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DISTRIBUTION OF TRANS-ANETHOLE AND ESTRAGOLE IN FENNEL (*Foeniculum vulgare* MILL) OF CALLUS INDUCED FROM DIFFERENT SEEDLING PARTS AND FRUITS.

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ABSTRACT

In the present study, seeds from local cultivar of fennel were germinated on Murashige and Skoog medium (MS) without plant growth regulators. Different types of explants from the growing seedling such as cotyledonal leaves, hypocotyls, epicotyls and roots were cultured on MS medium. The medium contained different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) either alone or with kinetin. Differential responses in the essential oil were observed in the induction and development of callus. The major components of essential oils included estragole, trans-anethole, limonene and fenchone were proposed to be used in chemical classification on callus.

INTRODUCTION

Fennel is a plant belonging to the *Umbelliferae* (*Apiaceae*) Family, known and used by humans since antiquity. Because of its flavor, it was cultivated in every country surrounding the Mediterranean Sea. Fennel is one of the oldest field crop used by the Egyptian in food and for medicinal purposes. Most of the area cultivated with fennel is located in Mid-southern Egypt (mainly, Elfayom, Menia and Assiut Governerates). The total area cultivated with fennel in Egypt was 2209 feddan in 2006 which produced 3446 tons of ripe dry seeds. Assiut Governorate cultivated about 86% of this area. (A.E.B,2007). Fennel is an important medicinal plant growth well under Egyptian conditions and plays an important role for the export of the country.

Only one strain of common fennel (*Foeniculum vulgare* Mill.) is cultivated in Egypt. The cultivar yields big fruits and reasonable percentage of essential oil with particularly high estragole content, but it is poor in fenchonewhich is an important constituent of the fennel essential oil. In order to find other cultivars that maintain, under Egyptian environmental conditions, good yields with a higher fenchone content as published by Massoud(1992). The present study try to find cultivar that has a higher anethole or fenchone content and lower estragole content.

The ethanolic extracts of fruits show diuretic, analgesic and antipyretic activities as well as antimicrobial and mitodepressive activity (Tanira, *et al*. 1996). Essential oil of fennel also has an inhibitory effect on the activity of ileum, the urinary bladder and against spasmogens (Saleh, *et al*.1996). Trans-anethole, estragole, fenchone and limonene are the major constituents of the essential oil of bitter fennel fruits (Venskutonis, *et al*. 1996) and (Bernath., *et al*. 1996). These, along with some other components, provide the unique aroma and taste: trans-anethole accounts for the anise taste, estragole the sweetness, fenchone the bitterness, and limonene provides the citrus taste.. Trans-anethole, commonly used for flavor in the food and liquors industry, is considered non-toxic (Barazani, *et al.* 1999). The fresh leaves and dried fruits of this plant are used as a spice for meat, baked and confectionery products, and as a local m*ateria medica* in Turkey (Davis, 1972). Sweet fennel (*Foeniculum vulgare* Mill.var. *dulce*) is eaten as green salad and used in pharmaceutical, perfumery industries and to flavour of different food preparations. (Fenaroli, 1971). Fennel seeds are used as a popular flavouring agent in culinary preparation, bread, pastry, confictionery, liqueurs and as ingredients of compound powder of liquorice (Ashraf, and Bhatty, 1975).

MATERIALS AND METHODS

The present studies were carried out at the "Biotechnology laboratory" Horticulture Research institute. Agricultural researches center during 2002 -2006.

Explant Source

Fennel fruits (*Foeniculum vulgare.L*) were obtained from Medicinal and Aromatic plant Research Departement, Agricultural Researches Center and surface sterilized for 1 min in 70% ethyl alcohol then for 20 min in 30% commercial bleach (sodium hypochlorite 5.25%) with 2drops of Tween 20, after that rinsed thoroughly for 3 times with sterile distilled water . The sterilized seeds were germinated on MS medium (Murashige and Skoog, 1962) growth regulators-free medium supplemented with 0.7% agar and 3% sucrose. Glass shell vials (25x95 mm) with polypropylene closures were used. The pH of the medium was adjusted to 5.7±1.0. The medium was autoclaving sterilized for 20 minutes at 121ºC and 1.2 Kg.cm^{-2}. The cultures were maintained at 27ºC±1.0 under cool white florescent light (28 m mol S-1m-2, 16h/day). After germination seedlings were used as a source for explants.

Callus induction

When the seedlings were about 6 cm height, four types of explant (leaves, hypocotyls, epicotyls and roots segments) were excised. These explants (3-5mm long) were cultured on MS medium supplemented with kinetin at concentrations of 0. 0.5, 1 mg/l without or with 2, 4-D at concentrations of 0, 0.5mg/l . The medium contained 3% sucrose and 0.7% agar. The cultured were incubated at 23ºC under 16 h photoperiod. The factorial treatments of kinetin and 2, 4-D in the medium were arranged in randomized complete block experiment with five replicates. The callus tissues were formed after 28 days, and were maintained by sub culturing every four weeks on the best medium to obtain alarge amount of stock calli for experiments (Tawfik, 1995).

Extraction of Essential oil

The different types of calli (3g) which formed from the different parts of the seedlings (roots, epicotyls, hypocotyls and leaves which induced on MS medium free growth regulator) or seeds were extracted by crushing with hexane (20ml). The supernatant solution was then concentrated to 1ml before injection (Barazani, *et al.* 1999).

Method of analysis:

The pure volatile oil was injected in the Gas chromatography mass spectrometer (GCMS) HP-INNO Wax with (30m X 0.25 mm ID 0.250 Micron film thickness) as follows:-

Sample injected 0.5µl.   Carrier gas Helium at 1.8ml/ min flow.
Temperature programming was:
Initial temp. of oven 120°C;for 1 min
Rate 120-250°C at 10°C / min to 250°C.for 5 min
Injection: split 40: 1,250°C, 1µl
Detector MSD 280°C
Sample was 0.05 to 0.11% each in methylene chloride

RESULTS AND DISCUSSION

Over than 63.49% of the germinated fennel fruits developed normal seedlings on MS growth regulator-free medium (fig. 1) after 40 days. The largest amount of callus induced from hypocotyls tissues (fig. 2). Differential explant responses were found in umbelliferous plants (Halperin and Wetherell 1964 and Nadal, *et al.,* 1989). The data observed that large amount of proliferating callus was on the medium with 2, 4-D at 0.5 mg/l with or without kinetin at 0.5 ,1.0 mg/l (table1). The 2, 4-D with kinetin has been used to induced callus in celery (Nadal, *et al.,* 1989 and Saranga and Janick 1991). The callus was compact and had light yellow color.

Fig.(1): Germinated fennel seeds developing seedlings.

Fig. (2): Callus initiation after 4 weeks on MS medium containing 2,4-D with kinetin (0.5mg/l).

Table: (1) Effect of different concentrations of kinetin and 2,4-D as well as effect of some seedling parts on callusing formation (%) of fennel (*Foeniculum vulgare*).

Seedling parts / Growth regulators	Root	Epicotyls	Leaf	Hypocotyls	Mean
Control	63.21	68.50	54.88	57.50	60.70 B
0.5mg/l Kinetin	61.88	60.75	39.50	45.75	51.83 B
1.0mg/l Kinetin	72.65	55.25	52.75	63.00	50.55 B
0.5mg/l 2,4-D	75.46	76.00	58.00	76.50	87.50 A
0.5mg/l Kinetin +0.5mg/l 2,4-D	78.75	42.25	39.00	101.0	86.75 A
1.0mg/l Kinetin +0.5mg/l 2,4-D	6.069	69.75	52.50	101.0	85.00 A
Mean	68.40AB	75.50 A	55.05B	82.60 A	

LSD value Factor A = 15.26
LSD value Factor B = 18.69
LSD value = 37. 37 at alpha =0.05

LSD	5%
E	15.26
C	18.69
E x C	37. 37

E: explants
C: cytokinins concentrations.
E x C : Interaction between explants and cytokinins concentrations.

Chromatographic Analysis

The identified components and their percentages by GC Mass in bitter fennel volatile oils are listed in table (2).

Trans-anethole is an important component for the aroma, because of its sweet taste and many studies confirmed that trans-anethole is constituting about 90-95% of the total fennel volatile oil composition. Trans-anethole represents the major component of callus induced from root, leaf, hypocotyls and epicotyls which was 58.726,

Table (2) Relative percentage composition of the volatile oils from callus induced from different seedling parts of fennel (*Foeniculum vulgare*)

component	fruits	Callus induced from roots	Callus induced From leaves	Callus induced from hypocotyls	Callus induced from epicotyls
Trans-anethole	0.871	58.726	82.490	66.772	68.293
Estragole	85.599	-	7.263	4.114	16.098
Fenchone	3.215	8.310	-	-	-
Limonene	4.019	-	3.502	-	-
Fenchylacetate	0.268	-	-	-	-
Anisaldehyde	0.536	7.479	5.447	2.848	6.829
Camphor	0.201	4.155	-	-	-
Carene	3.516	7.202	-	-	-
P-cymene	0.6698	4.432	-	-	-
β-myrcene	0.402	3.878	-	-	-
α-pinene	0.301	-	-	-	-
Unknown	0.402	5.817	1.297	26.266	8.78

Values calculated as percentages of the total fennel volatile oil composition.

82.49, 66.772 and 68.293% respectively. On the other hand in fruit, the contents of trans-anethole was 0.871% which considered very low concentration compare to different induced of callus. The mass spectra of trans- anethole and estragole are quite similar fig. (3) but could be identified by considering retention times. Estragole represents the major components in fruits 85.599% and arranged 7.263, 4.114 and 16.098% in leaf, hypocotyls and epicotyls respectively and could not be detected in callus induced from roots.

Limonene is conferred a fresh smell, it was represent in fruit (4.019%) and in callus induced from leaf (3.502%) only but it was absent in callus induced from root, hypocotyls and epicotyls. Samples with a high trans-anethole content contained small amounts of estragole and vice-versa , while callus contained more trans-anethole and fruit contained more estragole. Miraldi (1999) found that fruits with an intense, sweet and aniseed taste contained more trans-anethole, while samples with adelicate and soft taste contained more estragole. Brazani, *et al.,* (1999) reported that fennel which grow in habitats with high summer temperatures and low humidity exhibited the highest trans-anethole content (55 and 74% respectively) with the lowest level of estragole (8% of the Pick area) there are in agreement with our findings.

Because many of the reference mass spectra for essential oils have been produced on quadrupole instruments, the ion trap spectra are comparable to quadrupole spectra. Most of the volatile plant oil components yield almost identical spectra on (8.310%). Fenchone has a pungent and camphorate odour; it is present especially in bitter fennel.

Noteworthy differences were also recorded in the precentage of anisaldehyde an autoxidation product of trans-anethole, ranged from 2.848% in callus induced from hypocotyls, 5.447% in callus induced from leaf, to the intermediate 6.829% in callus induced from epicotyls, up to the highest 7.479% in callus induced from root. While it was very low in fruits (0.536%). In callus induced from root the present work found the most essential oils which existed in fruits with high percentage like camphor (4.155%), carene (7.202%), p-cymene (4.432%) and β-myrcene (3.878%) but in fruits they showed 0.201%, 3.516%, 0.6698%, 0.402% respectively. α-pinene was the monoterpene hydrocarbon (0.301%) which identified only in fruits Arslan *et al.* (1989) reported the percentage of anethole to be 86 to 88% in sweet fennel oil and 74% in bitter fennel oil while limonene was only 4 and 2% respectively in sweet and bitter fruits. fennel oil. Embong *et al.* (1977) working on *Foeniculum vulgare* var. dulce reported that anethole as 69%in fruits and 39% herb oil, the relative percentage of limonene being 8 and 20% in the fruits and herb oil. The volatile oils of callus which induced from root was relatively difference from other callus seven constituents were detected of which the major compound was trans-anethole at 58.726%. And the other compounds were relatively high concentrations from the other callus. They are fenchone, anisaldehyde, camphor, careen, p-cymene, β-myrcene and α-pinene (8.310, 7.479, 4.155, 7.202, 4.432and 3.878% respectively). However estragole contents was not available in callus induced from root .

F3

f4

Gross. *et al.,* (2002) reported that estragole and trans-anethole are the major constituents of the essential oils of bitter fennel chemotypes (*Foeniculum vulgare* Mill. var. *vulgare*). Cell free extracts from bitter fennel tissues display o-methyltransferase activities able to *invitro* methylate chavicol and trans-anol to produce estragole and trans-anethole, respectively. Their results indicated that an apparent association between the levels of estragole and trans-anethole in the different plant parts. Thus the present results indicate that fruits had the most substantial content of estragole and the callus had the most substantial content of trans-anethole. Muckensturm, *et al.,* (1997) obtained that *Foeniculum vulgare* var. *vulgare* presents great composition differences with varying populations with the aim of clarifying the status of var. *vulgare*, the proposed to subdivide it into three chemotypes according to their relative compositions. They called them chemotype estragole, chemotype estragole/anethole and chtyp. anethole. According to that our results divided into two chemotypes in the following .

1-	Chtyp. estragole	Estragole is the major compound	The fruits
2-	Chtyp. anethole	t-anethole is the major compound	The callus

CONCLUSION

The results showed that 2, 4-D at 0.5 mg/l plus kinetin at 1mg/l produced the best response in callus induction. For the first time, the volatile oil of fennel fruits and callus tissue cultures have been reported here. The concentrations of volatile oil found in the *invitro* samples were not close to the levels in fruits. The analysis of the callus samples essential oils showed that they are of superior quality, because they are more enriched in anethole, which is the most valuable constituent of this oil. Moreover, the fennel seeds had the highest level of estragole which is carcinogenic(McDonald, 1999). The chemical composition of the callus induced from root is similar for the fruits but it was more valuable because it did not contain estragole. From the presented data, it can be concluded that the seedlings which produced from the callus induced from root were the best for cultivation, and it could be induced into a breeding program in the future for commercial production.

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توزيع الأنيسول المخالف والأستراجول في نسيج الكالس الناتج من أجزاء مختلفة من بادرات وبذور الشمر البلدي

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اجريت هذه الدراسة بمعمل التكنولوجيا الحيوية بمعهد بحوث البساتين- مركز البحوث الزراعية خلال الفترة2002-2006.

فى هذه الدراسة تم انبات بذور الشمر البلدى على بيئة MS خالية من منظمات النمو النباتية و بعد الحصول على البادرات تم تقطيع اجزاءها (الاوراق – السويقة الجنينية السفلى- السويقة الجنينية العليا- الجذر) الى قطع صغيرة و تنميتها على بيئة MSتحتوى على تركيزات مختلفة من 2.4-D بمفردة او مع تركيزات مختلفة من الكينتين لتكوين الكالس لاستخلاص الزيوت الطيارة منه و الزيوت الطيارة التي امكن التعرف عليها كانت الفينشون والليمونين والأستراجول والأنيسول المخالف.

ومما يذكر أن استخدام 2.4-Dبتركيز 0.5 مليجرام/لتر مع الكينتين بتركيز 1 مليجرام /لتركان له افضل استجابة لتكوين الكالس .

- وجد اختلاف فى نوعية ونسبة الزيوت الطيارة الموجودة فى عينات الكالس المتكون من الاجزاء المختلفة من البادرات بين بعضها والبعض وبين الزيوت الطيارة المستخلصة من البذرة حيث وجد أن الأنيسول المخالف يوجد بنسبة أكبر في الكالس عنه في البذرة ففي الكالس الناتج من الأوراق يوجد بنسبة 82.490 % أما في البذرة فيوجد بنسبة 0.871% بينما الأستراجول يوجد بنسبة 85.599% في البذرة ولكن يوجد بنسبة 4.114% في الكالس الناتج من السويقة الجنينية السفلى وغير موجود في الكالس الناتج من البذور.
- باستخدام جهاز GC mass فى تحليل العينات وجد ان الزيوت الطيارة الناتجة من الكالس الناتج من الجذر المتكون على بيئة MS خالية من منظمات النمو النباتية ذات جودة عالية لعدم احتوائها على الأستراجول واحتوائها على كمية عالية من الأنيسول المخالف الذى له فائدة كبيرة حيث انه يعطى الرائحة المميزة للشمر لذا نوصى بتصميم برنامج تربية للبادرات الناتجة من هذا الكالس.