INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 5, No. 4, p. 7-17, 2014

RESEARCH PAPER OPEN ACCESS

A survey of the best growing conditions of in vitro culture in fennel different genotypes for mass production

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Key words: Fennel, genotype, growth regulators, in vitro culture, medicinal plants.

http://dx.doi.org/10.12692/ijb/5.4.7-17 Article published on August 18, 2014

Abstract

To determine the best growing conditions for *in vitro* culture of different genotypes of fennel to mass production, we compared different explants in various culture mediums for eight fennel genotype groups. Based on completely randomized plan, factorial experiment was done with four replications. Leaf, petiole, hypocotyl and root explants were isolated for 20 days, and seedlings were cultured on callus induction medium, 1/2 MS basal medium with 2,4-dichlorophenoxyacetic acid (2, 4-D) (2, 4 and 6 mg l^{-1}) and 6-benzyaminopurine (BAP) (0.5, 0.25 and 0.1 mg l^{-1}) combination. Color, Size, Percentage of callus induction, dry and fresh weight and Volume of callus were evaluated 3 month later. Through most of the genotypes, Hypocotyls and petiole explants in callus induction were better than the others. Callus induction mediums with 6 mgl^{-1} 2-4-D and BAP combinations had more appropriate responses. Leaf explants showed the highest callus size in the German genotype in callus induction medium with 4mgl^{-1} 2-4-D and 0.1mgl^{-1} BAP. Embryogenic calli were placed on regeneration medium with 1/2MS of a non-hormonal treatment and hormonal treatment with a level of 2-4-D (10 mgl^{-1}) and two levels of BAP (2 and 5 mgl^{-1}). The medium without hormones was recognized as the best medium for regeneration and hypocotyl and petiole had the highest rate of regeneration. Respectively, Tabriz and Gonabad genotypes were shown highest and lowest rate of regeneration.

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Introduction

The fennel (*Foeniculum vulgare)* is from the Apiaceae family, this plant is one of the oldest, important and popular medicinal plant. Fennel, a hardy, perennial herbwith branched, circular and without crack stems that subtle indentations are visible on them (Davis P H, 1972.REchinger K H. *et al* .1985) grows wild in most parts of temperate Europe, but is generally considered indigenous to the shores of the Mediterranean and it is cultivated and adapted in the most areas of Iran now (*Bruneton,* 1987. Stahl *et al*., 1975; Piccaglia *et al.,* 2001; Zahid *et al*., 2009; *He& Huang*, 2011). Just one species, *Foeniculum Vulgare*, of both cultivated and wild plant has been found in Iran (*Mozaffarian* 1375). The fennel has two specific and important varieties which belong to capillaceum subspecies. The one of them is Vulgare variety (Bitter fennel oil) and the other variety is Dulce (Sweet fennel oil). Bitter Fennel is considered indigenous to Mediterranean regions and grows wildly in France, Spain, Portugal and Northern Africa. This species has been collected of the Northern parts (Gorgan, Haraz Valley), Azerbaijan (Tabriz), Kurdistan, Kerman (Deh Bakri) and Khorasan in Iran (*Rechinger*, *et al*.1986). Different aspects of medicinal Fennel such as Plant tissue culture and micropropagation. Callus and suspension cultures, the ability of regeneration particularly through somatic embryogenesis, callus induction and regeneration and its relation to explants and growth regulators, have been studied (*Paupardin et al.,* 1980; *Hunault*, 1981; *Miura et al*., 1987; *Hunault et al*., 1989, *Maheshwari and Gupta*, 1965; *Benici et al*., 2004).

Production and regeneration Differences in callus are depend on both genotype and explants source (*Ganeshan et al*., 2003; *G*. Hunault, 1984; *Sarkheyl et al*., 2009; *Jiang et al*., 1998; *Anzidei et al*., 2000; Maatar, 1997; *Anzidei et al*., 1995). In the present study, different genotypes, explants and different hormone combinations were tested on medium response, callus induction and fennel regeneration. The aim of this study is to evaluate the effect of genotypes on callus induction, somatic embryogenesis and response to *in vitro* culture and introduction the best genotypes, explants and hormonal treatments for callus induction and embryogenesis of fennel. The effect of physical properties like color, size and callus fragility of callus embryogenesis potential is studied too.

Material and method

A. Plant Material

Previous studies had been evaluated the genetic diversity of Fennel germplasm by using molecular markers of Fennel seed samples from 30 different locations of Asian and European countries and 20 AFLP selective primer combinations. In classification of samples by using the 2/02e version of NTSYS software, The 30 samples of seeds were divided into 8 groups (Hassani *et al*., 2009). In this study, six genotypes of eight different groups of fennel that had been analyzed and clustered based on genetic diversity, were selected. Genotypes included, Hamadan of first group, Isfahan (Natanz) of second group, Gonabad of fourth group, Germany of fifth group, Turkey of seventh group and Tabriz of eighth,that these genotypes were analyzed based on growth medium responding by hormonal treatment in different explants.

Seeds were placed in 70% ethanol and shaken for 30 s to sterilization, then were washed two times with sterile double-distilled water. Then seeds were placed and shaken in 5% (w/v) NaOCl solution by adding 2-3 drops of tween for 8 min and subsequently were washed at least three times with sterile water. The seeds were placed on MS/10 (Murashige and Skoog 1962) basal medium consist of 30 gl^{-1} sucrose and 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. seed were maintained in 16 h light and 8 h dark photoperiod at 25 ℃± 20 ℃ temperatures for 20 days.

B. Callus induction stage

About 20 to 30 days after germination, sterile seedlings parts including leaves, petioles, hypocotyls and roots were used for callus formation (figure1). At

this stage a factorial experiment is conducted in a completely randomized design with four replications. 5 explants were placed in each Petri dish and the Petri dish was regarded as a replicate. Accordingly 1 / 2 MS culture medium was chosen for fennel by applying 9 hormonal treatments including 2, 4, 6 mgl^{-1} of 2,4-dichlorophenoxyacetic acid (2, 4-D), and 1, 0/25, 0/1 mgl^{-1} of BAP (Table 1). Also different explants of different genotypes are analyzed. After pH adjusting, about 5.8-6, definite amount of agar powder (7 mgl^{-1}) were added to the flasks. Then the callus inductions were placed in incubator under continuous darkness. Then calli were subcultured after one month.

Some of the characters that were analyzed in callus induction phase are, area, volume, fresh and dry weight of callus and callus percentage. Statistical analysis of the data, after data normality test and without data converting, is used by Microsoft Excel 2010 software and Spss Statistics 19. Mean comparison is performed by using Duncan's test too.

C. Regeneration stage

In this phase, we used 1 / 2 MS solid basal medium (7 gl^{-1} agar). For regenerating phase, two hormonal, 2, 4-D (0.1 mgl^{-1}) and BAP (2 and 5 mgl^{-1}), and a non-hormonal level were considered (Table 2). We incubated the cultures at 25± 2 ° C, in white fluorescent light, with day/night regime of 16/8. At this point, only callus regeneration and Embryogenesis feature were examined and we studied callus in terms of color, fragility and impact of these two traits on Embryogenesis too.

Results and discussion

About 1 to 2 weeks after explants exposure on callus induction medium, they significantly swallowed (especially in areas of cut). Complete callus masses emerged in second and third weeks. Callus Masses on G, H and I induction medium, were seen after one to two weeks delay. According to medium nutrient intake, during the callus induction stage we subcultured explants 3 times a month. Observations were recorded in second and third subculture were analyzed to assess characters. In callus induction phase, 1 / 2 MS medium with 2, 4-D and BAP hormones, obtained acceptable results in callus induction that had matched to *Sarkheyl & et al.* (2009) results. *Hunault & maatar*(1995)and Miura & *et al.*(1987) could receive appropriate response of fennel petioles as explants. Anzidei &*et al.* (2000) worked in Fennel hypocotyl in laboratory and also in the present study had obtained favorable results of fennel petiole and hypocotyls in tissue culture environment. In addition Sarkheyl & *et al* (2009) examined leaf and root explants, these explants have studied in this research too, but leaf explants has a different response to the callus induction phase, the results will show these differences. Similar researches were conducted by *Azza* (2002) on the *Cuminum cyminum Wakhlu* (1990) and *ValiZadeh* & *et al.* (2007) on *Buniumpersicum* B. And *Anzidei* & *et al.* (1996) on fennel had used 2,4-D and Kin hormonal combination . In the present study 2,4-D and Cytokinin were applied. Also *Panizza*(1997) and *Dronno*(1999) reported a successful combination of BAP & NAA for callus induction on *Lavandulavera*. *Anzidei & et al.* (2000) took a good response of 1/2MS basal medium in callus induction phase and also *Ade R, Rai M* in 2011 obtained acceptable results of callus induction by using MS basal growth medium, 2,4,D(Auxin) and BAP(Cytokinin) in Gloriosasuperba. Based on the variance analysis tables (table 4-7), all the traits in every 4 explants, leaf, petioles, hypocotyls and roots, the effect of genotype and hormonal levels and their interaction to each other were significant at 1%,and the results have been matched to *Anzidei* in 1996 and *Sarkheyl et al* in 2009. According to Duncan's test for callus size trait on leaf explants, the German's Genotype in hormonal treatments D (4 mg/ l 2,4-D and 0.1 mg/ l BAP) had the highest callus size (Figure 4). Leaf explants in the German's Genotype influenced by hormonal treatments more than other genotypes. Isfahan's petioles had the highest callus size in hormonal treatment F that it is in the same statistical group with hormonal treatment E (Figure 5). Also in hypocotyl explants, turkey's hypocotyl had the highest callus size in hormonal treatment F and Gonabad's genotype in hormonal treatment I had the lowest

(Figure 6). genotype and hormone interaction showed that Tabriz's root is the best in treatment A and Gonabad's root is the worst in treatment I (Figure 7). According to the results on callus size with regarding to the highest measured for each explants, Germany's leaf genotype in hormonal treatments D with 150.85mm² size; Turkey's hypocotyl genotype in hormonal treatments With 128.55mm² size; Isfahan's petiole genotype of the hormonal treatments F with 109.55 mm² size and Tabriz's root genotype in hormonal treatments A with 94 mm² size, respectively had the highest callus size. As Anzidei &*et al.* (2000) research on fennel and the obtained results of this study showed that the effect of genotype on tissue culture responding is very important. The results of this research are corresponding to (Hanault and Du Manoir 1992) and (Theiler-Medtrich & Kagi 1991) founds that the genotypic differences are the reason of not responding fennel to the culture medium in some populations. All genotypes and explants had appropriate response to callus induction, and only they were different in the effect of explants, genotype and hormone levels in callus induction samples that the amount of G, H and I hormone compounds (6 mgl^{-1} 2,4-D) were the lowest. In the 6 hormonal treatments, A to F, most genotypes and explants showed a good response (table 3).

Table 1. The amount of hormonal treatments used in the callus induction phase (mgl^{-1}).

Row	Medium name	BAP	2,4 –D
1	A	0.25	2
2	B	0.5	2
3	C	1	2
4	D	0.25	4
5	E	0.5	4
6	F	1	4
7	G	0.25	6
8	H	0.5	6
9	I	1	6

Table 2. Hormonal treatments were used in the regeneration phase (mgl^{-1}).

Medium name	BAP	2,4 –D
A	0	0
B	2	0.1
C	5	0.1

Table 3. Explants with 100% callus induction in the interaction of genotype and hormone treatment.

Genotype / Hormone	Isfahan	Germany	Tabriz	Turkey	Gonabad	Hamadan
A	Leaf, petiole, hypocotyl	Leaf, hypoctyl	Hypocotyl,root	------	Leaf, petiole, hypocotyls	-----
B	Petiole,hypocotyl	-----	Hypocotyls	Petiole,hypocotyl	Petiole,hypocotyls	-----
C	petiole	Petiole ,root	Hypocotyl, Root	Hypocotyls	Petiole,hypocotyl, root	Hypocotyls
D	hypocotyl	-----	Hypocotyls	Root	------	Petiol,hypocotyls
E	Petiole	------	-----	------	------	Petiole, root
F	Petiol,hypocotyl		Petiole	Petiol,hypocotyl	Root	Hypocotyle,root
G	------	------	------	------	------	------
H	------	------	------	------	------	------
I	------	------	------	------	------	------

Leaf callus of German Genotype in hormonal treatment D had the highest fresh weight in comparison to other genotypes. Isfahan genotype on hormonal treatment D, Tabriz genotype on hormonal treatment C and Turkey genotype in hormonal treatment F had the highest callus fresh weight. Gonabad's genotype in hormonal treatments A had the highest callus fresh weight That was not observed statistically significant differences between the first and second levels of 2,4-D. Callus of Hamadan genotype had an appropriate weight at all three levels of 2,4-D and BAP but by 2,4-D level increasing they were diminished a bit of weight. Petiole explants callus of Isfahan genotype on hormonal treatment F (4 milligrams per liter 2,4-D and 0/5 milligrams per liter BAP) had the highest fresh weight. Turkey genotype on hormonal treatments F, had the highest callus fresh weight in hypocotyl explants and Tabriz root explants on hormonal treatment A had the best results in comparison to other genotypes in callus fresh weight, and Gonabad roots in hormonal treatments I was the worst for this trait.

Table 4. Variance Analysis (ANOVA) for different traits of leaf explants.

Leaf	Df	Callus surface	%callus induction	Callus fresh weight	Callus dry weight	Callus volume
Genotype	5	7808/021**	0/476**	0/121**	0/001**	0/482**
Hormon	8	10862/128**	0/325**	0/171**	0/001**	0/729**
Gen×Hor	40	1679/569**	0/125**	0/24**	0/000**	0/102**

Table 5. Variance Analysis (ANOVA) for different traits of petiole explants.

Petiol	Df	Callus surface	%callus induction	Callus fresh weight	Callus dry weight	Callus volume
Genotype	5	2247/416**	0/268**	0/044**	0/000**	0/159**
Hormon	8	9018/301**	0/485**	0/141**	0/001**	0/595**
Gen×Hor	40	518/875**	0.063**	0/009**	0/000**	0/034**

Table 6. Variance Analysis (ANOVA) for different traits of hypocotyl explants.

Hypocotyls	Df	Callus surface	%callus induction	Callus fresh weight	Callus dry weight	Callus volume
Genotype	5	2201/324**	0/078**	0/026**	0/000**	0/103**
Hormon	8	15718/977**	0/784**	0/233.**	0/002**	0/930**
Gen×Hor	40	509/769**	0/065**	0/009**	0/000**	0/034**

Table 7. Variance Analysis (ANOVA) for different traits of root explants.

Root	Df	Callus surface	%callus induction	Callus fresh weight	Callus dry weight	Callus volume
Genotype	5	1303/324**	0/455**	0/020**	0/000**	0.085.**
Hormon	8	7200/838**	0/783**	0/104**	0/001**	0/424**
Gen×Hor	40	590/461**	0/179**	0/010**	0/000**	0/034.**

Panizza (1997) on lavender plants (Lavandulavera), used BAP and NAA on callus induction phase and gained good weight calli. Also in this study, by using growth regulators 2,4-D and BAP, were obtained appropriate weight calli and it was same as (*Sarkheyl et al* 2009) results. Based on Duncan test the German genotype had the highest dry weight in leaf explants, Petiole explants callus of Isfahan genotype on hormonal treatment F (4 milligrams per liter 2,4-D and 0/5 milligrams per liter BAP) had the highest dry weight. In hypocotyl explants the callus dry weight in Isfahan genotype on hormonal treatment F was better

than other treatments. In hypocotyl of German genotype, in the first and second levels of 2,4-D and second level of BAP we had an appropriate response on dry weight of callus. Hormonal treatment C in Tabriz and hormonal treatment F in Turkey had the highest dry weight ,trough other genotypes Hamadan genotypes in Hormonal treatment E and F, and Isfahan genotype in Hormonal treatment E in terms of hypocotyl dry weight were in a statistical group also Tabriz root in hormonal treatment A had the better results in comparison to others .

Fig. 1. Sterile seedling 20 to 50 days after germination and sterile seedlings parts including leaves, petioles, hypocotyls and roots.

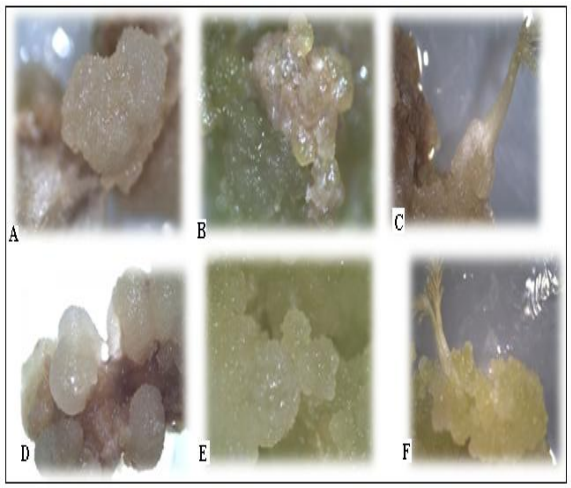

Fig. 2. Embryogenesis, after callus exposure in regeneration area. A: Germany Leaf Callus placed on callus induction hormonal treatment A and regeneration B: Hamadan petiole callus placed on callus induction hormonal treatment C and regeneration, C: Hamadan hypocotyl callus placed on callus induction hormonal treatments B and regeneration , D:Turkey petiole callus placed on callus induction hormonal treatments B and regeneration , E:Hamadan leaf callus placed on callus induction hormonal treatments B and regeneration , F: Gonabad Hypocotyl Callus placed on callus induction hormonal treatment A and regeneration.

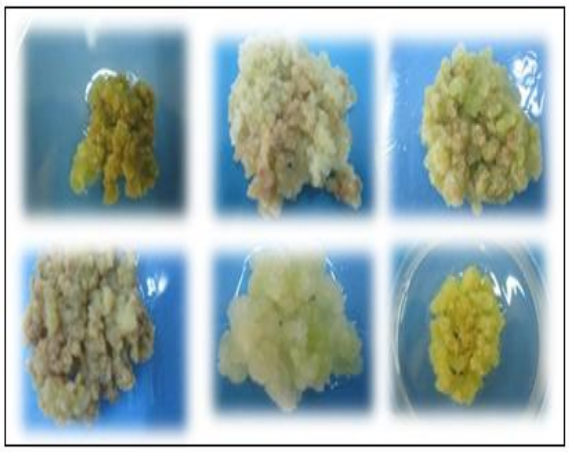

Fig. 3. Different colors of callus.

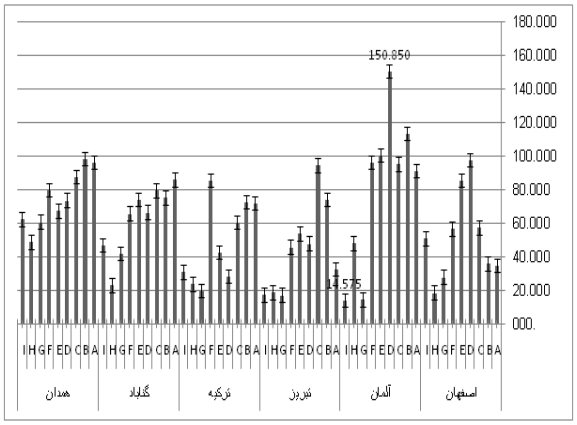

Fig. 4. The interaction of hormone and genotype in leaf explants callus size.

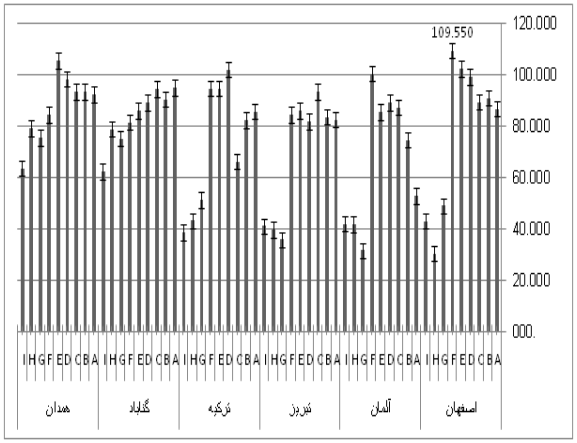

Fig. 5. The intraction of genotype and hormone in petiol explants callus size.

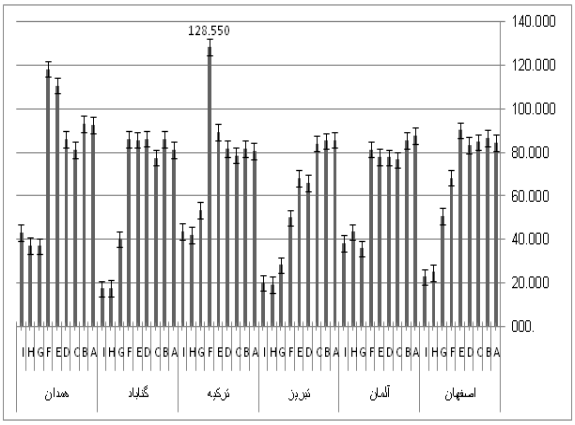

Fig. 6. The intraction of genotype and hormon in hypocotile explants callus size.

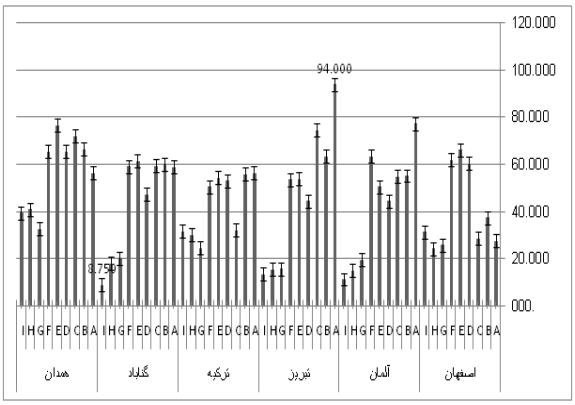

Fig. 7. The intraction of genotype and hormone in root explant callus size.

Due to the interaction effect, the maximum volume in leaf explants were about German Genotype in hormonal treatments D. Isfahan genotype in second level of 2,4-D was better as compared to other levels that callus volume reduced by BAP levels increasing in the genotype. Tabriz genotype in hormonal treatments C and Turkey genotype in hormonal treatments F had the highest callus volume. Generated callus explants of Gonabad genotypes had relatively good volumes in the first and second level of 2,4-D , Hamadan genotypes were less impressed by hormonal treatments and every three levels of 2,4-D and BAP was appropriate in callus volumes, However callus volume reduced slightly by increasing 2,4-D levels. In petiole explants of Isfahan, Germany and Tabriz genotypes, second level of 2,4-D and third level of BAP were the best combination hormone. In Turkey Genotype, except that reducing callus volume by increasing BAP level, second level of 2,4-D was better. Isfahan genotype in hormonal treatment F had the highest callus volume in the explants that were in a statistical group with Hamadan genotypes in hormonal treatment E. Petiole explants of Isfahan callus had the lowest volume in hormonal treatments H. Hypocotyl of Turkey genotype was the most in callus volume in hormonal treatment F and Tabriz root callus volume in hormonal treatment A was better than other genotypes. *Panizza* (1997), in a study of callus induction on lavender plant (Lavandulavera), reported that use of BAP in combination with NAA would be successful in producing callus size, on percentage of callus induction and weight and volume of created calli and also producing calli with good regeneration ability (the quality of calli). In this study, to develop Embryogenesis and plant regeneration ability of fennel, one MS / 2 medium with both 2,4-D and BAP combination hormone and a hormone-free medium,were used. Live callus with appropriate physical characteristics from leaf, petiole, hypocotyl and root explants of fennel (*Foeniculum vulgare*), almost a week after transfer to regeneration medium, form white nodules and their signs of embryogenesis appeared. Between one to two weeks after exposure in this medium in different genotypes heart shape embryos were visible. At least some of the calli find a rapid growth in these medium and calli size increased. There is remarkable point that the obtained calli of callus induction medium by containing low levels of the hormone are found more successful in embryogenesis. Mattar (1997) by studying on the effect of growth regulators Auxin(2,4-D)and Cytokinin (BAP, Kin) on the petiole, fennel, concluded that the use of high levels of these hormones in the culture medium, could prevent the induction of embryogenesis. Experiments on three of the proposed treatment for fennel regeneration have showed that the MS / 2 without hormones were the best medium for the regeneration of leaf, petioles, hypocotyls and roots. Some of the explants and genotypes are regenerated in hormonal treatments B 'and C' (Table 2) However they had far from the percentage of regeneration in Medium (A hormone-

free) (figure2). *Anzidei et al.*(2000), And *Sarkheyl et al.* (2009) said that hormone free medium is suitable for regeneration of the fennel. Also in this study, we were succeeded in leaf and petioles Embryogenesis. Haensch(2007) used 2,4-D and BAP Combined hormone On Madame Layal (Pelargonium × domesticum) petiole in growth medium for somatic embryogenesis and obtained acceptable results and Rajabpoor *et al.*, (2007) used of these two hormones on the Saffron (Crocus sativus L.) plant for callus induction and embryogenesis, and the results were satisfactory. Fotso *et al.*(2008) used 2,4-D and BAP hormonal composition on the Irvingiagabonensis plant, in MS / 2 medium for callus induction and somatic embryogenesis , Kamad *et al.* (1989) and also Pant *et al.* (2007) reported in their research that *in vitro* samples of carrot leaf ,hypocotyls and roots in MS medium by different hormonal levels have good percentage of propagation. The obtained results in a study on carrots (Daucuscarota) by Krzyzanowska (2006) emphasized the positive effect on hormone-free medium in Embryogenesis and plant regeneration in Apiaceae family, which is consistent with the obtained results in this study. According to these observations and notes eventually become clear that the quality of the callus will determine Embryogenesis So that the callus with a grainy, compact and fragile surface in comparison with the watery and non granular callus has more embryogenesis. The colors of the callus were visible almost in three classes, milky cream, bright greenish cream and cream leaning to dark brown (figure 3). Those colors that were too bright greenish-cream and milky cream had been recognized embryogenic with good regeneration and calli with very dark cream and the cream leaning to brown were not suitable. In most of the nodes after transferring to regeneration medium and after a few days white callus were visible, In fact, embryogenic parts under binoculars were visible as heart-shaped embryos in these areas. Everything that was good about the impact of callus quality on Embryogenesis and regeneration capability have matched to *Zeba* (1997) and *Sarabadani et al.* (2008) results. Finally, the different genotypes of fennel as a medicinal plant that are evaluated in this study had genetic variation in callus induction and responded to *in vitro* growth medium too. Selected explants responding not only influenced by different genotypes but also same explants culture from grown plants in the same conditions and belonging to a genotype had different responses in tissue culture. These results indicate that the response of cultured explants like most traits influenced over genetic and environmental factors. The interaction between genotype and hormone treatments in all explants and traits has showed significant differences. According to studies purposes created callus were embryogenic and had acceptable results either in terms of quality and regenerated stamina, and hormone-free medium have been recognized as the best medium to regeneration. Hypocotyl had the highest regeneration levels that had not great differences to petiole. Callus embryogenesis and regeneration ability of different genotypes had not differed much but Tabriz was more appropriate than others. By Color and fragility studying of the callus physical characteristics were determined that these were important in callus regeneration. by surveys that were conducted, the genotypes of Hamadan and Gonabad in terms of callus induction ,in comparison to other genotypes, have impressed less to explants effects and hormonal treatments while the German Genotype influenced more than others, and finally Hypocotyl and petiole explants were introduced as the best explants for callus formation. Based on the studies, obtained results were suggested According to appropriate response of *Foeniculum vulgare miller* medicinal plant to *in vitro* condition, studying more genotypes and the amount of active ingredient were assessed in the process of tissue culture up until finding genotypes for callus induction and regeneration. Also by integrating the marker and tissue culture studies, we could obtain higher active ingredient and better respond to *invitro* medium.

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