Study of Callus Induction and Cell Culture to Secondary Metabolite Production in *Hyssopus officinalis* L.

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A B S T R A C T

The *Hyssopus officinalis* L. is an important medicinal plant. Antimicrobial and antifungal activities of the essential oil of hyssop have been reported. In this article the effects of explants types, plants growth regulators and elicitors on callus induction and cell culture conditions were studied. In this experiment it was found that there were non- significant differences among hypocotyl and leaf *H. officinalis* explants for callus induction and callus growth rate. There were significant differences among levels of growth regulators and interaction effect between growth regulators and expellant types for callus induction and callus growth rate. There were significant differences among plant growth regulators levels for callus induction and callus growth rate. Results showed that medium supplemented by N_2B_1 and $N_{0.5}B_1$ showed the highest callus induction and callus growth rate respectively. In this paper it was demonstrated the important role of plant growth regulators and explant types on callus induction in *H. Officinalis* as a medicinal plants. Also the most important secondary metabolites in *H. officinalis* were increased in cell culture presence of salicylic acid, citric acid and yeast extract elicitors.

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Hyssopus officinalis L. is an important medicinal plant of the family *Lamiaceae*. As in other members of the family, the plant produces essential oil in its aerial parts [1]. It commonly known as 'hyssop' is a polymorphous species that grows as a subshrub on dry, rocky, calcareous soils in Europe, southwest- ern and central Asia and north-western India [2].
It is cultivated in the USA and former Soviet Union. This species is morphologically and genetically complex, with high variability between populations growing in different areas. So far, several subspecies have been recorded especially for Europe and northern Africa [3].
Antimicrobial and antifungal activities of the essential oil of hyssop have been reported in a number of studies [4, 5] as well as anti-inflammatory effects [6, 7], but only negligible antioxidant activity has been reported [8].
Skrzypek and Wysokińska (2003) [1] reported that "Analysis of dichloromethane extracts of cultured cells by TLC revealed the presence of sterols and triterpenes."
Triterpenoids are one of the most abundant classes of compounds in plants. It has frequently been suggested that triterpenoids play a defensive role against pathogens and herbivores. They also have several interesting pharmacological activities that include anti-inflammatory [9], antimycobacterial[10], antiviral [11] and cytotoxic [9] properties.
Among the biological activities of hyssop essential oil, its antimicrobial activity against pathogenic and spoilage bacteria has been most intensively studied [12, 5]. Most reports about the antifungal activity of hyssop oil are focused on its inhibitory effect against phytopathogenic and mycotoxin-producing fungi [13].
In vitro conditions, not only component production was increased but new product also plant tissue and cell suspension cultures have been investigated by biotechnological methods and provide a promising bioproduction platform for desired natural products. Tissue cultures of shoots or roots display undifferentiated metabolite characteristics compared to their parent plants, whereas similar cultures often accumulate target compounds to a less level [14].
We have established cell cultures of *H. officinalis*in order to study their ability to biosynthesize secondary metabolites.
The few investigators were reported on *H. officinalis* cell culture. The aim of this research was performing cell culture for investing secondary metabolites of *H. officinalis*and compares it with those in native one.

Materials and Methods

The abundant of calli are necessary to cell culture. In order to optimize the conditions of growth regulators and explant types for obtaining calli, two factors including factor A: two types of explants (leaf and stem) and factor B: different levels of benziladenin (BAP) in three levels (0, 0.5, 1 mg/L), Naphthalene Acetic Acid (NAA) in four levels (0.0, 0.5, 1, 2 mg/L) that were evaluated. Two grams of calli was inoculated to 200 mL liquid media with best concentrations of regulators based on the previous test (NAA: 2 mg/l & BAP: 1 mg/l). After cell growing, to study of elicitors effect on cell compositions, we used five levels of yeast extract elicitor (0, 5, 10, 20 and 40 mg/L), salicylic acid in five levels (0, 2, 4, 8 and 16 mg/L) and citric acid in five levels (0, 2, 4, 8 and 16 mg/L). These elicitors were filtered to media after autoclaving. Then they were placed on incubator shaker with 100 round per minute in 25± 1°C. This study was performed in completely randomized design (CRD). After seven days, cell masses were filtered by filter paper and were dried by freeze dryer and then were extracted by micro-Clevenger. Obtained extracts were analyzed by GC-MS to determine the amount of secondary metabolites in cells.

Results and Discussion

The *H. officinalis* L., is described for their antifungal, antibacterial, larvicidal and insect biting deterrent activities [1].
Many factors could affect tissue culture responses of plants, particularly formation of embryogenic callus and plant regeneration. These factors include genotype, explant tissue, culture medium and its supplements [15].

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After recording of data related to callus diameter and growth rate, the statistical analysis of data including analysis of variance and mean comparison were done.
Analysis of variance results demonstrated that there was non-significant difference among explant types for callus induction and callus growth rate, but there were significant differences ($p< 0.01$) among levels of growth regulators and reciprocal effect between growth regulators and expellant types for callus induction and Callus growth rate (Table 1).

Table 1. Analysis of variance effects of plant growth regulators (PGRs) and explant types on callus induction and callus growth rate in *H. officinalis*.

Source of variations	Df	Mean squares	
		Callus induction	**Callus growth rate**
Explant	1	2.500ns	3.589^{ns}
PGRs	11	1002.70**	1415.58**
Explant × PGRs	11	116.40**	154.518**
Error	96	37.410	52.257
CV(%)		7.86	12.95

Where ns (non-significant), *(significant at P<0.05) and ** (significant at 0.01)

The best plant growth regulators must not only induce callus but, the degree of callus induction must be considerable.
Many factors including the choice of growth regulators and explants were responsible for successful callus induction. Different plant species or genotypes will react differently to different plant growth regulators regime.

Mean comparison for effect of different media based on Duncan's test ($p<0.5$) on callus induction showed that N_2B_1, $N_{0.5}B_1$, $N_1B_{0.5}$, $N_2B_{0.5}$ and $N_{0.5}B_{0.5}$ media had the highest callus induction respectively and N_2B_0 and N_0B_0 had the lowest callus induction. Also the Callus growth rate was the highest in $N_{0.5}B_1$ media and it was the lowest in N_0B_0 media (Table 2).

Table 2. Mean comparisons for effects of plant growth regulators (PGRs) on callus induction and Callus growth rate in *Hyssopus officinalis* [Where N (NAA), B (BA)].

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PGRs	Callus induction (%)	PGRs	Callus growth rate (mm/d)
N_2B_1	98[a]	$N_{0.5}B_1$	0.276[a]
$N_{0.5}B_1$	96[a]	$N_2B_{0.5}$	0.247[ab]
$N_1B_{0.5}$	96[a]	$N_{0.5}B_{0.5}$	0.231[ab]
$N_2B_{0.5}$	92[a]	N_1B_0	0.223[b]
$N_{0.5}B_{0.5}$	88[a]	$N_1B_{0.5}$	0.209[bc]
N_1B_0	82[ab]	N_2B_1	0.209[bc]
$N_0B_{0.5}$	68[b]	$N_0B_{0.5}$	0.192[bc]
$N_{0.5}B_0$	42[c]	N_1B_1	0.166[cd]
N_1B_1	42[c]	$N_{0.5}B_0$	0.165[cd]
N_0B_1	36[c]	N_0B_1	0.125[d]
N_2B_0	14[d]	N_2B_0	0.061[e]
N_0B_0	0 [d]	N_0B_0	0.000[f]

The same letter in the each column show the non-significant difference at P<0.05

Many authors reported that the analysis of variance showed that there are significant differences among plant growth regulators (PGRs) levels and explant types and their interactions for callus induction percentage and callus growth rate [15, 16, 17, 18,19]

Duncan's mean comparison showed that there were no significant differences different explants for callus growth rate. This Duncan's results confirmed the ANOVA results.

Means comparison via Duncan's test ($p<0.5$) showed a significant differences for reciprocal effect between growth regulators and expellant types for callus induction and callus growth rate. Based on this test, leaf explants (L) had the highest callus induction in $N_{0.5}B_{0.5}$ and $N_1B_{0.5}$ media, and hypocotyl explants had the highest callus induction in N_2B_1 media. Also this test showed that leaf explants in $N_{0.5}B_{0.5}$ and $N_2B_{0.5}$ media had the highest and in N_0B_0 had the lowest Callus growth rate, and hypocotyl explants in $N_{0.5}B_1$ media had the highest and in N_0B_0 had the lowest Callus growth rate (Table 3).

Table 3. Mean comparisons for reciprocal effect between plant growth regulators (PGRs) and expellant types on callus induction and Callus growth rate in *H. officinalis* [Where N (NAA), B (BA), L (Leaf) and H (Hypocotyl)].

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PGRs*Explants	Callus induction (%)	PGRs*Explants	Callus growth rate(mm/d)
$N_{0.5}B_{0.5}$ L	100 [a]	$N_{0.5}B_{0.5}$ L	0.318[a]
$N_1B_{0.5}L$	100 [a]	$N_2B_{0.5}L$	0.318[a]
N_2B_1H	100 [a]	$N_{0.5}B_1L$	0.292[ab]
$N_{0.5}B_1H$	96 [b]	N_2B_1L	0.274[abc]
N_2B_1L	96 [b]	$N_1B_{0.5}L$	0.270[abc]
$N_{0.5}B_1L$	96 [b]	$N_{0.5}B_1H$	0.260[abcd]
$N_2B_{0.5}L$	96 [b]	N_1B_0L	0.258[abcd]
$N_0B_{0.5}H$	96 [b]	N_1B_1L	0.234[bcde]
B_0N_1L	92 [c]	$N_0B_{0.5}H$	0.212 [cdef]
$N_1B_{0.5}H$	92 [c]	$N_{0.5}B_0H$	0.188 [defg]
$N_2B_{0.5}H$	88 [d]	N_1B_0H	0.188 [defg]
$N_{0.5}B_{0.5}H$	76 [e]	$N_2B_{0.5}H$	0.176[efgh]
B_0N_1H	72 [f]	N_0B_1L	0.174 [efgh]
$N_{0.5}B_0H$	48 [g]	$N_0B_{0.5}L$	0.172 [efgh]
N_0B_1L	48 [g]	$N_1B_{0.5}H$	0.148 [fghi]
N_1B_1L	44 [h]	$N_{0.5}B_{0.5}H$	0.144 [fghi]
$N_0B_{0.5}L$	40 [i]	N_2B_1H	0.144 [fghi]
N_1B_1H	40 [i]	$N_{0.5}B_0L$	0.142 [fghi]
$N_{0.5}B_0L$	36 [j]	N_2B_0H	0.122[ghi]
N_2B_0H	28 [k]	N_1B_1H	0.098 [hi]
N_0B_1H	24 [l]	N_0B_1H	0.076 [i]
N_2B_0L	0 [m]	N_2B_0L	0.000[j]
N_0B_0L	0 [m]	N_0B_0L	0.000 [j]
N_0B_0H	0 [m]	N_0B_0H	0.000 [j]

The same letter in the column show the non-significant difference at P<0.05

In previous investigations [15，16], the most researchers used 2,4-D for callus induction. The 2,4-D has been the commonly and widely auxin in plant tissue culture. However it is defined as the use of high doses of this auxin leads increase in cell division and cell elongation.

The beta-Pinene (β-pinene) is amonoterpene, an organic compound that is found in plants. It is one of the two isomers of pinene, the other beingα-pinene. The β-pinene is colourlessliquid soluble inalcohol, but not water. It has a woody-greenpine-like smell [2].

According to Table 4 and Fig. 1, 2 and 3, it was defined that percentage of Beta- pinene as a secondary metabolite in citric acid 0 and 1 mg/L and salicylic acid 0 mg/l was more than other concentrations of elicitors, and the amount of this metabolite increased in concentration more than 8 mg/l of these elicitors.

Table 4. Mean comparison for the effect of the different concentrations of elicitor on the percentage of secondary metabolites in *H. officinalis*

Elicitor (mg)	β-pinene	1,8-Cineol	Pinocarvone	Myrtenal	Cis-	Germacren-

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	(%)	(%)	(%)	acetate (%)	pinocomphone (%)	D (%)
Free	8.867[a]	7.800[a]	8.700[a]	8.00[d]	19.13[d]	5.600[a]
Citric acid 2	9.000[a]	7.930[a]	8.870[a]	7.90[d]	18.90[d]	5.530[a]
Citric acid 4	6.030[c]	3.530[d]	5.130[d]	12.50[a]	22.13[b]	3.230[c]
Citric acid 8	5.130[d]	5.000[c]	6.470[c]	10.40[b]	22.83[a]	2.570[c]
Citric acid 16	7.100[b]	6.600[b]	7.700[b]	9.03[c]	21.20[c]	4.670[b]
Salicylic acid 2	7.000[e]	4.130[d]	5.770[d]	11.40[b]	21.20[a]	4.000[e]
Salicylic acid 4	7.930[d]	2.730[e]	4.400[e]	13.20[a]	19.83[b]	4.800[d]
Salicylic acid 8	9.870[b]	5.630[c]	7.100[c]	9.70[c]	18.13[d]	6/330[b]
Salicylic acid 16	10.470[a]	7.000[b]	8.170[b]	8.47[d]	17.20[e]	7.000[a]
Yeast 5	8.133[b]	6.467[b]	7.433[b]	9.23[d]	20.03[c]	4.933[b]
Yeast 10	6.100[c]	5.000[c]	6.633[c]	10.63[c]	21.83[b]	3.300[c]
Yeast 20	4.233[d]	3.333[d]	5.300[d]	12.37[b]	23.67[a]	1.833[e]
Yeast 40	4.267[d]	2.233[e]	3.667[e]	13.77[a]	23.90[a]	2.267[d]

Amount of 1, 8 cineol as a secondary metabolite in citric acid 0 and 1 mg/L and salicylic acid 0 mg/l was more than other concentrations of elicitors, and the amount of this metabolite increased in concentration more than 8 mg/l of these elicitors.

The 1,8-cineol is one of the bicyclic epoxymonoterpenes, precisely theLimone oxides[2].

Percentages of this metabolite decreased with increasing concentrations of yeast extract elicitor. Also Cis- pinocamphone amount was highest in citric acid 4 mg/l and salicylic acid 1 mg/l and it had the highest amount in concentration 40 mg/l yeast extract elicitor, so we suggested these concentrations of elicitors for product cis-pinocamphone.

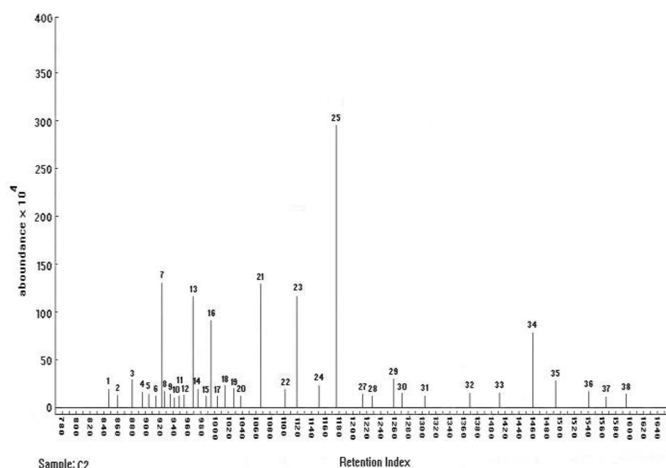

Fig. 1. GC-Mass results of the effect of citric acid on secondary metabolites in *H. officinalis*

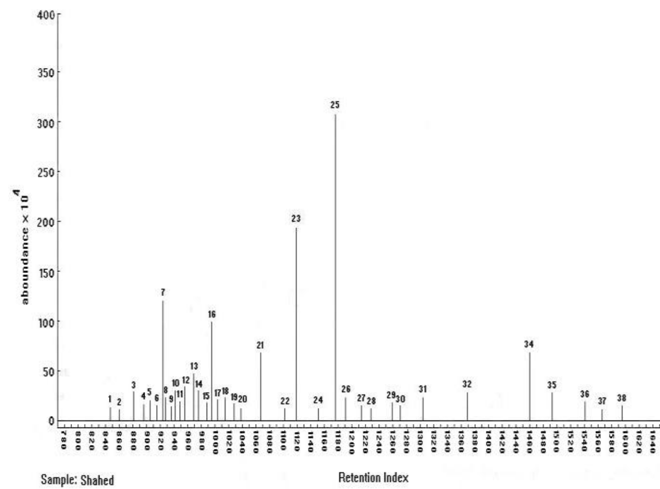

Fig. 2. GC-Mass results of the effect of salicylic acid on secondary metabolites in *H. officinalis*

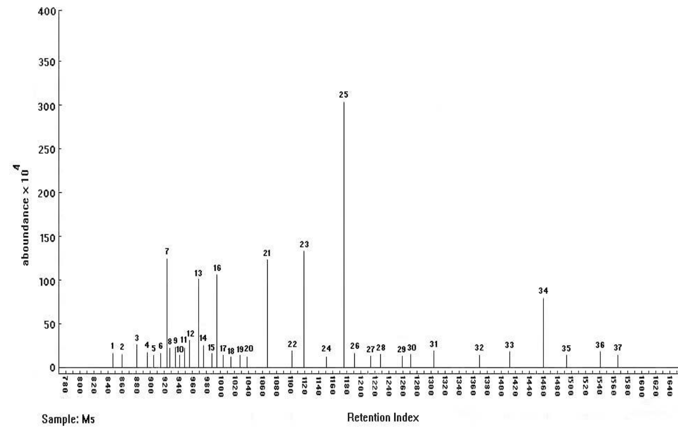

Fig. 3. GC-Mass results of the effect of yeast extract on secondary metabolites in *H. officinalis*

Yadegari and Shakeri (2014) increase amount of this metabolite in cell culture of *Salvia officinalis* L. by salicylic acid elicitor which it has conformity with this study.

Conclusion

In this experiment it was found that there were non- significant differences among hypocotyl and leaf of *H. officinalis* explants for callus induction and Callus growth rate but there were significant differences among levels of growth regulators and reciprocal effect between growth regulators and expellant types for callus induction and Callus growth rate. There were significant differences among plant growth regulators levels for callus induction and callus growth rate. Results showed that medium plants supplemented with N_2B_1 and $N_{0.5}B_1$ showed the highest callus induction and callus growth rate respectively. In this paper it was demonstrated the role of plant growth regulators and explant types on callus induction in stevia as a medicinal plants.

Also the most important secondary metabolites in *H. officinalis* were increased in cell culture presence of salicylic acid, citric acid and yeast extract elicitors.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

References

[1] Skrzypek Z, Wysokińska H. Sterols and triterpenes in cell culture of *Hyssopus officinalis* L. ZeitschriftfürNaturforschung C. 2003;58:308-12.
[2] Venditti A, Bianco A, Frezza C, Conti F, Bini LM, Giuliani C, et al. Essential oil composition, polar compounds, glandular trichomes and biological activity of *Hyssopus officinalis* subsp. aristatus (Godr.) Nyman from central Italy.Industrial Crops and Products. 2015;77:353-63.
[3] Govaerts R, Paton A, Harvey Y, Navarro T, del Rosario García Peña M. World Checklist of Lamiaceae. Facilitated by the Royal Botanic Gardens, Kew. 2015.
[4] De Martino L, De Feo V, Nazzaro F. Chemical composition and in vitro antimicrobial and mutagenic activities of seven Lamiaceae essential oils. Molecules. 2009;14:4213-30.
[5] Kizil S, Hasimi N, Tolan V, Karatas h. Chemical composition, antimicrobial and antioxidant activities of hyssop (*Hyssopus officinalis* L.) essential oil. NotulaeBotanicaeHortiAgrobotanici Cluj-Napoca. 2010;38:99.
[6] Džamić AM, Soković MD, Novaković M, Jadranin M, Ristić MS, Tešević V, et al. Composition, antifungal and antioxidant properties of *Hyssopus officinalis* L. subsp. pilifer (Pant.) Murb.essential oil and deodorized extracts. Industrial Crops and Products. 2013;51:401-7.
[7] Ma X, Ma X, Ma Z, Wang J, Sun Z, Yu W, et al. Effect of *Hyssopus officinalis* L. on inhibiting airway inflammation and immune regulation in a chronic asthmatic mouse model. Experimental and Therapeutic Medicine. 2014;8:1371-4.
[8] Yadegari M, Shakerian A. The effect of salicylic acid and jasmonic acid foliar applications of essence and essential oil of *Salvia officinalis*L. Journal of Applied Science and Agriculture. 2014; 9, 1578-1584.
[9] Rios MY, Gonzalez-Morales A, Villarreal ML. Sterols, triterpenes and biflavonoids of Viburnum jucundum and cytotoxic activity of ursolic acid. Planta Medica. 2001;67:683-4.
[10] Cantrell CL, Franzblau SG, Fischer NH. Antimycobacterial plant terpenoids. Planta Medica. 2001;67:685-94.
[11] Ohigashi H, Takamura H, Koshimizu K, Tokuda H, Ito Y. Search for possible antitumor promoters by inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation; ursolic acid and oleanolic acid from an anti-inflammatory Chinese medicinal plant, Glechoma hederaceae L. Cancer letters. 1986;30:143-51.
[12] Dehghanzadeh N, Ketabchi S, Alizadeh A. Essential oil composition and antibacterial activity of *Hyssopus Officinalis* L. grown in Iran. Asian Journal of Exprimental and Biological Sciences. 2012;3:767-71.
[13] Hristova Y, Wanner J, Jirovetz L, Stappen I, Iliev I, Gochev V. Chemical composition and antifungal activity of essential oil of *Hyssopus officinalis* L. from Bulgaria against clinical isolates of Candida species. Biotechnology & Biotechnological Equipment. 2015;29:592-601.
[14] Yue W, Ming Q-l, Lin B, Rahman K, Zheng C-J, Han T, et al. Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites. Critical Reviews in Biotechnology. 2016;36:215-32.
[15] Chenar HM, Kahrizi D, Zebarjadi A. Effect of Plant Growth Regulators and Explant Type upon Cell Dedifferentiation and Callus Induction in Chickpea (Cicer arietinum L.). Journal of Applied Biotechnology Reports. 2015;2:241-4.
[16] Darvishi E, Kazemi E, Kahrizi D, Chaghakaboudi SR, Khani Y. Optimization of callus induction in Pennyroyal (*Mentha pulegium*). Journal of Applied Biotechnology Reports. 2014;1:97-100.
[17] Soorni J, Kahrizi D. Effect of Genotype, Explant Type and 2, 4-D on Cell Dedifferentiation and Callus Induction in Cumin (Cuminum cyminum) Medicinal Plant. Journal of Applied Biotechnology Reports. 2015;2:265-70.
[18] Soorni J, Kahrizi D, Molsaghi M. The effects of photoperiod and 2, 4-D concentrations on callus induction of Cuminum cyminum's leaf explant: an important medicinal plant. Asian Journal of Biological Sciences. 2012;:378-83.
[19] Kazemi N, Kahrizi D, Mansouri M, Karim, H, Vaziri S, Zargooshi J, Khanahmadi M, Shokrinia M, Mohammadi N. Induction of linalool as a pharmaceutical and medicinal metabolite via cell suspension culture of cumin (*Cuminum cyminum* L.). Cellular and Molecular Biology. 2016; 62:65-68.

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