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Callus formation, regeneration and volatile constituents in *Teucrium chamaedrys*

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Abstract

***Teucrium chamaedrys* is regarded as an herbaceous perennial plant from Lamiaceae family that is used as medicinal plants and food from ancient times. Due to the recent developments in tissue culture techniques and extraction of secondary metabolites, this study aimed to investigate the explants of the *Teucrium* for callus induction and secondary metabolites. The interaction of Auxin hormones including 2,4-dichlorophenoxy acetic acid (2,4-D) and naphthalene acetic acid (NAA) with cytokinin hormones including benzyl amino purine (BAP) or kinetin (KIN) was used as hormonal treatments for callus induction. At the second stage, the presence of secondary metabolites in the NAA/KIN, NAA/BAP and 2,4-D/KIN -treated callus was investigated. Finally, regeneration process from the callus tissue using different hormonal combinations was performed. Our results indicated that NAA/KIN (0.5:0.5mg/L) was the best hormonal combination for callus formation. On the other hand, the obtained calli were subjected to hydrodistillation for essential oil isolation. Chemical composition of the obtained oil was analysed using GC-MS apparatus. The results showed the presence of hexanone, oxalic acid, oxime, dodecane, tetratriacontane, dotriacontane, nonadecene, neophytadine, tetradecanol, dodecane and kalaren as main callus essential oil components. Regeneration process took place in NAA/BAP (0.1:0.5 mg/l) and NAA/KIN (0.5:0.5 mg/l) for shoot and root formation, respectively.**

Keywords: Tissue culture, Essential oil, Teucrium

Introduction

Teucrium genus belongs to Lamiaceae family and has more than 300 species and 4 subspecies with distribution mostly in the Mediterranean regions. This genus consists of 13 species, 10 subspecies and one variety in Iran. *Teucrium chamaedrys* is regarded as a most known species of the genera in Mediterranean region. In the early 19th century, it was considered as a substitute for Cinchona and was used for treatment of gout. Previous chemical studies more conclusively demonstrated the therapeutic effects of this plant (Pourmotabbed *et al.*, 2010). On the other hand, essential oil of this plant has a combination of secondary metabolites which are widely used in food, pharmaceutical and healthcare industries as compounds with antimicrobial and antioxidant traits. Maryam Nokhodi is the common name of this plant in Iran where the plant was used as spice and medicinal plant (Rechinger, 1982).The special materials which can be used to treat some diseases are also generated and stored in these plants. These compounds belongs to secondary metabolites class of the plant chemicals (Omidbeige, 2008). More than 25 percent of medicines available in the world are obtained from the secondary compounds of plants (Bourgaud *et al*.,2001). At the recent decades, tissue culture technique was used for preparation of plant derived dugs and propagation of important medicinal plants.

A survey of literature indicated that some species of the genus were subjected to tissue culture, callus formation and volatile compounds analyses. It was previously pointed out that callus tissue of *Teucrium polium* produce high amount of beta caryophyllen as a volatile compound (Qudah *et al*., 2011). Due to high pharmaceutical and economic importance of *Teucrium chamaedrys*, recent work was focused on callus formation, regeneration and callus volatile constituents of the plant that to our knowledge is the first report in this regards.

Material and methods

Hormonal treatments for callus induction and plant regeneration: To breake physical dormancy of *Teucrium chamaedrys* seeds, the seeds were sterilized in vitro by a dissection needle with 70% ethanol for 35-30 seconds, then with 10% hypochlorite solution for 10-15 minutes as well as a few drops of tween 80. Then, the seeds were rinsed with sterile distilled water and were

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transferred onto a MS medium containing different concentrations of different hormonal combinations independently: 2,4-D (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l): KIN (0.1, 0.5 mg/l), as well as 2,4-D (0.1, 0.5 mg/l): KIN (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l), and NAA (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l): KIN (0.1, 0.5 mg/l), as well as NAA (0.1, 0.5 mg/l): KIN (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l) and NAA (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l): BAP (0.1, 0.5 mg/l), as well as NAA (0.1, 0.5 mg/l): BAP (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/l). The pH of the medium was adjusted to 5.8. Plates were transferred to a germinator at 25 °C with 16/8 hours of photoperiod. The fresh weight of the callus produced six weeks after the subcultured was measured.

Plant regeneration: 40-day old calli were passaged on new MS medium supplemented with 2% sucrose, 0.8% agar and combinations of hormones NAA/BAP (0.1/0.5) for shoot formation. The generated shoots (5-10 mm) were excised and individually transferred to sterile petri dishes with half-strength MS medium contained 2% sucrose, 0.8% agar and combinations of hormones NAA/KIN (0.5/0.5) for rooting. Rooted plants were transferred to pots containing 3:1 mixture of soil and vermiculite for further acclimation and growth in the greenhouse (16/8 hours L/D photoperiod, 16-24ºC and 80-90% humidity).

Essential oil distillation and analysis: The essential oil of six week old calli was distilled with micro-clevenger apparatus (Kamali Co, Iran). Then, it was dehydrated with sodium sulfate and was injected into the gas chromatograph-mass spectrometer (GC-MS) device (Thermoquest-Finnigan model) to determine its composition. The column used in HP-5 device had the dimensions of 30 meters in 0.25 mm with the components diameter of 0.25 micron. Thermal plan of the device was set from 50° C to 320° C with a speed of 4° C per minute. Helium with flow rate of 0.8 ml/min was used as carrier gas. Compounds were identified by comparison of their retention indices (RI) with those reported in the literatures and their mass spectrum with the Wiley Library (Adams, 2004).

Statistical analysis: The experiments were conducted in a completely randomized design with four replications. The software SPSS 19 was applied to perform statistical analysis. Analysis of variance (ANOVA) was done with Duncan's multiple range test at 0.05 probability of significance.

Results

The results indicated that an amount of 0.1 gr of callus was produced in control group (group without any hormonal treatment). It was much less than those of hormonal containing groups. The results also showed that the callus induction quantity was different at various medium containing different auxin and cytokinin based hormonal combinations. In the hormonal composition of NAA/KIN (0.5:0.4 mg/L) and NAA/BAP (0.1:0.5) the maximum production rate of callus was observed to be 320% higher than the control group (Figure 1).

This study tends to indicate that some hormonal combination might be effective for regeneration and can promote callus for organogenesis. It was also revealed that shoot induction in callus happen at hormonal treatments of NAA/BAP (0.1:0.5 mg/l). Other hormonal combination was not capable to promote shoot organogenesis in the callus. Root formation can be induced using NAA/KIN hormonal combination (Figure 2, Table 1). High rooting obtained in media supplemented with NAA/Kin (0.5:0.5 mg/l). Rooted shoots exhibited 80% viability after 4 weeks of transferring to soil/ vermiculite contained pots.

According to data and results obtained from the GC/MS analysis, there was a considerable difference in oil profile of callus obtained from different hormonal treatments. Whereas, callus oil from MS medium supplemented with of NAA/Kin (0.5:0.4 mg/L) was dominated with Tetratriacontane, the callus oils of the media enriched with NAA/BAP (0.1:0.5mg/L) and 2,4-D/Kin (0.5:0.5 mg/L) have Neophytadine and Hexanone as major compounds, respectively (Table 2).

Discussion

It was previously pointed out that explants from organs of some of Teucrium species like *T. polinum* and *T. chamadris* might be induced to callus production in the presence of 2,4-D and kinetin (Kintzios *et al*., 2008; Qudah *et al*., 2011). However, our results showed that hormonal combinations consisted of NAA/Kin are more effective than 2,4-D/kin combinations for callusing process. It is also obvious that like most other plants, the callus induction process in *Teucrium chamaedrys* takes place in a medium containing the approximately same amounts of all auxin and cytokinin based hormones. Callus generation was higher in medium supplemented with Kinetin than other cytokinin base hormone, and BAP. Another previous research demonstrated that callus formation in this plant induced with 2,4-D/ Kinetin (0.5:0.2 mg/l) (Antognoni *et al*., 2012). Our results indicated that a combination of NAA:KIN (0.1:0.5) was the most putative hormonal combination in callusing process of *Teucrium chamaedrys.* On the other hand, some reports have previously indicated that callus was produced in the medium without phytohormones or with auxin alone, as well (Hohtola, 1988). The results of present work indicated that in culture of *Teucrium chamaedrys* segments without phytohormones callus generation takes place in less quantity.

The results of this study also showed that the growth of callus was properly following the sub-culturing. And this was probably related to compatibility phenomenon. This process often occurs in the callus culture, because the callus is becoming autotroph in terms of growth regulators. Although the callusing process initially needs the growth regulator, it needs no growth regulator after a few sub-culturing (Bagheri, 1998).

On the other hands, it was depicted from the results

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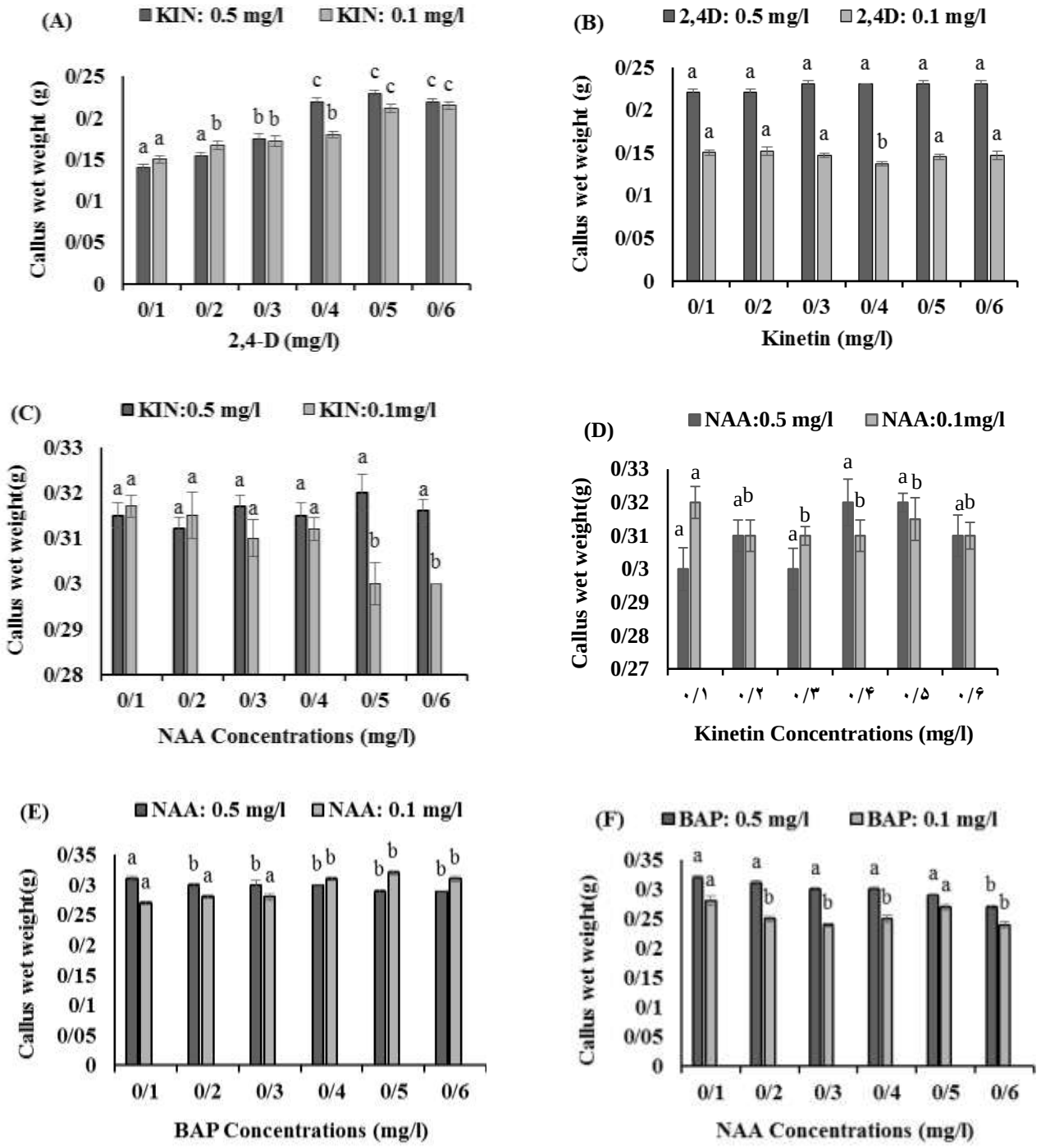

Figure 1. Effects of different concentrations of 2,4-D and Kinetin (A,B), NAA and Kinetin (C,D), BAP and NAA (E,F), on the production of callus tissue of *Teucrium chamaedrys.* The same letters in each column represent no significant differences according to Duncan test at 5 percent.

that NAA/BAP (0.1:0.5 mg/l) and NAA/KIN (0.5:0.5 mg/l) was regarded as suitable hormonal combination for shooting and rooting of the callus, respectively. Whereas, BAP was capable for shoot formation, root generation was promoted by KIN. NAA was more effective auxin base hormone for regeneration process of callus in *Teucrium chamaedrys* than other auxins. NAA efficiency in shoot formation in some Teurium species such as *T. polium* was previously descriped by other researchers (Qudah *et al*.,2011).

Our results indicated that there was a considerable difference in essential oil profile of the calli obtained at different hormonal combinations. It may be attributed to metabolic pathways shifting that was related to various hormonal treatments.

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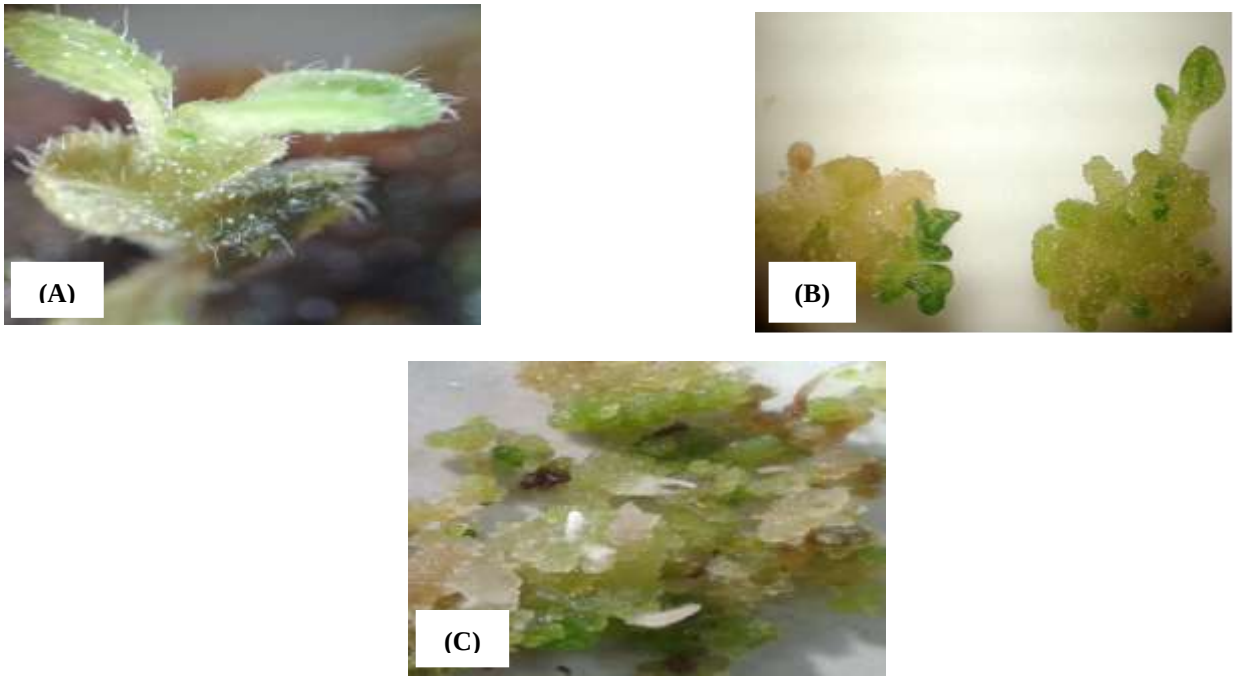

Figure 2. Regeneration process in the callus at the cetin hormonal combinations, shooting (A, B) and rooting (C)

Table 1: Shoot and root length in regenerated callus at different hormonal combinations

Mean / Hormone	Root length (mm)	Shoot length (mm)
NAA(0.5) , *KIN* (0.5)	7	0
NAA (0.1), BAP (0.5)	0	9
Other hormonal combintions	0	0

Table 2. Chemical composition of essential oils obtained from callus tissue obtained from different hormonal combinations

NO	Compounds	RT	RI	Abundance (%) in callus of different hormonal combinations		
				NAA/BAP (0.1:0.5mg/L)	NAA/Kin (0.5:0.4mg/l)	2,4-D/Kin (0.5:0.5 mg/L)
1	Hexanone	3.29	321	-	-	44.53
2	Hexanal	4.300	801	3.15	-	-
3	Decane	5.083	1000	3.83	-	-
4	Oxime	8.75	1148	-	-	9.31
5	Dodecane	7.72	1200	4.83	-	.-
6	Kalaren	8.60	1611	4.20	-	-
7	Tetradecanol	9.54	1672	8.16	-	-
8	Neophytadine	10.64	1839	70.90	-	-
9	Oxalic acid	11.00	1880	1.18	-	34.26
10	Docosane	14.32	2200		-	11.38
11	Octacosane	14.359	2988	1.90	-	-
12	Benzen acetic acid	16.358	nd	1.44	-	-
13	Nonadecene	32.19	2990	-	22.23	-
14	Dotriacontane	33.78	3200	-	25.78	-
15	1,2-benzenedicarboxylic acid	34.10	nd	-	15.76	-
16	Tetratriacontane	35.00	3413	-	36.22	-

RI= retention indices on DB-5 capillary column, RT= standard retention time

Conclusion

Callus induction in *Teucrium chamaedrys* occurs on medium containing both hormones, KIN and 2,4D as well as KIN and NAA. The best treatment to increase

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fresh weight of callus was the use the medium containing NAA (0.1 mg/l) and BAP (0.5 mg/l). It was revealed from the results that NAA/BAP (0.1:0.5 mg/l) and NAA/KIN (0.5:0.5 mg/l) were regarded as suitable hormonal combination for shooting and rooting of the callus, respectively. There are some compounds in the resulted callus which are absent in the essential oil of the plant aerial parts. However, the comparison of literature with the results of this study indicates that the essential oil compounds profile obtained from the callus of *Teucrium chamaedrys* is quite different from the essential oils distillated from the aerial parts of the plant. Therefore, biological and medical effects of the essential oils obtained from the callus might be quite different from the essential oils obtained from the plant aerial parts.

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