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CROP PRODUCTION

The growth, photosynthetic pigments and essential oil composition of monocropped and intercropped lemon balm with yarrow

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ABSTRACT. The purpose of this study was to assess the influence of lemon balm intercropping with yarrow on the vegetative growth, photosynthetic pigments and essential oil contents as well as the chemical composition of essential oils. Accordingly, the lemon balm and yarrow monocroppings were compared to the intercropping of both species. The plants were cultivated for 120 days under field conditions. The experimental design followed randomized blocks with seven repetitions. Growth was assessed through the accumulation of shoot dry matter and through the leaf area. The photosynthetic pigment content was set based on the fully expanded leaves from five plants in each treatment. The essential oil was extracted through the hydrodistillation of the lemon balm leaves and yarrow leaflets dehydrated in a forced ventilation oven at 40°C. The chemical analyses of the essential oils were performed using GC-FID and GC-MS. Intercropping showed a reduction of vegetative growth. However, intercropping increased the lemon balm essential oil content. On the other hand, these parameters did not change in yarrow. There were variations in the level of chemical constituents in lemon balm and yarrow essential oils, which were more evident in the major constituents.

Keywords: *Melissa officinalis* L.; *Achillea millefolium* L.; intercropping; gas chromatography.

Crescimento, pigmentos fotossintéticos e composição do óleo essencial de melissa em monocultivo e consorciado com mil folhas

RESUMO. Objetivou-se avaliar a influência do cultivo consorciado de melissa com mil folhas, no crescimento vegetativo, teores de pigmentos fotossintéticos e de óleos essenciais e composição química de óleos essenciais. Para isso, foram comparados os cultivos solteiros de melissa e de mil-folhas com o cultivo consorciado de ambas. As plantas foram cultivadas por 120 dias em condições de campo. O delineamento experimental foi em blocos casualizados com sete repetições. O crescimento foi avaliado através do acúmulo de matéria seca da parte aérea e pela área foliar. Os teores de pigmentos fotossintéticos foram determinados a partir de folhas completamente expandidas de cinco plantas de cada tratamento. O óleo essencial foi extraído por hidrodestilação das folhas de melissa e de folíolos de mil folhas desidratados em estufa com ventilação forçada a 40°C. As análises químicas dos óleos essenciais foram realizadas por CG-DIC e CG-EM. O cultivo consorciado indicou redução no crescimento vegetativo, mas aumentou o teor de óleo essencial da melissa. Por outro lado, esses parâmetros não foram alterados para a mil-folhas. Houve variações nos teores dos constituintes químicos de ambos os óleos essenciais, que foram mais evidentes nos constituintes majoritários.

Palavras-chave: *Melissa officinalis* L.; *Achillea millefolium* L.; consórcio; cromatografia gasosa.

Introduction

Melissa officinalis L. (Lamiaceae), also known as lemon balm, is an aromatic plant that has a smell similar to lemons (Reis, Pinto, Rosado, & Corrêa, 2009). It is one of the most important medicinal plants since it has antioxidant, antimicrobial, spasmolytic, astringent and specific sensorial properties (Yadegari, 2016). The essential oil produced by the species has neral (α-citral) and geranial (β-citral) as major compounds, which are attributed to the plant's medicinal properties (Luz, Silva, Habber, & Marquez, 2014; Sorensen, 2000). However, its essential oil productivity is low, and the oil content varies from 0.01% to 0.52% (Argyropoulos & Müller, 2014; Avci & Giachino, 2016; Szabó et al., 2016). The species *Achillea millefolium* L. (Asteraceae), commonly known as yarrow, has medicinal properties and is used as a painkiller, anti-inflammatory, diuretic and antispasmodic. It is also used in cosmetics and food seasonings and it is considered to be an ornamental plant (Brasil, 2009; Pinto et al., 2014).

Yarrow intercropping with lemongrass (*Cymbopogon citratus* DC.) or rosemary (*Rosmarinus officinalis* L.) has increased the essential oil yield, and this was previously observed by Santos et al. (2009) and Arashiro et al. (2011).

Intercropping can be defined as a cultivation system based on the simultaneous growth of two or more plant species in the same area (Gao et al., 2009). This method is more acceptable for plant cultivation according to the agro-ecological models because it presents countless environmental, productive and economic advantages due to the increased productivity and decreased number of weeds, plagues and diseases (Grangeiro et al., 2008; Wang et al., 2014).

The intercropping of medicinal plants aimed at increasing essential oil production in low productivity species may be an alternative for family farmers. However, the awareness regarding the influence of intercropping of medicinal plants on the production of active ingredients is limited, and previous studies focused on greenery species (Chamoli, Varshney, Srivastava, Pandey, & Dayal, 2013; Singh, Singh, Ram, Yadav, & Chanotiya, 2013). In addition, these studies that focused on the use of medicinal plants did not demonstrate the influence of this cultivation technique on the quality of the essential oil.

Therefore, by considering the value given to the use of plants for medicinal purposes and the increased demand for raw materials, studies that are able to generate knowledge regarding intercropping of medicinal plants are needed because these studies will demonstrate the advantages of this technique for vegetal production. Accordingly, the purpose of the current study is to assess the influence of yarrow-lemon balm intercropping on the growth, photosynthetic pigment production and content, and qualitative and quantitative chemical composition of essential oils.

Material and method

The experiment was conducted in the experimental site at the Agricultural Department (DAG) of Federal University of Lavras (UFLA) from November 2013 to March 2014. The experimental site is located at 21° 14' S and 45° 00' W at an altitude of 918 m. The Cwa type is the prevailing climate for the area, which consists of dry winters and warm summers (Sá Júnior, Carvalho, Silva, & Alves, 2012).

The soil type in the experimental site is classified as Dystrophic Red Latosol (oxisol) (Santos, 2013). The soil was analyzed in the Soil Chemical Analyses Laboratory of the UFLA Soil Science Department in order to establish the soil's fertility attributes. The physical-chemical properties of the soil (0-20 cm) are as follows: pH: 5.7, K: 142.0 mg dm^{-3}, P: 2.84 mg dm^{-3}, Ca: 2.60 $_{c}$mol dm^{-3}, Mg: 0.90 cmol$_c$ dm^{-3}, Al: 0.10 cmol$_c$ dm^{-3}, H+Al: 2.90 cmol$_c$ dm^{-3}, SB: 3.86 cmol$_c$ dm^{-3}, t: 3.96 cmol$_c$ dm^{-3}, T: 6.76 cmol$_c$ dm^{-3}, V: 57.16%, M: 2.535, organic matter: 2.87 dag kg^{-1}, and P-Rem: 24.34 mg L^{-1}. The climatological data for the assessed period was provided by the climatology station at the Engineering Department of UFLA, which had a mean record for a minimum temperature of 18.1°C, maximum temperature of 29.5°C, rainfall of 139.5 mm, and relative humidity of 69.5%.

The seedlings were produced from yarrow and lemon balm plant matrices found at the Medicinal Garden of UFLA. The lemon balm vegetative propagation was conducted by using apical cuttings, and the yarrow vegetative propagation was completed through the use of stolons. The seedlings were transferred to the field and then planted 30 days after cultivation with a 0.40 x 0.40 m spacing in November 2013. One kilogram of bovine manure was added to each pit (15 x 15 x 20 cm) at planting. The culture handlings necessary for plant maintenance were performed throughout the experiment.

The experimental design consisted of randomized blocks with four treatments placed in seven blocks with nine useful plants from each species per experimental unit including the border. The treatments consisted of lemon balm monocropping, yarrow monocropping and lemon balm/yarrow intercropping.

Harvest occurred 120 days after cultivation. Lemon balm stems and leaves and yarrow petioles and leaflets were individually dehydrated in a forced ventilation oven at 40°C until they reached a constant weight. Growth was assessed in nine lemon balm and yarrow plants per treatment by using shoot dry matter. The leaf area of the lemon balm was also determined by 50 leaves plant^{-1}, which was standardized from the third pair of leaves using Li-Cor model Li-3100 leaf area meter (Li-Cor, Inc. Lincoln, Nebraska, USA).

The analyzed photosynthetic pigments were carotenoids and a, b and total chlorophylls. The extraction was performed according to the methodology described by Lichtenthaler and Buschmann (2001). The fully expanded leaves located in the third node of the five plants were collected per treatment. To extract the pigments, 200 mg of fresh leaves were homogenized with 20 mL of 80% acetone (v/v). The mixture was then filtered through glass wool, and the volume was increased to 30 mL with the addition of 80% acetone. Immediately following this procedure the absorbance was measured at 663 nm, 646 nm and 470 nm. All procedures were done in the dark to preserve the integrity of the pigments. The results were expressed as milligrams of pigment per gram of foliar tissue fresh matter (FM).

The data were subjected to an F test analysis of variance and the means were compared by using the Scott-Knott test as well as through orthogonal contrasts via the Scheffé test at 5% probability using the SISVAR® software version 5.3 (Ferreira, 2010). The performed orthogonal contrasts were Y1, lemon balm monocropping versus lemon balm intercropping, and Y2, yarrow monocropping versus yarrow intercropping.

To proceed with the essential oil extraction, 45 g of lemon balm dry leaves and 45 g of yarrow leaflets (without the petiole) were subjected to hydrodistillation in a modified Clevenger for 90 minutes. The essential oil was collected and purified through a liquid-liquid partition with dichloromethane (3 x 5 mL). The collected organic portion was treated with anhydrous magnesium sulfate. The salt was removed by simple filtration and dry organic fraction at room temperature in a gas exhaustion chapel. The essential oil content (mg 100 g^{-1} leaf dry matter) and yield (mg $plant^{-1}$) were determined. Next, the samples were stored under refrigeration at 4°C until the chemical analyses were conducted.

The chemical composition analyses were performed by using samples composed of aliquots of equal volumes of essential oil from the repetitions in each treatment. The chromatographic analyses were conducted in the Agilent 5890A chromatographer equipped with a flame ionization detector (GC-FID) and with an Agilent 5975C mass selective detector (GC-MS), using an electron-impact ionization of 70 eV in scan model at a 1.0 scan s^{-1} speed with a mass-acquisition interval of 40 - 400 m/z.

The essential oil analyses were performed in a HP-5MS column (30 cm long x 250 μm internal diameter x 0.25 μm thick). Helium was used as the carrier gas with a flow of 1.0 mL $min.^{-1}$. The temperatures in the injector and in the detector were 220 and 240°C, respectively. For the lemon balm oil, the initial temperature in the oven was 60°C with a temperature ramp of 3°C $min.^{-1}$ up to 150°C. This ramp was followed by the temperature ramp of 10°C min^{-1} up to 250°C. For the yarrow oil, the initial temperature in the oven was 60°C with temperature ramp of 3°C $min.^{-1}$ up to 200°C. This ramp was followed by a temperature ramp of 10°C $min.^{-1}$ up to 270°C, and the yarrow oil was maintained under isothermal conditions for 1 minute. The essential oil samples from both species were diluted with ethyl acetate (1%, v/v). One microliter of each sample was automatically injected using the split mode at a ratio of 1:50.

The chemical components were identified using the comparison of their retention indices concerning the co-injection of an n-alkanes standard solution (C_8-C_{20}, Sigma-Aldrich®, St. Louis, USA) and through the comparison of mass spectra in the database of the NIST/EPA/NHI (Nist, 2008) library. The retention indices were calculated using the Van den Dool and Kratz (1963) equation. The attributions of the chromatographic peaks were set based on the retention indices described in the literature (Adams, 2007).

Result and discussion

The means of the analyzed growth variables of the lemon balm were significantly influenced by the intercropping system when the variables were compared against the monocropping system (Table 1). The intercropping system presented the lowest leaf area (486.76 cm^2), stem dry matter (24.43 g) and leaf dry matter (27.99 g) values. On the other hand, there were no significant differences in the yarrow growth variables.

Table 1. Mean leaf area★ and shoot dry matter values, and the orthogonal contrast estimates of the lemon balm and yarrow monocropping and intercropping in the field.

Plant	Cultivation	Leaf area (cm^2)	Dry matter (g $plant^{-1}$)	
			Stem	Leaf
Lemon Balm	Monocropping	537.88	35.13	36.12
Lemon Balm	Intercropping	486.76	24.43	27.99
Contrast	MS *vs* MC	51.121**	10.694	8.121**
Yarrow	Monocropping	-	41.11	21.43
Yarrow	Intercropping	-	42.60	22.28
Contrast	MFS *vs* MFC	-	-1.497	-0.856
CV (%)		10.85	24.65	17.99

** Statistically significant in the Scheffé test at 5% probability. MS (lemon balm monocropping), MC (lemon balm intercropping), MFS (yarrow monocropping), MFC (yarrow intercropping).

Brandão, Silva, Jaruche, Santos, & Martins (2014) have analyzed yarrow intercropping in pots planted with lemon balm and lettuce. They found satisfactory results in the dry matter production, and their results demonstrated that the field conditions may lead to the competition for resources. A study conducted using yarrow/rosemary intercropping demonstrated that rosemary reduced growth (Arashiro et al., 2011). Other reports using yarrow intercropping have also found reduced growth of other aromatic species (Corrêa Júnior, Ming, &

Scheffer, 1998, Santos et al., 2009; Scheffer & Corrêa Júnior; 2006). However, Arashiro et al. (2011) discovered an increase in rosemary growth when it was intercropped with yarrow in fertilized treatments because it increased the competition for nutrients. Thus, the negative effect of intercropping with other aromatic species, such as rosemary and lemon balm, on the yarrow growth variables may be related to an interspecific competition associated with nutrient availability.

The photosynthetic pigment production was also influenced by the intercropping of both studied species. The intercropping system has promoted an increase in chlorophyll *a* (0.38 to 0.66), chlorophyll *b* (0.57 to 0.76), total chlorophyll (0.95 to 1.43) and carotenoid (0.43 to 0.50) amounts in lemon balm, and there was a reduction in the ratio of b/a (Table 2). Such a reduction results from more production of chlorophyll *a*. The yarrow pigments have also presented a significant increase in the intercropping system, except for the amount of chlorophyll *a*, which remained the same.

Table 2. Mean quantification values of photosynthetic pigments and the orthogonal contrast estimates in the lemon balm and yarrow monocropping and intercropping in the field.

Plant	Cultivation	Chlorophyll (mg/g MF^{-1})				Carotenoid
		a	*b*	*b/a*	Total	(mg g^{-1} MF)
Lemon balm	Monocropping	0.38	0.57	1.47	0.95	0.43
Lemon balm	Intercropping	0.66	0.76	1.17	1.43	0.50
Contrast	MS *vs* MC	0.286**	0.196**	-0.301**	0.483**	0.138**
Yarrow	Monocropping	0.63	0.61	1.00	1.23	0.56
Yarrow	Intercropping	0.65	1.13	1.81	1.78	0.58
Contrast	MFS *vs* MFC	0.21	0.526**	0.809**	0.555**	-0.789**
CV (%)		13.96	15.91	22.39	10.7	11.34

** Statistically significant in the Scheffé test at 5% probability. MS (lemon balm monocropping), MC (lemon balm intercropping), MFS (yarrow monocropping), MFC (yarrow intercropping).

Plants often cultivated in shady environments receive longer wavelengths in the far-red region. Therefore, in order to increase light absorption and maintain the energetic balance between photosystems, the plants invest in more chlorophyll *b*, total chlorophyll and the ratio of *b/a* (Cao, 2000). Accordingly, the intercropping of the studied species may have promoted a competition for light, and it may have promoted the shady plant features in yarrow. Although the chlorophyll *b* contents were higher in lemon balm, the plant showed features of plants cultivated under sunlight with a low ratio of *b/a*, more carotenoid production and a smaller leaf area. Thus, it seems that intercropping may have promoted competition for light, nutrients and water, which has stressed the lemon balm.

Intercropping increased the content and yield of lemon balm essential oils (Table 3). On the other hand, yarrow did not show significant differences between the monocropping and intercropping scenarios. The increased essential oil content in plants intercropped with yarrow was also observed in lemongrass and in rosemary (Arashiro et al., 2011; Santos et al., 2009). In addition, Corrêa Júnior et al. (1998) have stated that yarrow presents allelopathy over aromatic plants and that it favors the production of essential oils in these plants.

Table 3. Mean values of the content and yield of the essential oils and the orthogonal contrast estimates of lemon balm and yarrow monocropping and intercropping in the field.

Plant	Cultivation	Content (%)	Yield	
			(g $plant^{-1}$)	(g ha^{-1})
Lemon balm	Monocropping	0.111	0.041	2561
Lemon balm	Intercropping	0.221	0.061	1898
Contrast	MS *vs* MC	0.110**	-0.019**	663.714
Yarrow	Monocropping	0.109	0.045	2816
Yarrow	Intercropping	0.121	0.052	1608
Contrast	MFS *vs* MFC	0.012	-0.006	1208,14**
CV (%)		21.75	25.25	30.73

** Statistically significant in the Scheffé test at 5% probability. MS (Lemon balm monocropping), MC (lemon balm intercropping), MFS (yarrow monocropping), MFC (yarrow intercropping).

The increased essential oil production may be associated with the deviation of carbon skeletons from photosynthesis to produce the secondary metabolites in lemon balm. Since the production of chlorophylls a and b improved, there is a higher proportion of PSI and PSII, respectively (Nakazono, Dacosta, Futatsugi, & Dias, 2001). Thus, the intercropping system has apparently promoted a balanced ratio between the photosystems in the lemon balm because there was increase in both chlorophylls *a* and *b*. This response in the intercropping conditions must have helped increase the photosynthetic rates and promote higher growth; this is a fact that was not observed in the present study. Accordingly, the photosynthetic products may have been used in secondary metabolism at the expense of growth and may be the response of lemon balm to survive in competitive environments.

There were no qualitative differences in the chemical composition of lemon balm essential oils between the monocropping and intercropping systems (Tables 4 and 5, respectively). Regardless of the cultivation system, the chemical nature of the lemon balm constituents was mostly monoterpene, and the lemon balm presented neral (29.68 - 27.29%) and geraniol (41.04 - 38.03%) as major constituents. However, the monocropping presented higher total monoterpene and citral (neral + geraniol) contents, with differences regarding the intercropping of 3.03 and 5.4%, respectively. The prevalence of monoterpene, geraniol and neral compounds as major constituents was recognized by other authors for the species (Brant et al., 2009; Martins & Pastori, 2004).

Table 4. Chemical composition of lemon balm leaf essential oil in monocropping and intercropping.

Constituents	Area (%) ± SD	
	Monocropping	Intercropping
Geranial	41.04 ± 1.95	38.03 ± 4.36
Neral	29.68 ± 0.77	27.29 ± 2.16
Citronellal	6.54 ± 2.47	3.89 ± 0.61
Trans-6-hydroxy-α-terpineol	2.23 ± 0.74	5.29 ± 2.28
Geranic acid	1.96 ± 0.24	2.18 ± 0.23
Methyl geranate	1.24 ± 0.44	1.74 ± 0.20
Isogeranial	1.06 ± 0.19	0.70 ± 0.15
Methyl citronellate	0.92 ± 0.19	0.61 ± 0.06
Valerate citronellate	0.90 ± 0.00	3.89 ± 1.83
Geranyl acetate	0.82 ± 0.25	0.64 ± 0.17
Iso-pinocamphone	0.73 ± 0.14	0.49 ± 0.10
Citronelol	0.51 ± 0.07	0.40 ± 0.05
Geranyl vinyl ether	0.49 ± 0.14	0.35 ± 0.09
Photocitral A	0.40 ± 0.15	0.32 ± 0.08
Prenyl angelate	0.31 ± 0.01	0.35 ± 0.05
1,8-Cineole	0.30 ± 0.11	0.26 ± 0.07
Trans-geraniol	0.29 ± 0.08	0.19 ± 0.00
Cis-pinocamphone	0.28 ± 0.09	0.29 ± 0.04
Linanool	0.23 ± 0.06	0,22 ± 0,06
α-Cyclogeraniol	0.23 ± 0.06	nd
Total Monoterpenes	90.16	87.13
Caryophyllene oxide	7.02 ± 1.52	7.32 ± 1.56
4(12),8(13)-Carophyl dien-5α-ol	0.30 ± 0.11	0.33 ± 0.10
Humulene epoxide II	0.22 ± 0.00	0.22 ± 0.01
Total Sesquiterpenes	7.54	7.87
Octen-3-ol	0.29 ± 0.12	0.22 ± 0.05
Benzaldehyde	0.16 ± 0.03	nd
Non-identified	nd	4.10 ± 0.83
Others	0.45	4.32
Total Area	98.15	99.91

SD: Standard deviation (n = 3); nd =non-detected.

Table 5. Chemical composition of yarrow leaflet essential oil in monocropping and intercropping.

Constituents	Area (%) ± SD	
	Monocropping	Intercropping
Borneol	9.74 ± 1.49	9.29 ± 1.37
Terpinen-4-ol	6.96 ± 1.40	6.76 ± 1.15
1,8-Cineol	3.18 ± 0.89	5.29 ± 1.85
α-Terpineol	2.66 ± 0.41	2.59 ± 0.35
Trans-sabinene hydrate	1.64 ± 0.22	1.58 ± 0.15
Sabinene	1.53 ± 1.02	1.70 ± 0.24
Cis-sabinene hydrate	1.62 ± 0.20	1.60 ± 0.19
α-Pinene	1.28 ± 0.48	2.23 ± 0.00
D-Limonene	0.75 ± 0.25	1.22 ± 0.48
Bornyl acetate	0.67 ± 0.09	0.64 ± 0.10
trans-2-Mentenol	0.51 ± 0.09	0.51 ± 0.08
α-Terpinene	0.34 ± 0.09	0.51 ± 0.14
Eugenol	0.29 ± 0.04	0.25 ± 0.04
Culminaldehyde	0.21 ± 0.01	0.24 ± 0.03
Total Monoterpenes	31.38	34.41
Chamazulene	33.81 ± 2.93	26.25 ± 4.75
β-Caryophyllene	12.35 ± 1.77	11.56 ± 1.82
Caryophyllene oxide	6.89 ± 0.73	6.04 ± 0.66
Spathulenol	2.47 ± 0.19	2.18 ± 0.30
α-Caryophyllene	1.55 ± 0.21	1.45 ± 0.23
D-Germacrene	0.57 ± 0.18	0.61 ± 0.17
Total Sesquiterpenes	57.64	48.09
Non-identified	1.77 ± 0.06	1.51 ± 0.25
1-Octen-3-ol	1.32 ± 0.06	0.94± 0.44
1-Nonen-3-ol	0.50 ± 0.07	nd
Non-identified	nd	6.91 ± 3.67
Others	3.59	9.36
Total Area	92.61	91.86

SD: Standard deviation (n = 3); nd = non-identified.

Regarding the quantitative analyses of yarrow leaf chemical composition, the most evident variations between the assessed cultivation systems were a 9.55% difference in the total sesquiterpenes and a 7.56% difference in the chamazulene content. The main constituents of this oil were the following: chamazulene (33.81 - 26.25%), β-caryophyllene (12.35 - 11.56%), borneol (9.74 - 9.29%), 4-terpineol (6.96 - 6.76%) and caryophyllene oxide (6.89 - 6.04%). The presence of these compounds as main constituents in yarrow was already described in other studies (Nadim, Mali, Ahmad, & Bakshi, 2011; Rahimmalek et al., 2009).

Intercropping led to a reduced amount of monoterpene compounds identified as major compounds in the lemon balm, as well as a reduction of the major constituents of sesquiterpene in yarrow. The medicinal properties attributed to both species are associated with the presence of these major compounds (Reis et al., 2009). Although intercropping has promoted an increase in essential oil production, the technique that promoted essential oil quality decreased in both species under field conditions. Thus, the intercropping between yarrow and lemon balm is not indicated under field conditions. In addition, the results found in this study have created the need for carefully assessing the quality of essential oils in future studies regarding yarrow intercropping with different aromatic species.

Conclusion

Intercropping of lemon balm and yarrow decreases growth in lemon balm, but it has no significant effect on yarrow.

The photosynthetic pigment contents change by the crop systems. Intercropped lemon balm exhibits an increase content of chlorophylls *a, b*, and total, and in carotenoids. While, intercropped yarrow has a significant increase in chlorophylls *b* and total, remaining the same content of chlorophyll *a* and carotenoids in comparison with monocropping system.

The content and yield of lemon balm essential oil increases in the intercropping system, whereas yarrow does not change the essential oil content. The amount of major constituents in both species drop in the intercropping system.

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