MS mineral salts and vitamins, 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. BA, IBA, 2,4-

Dand kin were added to media before autoclaving, while methyl jasmonate and chitosan was added to media after autoclaving through filter sterilization.

Essential oil extraction and analysis procedure

The proliferated *in vitro* shoots in medium with 0.1 mg l^{-1} BA and 0.05 mg l^{-1} IBA were used for essential oil extraction so 300 g of chopped fresh shoots subjected to hydro distillation for 4 h using the Clevenger apparatus. The oil phase was separated, dried over anhydrous sodium sulfate. The essential oils kept in a dark glass bottle at 4°C for the analyses.

Chemical compositions of the essential oil were determined by gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS). The GC analysis was done on an Agilent Technologies 7890 GC (Agilent Technologies, Santa Clara, CA) with a medium-polar HP-5MS capillary column (30 m×0.25 mm, 0.25 µm film thicknesses). The flow of the carrier gas Helium was 1 ml/min. The oven temperature was programmed from 60 to 240 ºC at 3 ºC/min and held isothermal at 240 ºC for 8.5 min The injector temperature was set at 280 °C.

GC/MS analyses were performed on an Agilent Technologies 5975 C mass selective detector (MSD) and quadruple EI mass analyzer. Mass spectra were recorded at 70 eV. Mass and the ion source were maintained at 230°C.The individual constituents were identified by retention indices and compared with constituents known from the literature [14].

Data analysis

Data were analyzed statistically using the Statistical Analysis Software (SAS Ver.8). The mean values were calculated and compared by Duncan's multiple range tests ($p \leq 0.05$).

Results and Discussion

Effect of methyl jasmonate on shoot and callus growth

Analysis of variance showed that methyl jasmonate levels significantly influenced the shoot regeneration of *H. perforatum* (Table 1).Treated shoots with high concentrations of methyl jasmonate (125 to 500 µM) showed necrosis and lost their viability along the three weeks after culture. Also in lower concentrations of MeJA (0-100 µM) with increasing concentration the formed shoots number and their weight significantly decreased so that in 100 µM the shoot regeneration was cessation. The necrotic shoots in 100 µM MeJA significantly were higher than control (Table 2). Reduction of shoot weight in MeJA treatment was reported for different *Hypericum* species. Coste *et al*. [15] confirmed that high concentration of jasmonic acid significantly reduced the plant growth of shoot culture of *H. hirsutum* and *H. maculatum* so the weight of regenerated shoots of treated explants was lower than control. Kim *et al*. [16] reported that the growth of threated explants of *Centella asiatica* (L.) Urb. with high concentrations of MeJA (>0.1 mM) decreased. As well as in *Withania somnifera* (L.) Dunal the shoots biomass production of MeJA treatments significantly was lower than control [17]. In addition, the number of necrotic regions in elicited callus of *H. perforatum* L. with ancymidol progressively increased, which led ultimately to the culture death. It seems that the necrotic regions become secondary elicitors and prevent further penetration of the primary elicitor [8]. According to table 3methyljasmonate significantly influenced the callus growth of *H. perforatum*. In spite of shoots, the calli maintained their viability in high concentrations of MeJA. The growth of treated calliwas significantly decreased in comparison with control and was sensible with increasing MeJA concentrations. The calli with lowest weight and diameter were observed in 500 µ MMeJA treatment (Fig.1a-b).

Table 1 Mean square for effect of methyl jasmonate on *in vitro* shoot growth of *Hypericum Perforatum* L..

Source of variation	df	No. shoots per explants	Shoot fresh weight	No. necrosis shoots
Pretreatment	4	17.60**	0.55**	0.43**
Error	25	0.25	0.005	1.37

** is significant at 1 percent probability level.

Table 2 The effect of methyl jasmonate on *in vitro* shoot growth of *Hypericum Perforatum* L.

Methyjasmonate (µM)	No. shoots per explants	Shoot fresh weight (g)	No. necrosis shoots
0	5.91 a*	1.00 a	0.00 c
25	4.45 b	0.50 b	0.50 b
50	3.00 c	0.43 cb	0.67 b
75	2.87 c	0.32 c	0.83 b
100	1.41 d	0.16 d	1.83 a

* Values followed by the same letter within a column indicate they are not significantly different ($P \leq 0.05$).

Table 3 Mean square for effect of methyl jasmonate on callus diameter and callus fresh weight of *Hypericum perforatum* L. *in vitro* cultures.

Source of variation	df	Callus diameter	Callus fresh weight
Pretreatment	3	1.77**	0.040**
Error	16	0.14	0.002

** is significant at 1 percent probability level.

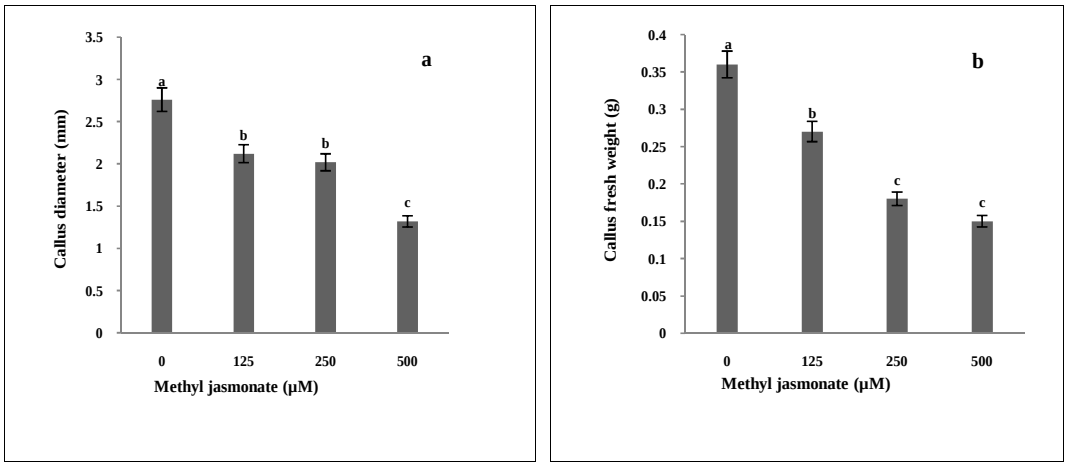

Fig. 1 The effect of methyl jasmonate on callus diameter (a) and callus fresh weight (b) of *Hypericum perforatum* L. *in vitro* cultures.

Such a result has already been reported in *H. perforatum* cell suspensions treated with jasmonic acid [5] and *Aspergillus* mycelia [9]. In elicited *H. perforatum* with jasmonic acid the fresh weight of the elicited cells was always below or equal to the control values [5]. It has been reported that the reduction in biomass might be due to membrane lipoxidation induced by elicitor treatment [9]. However Walker *et al*. [18] reported that used of 100 and 250 µM of methyl jasmonate improved cell growth in suspension cultures of *H. perforatum* but in 500 µM treatment cell growth was partly reduced. The elicitation of cell suspension culture of *Mentha piperita* L. with jasmonic acid and methyl jasmonic acid significantly decreased the cell growth especially in 200 µm [19]. The methyl jasmonate as elicitor in comparison with jasmonic acid had more negative effects on growth and biomass production of *M. piperita, Lavandula vera* DC. and *Salvia miltiorrhiza* Bunge cell cultures [19]. The concentration of jasmonic acid in plant tissue is low and it's exogenous application leads to accumulation of toxicconcentrations of jasmonic acid and suspension of plant growth [20].

Effect of chitosan on shoot and callus growth

Chitosan is a carbohydrate polymer that obtained via deactylation of chitin affects the plant growth and influences the plant resistance to biotic and abiotic stresses. Chitosan used as an exogenous elicitor to induction of growth and secondary metabolite production [21].

Analysis variance showed the significant effect of chitosan on studied parameters (Table 4).

Fig. 2 The effect of chitosan on *in vitro* shoot growth of *Hypericum Perforatum* L. ;a) 250 mg L^{-1} chitosan; b) control.

Table 4 Mean square for effect of chitosan on *in vitro* shoot and callus growth of *Hypericum perforatum* L. cultures.

Source of variation	df	No. shoots per explant	Shoot fresh weight	No. necrosis shoots	Callus diameter	Callus weight
Pretreatment	3	4.51*	0.13^{ns}	0.08^{ns}	0.06**	0.26*
Error	20 (shoot), 16 (callus)	0.79	0.05	0.07	0.005	0.43

^{ns}, * and** are non-significant, significant at 5 and 1 percent probability levels.

Table 5 The effect of chitosan on *in vitro* shoot and callus growth of *H. perforatum* cultures.

Chitosan (mg l^{-1})	No. shoots per explant	Shoot fresh weight (g)	No. necrosis shoots	Callus diameter (mm)	Callus weight (g)
0	0.78 b*	1.49 b	0.50 a	1.92 b	0.55 b
50	0.82 b	1.63 b	0.80 a	2.04 b	0.59 b
150	0.88 b	1.76 b	0.60 a	2.14 b	0.71 a
250	0.99 a	1.82 a	0.80 a	2.46 a	0.79 a

* Values followed by the same letter within a column indicate they are not significantly different ($p < 0.05$).

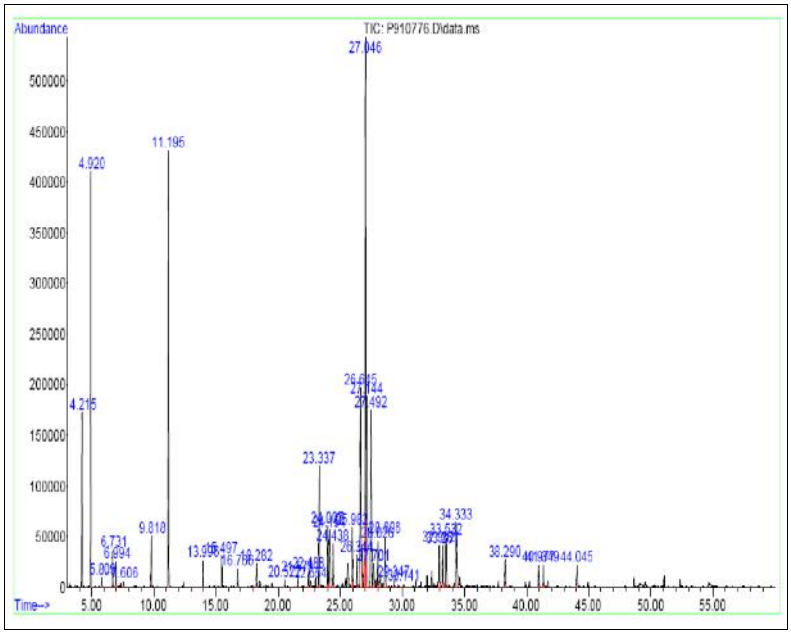

Fig. 3 Representative GC-MS chromatogram of *in vitro* grown *Hypericum perforatum* essential oil.

In the present study unlike to methyl jasmonate, chitosan showed positive effects on regeneration ability of treated explants and with increasing concentration, the formed shoots number and their weight significantly increased (Fig.2).

The highest regenerated shoots number (9.29 per explants) and shoot weight (1.82 g) were obtained in 250 mg l^{-1} of chitosan. Chitosan treatment did not have any effect on necrosis of *H. perforatum* shoots (Table 5).Early the growth induction effect of chitosan has been reported in *Vitisvinifera* [22]. The *in vitro* potato minituber production and their quality in the presence of chitosan in the media culture increased [23]. As well as in micro propagation of orchid, chitosan induced the new protocorm-like bodies regeneration [24]. The response of different species to chitosan is variable and depends on concentration and kind of used chitosan [25].

Chitosan also significantly improved the treated callus growth of *H. perforatum* so that the highest diameter and weight of callus were obtained by 250 mg L^{-1} treatment (Table 5). Tocci *et al*. [26] used the chitosan as elicitor in callus and cell suspension culture of *H. perforatum*. Their results showed that chitosan improvedthe growth of cells. Dornenburg and Knorr [27] reported that chitosan due to induction of protective mechanisms influenced the growth of cell cultures. Kanchanpoom *et al*. [28] also reported the effective impress of chitosan on regeneration capacity of oil palm. The growth induction mechanism of chitosan is attributed to its effect on physiological process of plant such as mineral absorbance, cell elongation and activity of enzymes as well as protein synthesis [29].

Essential oil content and chemical composition

One of bioactive compounds of *H. perforatum* is essential oil that was a little studied in comparison with phenolic compounds. In this study, the essential oil constituents of Iranian *H. perforatum* were investigated (Table 6). The essential oil content of *in vitro* proliferated shoots was 0.02% (ww^{-1}) that is very low in compression with 0.1% (ww^{-1}) of extracted essential oil of wild population which was reported by Morshedlu [30]. The condition of *in vitro* cultures and premature of shoots lead to low content of essential oil. The essential oil content of *in vitro* shoots of *H. androsaemum* was reported 0.74% that was lower than in vivo plants. However, the essential oil content of *in vitroH. undulatum* plants was higher than those in natural plants[31]. The characterization of essential oilby GC and GC/MS analyses, allowed the identification of 35 volatile constituents (Fig. 3), accounting for 97.01% of the total oil composition. Sesquiterpenes(51.72%) represented by γ–gurjunene (22.34%) were the main compounds in Iranian *H. perforatum* essential oil.

Table 6 Chemical composition of the essential oils of *in vitro* Iranian of *Hypericum Perforatum* L.

No.	Compound	RI[a]	(%)
1	2-Methyl-octane	857	2.721
2	*n*-Nonane	898	7.194
3	α-Pinene	932	0.184
4	3-Methyl-nonane	966	0.724
5	β-Pinene	975	0.549
6	*n*-Decane	998	0.101
7	2-Methyl-decane	1060	1.235
8	*n*-Undecane	1098	12.249
9	*n*-Nonanol	1167	0.729
10	*n*-Decanal	1203	0.868
11	*n*-Decanol	1269	0.691
12	*Methyl* decanoate	1322	0.189
13	α-Longipinene	1347	0.332
14	α-Ylangene	1369	0.546
15	α-Copaene	1373	0.165
16	β-Elemene	1389	3.926
17	Dodecanal	1406	1.977
18	(Z)-Caryophyllene	1409	2.117
19	(E)-Caryophyllene	1416	1.759
20	(E)-β-Farnesene	1415	1.904
21	*Alpha* -Acoradiene	1464	1.098
22	*n*-Dodecanol	1471	6.931
23	γ-Gurjunene	1481	22.342
24	γ-Himachalene	1483	6.322
25	α-Selinene	1492	6.805
26	β-Himachalene	1497	0.866
27	(E,E)-α-Farnesene	1506	1.505
28	Δ-Cadinene	1521	1.815
29	α-Calacorene	1540	0.219
30	(E)-Nerolidol	1561	0.083
31	γ-Eudesmol	1635	1.632
32	β-Acorenol	1643	1.439
33	α-Cadinol	1651	1.745
34	*n*-Tetradecanol	1673	3.244
35	*n*-Hexadecanol	1872	0.787
	Monoterpenes	-	0.733
	Sesquiterpene hydrocarbons	-	51.721
	Oxygenated sesquiterpenes	-	8.143
	Alkanes	-	24.224
	Alcohols	-	8.351
	Others	-	3.084

[a]RI: Retention index

In addition,alkanes represented by n-undecane (12.25%), n-nonane (7.19%) and alcohols (8.35%) represented by n-dodecanol(6.93%) were also other components. Gudes [10] reported the sesquiterpene hydrocarbons as the dominant compounds of *in vitro* essential oil of *H. anderosaemum* that including 80% of the total oil composition.

In the essential oil of in vivo *H. perforatum*the alkanes were reported as the dominant compounds and n-oktan (36.07%), α-pinene (23.66%) and 5-methyl-3-heptan (10.13%) were the main constituents [30].These results showed that the essential oil quantity and quality of *in vitro* and in vivo plants differed from each other. In agreement with our results Gudes *et al*. [31]; Gudes[12] reported the different essential oil quality and quantity of *in vitro* and in vivo plants of *Hypericum* species. Sesquiterpene hydrocarbons were as major group of *in vitro H. perforatum* with 40.3% and *n*-nonane (24.2%) was as a major compound [12]. In several studies, have been reported that growth conditions such as temperature, humidity and light affected the oil content and chemical composition of medicinal plants [12,30,31].

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