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THE EFFECT OF EXPLANT, BAP AND 2,4-D ON CALLUS INDUCTION OF *TRACHYSPERMUM AMMI*

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ABSTRACT

Ajowan (*Trachyspermum ammi*) has been considered as an important medicinal plant because it contains many alkaloids such as Thymol. In vitro culture of Ajowan provides new tissue sources such as callus, cell suspension and seedlings to produce secondary metabolites. The present study describes callus production optimization procedures experiment that was a factorial experiment based on completely randomized design at three levels with four explants (root, shoot, leaf and cotyledon) on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP (0.25, 0.5 and 1 $mg.L^{-1}$) and 2,4-D (2, 4 and 8 $mg.L^{-1}$). Comparison of means showed that the maximum callus production was obtained from shoot explants, the cotyledon and leaf explants were in the second orders. In overall, 0.25 $mg.L^{-1}$ BAP with 2 $mg.L^{-1}$ 2,4-D concentrations proved to be optimal for the production of maximum callus and also were more effect on callus weight, callus volume and callus color. The best explant based on callus weight was cotyledon explant and for callus volume was shoot explant. The result were shown that the effective hormone combination and explant was 2 $mg.L^{-1}$ 2,4-D with 0.25 $mg.L^{-1}$ BAP concentrations were more effect on callus induction and shoot explant, respectively.

Keywords: *Carum copticum* (L.); cotyledon; Plant Hormone; Thymol

INTRODUCTION

The consumption of herb and herbal medicines is increasing in different countries day by day and this is due to their proven effectiveness in scientific communities and admissibility in majority of human societies. Because of increasing concerns about the side effect of chemical medicines an ineffectiveness of number of them in long-term consumption, use of natural ingredients has been paid more and more attention alternatively or as a supplement treatment method. Use of herbs as medicament has continuing from the beginning of the human civilization **(Dattner, 2003)**. Herbal medicines are being used as an alternative treatment with less side effects and variety features and in some cases as the only effective treatment **(Huseini et al., 2006)**.

Like many plants which are important economically, Ajowan laboratory proliferation with using of tissue culture techniques can be considered as an alternative for traditional reproduction methods.

Ajowan, one of the herbal medications, is highly considered which due to secondary metabolic production and its high importance in medical consumption, cosmetic and hygienic industries. The utilization of Ajowan has been globally outspreaded **(Fazeli-nasab and Fooladvand, 2016)**. Therefore, applying correctional techniques is essential matter to breed Ajowan as well as improve of the quantitative and qualitative of important properties of the secondary metabolite. Given the economic and environmental benefits and also the intensive use of pasture and forest resource and limited crop cultivation and due to the requisite of use of the correctional optimization methods of preparatory courses of breeding a herb to be able take advantages or breeding methods and molecular agronomy to optimize achieving the secondary metabolite and related products of herbs **(Afolabi et al., 2018a; Afolabi et al., 2018b)**. According to not being presented any report of Ajowan tissue culture and even there is a little number of tissue culture of Apiaceae. in this research has been proceeded in finding cost-effective and economical methods to callus implantation in Ajowan in tissue culture condition to using of breeding methods such as somatic-clonal diversity, radiation and mutation, chimer and polyploidy production, production and extraction of essence and also molecular breeding be possible.

Karegar et al., (2011) obtained the highest weight of the callus of fennel plant from treatment 1 $mg.L^{-1}$ of 2,4-D and 0.2 $mg.L^{-1}$ BAP using root explant, **Anzidei et al., 2000; Anzidei et al., (1996)** obtained the best callus induction from hypocotyl explant and **Sarkheil et al., (2009)** obtained from the hypocotyl explant of apical meristem.

To form callus in different species of bean, the best explant fragment according to Malik and **(Malik and Saxena,**

1991) is leaf, **(Amiri and Fahimi, 2003)** hypocotyl, **(Ahmed et al., 2002)** cotyledon nodule, **(El-Shemy et al., 2002)** epicotyl and also **(Veltecheva et al., 2005)** is leaf.

In bee balm (*Monarda didyma*) the best percentage of callus induction obtained from the treatment of 1 mg.$L^{-1}$of 2,4-D hormone and 1 mg.L^{-1} of BAP for the explant of internode and petiole **(Soltani pol et al., 2011)** while in spinach the best obtained only from the 0.5-1 mg.L^{-1} of 2,4-D hormone for leaf explants **(Khayatzadeh et al., 2011)**.

In Silybum marianum the best percentage of callus induction obtained from 1 mg.$L^{-1}$of 2,4-D and 1.5 mg.L^{-1} KIN for root explant **(Arekhi et al., 2012)** and the best in Salsola arbuscula from the treatment of (0.5 and 1 mg.L^{-1}) of 2,4-D only for stem explant **(Amini et al., 2013)**. In fennel the best percentage of callus induction obtained from 2 mg.L^{-1} of 2,4-D hormone and 0.25 mg.L^{-1} of BAP for hypocotyl and apical meristem **(Sarkheil et al., 2009)**. In eucalyptus, the best percentage of callus induction obtained from 1 mg.L^{-1} of TDZ for stem explant **(Ghadiri Sardrood et al., 2012)**.

MATERIAL AND METHODS

Plant cultivation

To conduct the experiment, in year 2016, the Ajowan seeds including the Sistan local mass (origin of Ajowan **(Fazeli-nasab and Fooladvand, 2016))** was supplied from the Gene Bank of the Agricultural Biotechnology Research Institute of Zabol.

To the study germination used MS medium as well as instillation of the callus. In all condition, the density of sucrose was 30 g.$L^{-1}$and the density of agar was 7.5 g.L^{-1}.

The PH of the medium for germination and callus instillation was adjusted by in order 5.64 and 5.8

To sterilize seeds, submergence was done in sodium-hypo chloride 1% in laminar-flow-hood, after 10 minutes hypo chloride was evaporated and washing the seeds was done with sterilized distilled water in four step including a one minute pre-washing and three 5, 10 and 15 minutes sterilization washing with distilled water. After this stage, absorb the extra water and preparing for cultivation the seeds were located on filter paper. After cultivation, the medium was covered with parafilm and was kept in growth chamber under light condition, 16 hours light, 8 hours darkness and temperature of 15 °C. Finding appropriate germination environment in order that seeds be prepared for the wide cultivation In Vitro.

To carry out callus induction experiment, factorial in a completely randomized design was used with explant factors including root, stem, leaf and cotyledon, s, 4-D hormone in three levels of 2, 4 and 8 mg.L^{-1} and BAP hormone in three levels of 0.25, 0.5 and 1 mg.L^{-1} in 3 replication. The prepared explant from the 48 days grown plants growing In Vitro was inoculated on MS culture under different treatment. Containers containing the explants, were kept in growth chamber under temperature 25 ºC and a light period of 16 hours light and 8 hours darkness. Sub culturing every two weeks. After two months from growth explant some traits such as percentage of callus induction (generate or non-generate of callus), callus volume, callus weight, callus quality (fragile of watery) and callus color were recorded.

Data analysis

Data was analyzed with R and student statistic software and used Excel to draw the charts and Duncan's multiple range test (LSD) to compare the average means with probability of 1 and 5%

RESULTS

Germination

Based on qualitative observation, germination of Ajowan seeds on MS medium was more appropriate either the number of germination seeds or the required time for germination and development of the plant (Figure 1 (section A and B). However, 1/2MS medium has been found more appropriate rather than MS because it seems high levels of salt in medium can limit germination of different plants **(Reis et al., 2015)** but reported that Ajowan is resistant to salinity because it tolerates the saline environments up to 140 mg.L^{-1} and even in presence of Kinetin could increase the level of tolerance by 210 mg.L^{-1} of saltiness (highest level of the used treatment in the **experiment (Fazeli-Nasab et al., 2016)** consequently can say that Ajowan can growth well in MS medium because of higher level of Vitamin and macro and micro nutrition minerals as the result of the experiment approve that

Callus induction

To study the callus induction process, the explants segments including root, stem, leaf and cotyledon located on the base MS medium containing sucrose 3%, Vitamin B and 16 different density of a combination of 2,4-D and BAP hormones. The first reaction to forming callus was observed after 24 days and this completed after 60 days (Figure 1(section C and D)) and feature such as percentage of callus induction, callus color, callus volume and callus quality of the callus was recorded. After data analysis, results indicated that both hormone 2,4-D and BAP in most of the densities caused the callus induction in majority of explant segments except the root (Table 1 and Figure 2). So that the highest percentage of callus induction (100 %) was observed in stem explant and the least (zero percent) in root. Also regarding to callus induction a significant different of probability of 1 percent was between different level of BAP and different level of 2,4-D

BAP, singly, in density of 0.5 mg.L^{-1} had the highest effect (55%) on stem callus induction and the least role on root explant in density of 1 mg.L^{-1} (Figure 2). As well as, 2,4-D in a density of 2 mg.L^{-1} the most production of callus in stem explant segment (66%) and the least role in the density of 8 mg.L^{-1} in root explant. Meanwhile with increasing the density of 2,4-D its effect on the amount of callus was getting down as the highest percentage of callus in density of 2 mg.L^{-1} and the least in density of 8 mg.L^{-1} (Figure 3).

Effect of BAP, singly, on callus induction was less than 2,4-D but BAP could stimulate the callus induction in a lower density. In interaction of BAP (0.25 mg.L^{-1}) with 2,4-D (2 mg.L^{-1}) the most callus was obtained from stem explant and the least in density of BAP (1 mg.L^{-1}) with 2,4-D (8 mg.L^{-1}) in root explant (Figure 4).

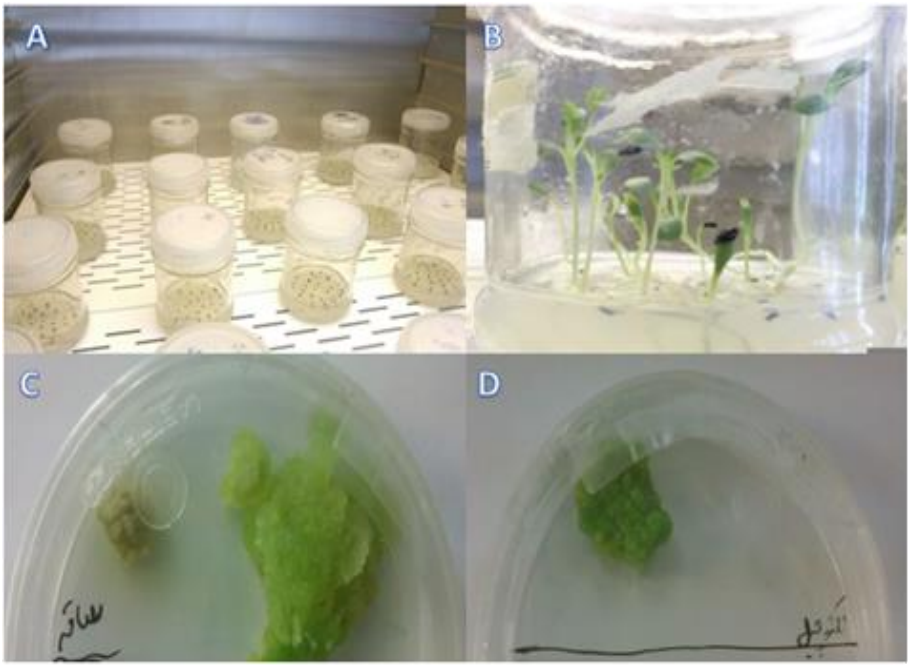

Figure 1 germination in ½ MS medium (A & B) and callus induction from Stem (C) and Cotyledon (D) explant.

Table 3 analysis variance of callus induction based on explants and hormones.

SOV	df	SS	MS	F
Explant	3	14074	4691.34	3.688**
BAP	2	2222.24	1111.12	8.7349**
2,4-D	2	2222.21	1111.11	8.7348**
BAP * Explant	6	10370.4	1728.4	1.3587**
2,4-D * Explant	6	1481.46	246.91	1.941**
BAP * 2,4-D	4	555.561	138.89	1.0918**
BAP * 2,4-D * Explant	12	9073.96	756.164	5.9445**
Error	72	9.159	1.272	
Total	107	39999.9		

Note: ** significant in p <0.01.

In means because of interaction, BAP reduced the effect of 2,4-D on callus induction. Therefor recommended first, to obtain the best and most callus in herb Ajowan, 2,4-D (2 mg.L^{-1}) should be used with BAP (0.25 mg.L^{-1}) on stem explant. Secondary, other admixture of 2,4-D with other different hormone such as NAA, IAA should be used to obtain a large and absolute hormone regarding to the most effective hormone or a combination of.

According to reported **(Schultz et al., 1990)** that callus induction begins from the cutting edges and spread out to other parts of the explant, so over the current study the explant were cut and the result indicated that the callus induction was appropriate so can be resulted the cutting in explant causes increasing in callus. So is recommended cut the surface of the explant before inoculation.

Weight, color and quality of the callus

There was a significant different of probability of 1% between explant, BAP and 2,4-D hormone, singly and interaction between hormone in the weight of the obtained callus of explant (Table 2). Meanwhile, LSD analysis indicated that there was a significant different between explant including root, stem, leaf and cotyledon based on callus weight and cotyledon was the best explant and then stem (Figure 5) The most effective level of BAP and 2,4-D on callus weight was in order 0.25 and 2 mg.L^{-1} (Figure 6).

Ki square test showed that explant regarding to color and quality had a significant different in probability of 1 percent 2,4-D also was effective on color and quality of callus at the probability of 5 percent. But BAP wasn't effective on color and quality of the callus as the obtained callus from green stem explant was lighter rather than other explant then cotyledon and after that leaf. On the other hand, treatment 2 mg.L^{-1} was more effective on color. As long as density of 2,4-D increased the color callus was getting darker obtained callus from stem, was more fragile and 2,4-D at level of 2 mg.L^{-1} was more effective on callus.

Callus volume

It was a significant different at probability of 1% between explant, BAP and 2,4-D hormone, individually and hormone interaction in volume of obtained callus of explant (Table 3). LSD results showed that there was a significant different between explant (root, stem. Leaf and cotyledon) regarding volume of callus and stem was the best explant then cotyledon (Figure 7). The most effective level of BAP and 2,4-D on volume was in order 0.25 and 2 mg.L^{-1} (Figure 8).

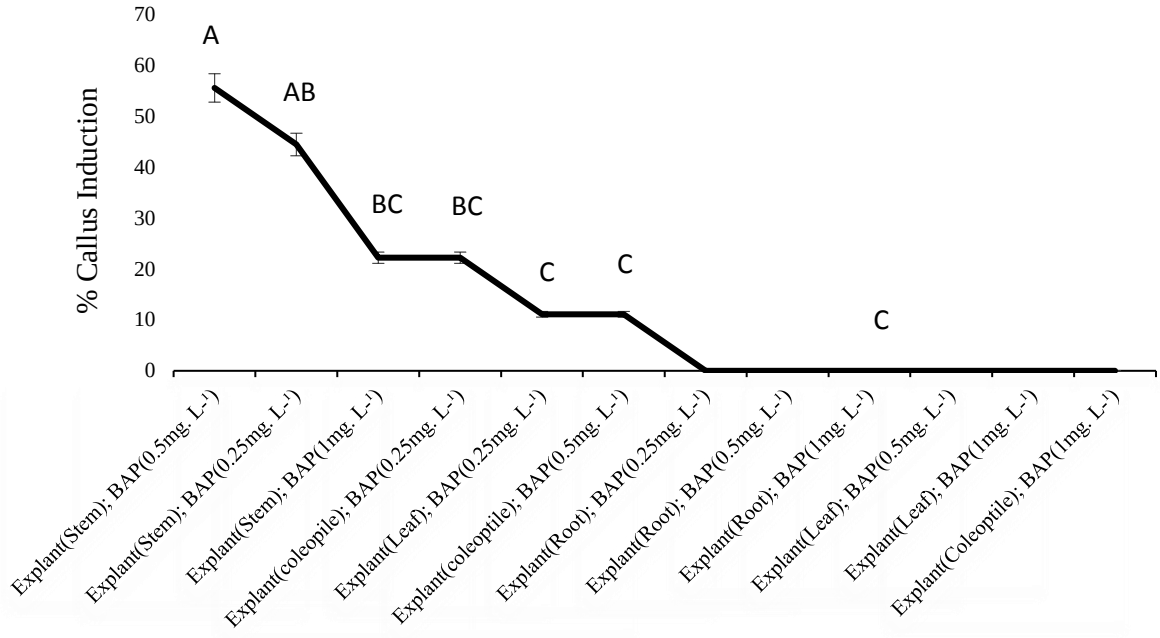

Figure 2 studying of different levels of BAP hormone and explant on percent of callus induction.

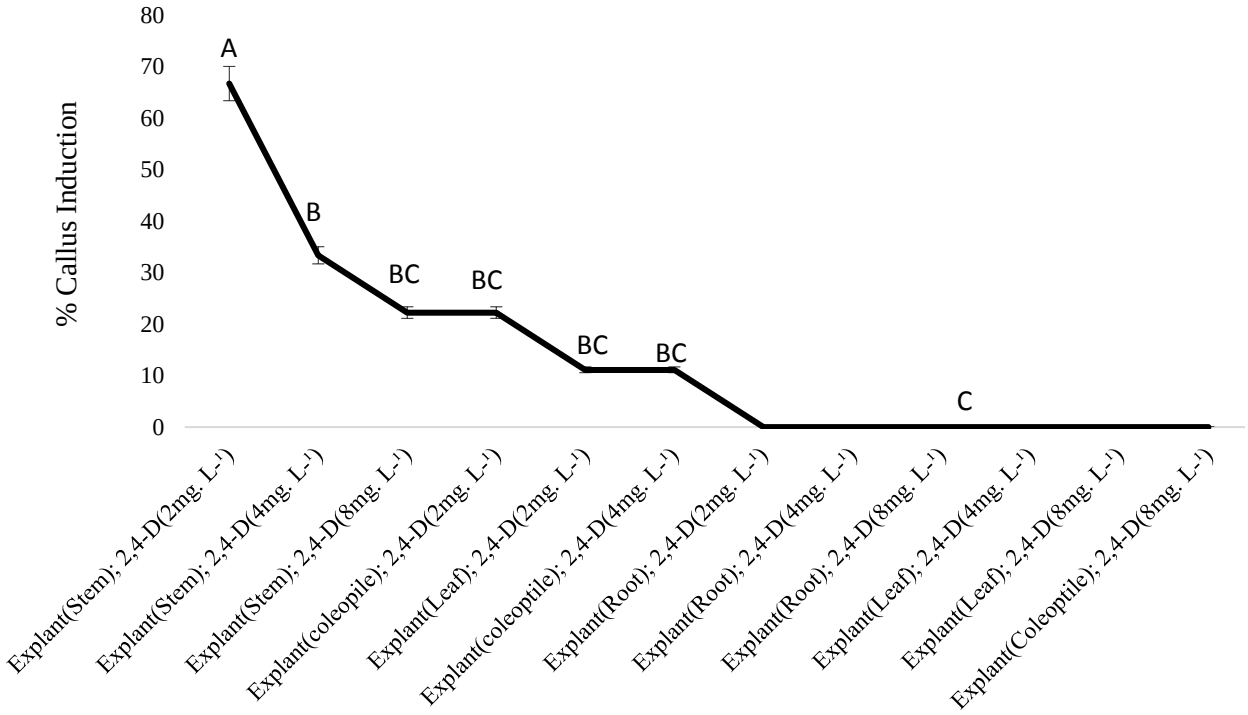

Figure 3 studying of different levels of 2,4-D hormone and explant on percent of callus induction.

DISCUSSION

Study of explants segments showed different results as root explant couldn't generate callus after a while necrotic and disappeared but the highest callus was in stem and then in order cotyledon and leaf. According to (**Malik and Saxena, 1991)** study the best explant was leaf, as well as hypocotyl by **(Amiri and Fahimi, 2003)**, Cotyledon node by **(Ahmed et al., 2002)**, hypocotyl by **(El-Shemy et al., 2002)** but has not been report regarding Ajowan so far the current report is the first report ever with regarding to Ajowan tissue culture. The root explant was the most effective explant.

In some results **(Karami et al., 2013)** that no callus in bean leaf explant was formed as well as the highest effect of BAP (in 4 $mg.L^{-1}$) for callus induction was 11 % but in combined form with NAA could increase the callus induction by 42 % while in this study effect of BAP on Ajowan callus was 55 % in a low density of 0.5 $mg.L^{-1}$.
The common point was that combination of BAP and 2,4-D in Ajowan and BAP with NAA in bean productized a callus of 42 %.

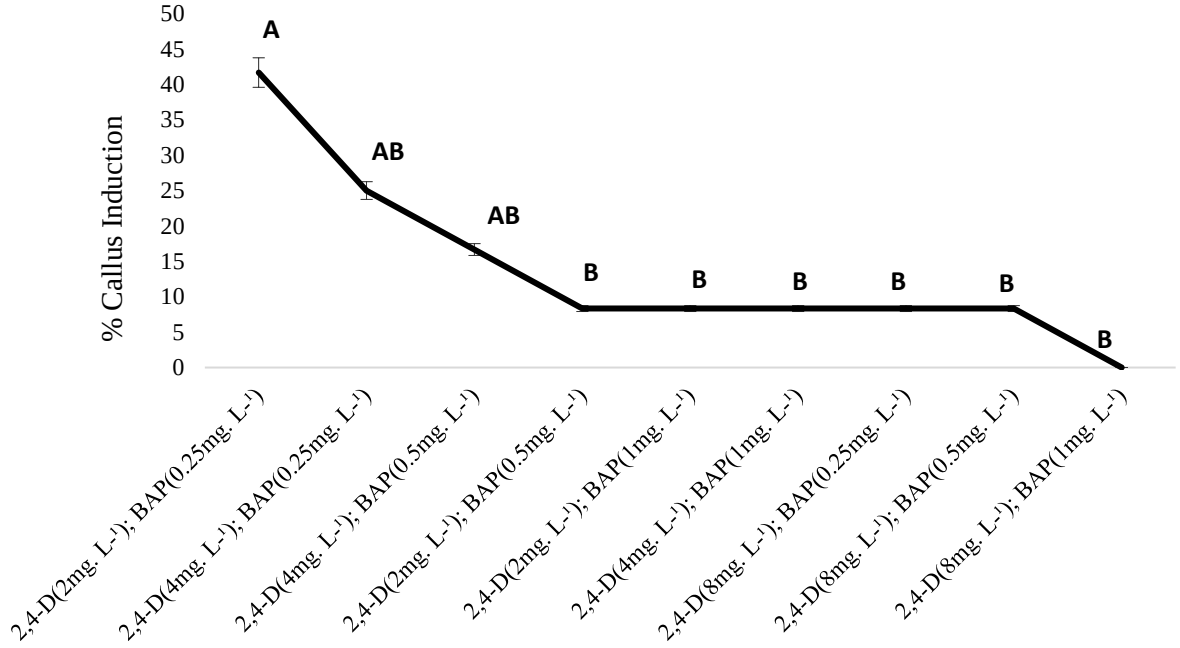

Figure 4 studying of the interaction effects of BAP and 2,4-D hormones on percent of callus induction.

Table 2 analysis variance of callus weight based on explants and hormones.

SOV	df	SS	MS	F
Explant	3	3.36	1.121	21.96**
BAP	2	0.888	0.444	8.69**
2,4-D	2	1.862	0.931	18.23**
BAP * Explant	6	1.994	0.332	6.51**
2,4-D * Explant	6	2.744	0.457	8.96**
BAP * 2,4-D	4	1.118	0.2797	5.48**
BAP * 2,4-D * Explant	12	3.713	0.3094	6.06**
Error	54	2.758	0.051	
Total	89	18.439		

Note: ** significant in $p < 0.01$

As reported that salts in medium has the negative effect on growth and callus induction to some extent **(Reis et al., 2015)** therefore the reason for callus induction in Ajowan can be due to salinity resistance **(Fazeli-Nasab et al., 2016)** and could product more appropriate callus in present of hormones.

In another results **(Ahmed et al., 2002)** obtained the best results in the MS medium contained BAP with the density of 1 $mg.L^{-1}$ and NAA with the density of 0.1 $mg.L^{-1}$. the highest product of callus either in **(Ahmed et al., 2002)** or **(Karami et al., 2013)** research was the result of interaction of Auxin and Cytokinin and also in current study the best result obtained from the combination of Auxinic and Cytokinin hormone but the result of this study was much higher that the mentioned researches, apparently seems that because the used explant in both experiments was partly different as well as the optimized densities that was applied. Naturally, can't be ignored the possibility of the main reasons of observed different in optimized densities of hormones caused by the different used species and laboratory conditions. In dissimilar results **(Amiri and Fahimi, 2003)** obtained the best callus in hypocotyl, NAZ variety in the density of 5 $mg.L^{-1}$ of 2,4-D and 2.5 $mg.L^{-1}$ of Kin. Noteworthy that NAA and Kin are weaker hormone and 2,4-D and BAP more appropriate in most studies.

Although 2,4-D normally considered as a strongest Auxin and the high level of Auxin causes the enlargement of cell length and increase the cell division **(Sobhanizadeh et al., 2017)** but in this study observed that increasing in 2,4-D didn't increase the callus induction so this disagreed with the result of **(Kurniati, 2013)** but agree with result of **(Soltani pol et al., 2011)**.

Regarding to the importance of hormones in callus induction **(Amini et al., 2013)** showed to product callus the presence is a necessity and in a medium without hormone and Auxin no callus obtained similar results related to this has been recorded by other scientist in a few species of plants **(Elaleem et al., 2009)**. Some studies indicated that in a medium with present of Auxin and not Cytokinin, callus can be produced but not without Auxin **(Hohtola, 1988)** in walnut cotyledon, also, reported that without Auxin callus couldn't be produced and also in this study showed that callus could inducted by collaboration of Auxin and Cytokinin that this achievement revealed similar to the former studies. Meanwhile, in a dissimilar study. **(Khayatzadeh et al., 2011)** reported that highest callus induction obtained from leaf explant, Orai variety with a

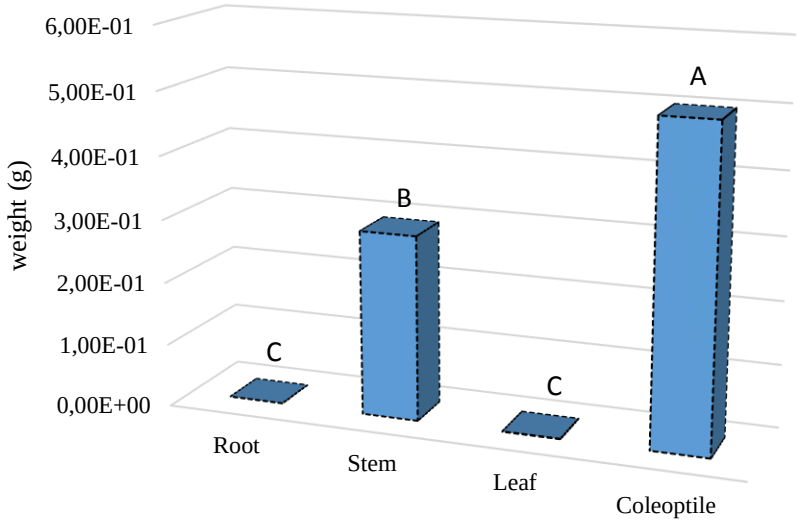

Figure 5 mean compare of effect of explant on callus weight in all explant of Ajowan.

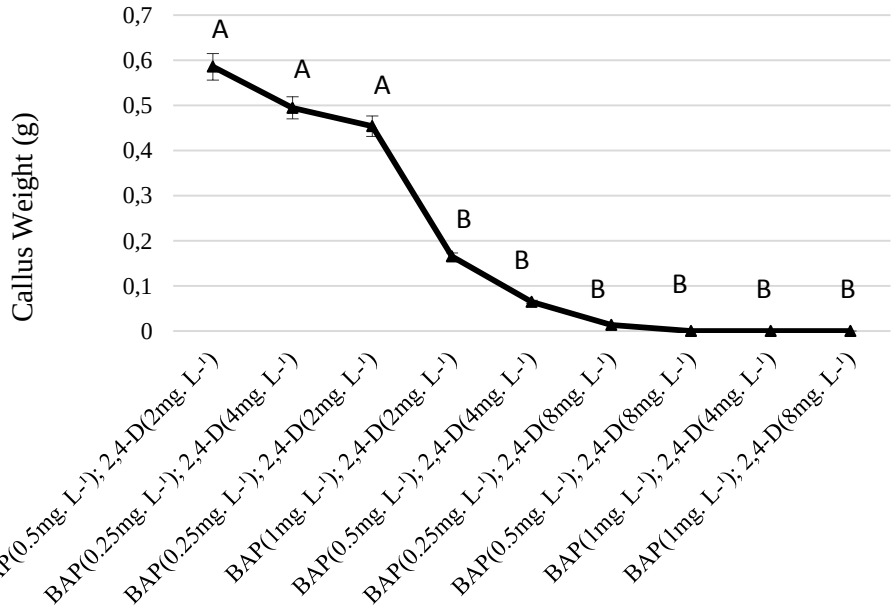

Figure 6 mean compare of interaction effect of BAP and 2,4-D hormones on callus weight in all explant of Ajowan.

density of 0.1 – 0.5 mg.L^{-1} of 2,4-D and Viroflay variety with a density of 0.5 – 1 mg.L^{-1} of 2,4-D.

Regarding to hormone combination, to induce an effective callus in Lemon balm, the best treatment obtained from applying 1 mg.L^{-1} of 2,4-D with 1 mg.L^{-1} of BAP for internodes and petioles explants under light condition as well as the treatment of 0.5 mg.L^{-1} it of 2,4-D with 1 mg.L^{-1} of BAP in the dark and then light for leaf explant **(Soltani pol et al., 2011)**. **Khayatzadeh et al., (2011)** reported that the highest callus induction obtained from leaf explant of Orai variety with a density of 0.1 – 0.5 mg.L^{-1} of 2,4-D and Viroflay variety with a density of 0.5 – 1 mg.L^{-1} of 2,4-D. **(Arekhi et al., 2012)** reported that the highest percentage of callus induction (98%) observed in root explant in a medium containing 1 and 1.5 mg.L^{-1} of 2,4-D and Kin. They also reported that the highest percentage of callus induction (97%) observed in root explant in a medium containing 1.5 and 1.5 mg.L^{-1} of NAA and Kin hormone. **Amini et al., (2013)** reported that the best explant was root and the most appropriate medium was the 1mg.L^{-1} of 2,4-D and 1 mg.L^{-1}of Kin as well as direct regeneration in stem in medium with density of 0.5 and 1 mg.L^{-1} of BAP. In separate researches, **Ebrahimie et al., (2003)** and **Mafavi Fard et al., (2010)** studied the effect of different explants, combination of different hormone and medium for callus generation in cumin. They were able to callus induction and regeneration in cumin with a combination of BAP, NAA and IAA in order 0.1, 0.2 and 0.4 mg.L^{-1} of each.

Sarkheil et al., (2009) studied the effect of 2,4-D and BAP on callus induction and regeneration on leaf, Hypocotyl, apical meristem, root and crown in fennel reporting 2 mg.L^{-1} of 2,4-D and 0.25 mg.L^{-1} BAP as the best combination as well as hypocotyl and apical meristem as the best explant **(Fatahi moghadam et al., 2011)**.

Table 3 analysis variance of callus volume based on explants and hormones

SOV	df	SS	MS	F
Explant	3	75.977	25.325	785.15**
BAP	2	10.322	5.161	160.01**
2, 4-D	2	21.096	10.548	327.02**
BAP * Explant	6	18.1482	3.024	93.77**
2, 4-D * Explant	6	15.278	2.546	78.94**
BAP * 2, 4-D	4	5.05	1.262	39.14**
BAP * 2, 4-D * Explant	12	42.365	3.53	109.45**
Error	54	1.741	0.032	
Total	89	189.9772		

Note: ** significant in p <0.01.

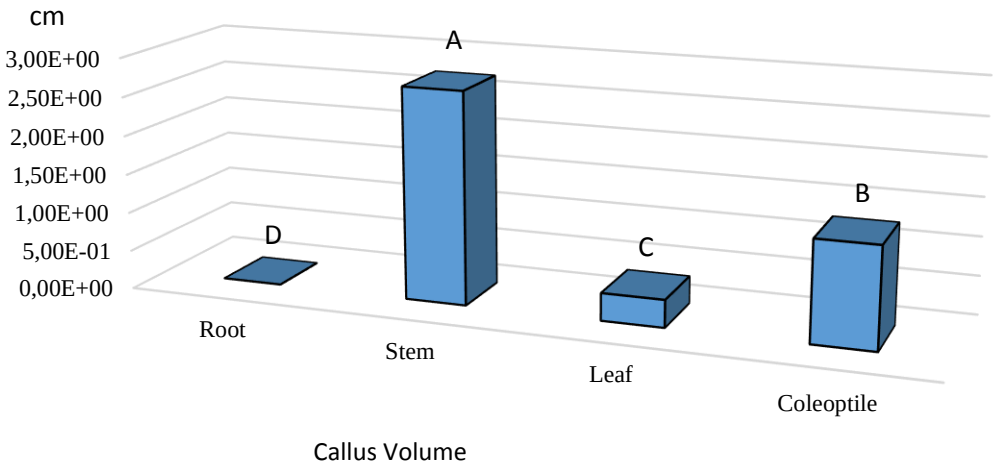

Figure 7 mean compare of callus volume based on Stem, Root, Leaf and cotyledon explants of Ajowan.

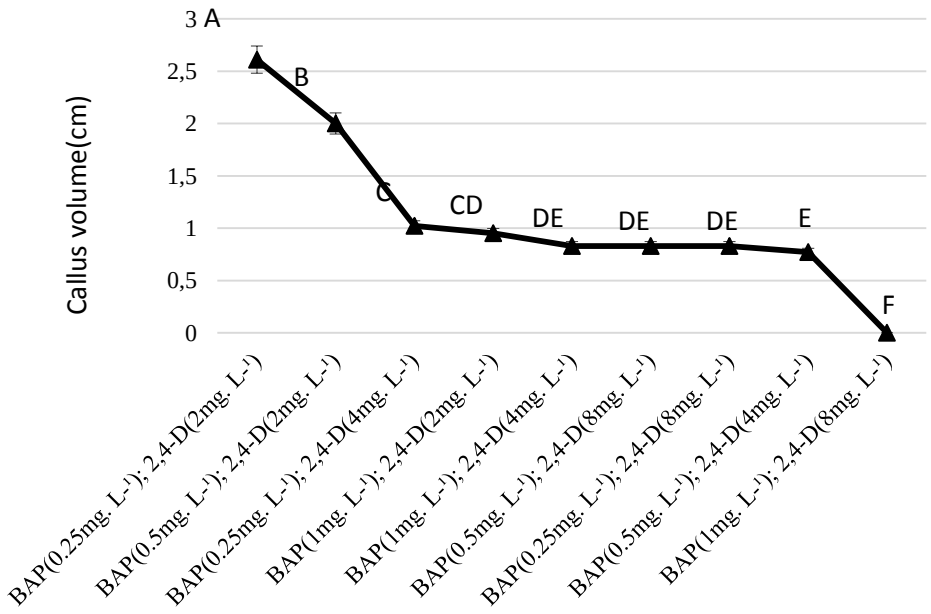

Figure 8 mean compare of interaction effect of BAP and 2,4-D hormones on callus volume.

Considered the effect of TDZ and 2,4-D on callus induction and regeneration in eucalyptus concluding 1 $mg.L^{-1}$ of 2,4-D and 0.5 $mg.L^{-1}$ TDZ the best combination of hormone and stem the best explant. In the present study, both 2,4-D and BAP in most often of density caused the callus induction process in majority of the explant except the root. Therefor the highest (100 %) callus induction was observed in stem explant as well as BAP in the density of 0.25 $mg.L^{-1}$ and 2,4-D in density of 2 $mg.L^{-1}$ had the highest callus generation.

CONCLUSION

Nowadays, indiscriminate and unprincipled harvesting on the one hand and the other hand the difficulties and complexities of germination of the herb seeds, put this plants in the danger of inexistence. On the other side, variability of drug compounds from clone to clone, enforced scientists to propagate after finding appropriate and prevalent clone and this micro-reproduction can be achieved only by applying tissue culture methods. Ajowan is one of the most important and local plant of Iran **(Fazeli-nasab and Fooladvand, 2016)** that in recent years a particular attention has been paid to so in the present study, focused on micro propagation. Obtained results indicated that the most effective treatment in Ajowan callus induction was 2 $mg.L^{-1}$ of 2,4-D with 0.25 $mg.L^{-1}$ of BAP and the best explant was stem.

Recommendations based on this study:

Advised study the biochemical pathway responsible for the production of valuable secondary metabolites of the herb such as Thymol and finally, application of engineering techniques to optimize cell suspension culture in terms of production of most of valuable secondary metabolites and also studying of secondary metabolites of the herb through the different stages of the tissue culture and preparing of the suspension medium.

It should be noted that given that Ajowan is one of the best herbs of Iran and rich of thymol and other secondary metabolites compounds.

The author of this article is on a comprehensive plan of action describing the optimization of production of secondary metabolites in Ajowan including studying the amount of generation of secondary metabolites in different vegetative stages and surface of the tissue culture, evaluation of level of secondary metabolites in Ajowan based on duplicated chromosomes then diploid state, evaluation of different genotypes of Ajowan in terms of secondary metabolites generation, the expression of genes involved in production of secondary metabolites and so on.

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