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**Research Article**

# Screening of several important compounds production in fennel (*Foeniculum vulgare* Mill.) populations

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ABSTRACT

**Background:** The perennial fennel plant is one of the most important and widely used medicinal plants of the Apiaceae family, which is mainly cultivated for the purpose of using the essential oil obtained from it in various pharmaceutical, food, cosmetic and health industries. **Objective:** To investigate callogenesis and secondary metabolites production from calli, 13 populations of Iranian fennel as well as two populations from Germany and two populations from Turkey were evaluated by GC-MS. **Methods:** Five types of explants (leaf, hypocotyl, epicotyl, cotyledon, and root segments) were cultured in MS medium supplemented with combination of 2,4-D + Kinetin (1:1), and also NAA + BA (0.5:1). **Results:** Among five explants used in this study, only hypocotyl explant had appropriate response to the callogenesis. According to the GC-MS analysis, the highest *trans*-anethole content (67.23 %) was produced in the callus of Turkish population (TUR1) under NAA + BA treatment (1:1). Callus extracts of other Turkish population (TUR2) contained considerable amounts of limonene (67.70 %) under 2,4-D + Kinetin treatment (0.5:1). **Conclusion:** Callus induction with different plant regulators can have a significant contribution to the production of secondary metabolites, so callus that produce more secondary metabolites can be cultured in suspension or cell culture bioreactor systems. Based on the results of this research, Turkish fennel populations had the capacity to produce significant amounts of main secondary metabolites.

## 1. Introduction

Fennel (*Foeniculum vulgare* Mill.) is an open pollinated species belonging to the *Apiaceae* family and originating in the Mediterranean region where it is possible to observe a high genetic variability [1]. Traditionally, in Europe and Mediterranean areas, fennel is used as antispasmodic, diuretic, anti-inflammatory,

*Abbreviations:* GC-MS, Gas Chromatography-Mass Spectrometer; MS Medium, Murashige and Skoog Medium; 2,4-D, 2,4-Dichlorophenoxyacetic acid; NAA, Naphthalene Acetic Acid; BA, Benzyl Adenine

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analgesic, secretomotor, secretolytic, galactagogue, eye lotion, and antioxidant remedy [2]. It was suggested that essential oil of fennel can be used in food industries, aromatherapy and pharmaceutical aims [3].

Medicinal plants produce a wide variety of secondary metabolites. These metabolites have always been considered as plant responses to biotic (pests and disease) and abiotic stress (salt, drought, etc.), which play a major role in the adaptation of plants to their environment [4]. In natural conditions, secondary metabolites accumulation is affected by water availability, soil microorganisms, and variations in soil pH and elements [5].

Tissue culture of medicinal plants provides a continuous source of secondary metabolites round the year without the destruction of the entire plant. Tissue and cell culture may be a powerful tool for plant improving and enhancing the production of secondary metabolites in comparison to whole plant. *In vitro* technology is well-known for biodiversity conservation. Since the late 1960s, plant cell and tissue culture have been introduced as an appropriate tool to study and produce secondary metabolites in medicinal plants [6-7]. Researchers believed that the *in vitro* system is a useful tool to obtain plant genetic uniformity that can be as a desirable source of medicinal compounds [8-9]. Controlled cultivation systems offer the opportunity to optimize yield and achieve a uniform and high-quality product. Callus culture in medicinal plants have been carried out for the production of active pharmaceutical materials [10, 11, 12, 13]. Fennel has shown important aspects with regard to its culture behaviour *in vitro* during micropropagation [12], callus and suspension cultures [10], and its capacity to regenerate plants especially via somatic embryogenesis [13]. This study aimed to identify the chemical composition of fennel population's calli under treatments of several plant growth regulators (PGRs).

## 2. Materials and methods

### *2.1. Plant material source*

In this research, 13 populations of fennel (*Foeniculum vulgare* Mill.) from Iran as well as two populations from Germany and two populations from Turkey were evaluated. The collection sites such as country, province and the nearest city of the region and abbreviations of the studied fennel populations are shown in Table 1.

The present study was carried out at the "Jaber ibn Hayyan laboratory" in University of Tabriz during 2014-2015. Five types of explants (leaf, hypocotyl, epicotyl, cotyledon, and root segments) were cultured on MS medium for callus induction [14].

### *2.2. Callus culture*

Fennel seeds were sterilized for one min in 70 % (v/v) ethyl alcohol and then for 20 min in 2.5 % sodium hypochlorite with two drops of tween 20, afterwards rinsed thoroughly four several times with double distilled water. The sterilized seeds were germinated on MS medium with 0.8 % agar and 3 % sucrose. Glass shell vials (25 × 95 mm) with polypropylene closures were used.

The pH of the medium was adjusted to 5.7 ± 1.0. The medium was sterilized by autoclave for 20 min at 121 ºC. The cultures were maintained at 27º C ± 1.0 under cool white florescent light (28 mmol/S.$m^2$ and 16 h/day photoperiod). After three weeks, the germinated seedlings were used as a source of explants. The PGRs treatments were a combination of: 1) 1 mg/L 2,4-D + 1 mg/L kinetin (Kin); 2) 0.5 mg/L 2,4-D + 1 mg/L Kin; 3) 1 mg/L naphthalene acetic acid (NAA) + 1 mg/L benzyl adenine (BA); 4) 0.5 mg/L NAA + 1 mg/L BA. Five grams of powder from each callus sample was used to extract secondary metabolites by *n*-hexane. The color of the calli in separate cultures with different regulatory treatments was from light green to dark green and the texture of the calli was thick and depending on the different populations from soft calli to relatively hard calli. In this experiment, two cultivations were carried out, each with a time interval of one month, and after the second cultivation, the calli were harvested in order to isolation the essential oil.

**Table 1.** Collection sites and abbreviations of the fennel populations

| No. | Country | Province/City | Abbreviations |
|---|---|---|---|
| 1 | Germany | Salzlandkreis | GER1 |
| 2 | Germany | Dachwig | GER2 |
| 3 | Turkey | Izmir | TUR1 |
| 4 | Turkey | Gaziantep | TUR2 |
| 5 | Iran | East Azarbaijan/Tabriz | TAB |
| 6 | Iran | Razavi Khorasan/Torbat-e Jam | TOR |
| 7 | Iran | Isfahan/Khur and Biabanak | KHUR |
| 8 | Iran | Alborz/Karaj | KAR |
| 9 | Iran | East Azarbaijan/Bonab | BON |
| 10 | Iran | South Khorasan/Birjand | BIR |
| 11 | Iran | Isfahan/Tatmaj | TAT |
| 12 | Iran | Lorestan/Khorramabad | KHOR |
| 13 | Iran | Ardabil/Moghan | MOGH |
| 14 | Iran | Ardabil/Meshkinshahr | MESH |
| 15 | Iran | Isfahan/Ziar | ZIYA |
| 16 | Iran | Razavi Khorasan/Nishapur | NEY |
| 17 | Iran | Hamadan/Hamadan | HAM |

### *2.3. Essential oil isolation*

Dry materials (100 grams seed) were subjected to hydro-distillation for 4 h using a clevengertype apparatus. The essential oils were separated, dried over anhydrous sodium sulfate, and stored in dark glass bottles at 4 ºC until analysis.

### *2.4. Gas Chromatography (GC)*

The GC analysis was carried out on Agilent 7890A Network GC system equipped with a split less model injector (with 1.0 µm volume and 250 °C temperature) and a flame ionization detector (FID) (with 250 °C temperature). Helium was used as carrier gas (1.1 ml/min) and the capillary column used was HP 5 MS (30 m × 0.25 mm, film thickness 0.25 µm). The column pressure was fixed to 8.13 PSI. The oven temperature initially was kept at 40 °C for 4 min after injection and then increased to 250 °C with a rate of 8 °C/min heating ramp and kept constant at 250 °C for 5 min. The percentage of the compounds was obtained by calculating GC peak area without using any correction factor [15].

*2.5. Gas chromatography-mass spectrometry (GC-MS)*

Callus extracts also analysed by GC-MS using Agilent 7890 A Network GC system combined with Agilent 5975C Network mass selective detector. The GC analysis was carried out in the same analytical conditions which explained in the previous section. MS was performed with an ionization voltage of 70 eV, and mass range of 34-450 m/z. The 280˚ C and 250˚ C temperatures used as anion source and interface temperature, respectively. Constituents of the callus extracts were recognized by matching of their retention times, retention indices and mass spectra pattern with related available data [15] or with Wiley and NIST libraries and literature.

*2.6. Statistical Analysis*

The methods used in order to group the effective substances of the populations in terms of different compositions were performed by the method of principal component analysis (PCA) using Statgraphics software and cluster analysis using SPSS software.

**3. Results**

*3.1. Chemical composition*

Different explants including leaf, hypocotyl, epicotyl, cotyledon, and root segments were checked during callus induction. Only hypocotyl had appropriate response to the callogenesis and this experiment was conducted by hypocotyl. Other explants, i.e., cotyledon, root and epicotyl explants due to weak callogenesis and leaf explants due to lack of callus production were excluded from further studies. In addition, callus induction was not successful with control treatment (MS medium without PGRs). So, only the hypocotyl explants due to appropriate callogenesis and high secondary metabolites content was selected to continue (Fig. 1, 2, 3, 4, 5).

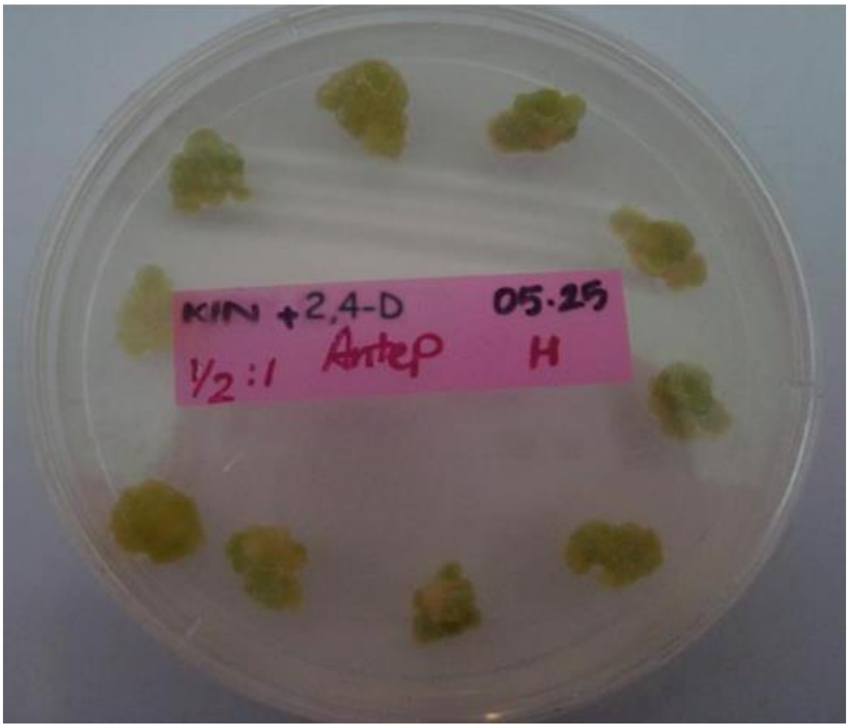


**Fig. 1.** Callogenesis in treatment 2,4-D + KIN and population of Antep

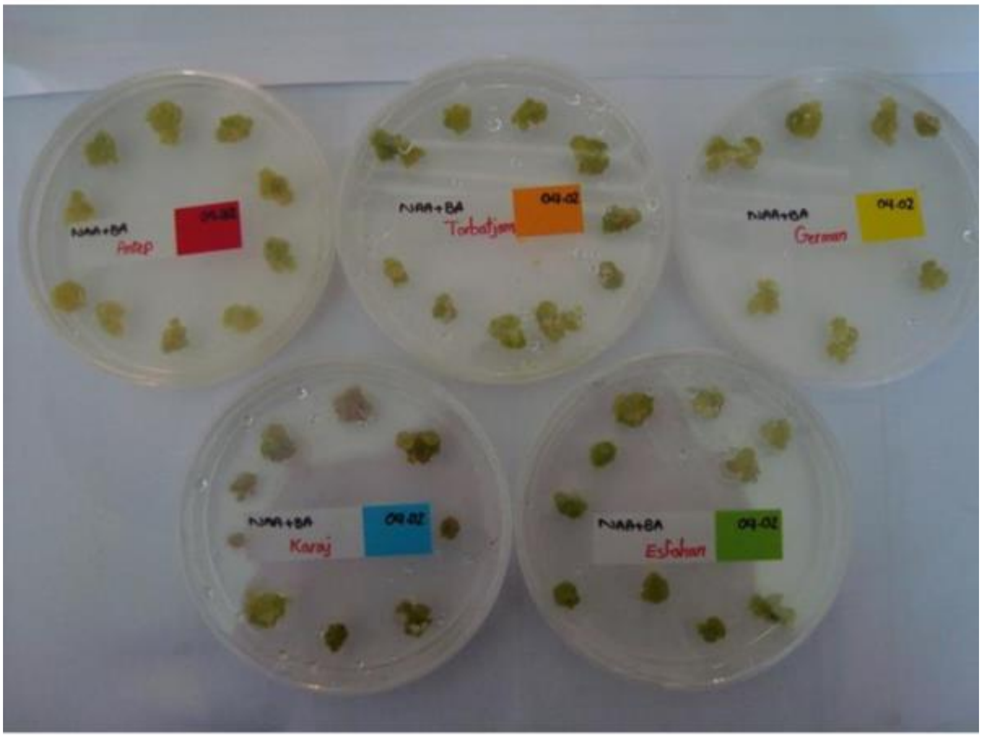


**Fig. 2.** Callogenesis with treatment NAA + BA in some studied populations

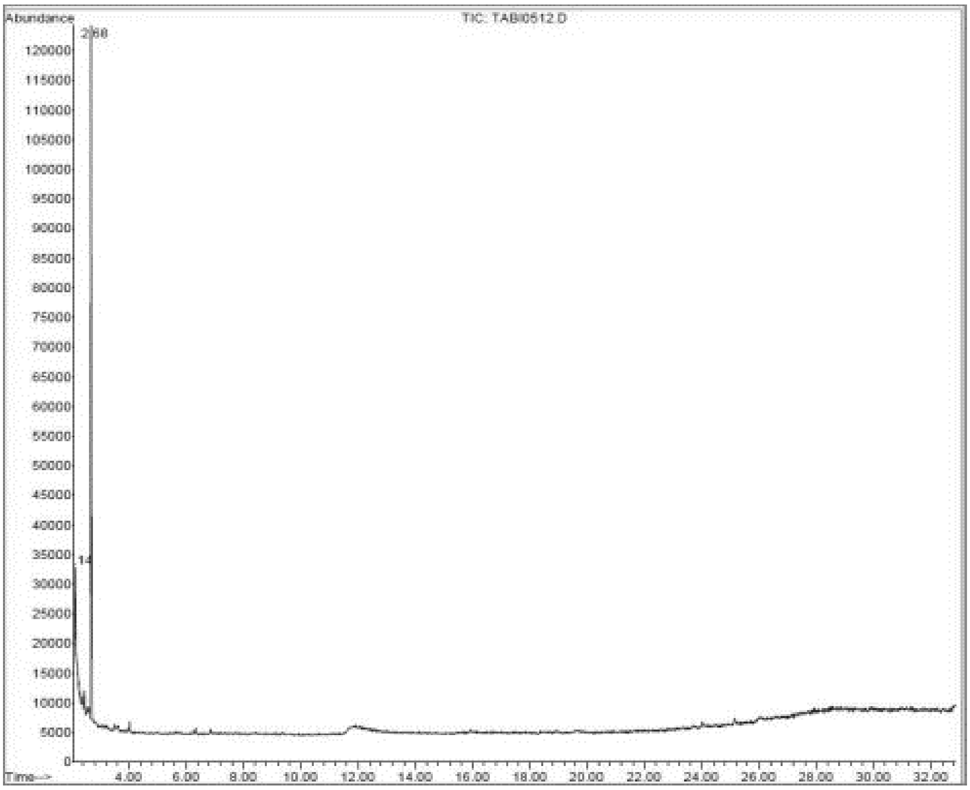


**Fig. 3.** GC-MS spectrum of callus extract of cotyledon explant of German population in NAA + BA treatment




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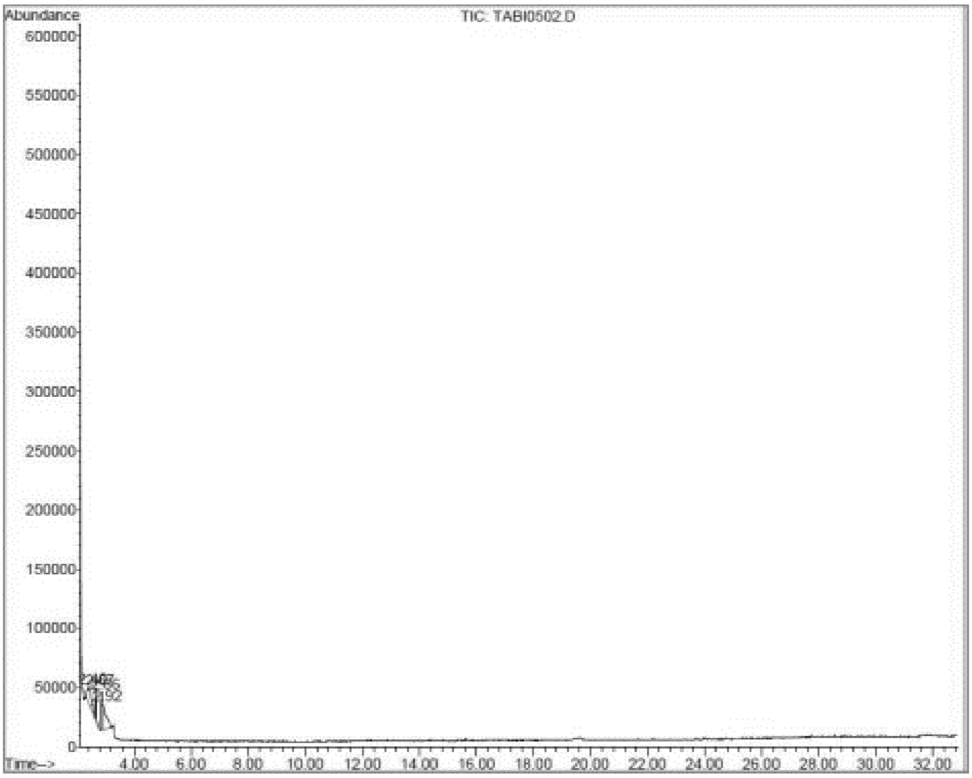


**Fig. 4.** GC-MS spectrum of callus extract of cotyledon explant of German population in 2,4-D + Kin treatment

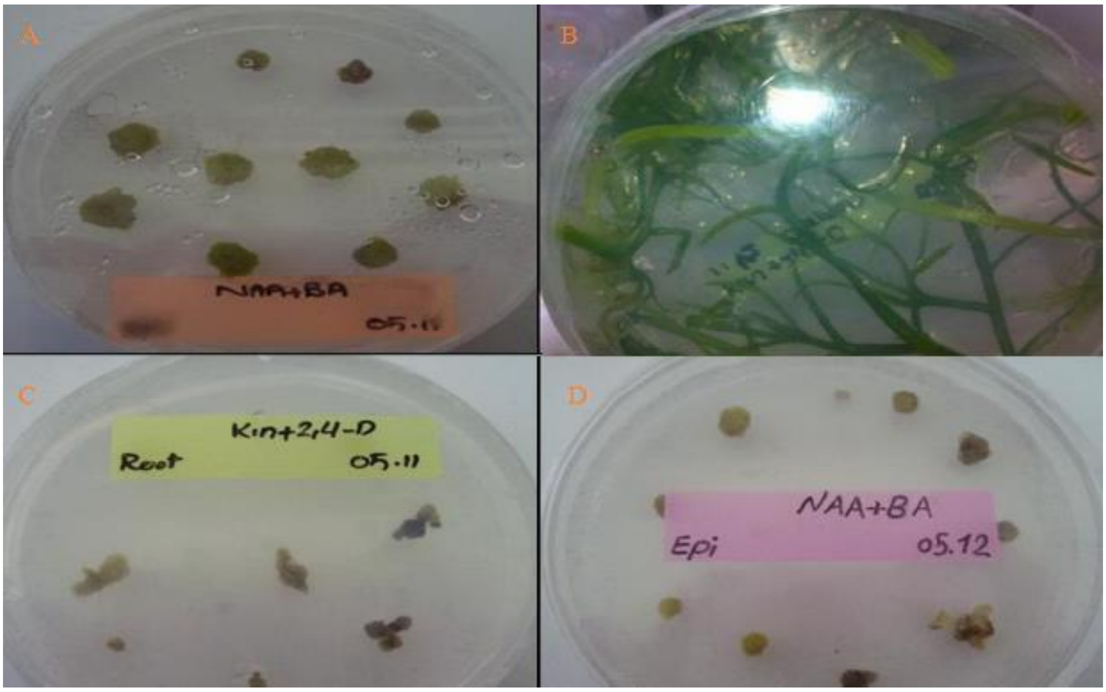


**Fig. 5.** Callus obtained from cotyledon explants of Torbat-e Jam population (A), Callus obtained from the leaf explant of Isfahan population (B), Callus obtained from root explants of Germany population (C), Callus obtained from epicotyle explants of Gaziantep population (D)

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Different PGRs treatments and various endemic and exotic populations' effects on production of secondary metabolites were checked to screen the most capable treatment and population combination (Table 2). According to the GC-MS analysis, a total of twenty-three compounds were detected in the examined extracts (Table 2). The highest *trans*-anethole content (67.23 %) was produced in the callus of exotic population; TUR1 under 1 mg/L NAA + 1 mg/L BA treatment. The callus of TUR2 population by the same treatment had only 0.37 % *trans*-anethole. The callus of TUR1 population under 1mg/L 2,4-D + 1mg/L Kin treatment produced 6.34 % *trans*-anethole. TUR1 population produced 9.11 % and 4.68 % *trans*-anethole under 0.5 mg/L NAA + 1 mg/L BA and 0.5 mg/L 2,4-D + 1 mg/L Kin respectively while the GER2 population produced 3.65 and 1.56 % *trans*-anethole at the mentioned treatments respectively.

TUR2 population callus extracts contained considerable amounts of limonene (67.70 %) under 0.5 mg/L 2,4-D + 1 mg/L Kin treatment and 18.3 % under 1 mg/L 2,4-D + 1 mg/L Kin treatment (Table 1) which it never been previously reported in fennel callus. Also, calli of seven Iranian populations including TOR, KHUR, KAR, KHOR, MOGH, ZIYA, and HAM contained limonene similar to German and Turkish populations. Among Iranian populations, KHOR population produced the highest limonene content (17.70 %). Significant amounts of α-pinene, camphene, sabinene, *p*-cymen, cineol, camphor, estragole, fenchone, linalool, and thymol were observed in some populations. In agreement with our findings, reported presence of estragole, fenchone, limonene, fenchyl acetate, camphor, caren, cymene, *α*-pinene, and anise aldehyde in fennel callus.

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments

| No. | Identified compounds | Retention indices | GER1 A | GER1 B | GER1 C | GER1 D | GER2 A | GER2 B | GER2 C | GER2 D | TUR1 A | TUR1 B | TUR1 C | TUR1 D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | 1.9 | 0.9 | 1.6 | - | 3.7 | **10.3** | 1.7 | 2.1 | 0.2 | 5.2 | 1.3 | 1.3 |
| 2 | Camphene | 951 | 0.8 | 2.1 | 0.3 | 0.4 | - | 0.5 | 0.9 | 0.5 | 1.2 | 3 | 2.3 | - |
| 3 | Sabinene | 973 | - | 4.8 | - | 5.3 | 0.1 | - | 0.4 | 3.1 | 1.3 | - | 0.9 | 0.1 |
| 4 | *β*-pinene | 976 | 1.7 | 0.8 | 2.3 | - | - | 2.3 | - | 0.9 | 0.4 | - | - | 3.8 |
| 5 | Myrcene | 988 | - | 0.7 | 0.6 | 0.4 | 1.6 | - | 3.7 | - | - | 0.1 | 3.9 | 1 |
| 6 | *p*-Cymene | 1021 | 0.8 | - | - | - | - | 4.4 | 2.4 | 4.1 | **18.2** | **24.6** | 9.7 | 0.5 |
| 7 | Limonene | 1026 | **20.1** | **11.2** | 6.3 | 3.7 | 5.3 | **17.9** | **19.3** | 9.3 | **25.6** | **32.6** | **12.3** | **67.7** |
| 8 | Cineol | 1028 | - | 2.1 | 1.6 | 0.7 | - | - | 0.7 | - | 1.2 | 5.7 | 3.7 | - |
| 9 | *γ*-Terpinene | 1056 | 2.5 | - | 3.4 | 0.7 | 0.4 | 2.4 | - | 2 | - | - | 1 | 9.1 |
| 10 | Camphor | 1144 | - | 1.4 | - | - | - | 1.2 | 0.6 | 4.1 | 5.2 | 6 | 2.1 | 3.3 |
| 11 | *trans*-Anethole | 1283 | 2.7 | 1.4 | 3.7 | 1.6 | 0.3 | 3.7 | 3.4 | 3.7 | 0.4 | 1.8 | 0.1 | - |
| 12 | Estragole | 1193 | 2.4 | - | 1.9 | 1.3 | - | 1.4 | 0.4 | 0.9 | 9.1 | 2.3 | 1.8 | 0.2 |
| 13 | Fenchone | 1083 | 1.2 | 1.8 | 7.8 | **10.5** | **52.3** | **25.6** | **14.3** | 7.6 | 1.9 | 3.6 | 3.2 | - |
| 14 | Borneol | 1174 | - | 0.9 | 0.3 | 5.1 | - | - | - | - | - | - | - | - |
| 15 | Fenchyl acetate | 1211 | 0.7 | 2.7 | 0.7 | - | 0.1 | 3.9 | 2.1 | 1.9 | 4.6 | 0.4 | 0.8 | 0.1 |
| 16 | Apiol | 1688 | **10.4** | 6.8 | **15.6** | **11.3** | - | - | 3.9 | - | 0.2 | 0.7 | 2.3 | 0.2 |
| 17 | Thymol | 1279 | 3.7 | 1.4 | 0.7 | 3.9 | 0.6 | 3.9 | 0.9 | 3.8 | 0.1 | 0.4 | 0.8 | - |
| 18 | *cis*-Ocimene | 1034 | - | 0.4 | - | 0.7 | - | - | - | - | - | 1.2 | 1.4 | - |
| 19 | Linalool | 1099 | 0.8 | - | 0.8 | 0.6 | **27.5** | 9.3 | 7.1 | 6 | 0.3 | - | 0.1 | 4.6 |
| 20 | *α*-Phellandrene | 1000 | 0.1 | 4.4 | 0.7 | 1.8 | 0.9 | 0.1 | 1.3 | 1.4 | 0.4 | - | 0.2 | - |
| 21 | Caryophyllene | 1402 | - | 1.2 | 0.5 | 0.4 | 0.7 | 0.9 | 0.8 | - | 0.8 | 0.7 | 3.4 | 1.1 |
| 22 | Germacrene D | 1488 | 3.1 | - | 1.4 | 2.3 | - | 1.1 | 2.1 | 0.7 | - | - | 0.7 | - |
| 23 | Spathulenol | 1554 | 0.4 | 0.2 | - | 1.9 | - | - | 4.1 | 0.7 | 0.4 | 0.4 | 0.6 | - |

Plant growth regulators treatments/Fennel populations.

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments (Continued)

| No. | Identified compounds | Retention indices | TUR2 A | TUR2 B | TUR2 C | TUR2 D | TAB A | TAB B | TAB C | TAB D | TOR A | TOR B | TOR C | TOR D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | 3.1 | 2.1 | 1.7 | 2.3 | - | - | 2.5 | 1.2 | 3.8 | 2.6 | 1.3 | 2.3 |
| 2 | Camphene | 951 | - | - | - | 6.3 | 6.5 | 5.6 | - | 0.4 | 0.8 | 6.3 | 4.3 | 0.6 |
| 3 | Sabinene | 973 | 1.3 | 3.4 | 0.5 | 1.5 | 1.2 | - | 1.7 | - | 1 | 0.7 | 0.1 | 0.4 |
| 4 | *β*-pinene | 976 | 2.1 | 2.8 | - | - | - | 0.9 | - | 5.3 | - | - | 0.2 | - |
| 5 | Myrcene | 988 | 2.1 | 1.4 | 1 | 2.8 | 0.9 | 0.2 | 1.3 | 1.4 | 2.3 | 2.7 | 3.1 | 5.2 |
| 6 | *p*-Cymene | 1021 | - | - | - | - | **49.6** | **25.1** | **18.5** | 8.6 | - | 8.3 | 4.3 | 3.1 |
| 7 | Limonene | 1026 | 9.7 | 5.3 | 4.3 | 1.8 | - | 0.6 | 0.4 | - | 9.8 | 4.7 | 1.2 | 0.5 |
| 8 | Cineol | 1028 | - | 0.1 | - | 5.5 | 0.3 | 0.5 | 3.7 | 2.9 | 0.8 | 2 | 5.2 | - |
| 9 | *γ*-Terpinene | 1056 | 2.1 | **12.2** | 0.6 | 4.1 | - | - | - | - | 1.8 | - | - | 5.1 |
| 10 | Camphor | 1144 | - | - | - | - | **18.3** | **10.7** | **35.4** | 8.2 | 4.3 | 5.7 | 7.3 | 3.8 |
| 11 | *trans*-Anethole | 1283 | **67.2** | 9.7 | 9.1 | 4.7 | - | - | 0.4 | 0.1 | - | 0.1 | 0.8 | 0.1 |
| 12 | Estragole | 1193 | 2.3 | 2.4 | 1.4 | 2.3 | - | - | - | - | 3.6 | - | 8.6 | 1.2 |
| 13 | Fenchone | 1083 | 3.1 | 5.1 | 2.6 | 2.8 | **12.4** | 6.3 | 0.4 | 0.8 | 0.4 | 1.8 | 0.2 | 2.8 |
| 14 | Borneol | 1174 | - | 1.2 | 3.2 | - | - | - | - | - | 1.3 | 5.3 | 1.7 | 0.6 |
| 15 | Fenchyl acetate | 1211 | - | - | 2.8 | 3.4 | 2.7 | 3.4 | 0.4 | 4.2 | 3.4 | 0.7 | - | - |
| 16 | Apiol | 1688 | 0.1 | 0.5 | 6.4 | 2.9 | - | 1.8 | 2.5 | 3.2 | - | 0.2 | 0.8 | 0.6 |
| 17 | Thymol | 1279 | - | 2.4 | - | - | 0.1 | - | - | 0.1 | 5.4 | - | - | 0.1 |
| 18 | *cis*-Ocimene | 1034 | 3.5 | 1.6 | 3.1 | 3.3 | 0.5 | 0.9 | 1.7 | 0.8 | 0.7 | 0.9 | 0.9 | 1.2 |
| 19 | Linalool | 1099 | - | 0.9 | 0.9 | 4.1 | 0.1 | 0.7 | 1.7 | 2.5 | 1.7 | 2.3 | 1.8 | 1.9 |
| 20 | *α*-Phellandrene | 1000 | 0.1 | 3.7 | 4.1 | 2 | 0.4 | 3.8 | 0.4 | - | 8.9 | 5.7 | 7.2 | 6.2 |
| 21 | Caryophyllene | 1402 | | 1.8 | 3.3 | 4.2 | 0.3 | - | 1.7 | 7.5 | 2.8 | 2.3 | 0.9 | 2.8 |
| 22 | Germacrene D | 1488 | 0.4 | 0.9 | 3.9 | 3.4 | 0.4 | **11.2** | 5.4 | 1.8 | - | 0.7 | 0.3 | 4.1 |
| 23 | Spathulenol | 1554 | - | 0.4 | 2.4 | - | - | - | 0.9 | 2.9 | 3.6 | 0.1 | 3.2 | 2.3 |

Plant growth regulators treatments/Fennel populations

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments (Continued)

| No. | Identified compounds | Retention indices | KHUR A | KHUR B | KHUR C | KHUR D | KAR A | KAR B | KAR C | KAR D | BON A | BON B | BON C | BON D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | - | 2.6 | - | - | - | - | - | - | - | 9.8 | - | 0.8 |
| 2 | Camphene | 951 | - | - | - | - | 2.3 | - | - | - | 6.3 | 4.6 | - | - |
| 3 | Sabinene | 973 | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | *β*-pinene | 976 | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | Myrcene | 988 | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | *p*-Cymene | 1021 | - | - | - | - | - | - | - | - | **16.1** | **20.7** | 1.1 | 1.4 |
| 7 | Limonene | 1026 | 3.7 | - | - | - | - | - | 4.4 | 2.4 | - | - | - | - |
| 8 | Cineol | 1028 | - | - | - | - | - | - | - | - | - | - | - | 0.2 |
| 9 | *γ*-Terpinene | 1056 | 3.5 | - | - | - | - | - | 0.8 | 0.7 | 0.9 | 1.0 | - | - |
| 10 | Camphor | 1144 | - | - | - | - | - | - | - | - | 8.3 | 7.4 | 0.5 | - |
| 11 | *trans*-Anethole | 1283 | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | Estragole | 1193 | - | - | 1.7 | 1.8 | - | - | 1.7 | 1.0 | - | 0.5 | - | - |
| 13 | Fenchone | 1083 | - | - | 1.7 | - | - | - | - | - | 8.2 | **15.2** | 1.2 | 0.1 |
| 14 | Borneol | 1174 | - | - | - | - | - | - | - | - | - | - | - | - |
| 15 | Fenchyl acetate | 1211 | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | Apiol | 1688 | 1.1 | - | - | - | - | - | 1.0 | - | - | - | 0.1 | - |
| 17 | Thymol | 1279 | - | - | - | - | - | - | - | - | - | - | - | - |
| 18 | *cis*-Ocimene | 1034 | - | - | - | - | - | - | - | - | - | - | - | - |
| 19 | Linalool | 1099 | - | - | - | - | - | - | - | - | - | - | - | - |
| 20 | *α*-Phellandrene | 1000 | - | - | - | - | - | - | - | - | - | - | - | - |
| 21 | Caryophyllene | 1402 | - | - | - | - | - | - | - | - | - | - | - | - |
| 22 | Germacrene D | 1488 | - | - | - | - | - | - | - | - | - | - | - | - |
| 23 | Spathulenol | 1554 | - | - | - | - | - | - | - | - | - | - | - | - |

Plant growth regulators treatments/Fennel populations

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments (Continued)

| No. | Identified compounds | Retention indices | BIR A | BIR B | BIR C | BIR D | TAT A | TAT B | TAT C | TAT D | KHOR A | KHOR B | KHOR C | KHOR D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | - | - | 0.2 | - | - | - | - | 0.2 | - | - | - | - |
| 2 | Camphene | 951 | - | - | - | - | - | - | - | - | - | 7.8 | - | 1.1 |
| 3 | Sabinene | 973 | - | - | - | - | - | - | - | - | 3.8 | 8.5 | - | 1.2 |
| 4 | *β*-pinene | 976 | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | Myrcene | 988 | - | - | - | - | - | - | - | - | 2.7 | - | - | - |
| 6 | *p*-Cymene | 1021 | - | - | - | - | - | - | - | - | - | - | - | - |
| 7 | Limonene | 1026 | - | - | - | - | - | - | - | - | **13.0** | **17.7** | 0.1 | 0.2 |
| 8 | Cineol | 1028 | - | - | - | - | - | - | - | - | - | - | - | - |
| 9 | *γ*-Terpinene | 1056 | 0.1 | - | 0.1 | - | 0.2 | - | - | - | 0.5 | - | - | - |
| 10 | Camphor | 1144 | - | - | - | - | - | **17.5** | - | 1.0 | - | - | - | - |
| 11 | *trans*-Anethole | 1283 | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | Estragole | 1193 | - | - | - | - | - | - | - | - | - | - | - | - |
| 13 | Fenchone | 1083 | - | - | - | - | - | - | - | - | **12.6** | 6.7 | 0.2 | 0.1 |
| 14 | Borneol | 1174 | - | - | - | - | - | - | - | - | - | - | - | - |
| 15 | Fenchyl acetate | 1211 | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | Apiol | 1688 | - | - | - | - | - | - | - | - | - | - | - | - |
| 17 | Thymol | 1279 | 6.8 | - | 0.4 | - | 5.3 | - | - | - | - | - | - | - |
| 18 | *cis*-Ocimene | 1034 | - | - | - | - | - | - | - | - | - | - | - | - |
| 19 | Linalool | 1099 | - | - | - | - | - | - | - | - | - | - | - | - |
| 20 | *α*-Phellandrene | 1000 | - | - | - | - | - | - | - | - | - | - | - | - |
| 21 | Caryophyllene | 1402 | - | - | - | - | - | - | - | - | - | - | - | - |
| 22 | Germacrene D | 1488 | - | - | - | - | - | - | - | - | - | - | - | - |
| 23 | Spathulenol | 1554 | - | - | - | - | - | - | - | - | - | - | - | - |

Plant growth regulators treatments/Fennel populations

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments (Continued)

| No. | Identified compounds | Retention indices | MOGH A | MOGH B | MOGH C | MOGH D | MESH A | MESH B | MESH C | MESH D | ZIYA A | ZIYA B | ZIYA C | ZIYA D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | 4.6 | **10.8** | 0.8 | 1.2 | - | - | - | - | - | **10.2** | - | - |
| 2 | Camphene | 951 | 3.4 | - | - | - | - | - | - | - | - | - | - | - |
| 3 | Sabinene | 973 | 1.1 | 0.4 | - | - | - | - | - | - | - | - | - | - |
| 4 | *β*-pinene | 976 | **16.3** | **20.1** | 1.1 | 1.5 | - | - | - | - | - | - | - | - |
| 5 | Myrcene | 988 | - | - | - | - | - | 3.0 | - | - | - | - | - | - |
| 6 | *p*-Cymene | 1021 | - | - | - | - | - | - | - | - | - | - | - | - |
| 7 | Limonene | 1026 | **11.3** | 9.6 | 0.2 | 0.2 | - | - | - | - | - | 4.4 | 0.3 | - |
| 8 | Cineol | 1028 | - | - | - | - | **16.0** | 8.1 | - | 0.1 | - | - | - | - |
| 9 | *γ*-Terpinene | 1056 | - | - | - | - | - | 2.6 | - | - | - | 2.4 | - | - |
| 10 | Camphor | 1144 | 4.6 | 7.6 | - | - | - | 1.9 | - | - | - | - | - | - |
| 11 | *trans*-Anethole | 1283 | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | Estragole | 1193 | **12.3** | - | - | - | - | - | - | - | - | - | - | - |
| 13 | Fenchone | 1083 | - | - | - | - | - | - | - | - | - | - | - | - |
| 14 | Borneol | 1174 | - | - | - | - | - | - | - | - | - | - | - | - |
| 15 | Fenchyl acetate | 1211 | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | Apiol | 1688 | - | - | - | - | - | - | - | - | - | - | - | - |
| 17 | Thymol | 1279 | - | - | - | - | - | **24.1** | - | - | - | - | - | - |
| 18 | *cis*-Ocimene | 1034 | - | - | - | - | - | - | - | - | - | - | - | - |
| 19 | Linalool | 1099 | - | - | - | - | - | - | - | - | - | - | - | - |
| 20 | *α*-Phellandrene | 1000 | - | - | - | - | - | - | - | - | - | - | - | - |
| 21 | Caryophyllene | 1402 | - | - | - | - | - | 6.1 | 4.6 | - | - | - | - | - |
| 22 | Germacrene D | 1488 | - | - | - | - | - | - | 1.0 | - | - | - | - | - |
| 23 | Spathulenol | 1554 | - | - | - | - | - | - | - | - | - | - | - | - |

Plant growth regulators treatments/Fennel populations

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

**Table 2.** Chemical composition of the callus of fennel populations under various plant growth regulators treatments (Continued)

| No. | Identified compounds | Retention indices | NEY A | NEY B | NEY C | NEY D | HAM A | HAM B | HAM C | HAM D |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *α*-Pinene | 937 | 3.5 | 3.2 | 0.3 | 0.2 | 4.1 | - | 0.1 | - |
| 2 | Camphene | 951 | - | - | - | - | - | - | - | - |
| 3 | Sabinene | 973 | - | - | 0.1 | - | - | - | 0.1 | - |
| 4 | *β*-pinene | 976 | - | - | - | - | 7.5 | - | - | - |
| 5 | Myrcene | 988 | - | 0.6 | - | - | - | - | - | - |
| 6 | *p*-Cymene | 1021 | - | - | - | - | **24.5** | **18.9** | 2.0 | 1.2 |
| 7 | Limonene | 1026 | - | - | - | - | - | **14.3** | - | - |
| 8 | Cineol | 1028 | 3.6 | 4.4 | 0.3 | 0.2 | - | 6.2 | - | - |
| 9 | *γ*-Terpinene | 1056 | 1.1 | 0.9 | - | - | - | - | - | - |
| 10 | Camphor | 1144 | - | 2.6 | - | - | - | - | 0.2 | - |
| 11 | *trans*-Anethole | 1283 | - | - | - | - | - | - | - | - |
| 12 | Estragole | 1193 | - | - | - | - | 6.9 | - | - | - |
| 13 | Fenchone | 1083 | - | - | - | - | - | 7.1 | - | - |
| 14 | Borneol | 1174 | 2.4 | 4.7 | 0.3 | 0.4 | - | - | - | - |
| 15 | Fenchyl acetate | 1211 | - | - | - | - | - | - | - | 0.1 |
| 16 | Apiol | 1688 | - | - | - | - | - | - | - | - |
| 17 | Thymol | 1279 | **39.3** | **38.4** | 2.2 | 2.0 | - | - | - | - |
| 18 | *cis*-Ocimene | 1034 | - | - | - | - | - | - | - | - |
| 19 | Linalool | 1099 | - | - | - | - | - | - | - | - |
| 20 | *α*-Phellandrene | 1000 | - | - | - | - | - | - | - | - |
| 21 | Caryophyllene | 1402 | 2.7 | 5.0 | 0.3 | - | - | - | - | - |
| 22 | Germacrene D | 1488 | 1.0 | 2.0 | - | - | - | - | - | - |
| 23 | Spathulenol | 1554 | 1.2 | 1.1 | - | - | - | - | - | - |

Columns: Plant growth regulators treatments/Fennel populations.

* **A:** 1 mg/L NAA + 1 mg/L BA, **B:** 1 mg/L 2,4-D + 1 mg/LKin, **C:** 0.5 mg/L NAA + 1 mg/L BA, **D:** 0.5 mg/L 2,4-D + 1 mg/L Kin

### *3.2. PCA and cluster analyses*

The principal component analysis (PCA) and clustering were used to identify possible relationships among the fennel populations based on the chemical composition of their calli. The calli extract compounds more than 10 % were subjected to the PCA and cluster analysis. The PCA analysis separated the fennel populations in four major groups based on their chemical compositions (Fig. 6, 7). The PC1 accounted positive correlation with *α*-pinene, camphene, sabinene, *β*-pinene, *p*-cymene, limonene, *γ*-terpinene, camphor, *trans*-anethole, estragole, fenchone and linalool and negative correlation with cineol, apiol, thymol and caryophyllene. The PC2 possessed positive correlation with camphene, *p*-cymene, cineol, camphor, fenchone, and thymol and the negative correlation with *α*-pinene, sabinene, -pinene, limonene, *γ*-terpinene, *trans*-anethole, estragole, apiol, linalool and caryophyllene. The PCA analysis confirmed the cluster analysis. According to the cluster analysis, fennel populations were divided to the four major groups. Group I consisted of MESH and NEY populations contained high amount of thymol. Group II formed of only GER2 population, containing high amount of linalool and fenchone. Group III made up of TUR1 and HAM with high amount of limonene. Other populations were clustered in group IV, containing other major compounds. The PCA and cluster analysis are the multivariate analyses which are used to determine the possible relationships among plant populations based on their morphological, phytochemical and molecular traits. This helps to select the superior populations of a species for multiple purposes in the breeding programs. Present study revealed that the studied populations of fennel clustered in four chemotypes.

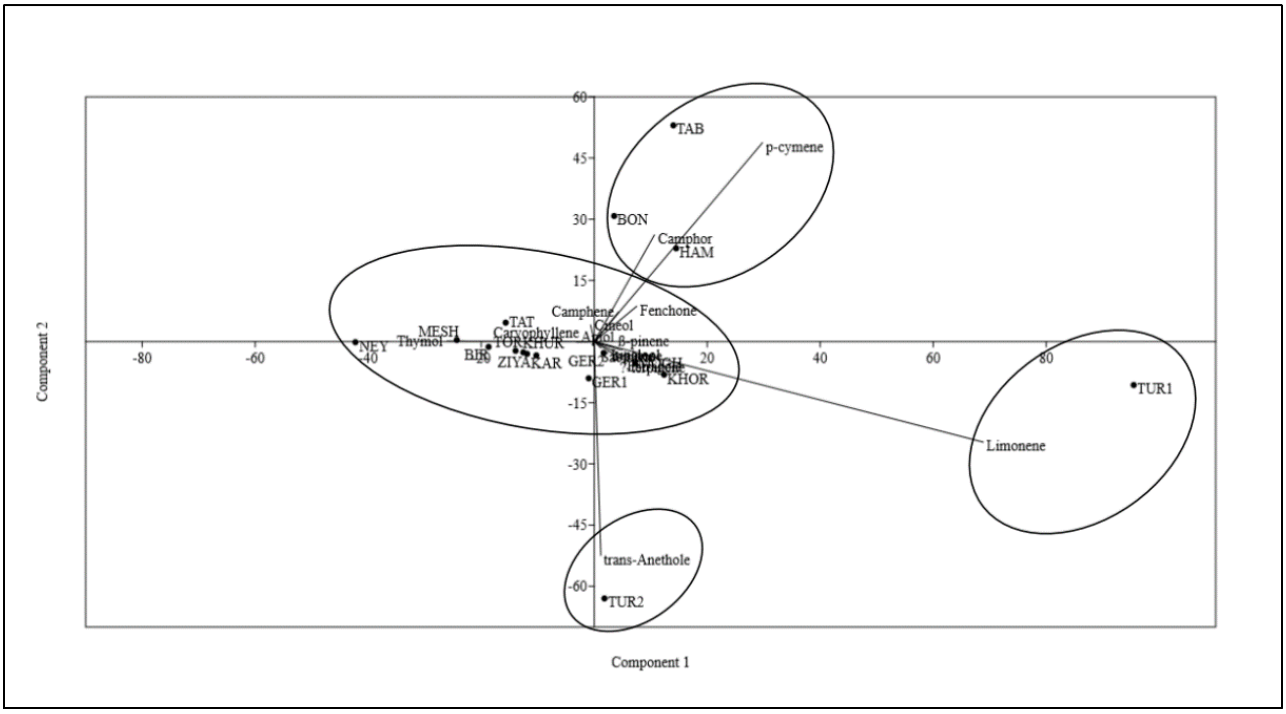


**Fig. 6.** Principal component analysis (PCA) of the fennel populations based on the chemical composition of their callus extracts

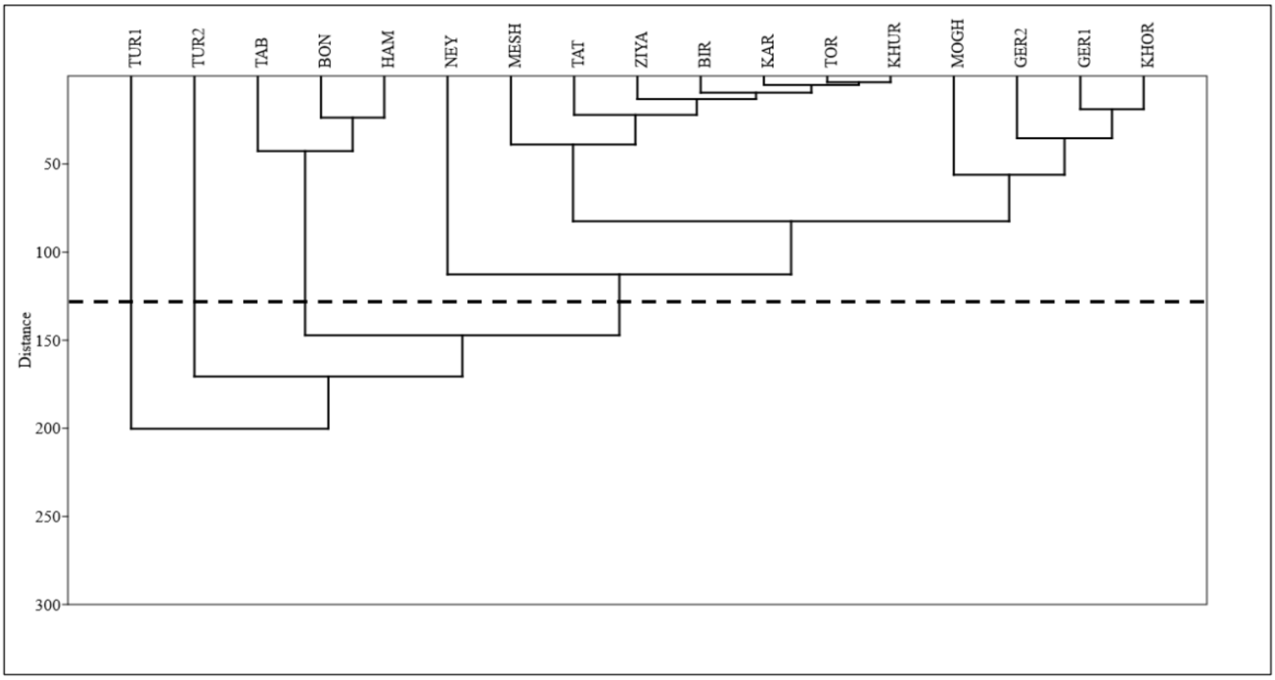


**Fig. 7.** Cluster analysis of the fennel populations based on the chemical compositions of their callus extracts

## 4. Discussion

In the present study, the proper callus was obtained from hypocotyl explant. Among the five root explants, hypocotyl, epicotyl, cotyledon and leaf of grown seedlings (up to 15 cm height), the hypocotyl explant had the best response to callus formation. The root explant and epicotyl were removed from the experiment due to weak callus formation, and the leaf explant due to lack of response. Cotyledon explants had acceptable callus formation, but due to the absence of secondary metabolites, they were excluded from the experiment. Therefore, the hypocotyl explant was selected due to its suitable callus production for the production of callus and secondary metabolites in all populations. In a study, the hypocotyl explant was introduced as the best explant in calligenesis [16]. In another study, hypocotyl was introduced as the best explant for callus formation, which was consistent with the results of this study [17]. In this study a high percentage of *β*-pinene, *p*-cymene, limonene, camphor, *trans*-anethole and thymol were found at least in a population. In a study, in the callus extract obtained from the hypocotyl, in the culture medium containing an equal combination of two growth regulators 2,4-D + Kin (0.5 mg/L), a value of 5.28 % of *trans*anthol was observed, while in a ratio of 0.5 to 1 This treatment obtained 98.72 % of this substance [17]. In another study, the highest amount of *trans*-antol was produced in fennel callus by applying growth regulator treatment NAA + BA (with a ratio of 0.1 to 1-0.1 mg/L) [18]. Callus extract of Izmir population contained a large amount (67.70 %) of limonene in the ratio of 0.5 to 1 mg/L of treatment 2,4-D + Kin. All four foreign populations contained limonene under all growth regulator treatments. The calli of seven Iranian populations, including Torbat Jam, Khor and Biabank, Karaj, Khorramabad, Khoroslari, Ziyar and Hamadan, had limonene, and Khorramabad has the highest percentage (17.70) with a ratio of 1 to 1 mg/L in the treatment 2,4-D + Kin. Alphapinene, Camphene, Sabinene, Paracimen, Cineol, Camphor, Estragole, Fanchone, Codeine, Linalool, Flavylium, Carne and Thymol were other effective substances that were observed in a relatively significant amount in the callus of some treatments. In the case of the main active ingredient of fennel (*trans*-anthole), the superiority of NAA+BA combination compared to 2,4-D+Kin was clearly evident. NAA in low doses increases the secretory channels and stimulates their activity. On the other hand, the type and amount of auxin or cytokinin, or the ratio of auxin to cytokinin, changes the formation and accumulation of secondary metabolites in cultured plant cells [18]. In a study, in addition to *trans*-anthole, estragole, fenchone, limonene, fenchyl acetate, anisaldehyde, camphor, carne, simene and alphapinene were also identified in fennel callus extract [17].

Several factors including type of the explants, media composition, environmental conditions and different population can affect callogenesis and regeneration process. Researchers have proved that hypocotyl is a suitable candidate explants for callogenesis in fennel population. Similar to previous works, our study showed that hypocotyl explants obtained from different endemic and exotic populations are more prone for callus production, in compared with other tested explants [19]. The positive response of hypocotyl explants to callogenesis would be an advantage, since high quality calli derived from that would be utilized in the next works such as suspension [20].

Previous works have been proved the effect of different plant regulators on the relative quantity structure of the essential oils from calli which they are originated from hypocotyl explants [19]. Our results showed a high variation among Iranian and exotic populations for the majority of the secondary metabolites [21,22]. The possible reasons for such variation in the accumulation of various classes of metabolites in *in-vitro* cultures have been discussed in many studies [19,21,23]. In contrast, in present study the highest *trans*-anethole percentage was obtained in the callus of exotic population; TUR1 population under 1 mg/L NAA + 1 mg/L BA treatment, which is disagreement with previous reports. Choice of population, composition of culture medium, callus friability, and explants origin from different organs are some of numerous factors which may affect the levels of target metabolites [6]. The results indicated an interaction among populations and growth regulator treatments. However, in both growth regulator treatments, (1:1 ratio) was more effective than (0.5:1 ratio) in production of secondary metabolites.

The PCA and cluster analysis are the multivariate analyses which are used to determine the possible relationships among plant populations based on their morphological, phytochemical and molecular traits. This helps to select the superior populations of a species for multiple purposes in the breeding programs.

## 5. Conclusion

In present study considerable amounts of limonene (67.70 %) under 0.5 mg/L 2,4-D + 1 mg/L Kin treatment and 18.3 % under 1 mg/L 2,4-D + 1 mg/L Kin treatment, which never been previously reported in fennel callus. For considering the main medicinal compound of fennel (*trans*-anethole), NAA + BA was more superior compared with 2,4-D + Kin treatment. According to the results, Turkish populations had the capacity to produce considerable amounts of main secondary metabolites.

## Author contributions

M.A.: contributed to the conception of the study, data collection, interpretation of data, drafting the manuscript. M.S.N.: supervised the study, formal analysis, reviewing and editing the manuscript. S.A.: helped in reviewing and editing the manuscript. S.A.M.: supervised the study, contributed in phytochemical analysis, data curation, writing and editing the manuscript.

## Conflict of interest

The authors declare that there is no conflict of interest.

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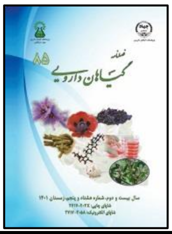

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تنظیم‌کننده رشد گیاهی

**چکیده**

**مقدمه**: گیاه چند ساله رازیانه یکی از مهم‌ترین و پرمصرف‌ترین گیاهان دارویی خانواده چتریان می‌باشد که عمدتا به منظور استفاده از اسانس حاصل از آن در صنایع دارویی، غذایی، آرایشی و بهداشتی کشت می‌شود. **هدف**: برای بررسی کالوس‌زایی و تولید متابولیت‌های ثانویه از کالوس‌ها، ۱۳ جمعیت رازیانه ایرانی و همچنین دو جمعیت از آلمان و دو جمعیت از ترکیه توسط کروماتوگرافی گازی متصل به طیف‌سنج جرمی مورد ارزیابی قرار گرفتند. **روش بررسی:** پنج نوع ریزنمونه (برگ، هیپوکوتیل، اپیکوتیل، لپه و قطعات ریشه) در محیط کشت MS همراه با ترکیب 2,4-D و کینتین (۱:۱)، همچنین NAA و BA (۰/۵: ۱) به عنوان مکمل، کشت داده شدند. **نتایج**: از بین پنج ریزنمونه مورد استفاده در این مطالعه، تنها ریزنمونه هیپوکوتیل پاسخ مناسبی به کالوس‌زایی داشت. بر اساس نتایج کروماتوگرافی گازی متصل به طیف‌سنج جرمی، بیشترین میزان ترانس- آنتول (۶۷/۲۳ درصد) در کالوس جمعیت ترکیه (TUR1) تحت تیمار NAA و BA (۱:۱) تولید شد. عصاره کالوس جمعیت دیگر ترکیه (TUR2) حاوی مقادیر قابل توجهی لیمونن (۶۷/۷۰ درصد) تحت تیمار 2,4-D و کینتین (۰/۵: ۱) بود. **نتیجه‌گیری**: القای کالوس با تنظیم‌کننده‌های مختلف گیاهی می‌تواند سهم قابل‌توجهی در تولید متابولیت‌های ثانویه داشته باشد، بدین ترتیب که از کالوسهایی که متابولیتهای ثانویه بیشتری تولید می‌کنند در سیستم‌های بیوراکتور کشت سوسپانسیون یا سلولی داده شود. براساس نتایج حاصل از این پژوهش، جمعیت‌های رازیانه مربوط به ترکیه ظرفیت تولید مقادیر قابل توجهی متابولیت های ثانویه اصلی را داشتند.

*مخففها:* GC-MS، کروماتوگرافی گازی متصل به طیف‌سنج جرمی؛ محیط MS، محیط کشت موراشیگه و اسکوگ؛ 2,4-D، ۲،۴- دی کلرو فنوکسی استیک اسید؛ NAA، نفتالین استیک اسید؛ BA، بنزیل آدنین

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