Phytochemical profiling of volatile components of *Echinacea purpurea* (L.) Moench multiplied by micropropagation technique

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ABSTRACT *Echinacea purpurea* (L.) Moench is a substantial herbaceous perennial species of ornamental and medicinal flowering plant that grows to 120 cm in maturity from the Asteraceae family. The development of *in vitro* protocols for this plant can have a significant impact on the global deployment of this beneficial germplasm. It will also facilitate the rapid multiplication, biotransformation, and genetic transformation of improved phytochemical production, particularly secondary metabolites. All stages of *E. purpurea* micropropagation *in vitro* and acclimatization to *in vivo* conditions were investigated in this study. Furthermore, the obtained volatile components of the plants were determined in this study. The research findings revealed that the best seed sterilization was observed with 15 % commercial bleach for 5 minutes. Murashige and Skoog (MS) plant tissue culture medium including 1.25 mg L^{-1} 6-benzyl amino purine was suitable for regeneration and MS medium containing 1.00 mg L^{-1} indole-3-butyric acid was appropriate for rooting. Furthermore, the findings show that peat is an appropriate substance for acclimation of rooted plantlets. Germacrene D, caryophyllene, Borneol, Caryophyllene oxide, and Myrtenal were identified as the main volatile components of *E. purpurea* by GC/MS and GC/FID analysis.

KEYWORDS Acclimatisation; Purple coneflower tissue culture; Volatile ingredient

1. Introduction

The perennial herb *Echinacea purpurea* (L.) Moench (purple coneflower) belongs to Asteraceae family, which is indigenous to the central and eastern United States and cultivated in Europe (Wu et al., 2018; Mokhtarzadeh et al., 2013a). *Echinacea* has nine plant species that *E. pallida* (Nutt.) Nutt, *E. angustifolia* DC var. *angustifolia* and, *E. purpurea* (L.) Moench are the most commonly used in medical research (McGregor, 1968; Wang and To, 2004; Waidyanatha et al., 2020; Burlou-Nagy et al., 2022). *Echinacea* species are mainly used for herbal medicine production (Wu et al., 2018; Cui et al., 2013; Percival, 2000). Encapsulated powders and extracts from the roots and aerial parts of *E. purpurea* and *E. angustifolia* have been used to treat wounds, snakebites, headaches, and flue in North America (Nieri et al., 2003). In addition, extracts of *Echinacea* species have antioxidant, antiviral, antibacterial, and antifungal properties and are widely used to treat flue, respiratory, and urinary tract diseases (Wu et al., 2018; Barrett, 2003). *Echinacea* sp. was introduced to Europe as a medicinal plant in the mid-twentieth century and the main product of that was made from the pressed juice of the aerial part of the purple coneflower or the hydroalcoholic extract of the root of the purple coneflower and was used to fight infections and stimulate the immune system. Currently, whole plants and extracts of *E. purpurea* supply one of the largest segments of the European and North American herbal medicine market (Wang and To, 2004; Chuang et al., 2009; Mokhtarzadeh et al., 2013a). For the large-scale production of beneficial plant metabolites, plant tissue culture has been proven to be an effective method for phytochemical biosynthesis and bioreactor systems (Wu et al., 2018; Jeong et al., 2009). Four economically significant purple coneflower species (*E. angustifolia*, *E. pallida*, *E. paradoxa* and *E. purpurea*) have recently undergone *in vitro* mass propagation using axillary buds, adventitious buds, and adventitious embryos (Lakshmanan et al., 2002; Wang and To, 2004). Due to the reported rapid decline in wild *E. purpurea* populations due to habitat loss and deforestation, medicinal *E. purpurea* is being grown to supply the market (Mechanda et al., 2004). There is also insufficient information on *Echinacea* species regeneration and tissue culture, compared to pharmacological data and clinical studies. For *in vitro* multiplication and regeneration from *E. purpura* leaf explants, employing tissue culture methods is advised (Choffe et al., 2000; Wang and To, 2004; Mokhtarzadeh et al., 2013a). However, *E. purpurea* plants frequently outcross and give rise to progeny that are largely heterozygous in the field conditions. Effective *in vitro* regeneration techniques for genetically comparable *E. purpurea* plants with desired agronomic and chemical properties must be created to fulfill the growing market demand (Abbasi et al., 2007; Jones et al., 2006; Chuang et al., 2009). Therefore, in this research all stages of micropropagation of *E. purpurea* in *in vitro* conditions and its acclimatisation to field conditions were investigated and the volatile components of the plants obtained via plant tissue culture method identified.

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2. Methods

2.1 Plant material

E. purpurea seeds used in the experiment were collected by farmers interested in the cultivation of medicinal and aromatic plants in the Mediterranean region of Turkiye in the agricultural year 2020-2021.

2.2 Methods of tissue culture, culture conditions and acclimatisation

To obtain sterilized plantlets, seeds of *E. purpurea* (L.) Moench were surface-sterilized with different concentrations of commercial bleach (Ace®, Turkiye) (5, 10, and 15 %) for five minutes and rinsed three times with sterile water for five minutes before germinating on medium (Murashige and Skoog, 1962). The primary leaf of the germinated seeds was then harvested and incubated in MS medium containing 0.75, 1.00, 1.25, 1.50 and 2.00 mg L^{-1} 6-benzyl amino purine (BAP). Shoots showing good regenerative development *in vitro* were aseptically cut and rooted in MS medium including 1.00, 1.50 and 2.00 mg L^{-1} Indole-3-butyric acid (IBA). In addition, all media pH was regulated to 5.6-5.8 before autoclaving and 35 ml of media was poured into each culture dish. All cultures were maintained at 16 h cool white fluorescent light photoperiod with 24 °C. Rooted shoots were adapted to *in vivo* conditions using four different growth materials. First, the base of microgrown sprouts was soaked in MS medium containing 1.00 mg L^{-1} IBA. To support the development and maintenance of established roots in micropropagated shoots, cultures were refreshed weekly. During testing, the pH was also adjusted to 5.6-5.8 before autoclaving at 121 °C for 20 minutes. Each culture contained ten explants, 24 °C under 5000 lux intensity light conditions provided by a growth chamber with a 16/8 h day/night photoperiod and 4 replicates. On the other hand, perlite was sterilized for two hours at 160 °C before being used in an experiment. To remove excess water, a hole was made in the bottom of each pot and watered well and humidified to the desired level. Each replicate contained ten explants and was incubated in four replications at 24 °C under 5000 lux intensity light conditions provided by a growth chamber with a 16/8 h day and night photoperiod. Third, a 1:1 mixture of clay and peat was sterilized at 160 °C for two hours. In addition, like the first and second methods, ten explants were included in each replication, which was incubated with four repetitions at 24 °C and 5000 lux of light intensity from a growth room with a 16/8 h day and night photoperiod. Also, the fourth ingredient, peat, was sterilized at 160 °C for two hours. Ten explants were used in each replication, which was carried out in four replicates at 24 °C and 5000 lux of light intensity provided by a growth environment with a 16/8 h day and night photoperiod. The seedlings were placed in pots on the substrate mentioned above, and then were covered with plastic bags. This bag assisted in maintaining optimal moisture conditions during plantlet acclimatization. In this study, the pots were carefully placed in 24 °C to receive the majority of the diffused light. During the trial the bags were opened each day to let fresh air into the cuttings and prevent mold growth, for a few minutes. Adapted plants were carefully removed from their pots without removing peat from their roots, and plants showing signs of growth (shoot elongation and sprouting of new leaves) were watered and developments were followed.

2.3 Isolation and analysis of the volatiles components

After aforementioned process, growing plants were transferred to *in vivo* condition and then samples were collected to analyze the volatile components. In order to make chemical analysis after the adaptation of the plants, the aerial parts of the plants (with and without flower) were dried at 30 °C for 14 days. Then, 100 g of the obtained dry materials were weighed and subjected to water distillation in the Clevenger apparatus for 3 hours according the PhEur 8.0 with modifications (Council of Europe, 2014). Also the organic layer (n-hexane) in the collecting vial was separated after distillation and then submitted to simultaneous analysis by GC-FID and GC-MS instruments.

2.4 Gas chromatography-mass spectrometry (GC-MS) analysis

In the course of the research a GC-MSD system from Agilent ®, model number 5973, was used for the Gas Chromatography-Mass Spectrometry analysis. An Innowax FSC column received helium as the carrier gas at a rate of 0.8 mL min^{-1} (60 m×0.25 mm, 0.25 mm film thickness). The temperature of the GC oven maintenance done at 60 °C for 10 minutes before being set to 220 °C at a rate of 4 °C min-1, also maintained at 220 °C for 10 minutes, and then increased to 240 °C at a rate of 1 °C min^{-1}. Throughout the analysis injector temperature was used as 250 °C. At 70 eV, mass spectra were captured and the mass range was M/z 35 to 450.

2.5 Gas chromatography-flame ionization detector (GC-FID) analysis

An Agilent 6890N GC system with a 300 °C FID detector temperature was used during the research for Gas Chromatography analysis. Simultaneous auto-injection was performed on a duplicate of the same column under the same operational parameters in order to acquire the same elution order with GC-MS. Relative percentage quantities of the separated chemicals were computed from FID chromatograms.

2.6 Identification of volatile compounds

Volatile components using inhibition indices and mass spectrometry of their combinations were identified and then compared with standard mass spectra available in computer libraries and authoritative sources.

3. Results

The results showed that the best seed sterilization was observed with 15 % commercial bleach for 5 min. After germination of primary leaf explant cultured on different concentration of MS medium including BAP high shoot regeneration was observed in MS medium including 1.25 mg L^{-1} BAP. In addition, well-developed *in*

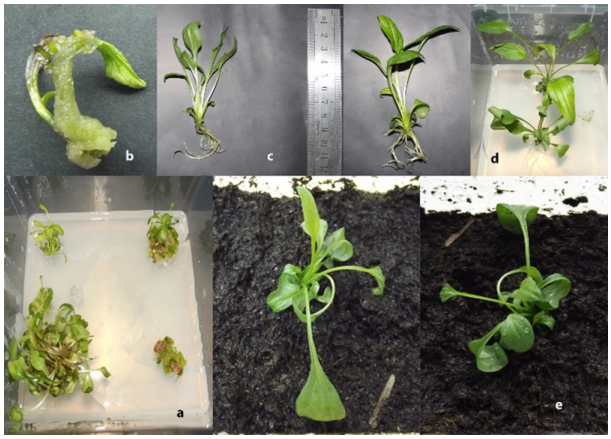

Figure 1: (a) Regeneration of leaf explant, (b) Calli and leaf regeneration, (c) Rooting (d) Regenerated shoots rooted on MS medium containing 1.00 mg/L IBA. (e) Acclimatisation.

vitro regenerated shoots were sprouted and rooted on MS medium containing 1.00 mg L^{-1} IBA.

In studies about plant tissue culture and *in vitro* research, acclimatization process and transferring of plants to a suitable substrate during acclimatization is necessary. In this research plantlets with roots were grown in liquid MS media including 1.00 mg L^{-1} IBA to establish a permissive *in vitro* micropropagation system of *Echinacea purpurea* L. Moench from regenerated primary leaf shoots. No progress on plantlets was noted and followed by chlorosis and necrosis. For a lengthy time period (4-5 weeks), the developing tissues were unable to survive, and they eventually perished. The plantlets died after ten days suffocated, since all treatments had a negative impact.

On the other hand, the optimal rooting medium for cuttings from ornamental and medicinal plants is perlite. Both the perlite and claypeat mix regenerated shoots in the study exhibited symptoms of degradation and perished 30 days after being cultivated. But since the soil substrate was retaining water, the lower end of the stem was unable to breathe properly, causing plant suffocation, browning, and the death of the roots in addition to the withering and destruction of the stems and the speedy death of the plantlets in 30 days. Peat, however, appears to be the most adaptable substance for the rooted plantlets. There were about 20 plants transferred to the soil, and all of them had evident signs of development and growth. Every plant received water after seven days (Fig. 1).

In the study, flowering and non-flowering samples were collected from the aerial parts of growing plants and were subjected to volatile components analysis. As a result, Thirty-four volatile compounds of aerial parts of micpropagated *E. purpurea* were identified representing 97.79 % for flowering samples and 74.38 % for non-flowering samples by GC and GC-MS systems, simultaneously. Major compounds for flowering samples included Germacrene D (44.25 %), β-Caryophyllen (6.05 %), Borneol (5.02 %), Caryophyllene oxide (4.81 %), Myrtenal (3.49 %). Also, Major compounds for non-flowering samples included Germacrene D (39 %), β-Caryophyllen (5.30 %), Borneol (2.09 %), Caryophyllene oxide (2.99 %), Myrtenal (2.57 %). Detailed information is given in Table 1.

Table 1: The composition of volatile compounds of aerial parts of micropropagated.

No.	Compound	RRI[a]	%[b]	%[c]
1	α-pinene	933	1.57	1.22
2	β-pinene	978	1.85	1.36
3	Myrcene	990	2.30	0.91
4	α-Phellandrene	1005	1.11	0.80
5	p-Cymene	1023	2.66	1.43
6	Limonene	1026	0.69	0.51
7	γ-terpinene	1059	1.89	1.39
8	cis-Sabinene hydrate	1067	0.38	0.30
9	Terpinolene	1089	0.92	0.74
10	Trans-pinocarveol	1136	1.83	0.98
11	Pinocarvene	1162	1.78	1.31
12	Borneol	1166	5.02	2.09
13	Terpinene-4-ol	1175	1.47	0.93
14	β-Caryophyllene	1182	6.05	5.3
15	α-terpineol	1192	0.86	0.78
16	Myrtenal	1196	3.49	2.57
17	n-Dodecane	1199	0.61	0.45
18	Carvacrol methyl ether	1241	0.42	0.36
19	Thymol	1293	0.54	0.49
20	Carvacrol	1297	0.90	0.86
21	α-Copaene	1377	0.82	0.76
22	β-Elemene	1392	0.56	0.48
23	Aromadendrene	1438	0.45	0.31
24	α-Humulene	1454	1.23	0.36
25	Germacrene D	1481	44.25	39
26	Bicyclogermacrene	1497	0.44	0.31
27	β-Bisabolene	1506	2.89	1.26
28	γ-Cadinene	1512	0.38	0.30
29	α-Cadinene	1539	2.63	1.58
30	Caryophyllenyl alcohol	1569	0.77	0.42
31	Spathulenol	1577	0.69	0.43
32	Caryophyllene oxide	1581	4.81	2.99
33	Humulene epoxide II	1610	0.82	0.77
34	α-Cadinol	1652	0.71	0.63
Total			97.79	74.38

RRI[a]: Relative retention indices calculated against n-alkanes; % [b]: The aerial parts of plant with flower calculated from FID data, % [c]: The aerial parts of plant without flower calculated from FID data.

The findings of this paper are similar to data of following articles:

Navarro et al. (1994) discovered that increasing CO2 concentration promoted dry matter accumulation *in vitro* in banana plantlets. After moving from laboratory circumstances to outdoor conditions, plantlets adjust to the aforementioned anomalies, according to Fila et al. (1998) the radiation on boats is substantially stronger than in greenhouses or fields, and the air is much less humid. The presence of sucrose gives the substrate a higher water potential than the medium, which causes the developing plants to wilt quickly and apt to unchecked transpiration from the

leaves. In addition, the low hydraulic conductivity of root and basal junctions can limit the amount of available water. According to Pospíšilová et al. (1992) after adaptation to field conditions, generally, leaf thickness expands, leaf mesophilia improves by differentiation into palisade parenchyma, stomatal form becomes elliptical from circular, stomatal density decreases, and the leaves of plantlets after being transferred to the soil showed vigorous growth. Koroch et al. (2002) reported that purple coneflower callus cultures produced from leaf tissue showed effective plant regeneration. Proliferating shoot cultures with a high number of shoots per explant (7.7 shoots/explant) were obtained by placing leaf explants on MS medium containing BAP (4.44 M) and NAA (0.054 M) combinations that were the most effective medium and provided high shoot regeneration frequencies (100 %). According to the results, plantlets were rooted on normal MS medium or with various concentrations of IBA and high rooting and survival were achieved using MS media without plant growth regulators. Lucchesini et al. (2009) reported that regenerated callus was formed from the leaves of shoots cultured under *in vitro* condition in CH medium including 3.00 mg L^{-1} BAP for rejenerating and 0.5 mg L^{-1} IBA or rooting. Also, shoots developed from callus cultures were subcultured on CH medium with 0.5 mg L^{-1} BAP concentration. Hanafy et al. (2010) described, shoot tips and leaves of *Echinacea purpurea* into small segments incubated in MS medium with varying concentrations of NAA and BAP. For two months, cultures were incubated in the same conditions. Regenerated shoots (3-4 cm in height) were rooted on MS containing varying concentrations of IBA ranging from 4.92-14.76 µm. Mokhtarzadeh et al. (2013b) suggested that every step after rooting, such as transferring the plantlets to pots and fields, and adaptation are of high importance. It is reported that the Lavenders were transferred to field after a difficult procedure of adaptation and optimization using hydroponic culture, sterilized sand, clay, peat, and their mixture. Chen et al. (2013) demonstrated that regeneration and growth of regenerated buds of *E. purpurea* plants could be effectively improved by using DA-6 at a concentration of 0.16 mg L^{-1}. Yang et al. (2015) discovered that MS medium containing 0.5 mg L^{-1} BAP was the best medium for adventitious buds. Furthermore, when MS medium contained only 0.5 mg L^{-1} BAP used for *in vitro* culture of purple coneflower, the multiplication efficiency of adventitious buds was high, and the regenerated buds were robust. According to Nyalambisa et al. (2017), GC/GC-MS analysis revealed that the number of compounds identified in the leaf and root oils of *E. purpurea* were 25 and 31, respectively. Also Geracrene D, naphthalene, caryophyllene oxide, phellandrene, and cadinol were among the major compounds identified in the oils. According to Liu et al. (2019), MS was the best basic medium for inducing adventitious bud regeneration from leaf explants in *E. purpurea*. Induction of adventitious buds was more effective (90 %) when the $CuSO_4$ concentration was 0.5 mg L^{-1} meanwhile, MS medium containing 0.5 mg L^{-1} 6-benzylaminopurine was found to be the best culture medium for the proliferation of adventitious buds. Lucchesini et al. (2019) reported that a liquid sugarless MS medium that contained 1 mg L^{-1} indole-3-butyric acid is appropriate for *in vitro* rooting phase of Echinacea angustifolia. Kaya et al. (2019) observed the identified volatile in Echinacea as total was 92 including monoterpenes, monoterpenoids, sesquiterpenes, sesquiterpenoids and other hydrocarbons. According to Maggini et al. (2020), the differences in the volatile profiles of *Echinacea purpurea* plants were analyzed and compared by GC-FID/GC-MS in *in vitro* condition and non-terpenes and sesquiterpene hydrocarbons were as the most abundant classes. Additionally, in the study, the primary substances recovered from EpS/L were (Z)-8-dodecen-1-ol and 1-pentadecene, whereas EpC and EpR's main substances were germacrene D and caryophyllene. (Yildiz et al., 2021) to determine aroma compounds of oven-dried and fresh thyme and investigate the drying effect in key aroma compounds of thyme samples have done an experiment. In that research according to Gas Chromatography-Olfactometry (GC-O) and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis results, lower number of volatile compounds for dried thyme identified, while more volatile components found in fresh thyme. It also observed that, based on GC-O data, the process of drying influenced the FD factors. Ahmadi et al. (2021) reported that the major volatile components of *E. purpurea* are germacrene D (51 %), myrcene (15 %), α-pinene (12 %), β-caryophyllene (11 %), and 1-Pentadecene (4.4 %). Soltanbeigi and Maral (2022) observed that sesquiterpene hydrocarbons were the most abundant chemical group compound of *E. purpurea* essential oil and Germacrene D (20.4-50.6 %) was the predominant constituent. According to this report other major compounds were β-pinene, β-myrcene, α-humulene, δ-cadinene, spathulenol, and α-cadinol.

4. Conclusions

There are a few breeding methods to obtain the high yield and yield components, as well as the variety of diseases and herbicides in this plant, however, a few studies have been carried out on it in Turkiye. Therefore, the findings of this paper about seed germination, shoot regeneration, rooting and acclimatisation can be used for further studies on purple coneflower. Moreover, the adaptation of purple coneflower plants in *in vitro* conditions and the use of obtained data will be effortless to cultivate and acclimate transgenic plants of *Echinacea purpurea*. In addition, determination of *Echinacea purpurea* volatile components in this study obtained by micropropagated samples could be attractive data for scientists interested in secondary metabolites.

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