Australian Journal of Crop Science AJCS

AJCS 13(10):1617-1624 (2019) ISSN:1835-2707
doi: 10.21475/ajcs.19.13.10.p1834

Inoculation with arbuscular mycorrhizal fungi alters content and composition of essential oil of Sage (*Salvia officinalis*) under different phosphorous levels

Rayane Monique Sete da Cruz[1], Glaucia Leticia Sete da Cruz[2], Douglas Cardoso Dragunski[3], Affonso Celso Gonçalves Junior[4], Odair Alberton[5*], Silvia Graciele Hulse de Souza[1]

[1]Postgraduate Program in Biotechnology Applied to Agriculture; Paranaense University – UNIPAR; Praça Mascarenhas de Moraes; 4282; 87502-210; Umuarama – PR – Brazil
[2]Agronomic Engineering; Paranaense University – UNIPAR; Praça Mascarenhas de Moraes; 4282; 87502-210; Umuarama – PR – Brazil
[3]Department of Chemistry, State University of Western Paraná, College street, 645, CEP 85903-000, Toledo – PR – Brazil.
[4]Center for Agricultural Sciences, State University of Western Paraná., Pernambuco street, 1777, CEP 85960-000, Marechal Cândido Rondon – PR – Brazil
[5]Postgraduate Program in Medicinal and Phyto therapeutic Plants in Primary Care. Laboratory of Preclinical Research of Natural Products, Paranaense University, Umuarama – PR – Brazil

***Corresponding author: odair@prof.unipar.br, or oalberton@yahoo.com.br (Alberton O.)**

Abstract

This study aimed at evaluating the growth and essential oil content of *Salvia officinalis* L. (sage) inoculated with two species of arbuscular mycorrhizal fungi (AMF) *Rhizophagus clarus* and *Claroideoglomus etunicatum* under different phosphorous (P) levels. The treatments were prepared in a sterile substrate (sand, vermiculite and organic compound (1: 1: 2, v:v) with high (200 mg kg^{-1}) and low (20 mg kg^{-1}) P levels at sowing, with and without AMF inoculation; the experiment was conducted in a greenhouse for four months. Plants were grown in pots with 3 kg of substrate in a 3 x 2 (3 mycorrhizal x 2 levels of P) factorial experiment, in a completely randomized design (with eight repetitions). Spore density, AMF root colonization, plant dry mass, P and N shoot content, yield and composition of essential oil (EO) were determined. Data were processed and submitted to analyses of hierarchical clustering and principal component. Plant biomass increased with addition of P in the substrate. EO content increased with AMF *Rhizophagus clarus* inoculation in high-P substrate. It was concluded that inoculation with *R. clarus* and the addition of P at sowing can boost the growth of sage and the content of its EO. Camphor, α-humulene, viridiflorol, manool, α-thujone and β-thujone were the main components of the EO.

Keywords: Manool; inoculum; sage; mycorrhiza; essential oil.
Abbreviations: AMF_arbuscular mycorrhizal fungi; EO_essential oil; GC/MS_Gas chromatography/mass spectrometry K_potassium; N_nitrogen; NS_nitrogen in shoot; P_ phosphorous; PS_phosphorus in shoot; RDM_root dry matter; SDM_shoot dry matter; TDM_total dry matter

Introduction

The Latin name *Salvia officinalis* L. (sage) means "healing", which implies great popularity in traditional medicine (Bors et al., 2004). It is a perennial herb that adapts better to clay-sandy, fertile and humid soils, requiring full lighting to develop (Farhat et al., 2016).

Aroma and essential oil production are striking features of this species (Grdiša et al., 2015). Tarraf et al. (2017) found forty-four different compounds in the essential oil of *S. officinalis* inoculated with Symbivit, *Septoglomus viscosum (*syn. *Glomus viscosum)* under doses of phosphorous. The major components of the sage's oil were: α -thujone (13.09-28.34%), manool (13,57-28,13%), camphor (8,37-13,51%), viridiflorol (9.41-16.94%), α -humulene (5.61 -8,49%), ß-thujone (2,22-5,58), 1,8-cineol (3,09-5,09%), trans-caryo-phyllene (1,37-4,48%), borneol (1,52-3,73%), α-humulene epoxide II (0,92-2,15%) and camphene (0,55-1,31%).

In the sage's leaves, other substances can be found such as flavonoids, tannins and proteins (Miladinović; Miladinović, 2000). Bors et al. (2004) reported an antioxidant potential of sage from rosmarinic acid, carnosic acid and derivatives. Alonso (1988) found a toxic effect of sage from camphor and thujones when the plant is incorrectly used.

Sage's aroma forms from the major compounds of EO, varying according to both qualitative and quantitative aspects involving year season, flowering time, plant age,

amount of circulating water resulting from rainfall, geography, climate, soil fertility and interactions with AMF present in soils (Grdiša et al., 2015). The AMF symbiosis benefits plant growth and development by improving water and P uptake in the roots (Lermen et al., 2015a; Urcoviche et al., 2015). According to Marschner (2012), the absorbed P composes the molecule of ATP, responsible for storing and transporting energy to endergonic processes such as synthesis of organic compounds and active absorption of nutrient, favoring the growth of plants.

Plant production can be optimized using bio-inputs such as AMFs, which make plants more resistant to biotic and abiotic stresses and potentialize the use of nutrients by the symbionts through nutrient exchanges in the system (Choi et al., 2018).

The AMF symbiosis between fungi and plant works as a three-phase heterogeneous mixture, in which all components – soil, plant and fungus - interact. Each component (soil, plant and fungus) interferes with mycorrhiza symbiosis in a way. However, the symbiosis success depends mainly on the plant mycorrhizal dependency, fungus symbiotic efficiency and P availability in soil (Choi et al., 2018).

Availability of P in soil is the major edaphic factor on the functioning of AMF symbiosis. Small amounts of applied P can positively raise AMF colonization; therefore, the most common and consistent effect on the AMF symbiosis formation is the application of high doses of P in the soil, which can sharply reduce the AMF colonization (Smith and Read, 2008).

Copetta et al. (2006) and Khaosaad et al. (2006) reported an increase in biomass and EO content of basil plants inoculated with AMF (*G. mosseae*). In another study, Geneva et al. (2010) demonstrated an increased production in the shoot biomass and EO of sage plants inoculated with AMF (*Rhizophagus irregularis* = *Glomus intraradices*).

However, few studies have investigated the AMF effects on the growth and production of medicinal, aromatic and spicy plants with different levels of P applied in the soil (Lermen et al., 2017; Urcoviche et al., 2015). Therefore, this study aimed at evaluating the plant growth and EO content of sage inoculated with AMFs (*Rhizophagus clarus* and/or *Claroideoglomus etunicatum*) under different levels of P, thus confirming the importance and novelty of the present study.

Results and discussion

Spore density and AMF root colonization

The spore density and root colonization by AMFs in sage showed significant differences among treatments that were un-inoculated and inoculated with AMFs and subjected to different P levels (Table 1).

The AMF root colonization was the highest (30.66%) when substrates were inoculated with *R. clarus* under low-P level. However, treatments in which the substrate was fumigated presented a small incidence of spores and AMF colonization, indicating a possible contamination during the experiment period. The AMF root colonization and spore density were low when the substrates were inoculated with AMF under high-P level (Table 1).

Urcoviche et al. (2015) observed an increased AMF root colonization in *M. crispa* inoculated with AMF under low-P level. Lermen et al. (2015a) also observed increases in spore density and AMF root colonization in *Cymbopogum citratus* inoculated with AMF, resulting in better biomass production. This finding corroborated with the result of the present study and Smith and Read (2008), who reported that AMF colonization and sporulation can increase with low P doses applied to P-poor soils. Both AMF colonization and sporulation are inhibited at high P doses and the magnitude of these effects seems to be related to the plant species.

Plant growth

The shoot dry matter (SDM) was significantly increased with the doses of P applied to the substrate ($p < 0.01$) (Table 2) but did not differ significantly among treatments with AMF inoculation. Similar results were verified by Silva et al. (2009), who found an increased biomass production in *Jaracatia spinosa* as a response to increasingly doses of P applied to soil, independently of AMF inoculation.

Root dry matter (RDM) and total dry matter (TDM) differed significantly among treatments in response to AMF inoculation and TDM differed significantly in response to P levels (Table 2). The inoculation with *C. etunicatum* in high-P substrate reduced RDM by more than 50% in comparison with the low-P control. The RDM increased in response to inoculation with *R. clarus* but did not differ among P levels (Table 2). However, the TDM decreased when plants were inoculated with *C. etunicatum* and/or *R. clarus* in high-P substrate. Similar results were observed in previous studies by Nell et al. (2009), who reported that AMF inoculation did not increase the SDM of sage plants but increased significantly their RDM.

Carneiro (2004) also found similar results in *Cecropia pachystachya* inoculated with AMF, the authors' verified increases in RDM as response to AMF inoculation but observed no differences in SDM.

Geneva et al. (2010) reported increases of 57% in RDM and 26% in SDM of sage plants inoculated with *Rhizophagus irregulars*. The authors also verified that the biomass production of AMF inoculated plants increased at 63% with foliar application of fertilizers, in comparison with un-inoculated plants.

In another recent study, Ghoushchi et al. (2013) observed a significant increase in the growth of sage plants inoculated with *R. irregularis* and/or *G. mosseae* in comparison with the un-inoculated control. Karagiannidis et al. (2012) also verified a 44.53% increase in biomass production of sage plants inoculated with *G. lamellosum*.

Urcoviche et al. (2015) found an increased production of shoot fresh mass of *M. crispa* inoculated with AMF in high-P soil, which does not corroborate with our findings. In other study, Lermen et al. (2015a) investigated the growth of *C. citratus* inoculated with *R. clarus* under different levels of lead (Pb) and observed that the AMF inoculation increased plant growth and reduced Pb accumulation in leaves.

Table 1. Spore density (number of spores g^{-1} dry soil) and root colonization (%) of AMFs in sage plants uninoculated and inoculated with *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low and high level of phosphorus.

Treatments	Number of Spores	AMF colonization
Uninoculated low-P (control)	0.04±0.01c	1.52±0.29c
Uninoculated high-P (control)	0.05±0.01c	0.96±0.02c
R. clarus and low-P	3.57±0.37a	30.66±4.97a
R. clarus and high-P	1.93±0.14b	17.71±2.76b
C. etunicatum and low-P	3.85±0.34a	24.64±5.54ab
C. etunicatum and high-P	2.23±0.19b	17.91±1.18b
Significance	<0.001	<0.001

Means ± standard error with eight repetitions. Means with different letters in the same column differ significantly by Duncan test at 5% probability.

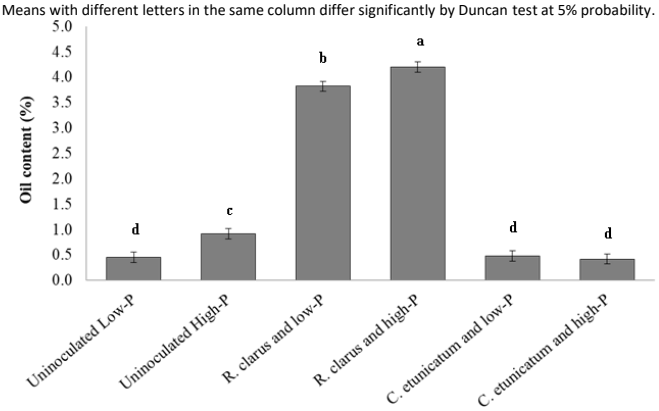
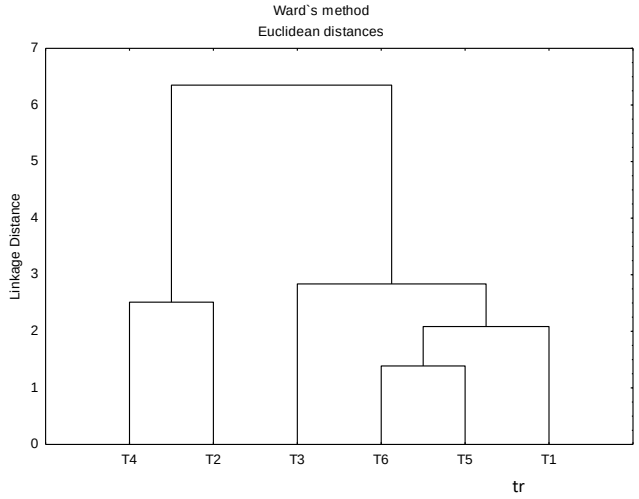


Fig 1. Essential oil content (%) of sage plants uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low and high level of phosphorus. Columns followed by the same letter are not statistically different (Duncan. $p \leq 0.05$). Bars = standard error.

Table 2. Shoot dry matter (SDM – g $plant^{-1}$), root dry matter (RDM – g $plant^{-1}$), total dry matter (TDM – g $plant^{-1}$), SDM/RDM ratio, P accumulated in the shoot (P-shoot – mg P g^{-1}) and N accumulated in the shoot (N-shoot – mg N g^{-1}) in sage plants uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low and high level of phosphorus.

Treatments	SDM	RDM	TDM	SDM/RDM	P-shoot	N-shoot
Uninoculated low-P	4.02±0.51ab	0.77±0.11b	4.79±0.59ab	0.19±0.02c	4.34±0.18ab	23.28±1.11d
Uninoculated high-P	4.78±0.57a	1.02±0.08a	5.79±0.57a	0.23±0.03bc	4.45±0.51ab	28.18±0.50bc
R. clarus and low-P	3.83±0.12abc	1.06±0.07a	4.89±0.09ab	0.28±0.03b	3.93±0.01bc	30.98±1.10a
R. clarus and high-P	3.28±0.15bc	1.17±0.09a	4.45±0.17c	0.36±0.03a	4.73±0.09a	27.82±0.91bc
C. etunicatum and low-P	2.77±0.34c	0.57±0.04b	3.34±0.32c	0.23±0.03bc	3.64±0.05c	25.90±0.01c
C. etunicatum and high-P	3.10±0.09bc	0.35±0.02c	4.45±0.10bc	0.11±0.01d	3.59±0.07c	29.50±0.01ab
Significance	**0.003**	**<0.001**	**<0.001**	**<0.001**	**0.020**	**<0.001**

Means ± standard error with eight repetitions. Means with different letters in the same column differ significantly by Duncan test at 5% probability.



ol high-P; T3: *R. clarus* and low-P; T4: *R. clarus* and high-P; T5: *C. etunicatum* and low-P and T6: *C. etunicatum* and high-P.

Fig 2. Hierarchical clustering dendrogram of essential oils of sage plants uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low and high level of phosphorus. Based on data of Table 3.

Table 3. Chemical composition of essential oil (%) of sage uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low (20 mg P kg^{-1} soil) and high (200 mg P kg^{-1} soil) level of phosphorus.

N°	[A]Substance	T_1	T_2	T_3	T_4	T_5	T_6	RI lit.	RI calc.	Methods of identification
Hemiterpene										
1	Cubenol	0.27	t	t	0.19	0.29	t	1645	1631	a.b.c.
Monoterpenes										
2	α-Pinene	0.10	0.94	t	0.41	0.06	t	932	931	a.b.c.
3	Camphene	0.15	0.94	t	0.62	0.09	t	946	943	a.b.c.
4	β-Pinene	0.30	1.61	t	0.78	0.19	t	974	972	a.b.c.
5	Myrcene	0.29	0.77	t	0.54	0.23	t	988	991	a.b.c.
6	α-Terpinene	0.06	0.18	t	0.13	0.13	t	1014	1018	a.b.c.
7	β-Ocimene	t	t	t	0.05	t	t	1032	1035	a.b.c.
8	γ-Terpinene	0.23	0.42	t	0.33	0.20	t	1054	1053	a.b.c.
9	α-Terpinolene	0.41	0.39	0.38	0.47	0.35	t	1086	1088	a.b.c.
10	α-Thujene	t	t	t	t	t	t	924	923	a.b.c.
Oxygenated Monoterpenes										
11	1.8 cineole	3.98	6.99	2.50	5.60	2.26	1.67	1026	1023	a.b.c.
12	β-Terpineol	0.22	t	t	t	0.19	t	1140	1071	a.b.c.
13	α-Thujone	16.53	17.68	16.48	20.03	15.85	16.54	1101	1102	a.b.c.
14	β-Thujone	9.67	10.89	4.89	10.32	4.19	7.53	1116	1113	a.b.c.
15	Camphor	13.92	12.94	11.67	16.67	10.89	14.65	1141	1141	a.b.c.
16	Isoborneol	0.06	t	t	t	0.07	t	1155	1171	a.b.c.
17	Pinocamphone	0.09	0.19	t	0.12	1.68	t	1172	1174	a.b.c.
18	Borneol	2.95	2.38	2.83	2.37	t	1.87	1165	1178	a.b.c.
19	terpineol 4	0.70	0.79	0.48	0.80	0.44	t	1174	1185	a.b.c.
20	Cymen-8-ol-p	0.05	t	t	t	t	t	1179	1188	a.b.c.
21	α-Terpineol	0.32	0.21	t	0.26	0.13	t	1186	1190	a.b.c.
22	Pinocarveol	0.05	t	t	t	t	t	1182	1195	a.b.c.
23	Carveol	0.12	t	t	0.10	t	t	1215	1208	a.b.c.
24	Mentha-1(7).8-dien-9-ol	0.13	t	t	0.11	t	t	1227	1221	a.b.c.
25	cis-Carveol	0.07	t	t	0.07	t	t	1226	1228	a.b.c.
26	Pinanediol	0.31	0.31	t	0.28	0.37	t	1318	1298	a.b.c.
27	Trans-Thujone	t	7.08	t	t	t	t	1112	1116	a.b.c.
28	Isopinocamphone	t	0.09	t	t	t	t	1176	1181	a.b.c.
29	Bornyl acetate	2.06	1.73	3.42	1.86	t	1.63	1287	1269	a.b.c.
30	Myrtenyl acetate	0.05		0.32	0.08	0.06	t	1324	1305	a.b.c.
31	cis-Carvyl acetate	t	t	t	t	t	t	1339	1310	a.b.c.
32	Prasterone	0.16	t	t	0.13	0.19	t		1913	a.b.c.
33	Palmitolactone	t	t	t	0.06	0.12	t		2026	a.b.c.
Sesquiterpenes										
34	Myrtenol	0.19	0.24	t	0.21	0.13	t	1194	1193	a.b.c.
35	α-Copaene	0.08	t	t	0.07	0.09	t	1374	1376	a.b.c.
36	β-Bourbonene	t	t	t	T	t	t	1387	1384	a.b.c.
37	Caryophyllene	4.90	3.19	t	T	5.12	6.21	1408	1418	a.b.c.
38	Aromandendrene	0.16	t	t	T	t	t	1439	1439	a.b.c.
39	α-Humulene	8.11	7.03	10.38	6.81	10.58	8.71	1452	1453	a.b.c.
40	γ-Muurolene	0.10	t	t	0.09	0.12	t	1478	1472	a.b.c.
41	Germacrene D	0.21	t	t	0.26	0.26	t	1484	1480	a.b.c.
42	Ledene	0.28	t	t	0.15	0.23	t		1492	a.b.c.
43	γ-Cadinene	0.06	t	t	0.07	0.09	t	1513	1512	a.b.c.
44	δ-Cadinene	0.16	t	t	0.17	0.27	t	1522	1523	a.b.c.
45	Patchoulane	0.07	t	t	T	0.13	t	1502	1534	a.b.c.
46	Guaia-1(5).7(11)-diene	0.07	t	t	T	t	t		1688	a.b.c.
47	Alloaromadendrene	t	0.11	t	T	t	t		1456	a.b.c.
48	Caryophyllene	t	t	4.97	3.04	t	t	1408	1418	a.b.c.
49	Patchoulane	t	t	t	0.07	t	t	1502	1534	a.b.c.
Oxygenated Sesquiterpenes										
50	Caryophyllene oxide	0.51	0.31	t	0.32	0.50	0.74	1582	1576	a.b.c.
51	Viridiflorol	6.64	5.49	6.43	5.54	8.77	10.06	1592	1595	a.b.c.
52	Humulene epoxide II	0.63	0.55	0.48	0.58	0.76	0.74	1608	1602	a.b.c.
53	Bulnesol	0.21	t	t	0.16	0.24	t	1670	1652	a.b.c.
54	α-Cadinol	0.19	t	t	0.10	0.18	t	1652	1655	a.b.c.
55	Ledene oxide-(II)	0.10	t	t	T	0.14	t		1658	a.b.c.
56	cis-Z-α-Bisabolene epoxide	0.23	0.21	t	0.27	0.32	t		1663	a.b.c.
57	α-Santalol	0.27	0.28	t	0.50	0.42	t	1674	1667	a.b.c.
58	Caryophyllene hydroxy	0.43	0.33	0.48	0.45	0.70	t	1668	1671	a.b.c.
59	β-Santalol	1.05	1.05	1.67	1.63	2.09	1.76	1702	1694	a.b.c.
60	Pentadecanone-2	t	t	t	T	t	t	1697	1703	a.b.c.
Diterpenes										
61	Cupressene	t	t	t	t	t	t		1980	a.b.c.
62	Trachylobane	0.08	t	t	t	t	t		1993	a.b.c.
63	Thunbergol	0.34	t	t	t	t	t		2035	a.b.c.
64	Trans-Totarol	0.11	t	t	t	0.14	0.20		2225	a.b.c.
Oxygenated Diterpenes										
65	Epimanool	t	0.65	1.66	1.00	1.55	1.17		2016	a.b.c.
66	Manool	21.13	13.78	30.89	14.95	25.26	26.69	2056	2021	a.b.c.
67	Sclareol	t	t	t	t	0.13	t		2031	a.b.c.
	Total identified %	99.79	99.74	99.92	99.58	99.55	99.93			

[a]**RI calc. -** Retention Index calculated by *n*-alkanes $C_8 - C_{26}$ on DB-5 column (phenylmethyl siloxane 5%)**.**

[b]**RI lit. -** Relative Retention Index found in literature on DB-5 column and comparison of Retention Indexes and/or Mass Spectra with literature (Adams, 2007).

[c]**MS -** Identification based on comparison of mass spectra with the Wiley 275 library.

[A] Compound listed in order of elution from a DB-5 column (phenylmethyl siloxane 5%)**.**

t- trace

T_1: Control low-P; T_2: Control high-P; T_3: *R. clarus* and low-P; T_4: *R. clarus* and high-P; T_5: *C. etunicatum* and low-P and T_6: *C. etunicatum* and high-P.

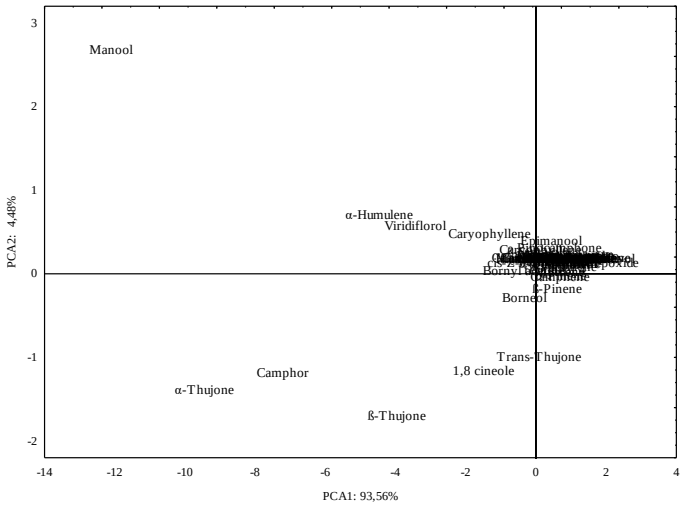


Fig 3. Biplot representation of a PCA (Principal Component Analysis) performed on essential oils of sage plants uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low and high level of phosphorus.

Table 4. Chemical characterization (%) of the main components of the essential oil of sage uninoculated and inoculated with AMF *Rhizophagus clarus* and/or *Claroideoglomus etunicatum* in substrate with low (20 mg P kg^{-1} soil) and high (200 mg P kg^{-1} soil) level of phosphorus.

Treatment	*α*-thujone	*β*- thujone	camphor	*α*-humulene	viridiflorol	Manool	Yield
T_1	16.53	9.67	13.92	8.11	6.64	21.13	0.45
T_2	17.68	10.89	12.94	7.03	5.49	13.78	0.92
T_3	16.48	4.89	11.67	10.38	6.43	30.89	3.82
T_4	20.03	10.32	16.67	6.81	5.54	14.95	4.20
T_5	15.85	4.19	10.89	10.58	8.77	25.26	0.48
T_6	16.54	7.53	14.65	8.71	10.06	26.69	0.42
Based on the literature							
Mean	19.80	5.76	16.35	4.31	6.18	8.49	1.33
SE	0.96	0.52	0.75	0.34	0.54	1.13	0.07
Amplitude	1.2-49.7	0.1-38.5	1.3-38	0.11-14.1	0.1-21.77	0.3-28.13	0.22-2.8
n	106	105	105	86	85	50	62

T_1: control low-P; T_2: control high-P; T_3: *R. clarus* and low-P; T_4: *R. clarus* and high-P; T^5: *C. etunicatum* and low-P and T_6: *C. etunicatum* and high-P.
SE: Standard error of the mean; Amplitude: difference between the highest and the lowest value; n: number of observations.

Freitas et al. (2004) verified an increased production of shoot fresh mass of *Mentha arvensis* inoculated with AMF and fertilized with P.

P and N content in the shoots

The P and N contents in shoots of sage showed significant differences among treatments that were un-inoculated and inoculated with AMFs and subjected to different P levels (Table 2).

The content of P in the shoot increased in response to inoculation with *R. clarus* and P levels applied to substrate. However, when plants were inoculated with AMF *C. etunicatum*, the content of P in the shoot decreased independently of the P level applied to substrate (Table 2). This result was not expected since AMF inoculation commonly increased the accumulation of P in plants' shoot, as reported by Lermen et al. (2015a) and Weber et al. (2004), who found an increased accumulation of N, P and K in leaves of cashew trees (*Anacardium occidentale* L.) inoculated with AMF and fertilized with P. In addition, Nell et al. (2009) also reported an increased content of P in leaves of sage inoculated with AMF and subjected to P levels applied to the soil, as observed in our study.

The N content in the plant increased for 30.98 mg N kg^{-1} dry mass with AMF inoculation with *R. clarus* in low-P substrate (Table 2). The lowest content of N (23.28 mg N kg^{-1} dry mass) was observed in the control with low-P level and un-inoculated plants. Carneiro et al. (2002) demonstrated that plants of *Medicago sativa* accumulated more N in the shoot when inoculated with *C. etunicatum*, thus corroborating our study.

Content and composition of the essential oil

EO content ranged from 0.42 to 4.2% among the different treatments (Figure 1), with an upper limit above the literature mean (1.33%) (Table 4), in which we reviewed 17 published studies with sage content and composition of the EO. Inoculation with *R. clarus* increased EO content to 4.2% under high-P level and decreased it to 3.8% under low-P level. However, inoculation with *C. etunicatum* did not interfere significantly with the EO content (0.45%) in comparison with the low-P control (un-inoculated plants) (Figure 1). Similar results were found by Geneva et al.

(2010), who reported a significant increased EO content from 0.44 to 0.63% in sage plants inoculated with *R. irregulars*. Arango et al. (2012) verified a significantly increased EO content from 40 to 50% in peppermints as P levels in the soil increased from 10 to 40 mg P kg^{-1} soil, disregarding mycorrhization. Urcoviche et al. (2015) reported a five-fold increase in the EO content of *M. crispa* inoculated with AMF *C. etunicatum* in low-P soil. Essential oils are five-carbon-based terpenes named isoprenoids. Related to our results are the facts that Acetyl-CoA, ATP and NADPH are molecules that participate in the synthesis of isoprenoids and are dependent of the P-inorganic (i) accumulated in the plant (Loomis and Corteau, 1972; Kapoor et al., 2002).

Ghoushchi et al. (2013) observed that EO content in sage plants ranged from 0.10 to 0.61% but did not show significant differences among treatments that were un-inoculated and inoculated with *R. irregularis* and/or *G. mosseae.* Karagiannidis et al. (2012) found a significant 55.56% increase in the EO content of sage inoculated with *G. lamellosum.*

According to Alonso (1998), the sage's EO content commonly ranges from 0.4 to 1.0%. However, Cvetkovikj et al. (2015) found EO content variation of 0.25 to 3.48% among twenty-five samples of sage plants across nine Southeast European countries. Govahi et al. (2015) observed an averaged EO content of 1.86% in sage plants cultivated under different doses of fertilizers and subjected to water stress. In the review of Grdiša et al. (2015), it was reported EO content variation of 1.93 to 3.70% among twenty-five samples of sage cultivated in Croatia.

All yield values mentioned above are lower than the upper limit (4.2%) of the EO content range found in this study (Figure 1 and Table 4).

The chemical characterization by GC/MS revealed sixty-seven compounds in the essential oil of sage (Table 3). Oxygenated monoterpenes were the predominant class. In all treatments, α-thujone (15,85 - 20,03%), β- thujone (4,19 - 10,89%), camphor (11,67 - 16,67%), α-humulene (6,81 – 10,58), viridiflorol (5,49 - 10,06%) and manool (13,78 - 30,89%) were the main components (Table 4).

The International Organization for Standardization ISO 9909:1997 – Oil of Dalmatian sage (*S. officinalis*) prescribes the following: α-thujone, 18.0 - 43.0%; β-thujone, 3,0 - 8,5%; camphor, 4,5 - 24,5%; 1,8-cineol, 5,5 - 13,0%; α-humulene, 0 - 12%; α-pinene, 1,0 - 6,5%; camphene, 1,5 - 7,0%; limonene, 0,5 - 3,0%; linalool and its esters, <1%; and bornyl acetate, <2,5%. The German Codex requirements for medicinal products are as follows: thujones (≥20,0%), camphor (14,0 - 37,0%), 1,8-cineol (6,0 - 16,0%), borneol (≤ 5,0%) and bornyl acetate (≤ 5,0%) (Teuscher, 2006). The ISO and Codex requirements signalize that the oils found in this study are slightly higher than prescribed.

Hierarchical clustering was employed to illustrate the chemical variability of EO among different treatments. The samples were grouped into two main groups and displayed in a dendrogram obtained by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Figure 2). The first cluster includes the un-inoculated plants in high-P substrate (T2) and plants inoculated with *R. clarus* in high-P substrate (T4), while the second cluster includes the other treatments (T1, T3, T5 and T6). The grouping result showed the order of the clusters according to the chemical composition of EO (Table 4).

The Principal Component Analysis (PCA) allows a conjoint evaluation of all variables. This multivariate analysis was applied to verify the behavior of the chemical EO constituents considering all treatments. The PCA analysis presented a total variance of 98.04% within which 93.56% is explained by PC1 and 4.48% is explained by PC2, the principal components (Figure 3). Some compounds of EO were displaced in relation to the others (Figures 2). Manool was the farthest compound. Although not much mentioned, manool is a diterpene and one of the main compounds found in the EO of sage (Farhat et al., 2009). Inoculation with *R. clarus* in 20 mg P kg^{-1} substrate increased manool synthesis (30.89%) compared to the control (without inoculation) (21.13%), in both treatments with values above the literature mean (8.49%). Recently, Tarraf et al (2017) observed variations in the content of manool in response to inoculation with *S. viscosum* (28.13%) in comparison with the control (13.57%).

Our findings indicate that inoculation of sage with AMF can positively interfere with yield of a chemical composition of the EO, and appears as a viable and sustainable alternative to produce medicinal plants, mainly for industrial proposes.

Materials and methods

Plant material and AMF inoculum

Ten seeds of *S. officinalis* were sowed in each pot. The AMF treatments were prepared inoculating 200 spores per pot of *Rhizophagus clarus* (=*Glomus clarum*) and/or *Claroideoglomus etunicatum* (=*Glomus etunicatum*), both obtained from the bank of Glomales of UNIPAR. The control treatments (without inoculation) were prepared according to Urcoviche et al. (2015).

Experimental design and set up

The experiment was carried out in the Laboratory of Botany at the University of Parana - UNIPAR, Umuarama / PR. The substrate for plant cultivation was prepared with a sand, vermiculite and organic compound (1: 1: 2 v:v) low P content and packed in sealed black plastic bags for fumigation with 10 mL of chloroform ($CHCl_3$) kg^{-1} substrate for three days (Endlweber and Scheu, 2006). Then the bags were opened inside a chemical exhaust chamber and left for a week before starting the experiments.

To the low P soils, 0.88 g KH_2PO_4 kg^{-1} soil was added just before sowing, corresponding to 200 mg P kg^{-1} soil (high P level). To the low P treatments, 0.38 g pot^{-1} KCl was added, according to Urcoviche et al. (2015). In this way, all treatments had approximately the same concentration of K in the soil, varying only for P doses.

Six treatments were prepared in forty-eight polyethylene pots containing 3 kg of substrate previously fumigated. A 2x3 factorial experiment (2 levels of P doses -high and low and 3 levels of AMF inoculation – two AMF species and control) in a completely randomized design (eight repetitions) was used in this study.

The treatments consisted of:
Treatment 1 (T_1): prepared substrate + 20 mg P kg^{-1}
Treatment 2 (T_2): prepared substrate + 200 mg P kg^{-1}
Treatment 3 (T_3): prepared substrate + *R. clarus* + 20 mg P kg^{-1}
Treatment 4 (T_4): prepared substrate + *R. clarus* + 200 mg P kg^{-1}
Treatment 5 (T_5): prepared substrate + *C. etunicatum* + 20 mg P kg^{-1}
Treatment 6 (T_6): prepared substrate + *C. etunicatum* + 200 mg P kg^{-1}
All treatments were fert-irrigated every two days for four months, which was conducted in a greenhouse, with half concentration of the solution by Hoagland and Arnon (1950), except for P applied at the beginning of the experiment (Lermen et al., 2017; Urcoviche et al., 2015).

Spore density and root colonization by AMF

The spores and root colonization by AMF were evaluated according to Lermen et al. (2017) and the method of wet sieving meshes (Gerdemann et al., 1963).
Fine roots were prepared to conform to the approach of Phillips and Hayman (1970). The count of colonized root segments was made (Giovanetti and Mosse, 1980) and after, we estimated the total root colonization by AMF.

Determination of plant dry masses, P and N in shoot

The plant material was dried in an oven with forced air at 65 ºC for 48 h. Then, shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) were determined by weighing with a semi-analytical balance. After that, the plant materials were ground to determine P and N in shoot as described by Silva (2009) and according to Lermen et al. (2017)

Extraction, content and chemical identification of essential oil by GC/MS

The EO extraction process of all individual treatments was done on 100 g fresh shoots that were crushed in a blender with 1 L deionized water. Then, the material was submitted to hydrodistillation in a modified Clevenger apparatus for 3 h according to Lermen et al. (2015b) and Urcoviche et al. (2015).
The extracted EO was transferred to amber flasks and solvent evaporation was expected to calculate the content (m / m%), considering the plant mass versus the EO mass. The EO was stored in a freezer at -20 ºC prior to determination of the EO chemical components. Chemical identification of the essential oil was obtained by GC/MS according to Lermen et al. (2015b).

Statistical analysis

Data were submitted for analysis of variance (ANOVA). Means were compared by Duncan´s test ($p \leq 0.05$) using the statistical program SPSS, version 22.0 for Windows (SPSS Inc. Chicago, IL, USA).

Cluster (CA) and Principal Component (PCA) analyses were performed to discriminate the composition of EO based on the different treatments and the variables were analyzed using ''Statistica v. 13,3" software (Statsoft, 2017).

Conclusion

The interaction between AMF inoculation and P applied to substrate interfered with the biomass production of sage plants. The high-P substrate resulted in the highest production of sage's biomass. EO content increased as a response to inoculation with *R. clarus*, mainly in plants cultivated in high-P substrate. The chemical composition of EO had α-thujone, β- thujone, camphor, α-humulene, viridiflorol and mannol as the main components in all treatments, being manool the chemical component more sensible among the treatments.

Acknowledgements

The authors thank the Universidade Paranaense – UNIPAR for supporting the research. Rayane Monique Sete da Cruz thanks PROSUP/CAPES for the scholarship. Odair Alberton, Douglas C. Dragunski, Affonso C. Gonçalves Junior and Odair Alberton acknowledge a research fellowship from the CNPq (National Council of Scientific and Technological Development). Silvia Graciele Hülse de Souza thanks the Fundação Araucária for supporting the research.

References

Adams RP (2007) Identification of essential oils by gas chromatography/mass espectroscopy. Carol Stream: Allured Publishing Corporation. USA.

Alonso JR (1998) Tratado de fitomedicina: bases clínicas y farmacológicas. Buenos Aires: Isis, 1038p.

Arango MC, Ruscitti MF, Ronco MG, Beltrano J (2012) Mycorrhizal fungi inoculation and phosphorus fertilizer on growth, essential oil production and nutrient uptake in peppermint (*Mentha piperita* L.). Rev Bras Pl Med. 14: 692-699.

Bors W, Michel C, Stettmaier K, Lu Y, Foo LY (2004) Antioxidant mechanisms of polyphenolic caffeic acid oligomers, constituents of *Salvia officinalis*. Biol Res. 37: 301-311.

Carneiro RFV, Evangelista AR, Tonelli MTL, Reis ST (2002) Inoculação com fungos micorrízicos arbusculares em alfafa (*Medicago sativa* L.) em solo com doses crescentes de fósforo. Ciênc Agrotec. 26: 618–625.

Carneiro MAC, Siqueira JO, Davide AC (2004) Fósforo e inoculação com fungos micorrízicos arbusculares no estabelecimento de mudas de embaúba (*Cecropia pachystachya* Trec). Pesq Agropec Bras. 34: 119-125.

Choi J, Summers W, Paszkowki U (2018) Mechanisms underlying establishment of arbuscular mycorrhizal symbioses. Annu Rev Phytopathol. 56: 1–26.

Copetta A, Lingua G, Berta G (2006) Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. Var. Genovese. Mycorrhiza. 16: 485-94.

Cvetkovikj I, Stefkov G, Karapandzova M, Kulevanova S, Satović Z (2015) Essential oils and chemical diversity of Southeast European populations of *Salvia officinalis* L. Chem Biodivers. 12: 1025-1039.

Endlweber K, Scheu S (2006) Establishing arbuscular mycorrhiza-free soil: a comparison of six methods and their effects on nutrient mobilization. Appl Soil Ecol. 34: 276–279.

Farhat BM, Jordán MJ, Chaouech-Hamada R, Landoulsi A, Sotomayor JA (2009) Variations in essential oil, phenolic compounds, and antioxidant activity of Tunisian cultivated *Salvia officinalis* L. J Agric Food Chem. 57: 10349–10356.

Farhat MB, Jordán MJ, Chaouch-Hamada R, Landoulsi A, Sotomayor JA (2016) Phenophase effects on sage (*Salvia officinalis* L.) yield and composition of essential oil. J Appl Res Med Aromat Plants. 3: 87-93.

Freitas MSM, Martins MA, Vieira IJC (2004) Yield and quality of essential oils of *Mentha arvensis* in response to inoculation with arbuscular mycorrhizal fungi. Pesqui Agropecu Bras. 39: 887–894.

Geneva MP, Stancheva IV, Boychinova MM, Mincheva NH, Yonova PA (2010) Effects of foliar fertilization and arbuscular mycorrhizal colonization on *Salvia officinalis* L. growth, antioxidant capacity, and essential oil composition. J Sci Food Agric. 90: 696-702.

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. Trans Brit Mycol Soc. 46: 235–246.

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring VA mycorrhizal infection in roots. New Phytol. 84: 489–500.

Ghoushchi MJ, Abbaszadeh B, Oraei M (2013) Effects of chemical and biological fertilizers on growth, yield and essential oil of *Salvia officinalis*. J Med Plants By-prod. 1: 31-37.

Govahi M, Ghalavand A, Nadjafi F, Sorooshzadeh A (2015) Comparing different soil fertility systems in Sage (*Salvia officinalis*) under water deficiency. Ind Crops Prod. 74: 20-27.

Grdiša M, Jug-Dujaković M, Lončarić M, Carović-Stanko K, Ninčević T, Liber Z, Radosavljević I, Šatović Z (2015) Dalmatian Sage (*Salvia officinalis* L.): A Review of biochemical contents, medical properties and genetic diversity. Agric Conspec Sci. 80: 69-78.

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. Calif Agric Exp Stn. 347: 1-32.

ISO 9909. (1997) Oil of dalmatian sage (*Salvia officinalis* L.).

Kapoor R, Giri B, Mukerji KG (2002) Mycorrhization of coriander (*Coriandrum sativum* L.) to enhance the concentration and quality of essential oil. J Sci Food Agric. 88: 1-4.

Karagiannidis N, Thomidis T, Panou-Filotheou E (2012) Effects of *Glomus lamellosum* on growth, essential oil production and nutrients uptake in selected medicinal plants. J Agric Sci. 4: 137-144.

Khaosaad T, Vierheilig H, Nell M, Zitterl-Eglseer K, Novak J (2006) Arbuscular mycorrhiza alter the concentration of essential oils in oregano (*Origanum* sp., Lamiaceae). Mycorrhiza. 16: 443-6.

Lermen C, Mohr FBM, Alberton O (2015a) Growth of *Cymbopogon citratus* inoculated with mycorrhizal fungi under different levels of lead. Sci Hortic. 186: 239-246.

Lermen C, Morelli F, Gazim ZC, da Silva AP, Gonçalves JE, Dragunski DC, Alberton O (2015b) Essential oil content and chemical composition of *Cymbopogon citratus* inoculated with arbuscular mycorrhizal fungi under different levels of lead. Ind Crops Prod. 76: 734-738.

Lermen C, Cruz RMS, Souza JS, Marchi BA, Alberton O (2017) Growth of *Lippia alba* (Mill.) N. E. Brown inoculated with arbuscular mycorrhizal fungi with different levels of humic substances and phosphorus in the soil. J Appl Res Med Aromat Plants. 7: 48-53.

Loomis WD, Corteau R (1972) Essential oil biosynthesis. Recent Adv Phytochem. 6: 147-185.

Marschner H (2012) Mineral nutrition of higher plants. 3. ed London: Elsevier, 643p.

Miladinović D, Miladinović L (2000) Antimicrobial activity of essential oil of sage from Serbia. Facta Universitatis. 2: 97–100.

Nell M, Voetsch M, Vierheilig H, Steinkellner S, Zitterl-Eglseer K, Franz C, Novak J (2009) Effect of phosphorus uptake on growth and secondary metabolites of garden sage (*Salvia officinalis* L.). J Sci Food Agric. 89: 1090-1096.

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Brit Mycol Soc. 55: 157–160.

Silva FC (2009) Manual de análises químicas de solos, plantas e fertilizantes. Embrapa informação tecnológica, Brasília, DF, 627p.

Silva APD, Garcia TL, Machineski O, Truber PV, Balota EL (2009) Resposta do *Jaracatia spinosa* à inoculação de fungos micorrizícos arbusculares em diferentes níveis de fósforo. Syn Scy. 4: 1–3.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, Academic Press, New York.

Statsoft. Electronic Statistics Textbook. Tulsa: StatSoft, 2017.

Tarraf W, Ruta C, Tagarelli A, De Cillis F, De Mastro G, 2017. Influence of arbuscular mycorrhizae on plant growth, essential oil production and phosphorus uptake of *Salvia officinalis* L. Ind Crops Prod. 102: 144–153.

Teuscher E (2006). Medicinal Spices: Medpharm Stuttgart, 324p.

Urcoviche RC, Gazim ZC, Dragunski DC, Barcellos FG, Alberton, O (2015) Plant growth and essential oil content of *Mentha crispa* inoculated with arbuscular mycorrhizal fungi under different levels of phosphorus. Ind Crops Prod. 67: 103-107.

Weber OB, de Souza CCM, Gondin DMF, Oliveira FNS, Crisóstomo LA, Caproni AL, Júnior OS (2004) Inoculação de fungos micorrízicos arbusculares e adubação fosfatada em mudas de cajueiro-anão-precoce. Pesqui Agropecu Bras. 39: 477–483.