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Determination of Essential Oil Bioactive Components and Rosmarinic Acid of *Salvia officinalis* Cultivated under Different Intra-row Spacing

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Abstract

Salvia officinalis, known also as sage, is a medicinal plant belonging to the *Lamiaceae* family that spreads all over the word in several countries. The demand for the raw material and extracts of this plant is increasing due to its numerous applications in pharmacy, food and herbal tea production. The present study investigated for the first time the effect of 15, 30 and 45 cm intra-row spacing (plant density) on the main constituents of sage essential oils and rosmarinic acid content. The highest content of essential oils (2.7%) and rosmarinic acid (2.0%) were obtained in plants grown using 15 cm planting space. Likewise, close spacing resulted also in a substantial content of 1,8-cineole (47-50%, GC/FID; 55-60%, GC/MS). This work indicated that 1,8-cineole chemotype was a dominant character of cultivated *S. officinalis* in south of Jordan. In general, the percent of α-thujone in essential oil was not affected by intra-row spacing. However, the percent of β-thujone decreased from (2-3%, GC/MS) in plants grown using 15 cm intra-row spacing to (1-2%, GC/MS) in plants grown using 30 and 45 cm intra-row spacing. The highest content of α-and β-pinene was recorded in plants grown using 45 cm planting space (8-10%, GC/FID; 5-6% GC/MS). Based on GC/MS, camphor compound was enriched (9-10%) in sage plants grown under 15 cm spacing and greater than in plants grown under 30 (6-7%) or 45 cm (5-6%) spacing. The results make the potential use of sage extracts in the treatment of some human disorders or illness an area of further research.

Keywords: 1,8-cineole, GC/FID, GC/MS, population density, sage

Introduction

The genus *Salvia* includes about 900 species that grow in several regions all over the world (Kintzios, 2000). *S. officinalis* L., one of the most important species of the genus Salvia, is a medicinal and aromatic plant that has been used as a flavoring agent as well as in cosmetic-, perfume- and folk medicine-industries since ancient times (de Vincenzi *et al.*, 1997; Lu and Foo, 2002; Tisserand and Balacs, 1995).

S. officinalis contains many biologically active substances like essential oils, phenolic acids, flavones, phenylpropanoid glycosides, tannins and others (Capek *et al.*, 2003; Hohmann *et al.*, 2003; Leung, 1980; Miura *et al.*, 2001; Ninomiya *et al.*, 2004). The healing and therapeutic properties of *S. officinalis* have been largely attributed to its content of essential oils. Antibacterial and antiviral activities of *S. officinalis* essential oils were mainly related to camphor, α-and β-thujone and 1,8-cineole (Sivropupoulou *et al.*, 1997) which have been also reported to be the major constituents of *S. officinalis* essential oils (Bettaieb *et al.*, 2009; Boelens, 1997; Chalchat *et al.*, 1998; Khalil *et al.*, 2008; Pino *et al.*, 1997). Other than the major compounds, α-and β-pinene were also reported to have antimicrobial activity (Abu-Darwish *et al.*, 2012a; Delamare *et al.*, 2007). Recent investigations have shown significant antimicrobial effects of several essential oil compounds against enteropathogenic organisms in farm animals (Franz *et al.*, 2010). Polyphenols and in particular romarinic acid are important compounds found in *S. officinalis* plants and reported to have antioxidant activity (Cuvelier *et al.*, 1994; Lu and Foo, 2002). Rosmarinic acid was also reported to have antithrombotic, anti-wrinkle and antiplatelet effects, in addition to its use as inhibitor against adenylate cyclase and xanthine oxidase (Lu and Foo, 2002).

ISO 9909 for medicinal uses stated the following percentages of *S. officinalis* essential oil constituents: cis-thujone (18.0–43.0%), camphor (4.5–24.5%), 1,8-cineole (5.5–13.0%), trans-thujone (3.0–8.5%), α-humulene (≤12.0%), α-pinene (1.0–6.5%), camphene (1.5–7.0%), limonene (0.5–3.0%), bornyl acetate (≤2.5%) and linalool + linalyl acetate (≤1.0%) (Mokuté *et al.*, 2003; Vera *et al.*, 1999). The German Drug Codex requirements differ from ISO specifications as follows: thujones (≥20.0%), camphor

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(14.0–37.0%), 1,8-cineole (6.0–16.0%), borneol (≤5.0%) and bornyl acetate (≤5.0%) (Teuscher, 2006). The Two world standards differ for *S. officinalis* essential oil composition with ISO 9909 regulating eleven constituents while the German Codex regulates only five compounds.

The composition of *S. officinalis* essential oils generally varied as reported in literature depending on factors like climate conditions (Mathé *et al.*, 1992), season (Grella and Picci, 1988) and culture site (Santos-Gomes and Fernandes-Ferreira, 2001). Research aiming at investigating properties of *S. officinalis* plants in Jordan included recently plants cultivated under suitable agricultural practices for massive production purposes. Essential oil production was studied for *S. officinalis* plants grown utilizing 15, 30 or 45 cm intra-row spacing in Shouback city south of Jordan, which is presumably a suitable place for the growth of high quality herbal and medicinal plants (Abu-Darwish and Abu-Dieyeh, 2009; Abu-Darwish *et al.*, 2011; Al-Ramamneh, 2009). In our previous work, it was found that *S. officinalis* plants grown South Jordan using 15 cm planting space and harvested during vegetative stage produced the maximum content of essential oils (Abu-Darwish *et al.*, 2011).

The aim of the present work conducted in Shouback city, South Jordan, was to investigate the content of essential oils and rosmarinic acid of *S. officinalis* aerial parts as influenced by three intra-row spacing (15, 30 and 45 cm). We have also determined the content of 1,8-cineole, α-and β-thujone, α-and β-pinene and camphor in *S. officinalis* using GC⁄FID and GC⁄MS instruments. The content of rosmarinic acid was analyzed by means of UV absorption measurement and comparison with authentic samples using HPLC.

Materials and methods

Study site and experimental procedures

The experiments were conducted in the research farm of Shoubak University College, Shoubak, Jordan. Tab. 1 shows site information and climatic conditions that prevailed during the studying period.

Tab. 1. Characteristics of the cultivation site and climatic conditions in Shoubak region south of Jordan during the study period

Criteria	
Altitude	1365 m asl
Longitude	32° 35′ E
Latitude	30° 31′ N
Soil pH	7.9-8.3
Soil EC	1.4-1.8
Soil texture	Clay loam
Mean temperature	12.9°C
Total Rainfall	145.6-207.3 mm

S. officinalis seedlings (10±2 cm) were obtained from a local nursery and transplanted in the experimental area in May in 50 cm-rows with 15, 30 and 45 cm intra-row spacing. Plants were irrigated using drip irrigation. After planting, irrigation was done every three days. One month later, irrigation frequency was reduced and plants were irrigated once every 15 days. Plants were grown without fertilizers and weed control was practiced when needed. Five plants were harvested from each intra-row spacing only during the vegetative stage because the highest oil content was reported during this stage as reported in our previous study (Abu-Darwish *et al.*, 2011). Plant samples were dried in shade at 23-25°C until used later.

Essential oil extraction

Twenty grams of aerial parts of *S. officinalis* were ground into a fine powder and hydro-distilled in triplicate for 2 h using a Clevenger-type apparatus according to the European Pharmacopeia. Content percentage was calculated as volume of essential oils per 100 g of plant dry matter.

Gas Chromatography⁄Flame Ionization Detection (GC⁄FID) and Gas Chromatography⁄Mass Spectrometry (GC⁄MS) analyses of essential oils

GC/FID: The essential oils were analyzed by Gas Chromatography (GC-FID) using a Hewlett-Packard 5890 Series II with FID, a split-split less system for injection, an HP-5 capillary column (50 m long × 0.20 mm i.d.) for constituent separation, and nitrogen as a carrier gas. The operating conditions were an injection temperature of 150°C, a detector temperature of 250°C and a temperature program beginning at 90°C (0 min), 10°C min^{-1} to 150°C (5 min), 5°C min^{-1} to 180°C (3 min), then 7°C min^{-1} to a final isothermal of 280°C for 25 min. The carrier gas flow velocity was 274 mm s^{-1}; auxiliary gases were nitrogen at 30 mL min^{-1}, hydrogen at 30 mL min^{-1} and air at 400 mL min^{-1}. Sample sizes were 1.0 μL and a manual injection was used. Peak areas and retention times were measured by electronic integration with a Hewlett Packard 3396 Series II integrator.

GC/MS: Samples were analysed using an HP 5890A gas chromatograph equipped with an HP 5971A mass detector and with a 30 m × 0.25 mm internal diameter, DB-5 column. The operating conditions were: injector temperature 260°C, column temperature 60°C (2 min), 4°C (1 min), 260°C (5 min), carrier gas He 1 mL/min, split 1:20, scan time 1 s. Electrons at 70 eV performed the ionization.

Most components were identified from their GC retention indices, with either those reported in literature (Adams, 2001; Davies, 1990; de Martino *et al.*, 2009; Jennings and Shibamato, 1980) or with standards available in our laboratories, purchased from Sigma Aldrich, Bratislava, Slovakia. The retention indices were determined in relation to a homologous series of n-alkenes (C_8-C_{24}) under the same operating conditions. Component relative

concentrations were calculated based on GC peaks without using correction factors. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of three injections from each oil sample, without using correction factors.

Quantification of rosmarinic acid

For a reliable determination of rosmarinic acid, identification of this compound was done by means of UV absorption measurement which is confirmed by comparing its retention time with authentic samples using HPLC method (Troncoso *et al*., 2005).

UV measurements: Rosmarinic acid content was determined using water phase with dilution then thickening at the vacuum evaporator followed by pH modification. Rosmarinic acid is consequently extracted into ethyl acetate which is neutralized and then the crystallization of rosmarinic acid from the salty dilution follows with crystal filtering out and dehydratation.

Five grams of homogenous sample in 50 mL of 60% ethanol were used for the isolation of rosmarinic acid and allowed to macerate for 2 h at the temperature of 70°C. After the filtration and dilution of the sample, the absorption was measured at 329 nm, and the content of rosmarinic acid was determined according to calibration line as follows:

$C_{KR} = (A_{329} - 0.03978)/0.04481. r$ (mg/L)

Content in the dry matter: $(C_{KR}. V)/1000.n$ (%)

where: r–dilution of the sample

V–capacity of extract in mL

n–embankment of the sample in g

1000–number mg per g

HPLC method: Rosmarinic acid determination was done by liquid chromatographic method using HPLC BREEZE septum from Waters Company. The structure of the septum: HPLC–Binary Pump 1525, Dual Alfa Absorbance Detector 2478, in-line Degasser AF, GPC Software, Columns: Waters HPLC Columns Symmetry C18, 3.5 micrometers (4.6 × 75 mm) and Nova-Pak C_{18}, 4 micrometers (3.9 × 150 mm). Identification of the main component of rosmarinic acid was carried out by comparing its retention time with authentic reference standard (rosmarinic acid, 97%, Aldrich).

Results and discussion

Content of essential oils and rosmarinic acid

Drying the plant samples in shade resulted in 9% weight loss. The content of essential oils varied between 0.8 and 2.7% reaching its maximum in plants grown using 15 cm intra-row spacing and the minimum in plants grown using 30 cm spacing (Tab. 2). The results of the present findings are in agreement with that of Abu Darwish *et al.* (2011) who found that the highest essential oil content was in *S. officinalis* plants cultivated at 15 cm intra-row spacing during vegetative stage (2%). According to that study, 30 and 45 cm intra-row spacing produced *S. officinalis* plants with essential oil content of 1.80 and 1.73%, respectively. Amr and Đorđević (2000) reported that an essential oil content ranged between 1.18 and 2.13% for aerial parts of *S. officinalis* collected from two locations in Jordan (Hfashiet Al Dbajbe and Al Fesalia), reaching its maximum in the blooming period and the minimum in samples picked during winter. The differences in essential oils between our study and previous study indicated that the influences of plant age, geographical location, climatic conditions and part of plant used may have played a trigger role as important factors on oil content. Also, essential oil content of *S. officinalis* has been studied in several countries. For instance, the amount of *S. officinalis* essential oils originated from various localities in Serbia was 1.1 to 2% in collected plant material from the surrounding of Niš, indicating climate variation from year to year (Miladinović and Miladinović, 2000; Veličković *et al*., 2003). The chemical composition of *S. officinalis* growing in Algeria revealed essential oils content of 0.9% based on dry weight of the plants (Dob *et al*., 2007).

The content of total substances in extracts obtained using 60% ethanol from herbal parts of *S. officinalis* was 22, 25 and 24% for plants grown using 15, 30 and 45 cm intra-row spacing, respectively (Tab. 2). Rosmarinic acid content, estimated on dry herb bases, was abundant in plants grown using 15 cm spacing (2%) whereas it was lower in plants grown using 30 or 45 cm spacing (1.3%). The same results were also obtained using ethanolic extracts where rosmarinic acid was present as 9, 6 and 5% in extracts obtained from plants grown using 15, 30 and 45 cm intra-row spacing, respectively (Tab. 2).

Rosmarinic acid content in *S. officinalis* plants grown in other countries was reported in literature. For instance sage plants cultivated North of Portugal contained rosmarinic acid as the major compound of the total phenolics (Areias *et al*., 2000). The presence of rosmarinic acid was also reported in field-grown as well as various in vitro cultures of *S. officinalis* plants originating in Poland (Grzegorczyk *et al*., 2007). The content of rosmarinic acid in that study was about 1.2-1.9% and 0.7-0.8% on dry weight basis for shoots (12-19 mg g^{-1}) and roots (7-8 mg g^{-1}), respectively.

Tab. 2. Essential oil and rosmarinic acid content of sage aerial parts collected from plants grown using 15, 30 and 45 cm intra-row spacing in Shoubak region of south Jordan

Intra-row spacing (cm)	Essential oils (%)	Rosmarinic acid%	
		Dry herbs	Extract substances (60% ethanol)
15	2.7 ± 0.2	2.0 ± 0.1	9.0 ± 0.5
30	1.8 ± 0.1	1.3 ± 0.1	6.0 ± 0.5
45	1.1 ± 0.1	1.3 ± 0.1	5.0 ± 0.5

Values represent the mean of each treatment ± SE

The high content of rosmarinic acid in ethanolic extract of the herbal parts of *S. officinalis* plants grown using 15 cm intra-row spacing in the present study may indicate its potential use as an inflammatory agent in traditional medicine. This is in agreement with Geller *et al.* (2010) who showed that ethanolic extract, with rosmarinic acid as its major compound; of *Cordia americana* support its wide use in traditional medicine in South Brazil to treat wounds and various inflammations.

Essential oil major constituents

Essential oils of *S. officinalis* as shown in this study was particularly rich in 1,8-cineole and the highest amount was recorded in plants grown under 15 cm intra-row spacing (47-50%, GC⁄FID; 55-60%, GC⁄MS) (Tab. 3). The content of 1,8-cineole in plants grown in 30 and 45 cm spacing was 18-20% less than that for plants grown in closer spacing (15 cm) as measured by GC⁄MS method (37-40%) (Tab. 3). α-and β-thujone represented only a small fraction of essential oils regardless of the planting space and ranged between 1-3% with the minimum (1%, GC⁄FID) recorded for plants grown using 45 cm spacing. α-pinene was 3-5% (GC⁄FID) in plants grown using 15 and 30 cm spacing. Higher content of α-pinene was measured with 45 cm intra-row spacing (8-10%, GC⁄FID). β-pinene followed the same trend with its content showing no real difference in plants grown using 15 or 30 cm spacing. A maximum of 5-6% (GC⁄MS) of β-pinene was measured in plants spaced 45 cm apart. The highest content of camphor (8-9%, GC⁄FID; 9-10%, GC⁄MS) was reported for plants grown using 15 cm spacing compared to plants grown using 30 cm (4-5% GC⁄FID, 6-7% GC⁄MS) or 45 cm spacing (5-6% GC⁄FID, 5-6% GC⁄MS).

The content of essential oil and its major constituents in *S. officinalis* can be influenced by their metabolic pathway that is regulated at the genetic level (Grausgruber-Gröger *et al.*, 2012; Sangwan *et al.*, 2001). However, this expression is modulated by so many factors like developmental stage (ontogeny), organ and tissue, glandular trichome morphology and environmental conditions such temperature, day length and light (Grausgruber-Gröger *et al.*, 2012; Sangwan *et al.*, 2001). Seasonal variation in essential oils and their main monoterpenes have been reported (Abu darwish *et al*., 2011; Arraiza *et al*., 2012). However, the variation in a specific metabolite in the present study for essential oil major constituents and rosmarinic acid in *S. officinalis* cultivated under different intra-row spacing during vegetative stage could be a result of plant physiological adaptation to space confinement in closer spacing or the availability of space in wider spacing (Al-Ramamneh, 2009). For thyme plants, the highest oil content was obtained in plants spaced 45 cm apart (Abu-Darwish *et al.*, 2012b; Al-Ramamneh, 2009). Thyme plants had higher leaf area in wider spacing than in close spacing during vegetative stage indicating better light interception. This, in turn, enhanced glandular trichomes production and essential oil accumulation in thyme shoots (Al-Ramamneh, 2009). Thus, the study of *S. officinalis* plant's phenological strategy in response to population density can provide more information about the observed variation in secondary metabolites in *S. officinalis* plants grown under different intra-row spacing. furthermore, understanding assimilate distribution between the plant's different organs in future studies may contribute to better understand the variation in metabolic pathway leading to the production of the different major constituents.

The high content of 1,8-cineole in all studied intra-row spacing in the present study can be due to the effect of some heavy metals presented in the cultivation soil (Tab. 4; Abu-Darwish *et al*., 2011). Abu-Darwish *et al.* (2011),

Tab. 3. Major constituents of the essential oils from *S. officinalis* plants grown using 15, 30 and 45 cm intra-row spacing in Shoubak region south of Jordan

Constituents	Intra-row spacing (cm)					
	15		30		45	
	GC/FID	GC/MS	GC/FID	GC/MS	GC/FID	GC/MS
α-pinene	3–4%	3–4%	4–5%	3–4%	8–10%	5–6%
β-pinene	2–3%	1–2%	1–2%	3–4%	3–4%	5–6%
cineole	47–50%	55–60%	40–43%	37–40%	37–40%	37–40%
α-thujone	2–3%	2–3%	1–2%	2–3%	1%	2–3%
β-thujone	2–3%	2–3%	1–2%	1–2%	1%	1–2%
Camphor	8–9%	9–10%	4–5%	6–7%	5–6%	5–6%

Tab. 4. Concentration of heavy metals (mg/kg) during the vegetative stage in *S. officials* L. cultivated under different intra-row spacing in Shoubak region south of Jordan (Abu-Darwish *et al.*, 2011)

Spacing (cm)	Ni	Zn	Fe	Cu	Mn
15	0.42±0.25	116.91±0.36	736.17±6.94	7.32±0.81	45.0±0.46
30	2.78±1.08	95.81±1.19	768.97±5.41	7.02±0.50	51.35±0.42
45	4.17±1.38	108.85±0.29	524.67±3.35	13.07±0.70	44.63±0.72

Values are the mean±SE. Pb, Cd, Cr and Co elements were not detected

showed the presence of Zn and Cu in *S. officinals* plants in the same experimental field of the present investigation. The results of the present study is in agreement with that of Stancheva *et al.* (2009), who reported that components like α-and β-thujone decreased, whereas 1,8-cineol, among other compounds increased in essential oils of *S. officinals* plants grown in Bulgarian heavy metal contaminated soils.

The composition of the major constituents in the present study, averaged over all spacing, showed a partial agreement with the requirement of ISO 9909 for camphor, thujone and α-pinene. On the other hand, *S. officinals* essential oils obtained from Shouback, South of Jordan were lower in thujone and camphor as compared to that of the German Drug Codex standard. However, the content of 1,8-cineole in *S. officinals* essential oil in the present study was higher than that specified in both ISO 9909 and German Drug Codex.

Studies on the oil composition of *S. officinals* from different origins showed variable results. In partial agreement with the result of the present study, the major constituent of essential oils extracted from the aerial parts of cultivated Tunisian *S. officinalis* L. was 1,8 cineole (33.27%) (Hayouni *et al.*, 2008). In contrast to our results, Tunisian *S. officinalis* essential oils had relatively high α-and β-thujone (13.5 and 18.4%, respectively) compared to the low percentage of these constituents determined in the present study. However, The major constituents of *S. officinalis* from other countries like Egypt and Brazil were α-and β-thujone, 1,8-cineole and camphor in that order respectively (Delamare *et al.*, 2007; Khalil *et al.*, 2008).

Conclusions

Growing *S. officinalis* using 15 cm planting space had increased essential oils and rosmarinic acid content up to 2.7 and 2% on dry herb basis, respectively. In fact, this confirms the beneficial effects that agricultural practices could have on the essential oil and rosmarinic acid content of *S. officinalis*. Furthermore, adopting suitable agricultural practices can enhance the qualitative industrial properties of plants like *S. officinalis* grown South of Jordan. The present findings regarding essential oils composition also suggested that the particular chemotype of *S. officinalis* cultivated in Shouback city is characterized by the presence of 1,8 cineole as a dominant component. This chemical composition of *S. officinalis* makes the potential use of its products and extracts in traditional medicine as anti-inflammatory and wound-healing agents the target of further research.

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