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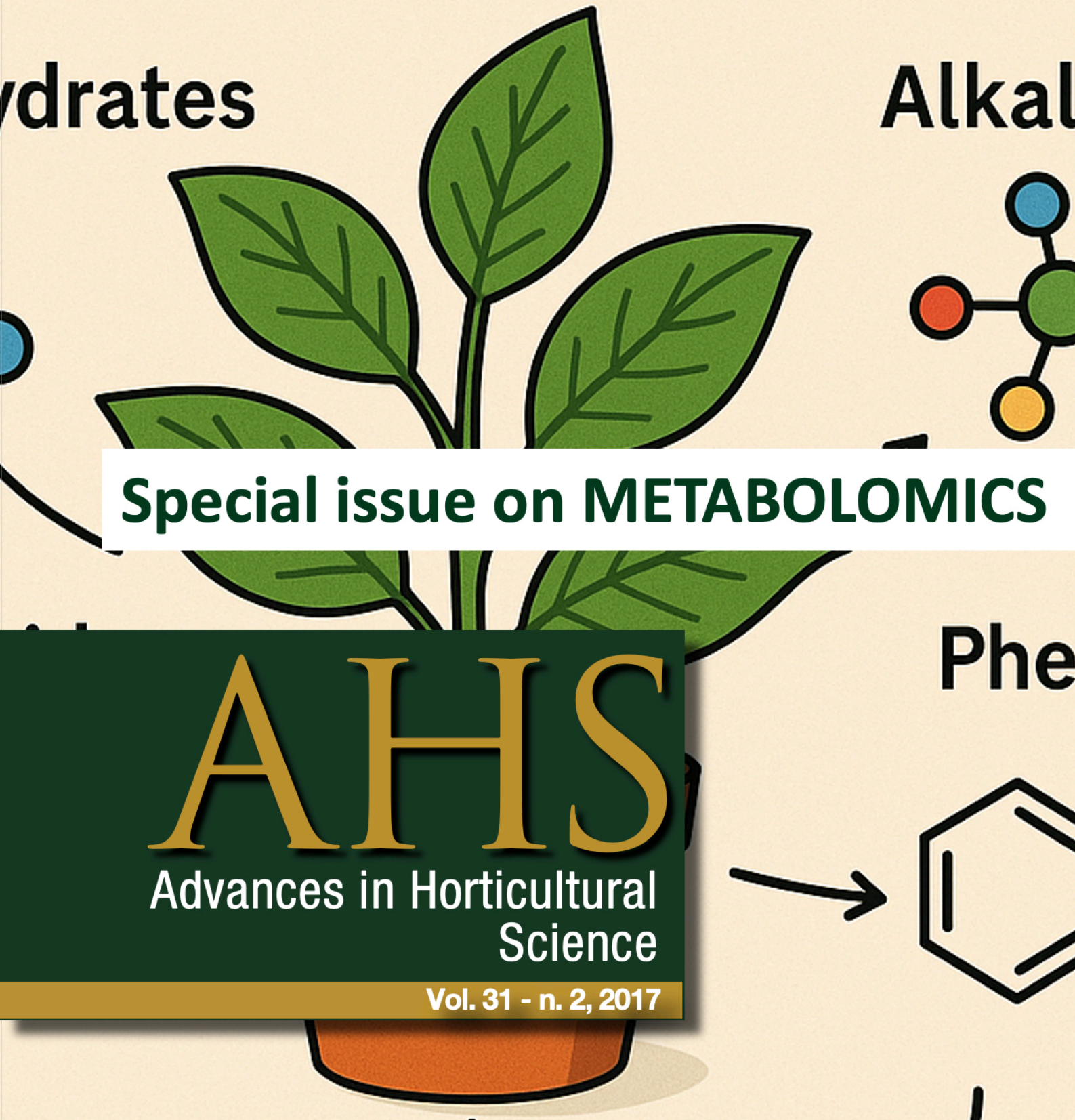

Special issue on METABOLOMICS

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SPECIAL ISSUE ON PLANT AND FOOD METABOLOMIC

This special issue has been realized with the contribution of the Regione Toscana "PRAF 2012-2015 misura 1.2 e)" program, "Profumi e sapori della Toscana: dalla caratterizzazione del volatoma alla valorizzazione dei prodotti alimentari di pregio (VOLATOSCA)" project.

Foreword

Throughout the nineteenth century and for much of the twentieth century, the definition of secondary metabolites simply meant that these substances, while present in plants, did not participate in the metabolic processes essential to the life of the organism. They were defined as by-products of primary metabolism or as excretory products or final products of metabolism, always pointing out that their irregular presence on plants meant that they were not indispensable. Over the last 50 years, knowledge in this area has been greatly expanded, especially following the development of new isolation, separation and structural identification technologies (spectroscopy, nuclear magnetic resonance and mass spectrometry). The ease of structural identification has expanded the number of secondary metabolites to reach the unthinkable number of 200000 for many of which, however, remain uncertainty about their biochemical and physiological role. In recent years, however, many researches have progressively clarified the role of these substances and some of them are known to have a fundamental function. Thus it has been for shikimic acid, for many years considered a simple metabolite of *Illicium anisatum*, then revealed as a key molecule for synthesis of aromatic amino acids and, in turn, precursors of phenylpropanoids (flavonoids, coumarin, tannins and lignin). Certainly these are not secondary functions!

Primary metabolism is essential to plants for growth and development, and secondary metabolism helps plants to interact with the environment. Many plant metabolites are also industrially important (e.g.: the physiologically active alkaloids used in modern medicine). These metabolites are produced by plants through complex metabolic pathways.

Among the researchers in this area it is inevitable to remember J.B. Harborne, who has been devoting some 50 years to the study of flavonoids and, more generally, to chemical ecology and plant chemosystematics. Harborne's work has called for experimentation on all other classes of secondary metabolites, alkaloids, non-protein amino acids, glucosinolates, lectins, terpenes, steroids, tannins, flavonoids, phenylpropanoids, lignins, coumarin, waxes, etc. Plant secondary metabolites are critical to various biological processes.

The plant's specific organization has led it to produce a large amount of secondary metabolites and probably selective pressure has left those molecules capable of conferring specific benefits to the plant: defense role against animal, fungal and bacterial parasites, but also those molecules that allow to establish relationships between plants of the same species or between different species.

Numerous contributions to this special number of AHS are devoted to ecological biochemistry and that explains the "non-secondary" role of so many metabolites; It is also hoped that the molecular biological tools will spread to the labs in order to have accurate information on the genes involved in the various secondary metabolism.

Plant genomes are variously estimated to contain 20000-60000 genes, and perhaps 15-25% of these genes encode enzymes for secondary metabolism. Still a lot of research work has to be done both physiologically and biochemically on the secondary metabolism of many species, but I believe it is essential that laboratories use data availability about the genes of *Arabidopsis*, an extraordinary model species, involved in secondary metabolism.

Thus, our knowledge of secondary metabolites, which have already made great advances over the last decades, will make further progress in both physiological and biochemical and technological terms, confirming that their traditional placement as "secondary metabolism products" was only due to lack of knowledge.

Amedeo Alpi

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Chemical characterization and sensory analysis by blind and visually impaired people of local peach varieties

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Key words: ancient varieties, physicochemical traits, *Prunus persica* (L.) Batsch, total antioxidant capacity, total phenols.

Abstract: The interest in locally produced foods by the reintroduction of old varieties is due to their environmental hardiness and suitability for low-input agricultural systems. These managements can often produce fruits with imperfections, preventing the consumer acceptance. We have settled a new sensory evaluation going beyond the appearances, involving blind and visually impaired people to provide a quality evaluation of fruits linked to intrinsic rather than exterior characteristics. The research was conducted over two consecutive harvest seasons on three peach old local varieties ('Alberta', 'Mora di Dolfo' and 'Regina di Weinberger' called *in loco* 'Regina di Bember') grown in central Italy. On fruits, physicochemical traits (fruit weight, peel and flesh color, flesh firmness, pH, total soluble solids, titratable acidity), antioxidant content (total antioxidant capacity and total phenols) and sensory analysis were assessed. The three local peach varieties showed interesting fruit attributes in both studied growth-ripening seasons. The white-fleshed 'Mora di Dolfo', characterized by the highest antioxidant contents, was particularly appreciated by panellists for its aroma. The new sensory analysis, providing an evaluation based on judgment of intrinsic characteristics of peaches, emerges as a valid tool to assess the interest and appreciation of fruits for a conscious consumer's choice.

1. Introduction

In these last years, more consumers are interesting in alternative food systems which have grown in tandem with a proliferation of diverse food attribute labels, such as organic, local, humane, fair trade, etc. (Berlin *et al*., 2009). In particular, locally products, such as fruits and vegetables, not offered by conventional grocery stores, are looking for niche markets, in country and urban areas. Studies indicate that many consumers associate the local foods with higher perceived quality as well as increased freshness and naturalness of the products (Denver and Jensen, 2014). The appeal for local varieties, represented by ancient genotypes, is due to the major environmental hardiness (Negri, 2005) able to tolerate extreme climate events such as an increase in temperatures and frequency or intensity of precipitation. This peculiarity makes ancient genotypes suitable for sustainable cultivations as support for farmers and consumers addressed to choose healthy products.

Fruits and vegetables are well recognized to be important source of vitamins, minerals, fibers and many hundreds of compounds with potential antioxidant activity which, acting against cellular oxidation reactions, may have beneficial effects on human health (Halliwell, 1996). The antioxidant activity of fruits is strongly affected by the species and, within species, by the variety which is the main factor in determining fruit nutritional quality (Scalzo *et al.*, 2005). Among other pre- and post-harvest factors influencing the antioxidant properties, such as cultivation techniques, ripening season, shelf-life and processing, the environmental conditions have an important role. In particular, it has been found that light intensity, temperature together with water availability are related to the antioxidant activity in different fruit species (Lee and Kader, 2000).

The fruit nutritional quality of many commercially important varieties has been characterized, but still scanty is knowledge on the antioxidant properties of ancient genotypes that can show high quality performances, in terms of flavor and nutritional properties (Donno *et al.,* 2012; Jakobek *et al.,* 2013). Recently, a trend to discover and to reintroduce into marketplace genotypes native of a restricted geographical

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area took place (Krawitzkyab *et al*., 2014)**.** The local varieties, mainly managed by low-input agricultural systems, often produce fruits with defects, preventing the consumer acceptance (Donno *et al*., 2012). It has been showed that the appearance of fresh fruits is the primary criterion in making purchasing decisions (Kays, 1991). To overcome this approach we have settled a new sensory evaluation going beyond the appearances, involving blind and visually impaired people (Bartolini *et al*., 2015 a), who having a higher sense of taste and smell (Luers *et al*., 2014), can provide a quality assessment of fruits linked to intrinsic rather than exterior characteristics.

The aim of this research was to determine some fruit quality traits of three peach local varieties originated from Lucca province (central Italy). Physicochemical and antioxidant parameters were studied, and the innovative sensory analysis involving people with visual disability was introduced.

2. Materials and Methods

Plant material and cultivation site

The research was conducted over two consecutive harvesting seasons (2014-15) on full bearing peach trees of old local varieties: 'Alberta', 'Mora di Dolfo' and 'Regina di Bember'. This latter variety is an example of synonymy; its name was modified by local citizen from the original 'Regina di Weinberger' (Bartolini *et al*., 2015 a), a Californian variety widespread at the end of fifties which nowadays has lost importance at national level.

The orchards were located in a traditional area at high agricultural vocation (Tuscany, Lucca province, lat. 44.02 N, long. 10.27 E) and trees were grown in similar soil conditions, using low input farming practices.

The main climatic parameters, minimum-maximum temperatures and rainfall data were provided by the Regional Agro-meteorological Service of Florence (SIR) (http://www.sir.toscana.it).

At physiological maturity stage, samples of peaches were collected and analyzed to determine the main physicochemical, antioxidant and sensory traits.

Physicochemical analysis

From each fruit (*N* = 30 per variety), measurements of weight, peel and flesh color, flesh firmness (FF), total soluble solids (TSS), pH and titratable acidity (TA) were determined. Fruit weight was expressed according to the following size categories (Della Strada *et al*., 1984): very small (≤90 g), small (91-125 g), medium (126-160 g), large (161-195 g), very large (>195 g). Peel and flesh color was evaluated using color charts according to UPOV Code (International Union for the Protection of New Varieties of Plants, Geneva) for peach. The red cover color area of peel (CC) was visually evaluated and expressed as percentage. Firmness was assessed with a manual penetrometer on two peeled opposite areas at the equatorial region of peach, using an 11-mm-wide plunger (Model 53200SP TR, TR-Turoni & C. Inc Forlì, Italy). TSS were measured using a refractometer (Model 53015C TR, TR-Turoni & C. Inc Forlì, Italy) and expressed in °Brix at room temperature. The fruit pH was recorded with the help of an electronic pH meter, and TA was determined on fruit juice by titrating a known volume of juice with 0.1N sodium hydroxide (NaOH) to pH 8.1. TA was expressed as milliequivalents of malic acid per 100 grams of fresh weight (meq malic ac. 100 g FW^{-1}).

Total antioxidant capacity (TAC) and total phenol (TP) analysis

Total Antioxidant Capacity (TAC) and Total Phenols (TP) analyses were carried out on the same fruits that had been previously subjected to the physicochemical determinations. Fresh fruit samples (flesh with peel) of 3 g (in triplicate) were immediately frozen and stored at -20°C until extraction. The samples were homogenized using an ultra-Turrax blender at 4°C to avoid oxidation. The extraction was performed in 80% ethanol for 1 h in a shaker in the dark and subsequently centrifuged at 4°C for 10 min at 2600 g. The supernatant was used for TAC (Total antioxidant capacity) and TP (total phenol) analysis.

TAC was evaluated using the improved Trolox equivalent antioxidant capacity (TEAC) method (Arts *et al*., 2004). The TEAC value was calculated in relation to the reactivity of Trolox, a water-soluble vitamin E analogue that was used as an antioxidant standard. In the assay, 40 µl of the diluted samples, controls or blanks added to 1,9690 µl $ABTS^{\bullet+}$ solution, resulted in a 20-80% inhibition of the absorbance. The decrease in absorbance at 734 nm was recorded 6 min after an initial mixing, and plotted against a dose-response curve calculated for Trolox (0-30 µM). Antioxidant activity was expressed as micromoles of Trolox equivalents per gram of fresh weight (µmol TE g FW^{-1}). Trolox was purchased from Sigma Chemical Co. (St. Louis, MO).

TP content was determined according to the improved Folin-Ciocalteu (F-C) method (Waterhouse, 2001). The assay provides a rapid indication of the

antioxidant status of the studied material and is valuable for different food samples. The standard compound for the calibration curve was gallic acid (GA, Sigma Chemical Co, - St. Louis, MO). Total phenol content was calculated as milligrams of GA equivalent (GAE) per gram of fresh fruit weight (mg GAE g FW^{-1}). The absorbance of the blue colored solutions was read at 765 nm after incubation for 2 h at room temperature.

Sensory evaluation

The sensory evaluation was carried out on fruits of the varieties 'Alberta' and 'Mora di Dolfo' as they have the same harvest time (end of July) but different characteristics, such as taste, ground peel (yellow and light-green) and flesh color (yellow and white), respectively. The test was performed without visual inspection, in collaboration with blind and visually impaired persons of the 'Blind and Visually Impaired People Italian Association' of Lucca (Italy). This is an original experiment on stone-fruits realized for 'going beyond the appearances' that, to our knowledge, is the only one after an experience carried out in Sicily (http://www.consorzioparsifal.it). Taking into account the disability of the assessors-consumers, a tentative of protocol was established using an acceptability test, as a pertinent option for specific applications (Varela and Ares, 2012). In both years, the same 10 blind and visually impaired persons, aged between 26 and 65, performed the evaluations on the selected varieties. In order to make the evaluation phase easier, prior the acceptability test a short training stage (5 hours) was provided outlining methodology and procedure of the sensory method.

A room equipped with individual sites was used. To each panellist, room-tempered and washed half-unpeeled anonymous peaches were served in randomized order, and samples were evaluated in one session. The panellist were invited to rinse the mouth with mineral water and eat saltless bread between samples, in order to avoid tiring effects. The fruit attributes evaluated were the following: shape, size, texture, aroma, sweetness, acid taste, juiciness (Shinya *et al*., 2014). Moreover, participants were asked to assess the fruit global appreciation. A continuous non-structured 9-point hedonic scale was utilized for evaluation: 1= dislike extremely; 2= dislike very much; 3= dislike moderately; 4= dislike slightly; 5= neither like nor dislike; 6= like slightly; 7= like moderately; 8= like very much; 9= like extremely (Porretta, 2000).

The blind judges were requested to indicate the score for each attribute and they were supported by sighted persons helping them to fill the data form. Judges were not informed about the characteristic of fruits such as peel and flesh color.

Statistical analysis

Physicochemical data of each variety were compared using a Student's *t*-test analysis with two treatments (2014 and 2015 harvest seasons); for each season, 30 repetitions/parameter were used, excluding TA and pH, where three composite samples containing ten fruit per sample were considered. A two-way ANOVA analysis were performed to test the effects of year and variety on the main physicochemical and antioxidant parameters. Differences on the content of antioxidants and phenolics between the three varieties were investigated with the analysis of variance (ANOVA), using Tukey's post hoc test at $P \leq 0.05$. A correlation analysis of antioxidant capacity versus total phenol content was calculated and correlation coefficient was reported in terms of goodness of fit. For the sensory evaluation, taking judges as factors, results were compared through the Student's *t*-test analysis between harvest seasons. Spearman's correlations were performed to estimate relationships among sensory and physicochemical traits of fruits. Data are reported as means ± standard errors of the means (SEM), and the analysis were all performed using the statistical package GraphPad Prism 5 (GraphPad Software, Inc.).

3. Results and Discussion

Weather conditions

During the fruit growth, from May to July, the considered harvest seasons were characterized by different weather conditions, in terms of temperatures and rainfall events (Table 1). In particular, at the final stages of fruit ripening the strongest differences were observed: the amount of precipitation was consistently higher in 2014 when, only on July, more than 300 mm of rainfall occurred. This condition was unusual for Lucca area in comparison to

Table 1 - Monthly minimum and maximum temperatures (°C) and cumulative rainfall (mm) from May to July, over a 2-year period (2014-2015)

Month	2014				2015			
	T. Min.	T. Max.	Average	Rainfall	T. Min.	T. Max.	Average	Rainfall
May	11.8	23.7	17.8	39.8	13.0	24.5	18.8	34.4
June	16.0	29.4	22.7	48.0	16.8	30.2	23.5	29.8
July	17.4	28.7	23.2	314.6	20.7	33.9	27.3	7.4
Average	*15.1*	*27.3*	*21.2*		*16.8*	*29.5*	*23.2*	
Total rainfall				*40.4*				*71.6*

average values of the last 10 years, ranging from 8.6 to 60.2 mm (SIR Toscana). The cumulative rainfall of the early summer seasons (May-July) was about 400 and 72 mm in 2014 and 2015, respectively. The second year was particularly drought and warm by mean temperatures of 4-5°C more than 2014.

Physicochemical and antioxidant fruit parameters

Harvest time and mean values of the main physicochemical traits of the peach varieties, recorded over a 2-year period, are presented in Table 2. Under the environment of Lucca area, the harvest time occurred between the first ('Regina di Bember') and third decade of July ('Alberta' and 'Mora di Dolfo'). Considering the physical traits of fruit, all varieties showed attractiveness: - 'Alberta' was characterized by fruit of medium size with yellow ground peel (10-50% red cover color) and deep yellow flesh; - 'Mora di Dolfo' had a large fruit size, light-green peel (30-50% red cover color) and white flesh; - 'Regina di Bember' had a medium-large fruit size, yellow ground peel, (50-90% extensive red cover color) and deep yellow flesh (Fig. 1).

Within each variety, values of chemical parameters showed variations between years, particularly for soluble sugars (TSS), titratable acidity (TA) and pH. 'Regina di Bember' had the greatest differences in relation to the weather conditions occurred during the ripening period of the considered years. TSS ranged from about 9 to 12 °Brix, and TA changed between 8 and 6 meq malic ac. 100 g FW^{-1} in the wettest (2014) and driest (2015) year, respectively. As a consequence, the TSS/TA ratio varied greatly with the lowest values in the wettest year. A similar trend was recorded in 'Alberta' where TA changed while TSS did not differ as much. No changes in chemical parameters were observed in 'Mora di Dolfo', performing well under the rainy climatic conditions of 2014. In both years, fruits exhibited similar values in TSS (11.8-13.9 °Brix), TA (9.6-9.8 meq malic ac. 100 g FW^{-1}) and, as a consequence, in their ratio.

The ANOVA results comparing harvest year and variety effect showed significant interactions 'year x variety' for TA and TSS/TA (Table 3). During the latter stages of fruit development, the influence of weather

Table 2 - Harvest time, peel and flesh color, red cover color (CC, %), and main physicochemical parameters recorded on three local peach varieties, over a 2-year period: fruit size, flesh firmness (FF, Kg/0.5 cm^2), total soluble sugars (TSS, °Brix), pH, titratable acidity (TA, meq malic ac. 100 g FW^{-1}), sugars/acids ratio (TSS/TA)

	'Alberta'		'Mora di Dolfo'		'Regina di Bember'	
Harvest time	end July		end July		early July	
Peel color	yellow, 10-50% CC		light green, 30-50% CC		yellow, 50-90% CC	
Flesh color	deep yellow		white		deep yellow	
Fruit size	medium		large		medium-large	
	2014	*2015*	*2014*	*2015*	*2014*	*2015*
FF	1.2±0.3	1.7±0.1	3.9±0.5	5.0±0.4	2.8±0.5	3.3±0.4
TSS	10.7±0.4	11.6±0.2	11.8±0.9	13.9±0.4	9.2±0.4	12.2±0.7 *
pH	3.80±0.1	4.15±0.1*	3.71±0.0	3.75±0.0	3.81±0.0	3.76±0.0
TA	10.2±0.1	6.1±0.1 *	9.6±0.1	9.8±0.1	8.3±0.1	6.2±0.1 *
TSS/TA	1	1.9 *	1.2	1.4	1.1	2.0 *

Mean±SEM. Within each variety, asterisk indicates significant differences between years by Student *t*-test ($P \leq 0.05$).

Fig. 1 - The peach old local varieties 'Alberta', 'Mora di Dolfo' and 'Regina di Bember' (originally 'Regina di Weinberger').

Table 3 - Two-way ANOVA results. Variables: TSS (°Brix), TA (meq malic ac. 100 g FW^{-1}), TSS/TA, TAC (µmol TE g FW^{-1}), and TP (mg GAE g FW^{-1})

Main effects	TSS	TA	TSS/TA	TAC	TP
			P		
Year	0.0001 ***	0.0015 **	< 0.0001 ***	0.6980 NS	0.2792 NS
Variety	0.0015 **	0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***
Interaction Year x Variety	0.5636 NS	0.0389 *	0.0016 **	0.0113 *	0.2500 NS

*, **, ***: significant at $P \leq 0.05$, 0.01, 0.001, respectively. NS= not significant.

conditions on pomological and quality properties has been proved: among fruit quality parameters, soluble solids content can vary substantially from year to year, and rainfall and summer sunshine have been identified as the main affecting factors (Choi *et al*., 2003). In peach, extensive rain may reduce the sweetness, while severe water stress or regulated irrigation deficit techniques caused a soluble sugar increased, as a recognized physiological response to stress (Kwon *et al*., 2008).

As regards TAC (Fig. 2A), related to several bioactive compounds such as phenolics, flavonoids, anthocyanins and vitamin C (Gil *et al*., 2002), the considered local varieties showed higher values than those reported in literature for commercial varieties of which, generally, only the flesh was analysed (Scalzo *et al*., 2005). By the contrary, our investigation assessed samples constituted by flesh with peel, fraction of fruit with a key role in determining the antioxidant properties of the whole fruit (Remorini *et al*., 2008; Zhao *et al*., 2015). In both years, significant differences were observed: 'Mora di Dolfo' was characterized by the highest TAC levels, on an average of about 14 µmol TE g FW^{-1}; 'Alberta' and 'Regina di Bember' had similar average values (8.5-9.5 µmol TE g FW^{-1}).

The TP content (Fig. 2B), likewise TAC results, was the highest in 'Mora di Dolfo' (about 2 GAE mg FW^{-1}), while the other varieties showed values from 0.7 to 0.9 GAE mg FW^{-1}. The relevant antioxidant content of the white-fleshed variety 'Mora di Dolfo' is in agreement with works reporting the tendency of white-fleshed cultivars to have significantly higher antioxidant content than yellow-fleshed peaches and nectarines (Cantìn *et al.,* 2009). A significant relationship between TAC and TP content was found (Fig. 3), confirming phenolic compounds as important contributors to the antioxidant activity of peach fruits (Karav and Eksi, 2012).

The similar TAC and TP values observed between years suggest that different rainfall amounts, occurred during the last growth-ripening period of fruit in 2014 (wet year) and 2015 (dry year), did not impact on the antioxidant power of varieties. These interesting results are not in agreement with studies carried out on several fruit species, where a variability in antioxidant levels in relation to climatic conditions has been found. In particular, the water availability markedly determined changes in fruit nutritional properties, and a linear significant inverse relationship between water status and antioxidant content was reported by several authors (Leccese *et al.*, 2010; Laribi *et al*., 2013; Bartolini *et al*., 2015 b). Also in peach (cv. Suncrest) the irrigation stress induced a

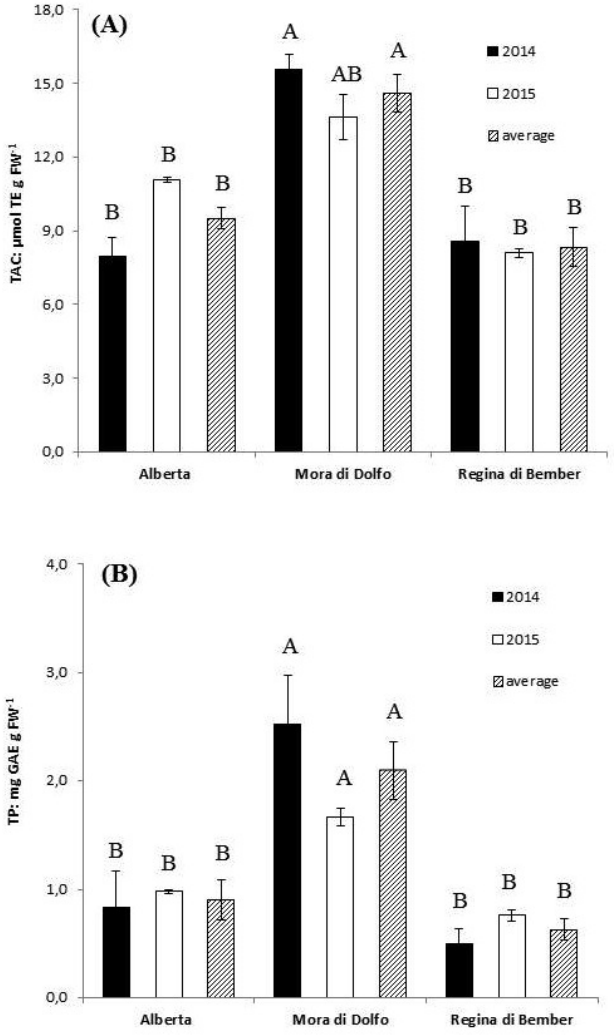

Fig. 2 - (A) Total antioxidant capacity (TAC) and (B) total phenols (TP) in peach fruits of local varieties recorded over a 2-year period. Values (means ± SEM) are expressed for TAC as µmol of Trolox Equivalent per g of fresh weight (µmol TE g FW^{-1}), and for TP as mg of Gallic Acid Equivalent per g of fresh weight (mg GAE g FW^{-1}). Different letters indicate significant differences at $P \leq 0.01$.

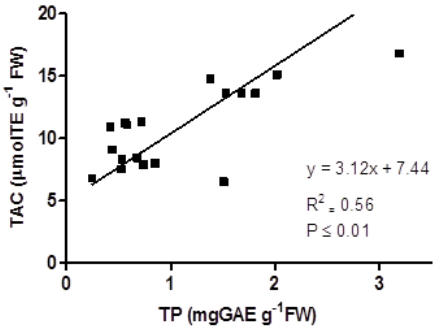

Fig. 3 - Linear regression between total antioxidant capacity (TAC) and total phenols (TP) of the studied varieties (2-year period).

higher biosynthesis of phytochemical compounds (Tavarini *et al.,* 2011).

Analysis of variance (Table 3) showed that variation in TAC and TP between varieties was much greater than between harvest years, indicating that genotype plays a more important role than growing season in influencing antioxidant content in peach, as found in other fruit species (Howard *et al*., 2003; Leccese *et al*., 2012). The significant interaction 'variety x year' for TAC revealed that environmental growing conditions may impact the capacity of genotype to synthesise antioxidants which are influenced by biotic and abiotic factors (Biesiada and Tomczak, 2012).

Although investigation on the behavior of these varieties has to be continued, the high antioxidant capacity of peaches can be considered as a part of cultivar value (Dalla Valle *et al*., 2007). This peculiarity would be crucial for breeding strategies selecting genotypes with enhanced antioxidant levels which may provide health benefits to consumers. Moreover, the availability of peach hardy genotypes will be a key factor for future, in view of the climate variability responsible of extreme event increases, such as drought or intense rainfall.

Sensory evaluation

The sensory evaluation, carried out by blind and visually impaired panellists over a 2-year period, was conducted on varieties 'Alberta' and 'Mora di Dolfo', characterized by yellow and white flesh color, respectively. Considering that color and exterior feature of fruits are nowadays important commercial sensorial traits to attract consumers (Kays, 1991), the judgment carried out by sightless assessors allowed to attain a real intrinsic quality evaluation of fruits. 'Going beyond the appearances' revealed its importance particularly under the wet growing season of 2014; in fact, this condition, favoring the presence of defects on peach peel, could have prevented the consumer's acceptance.

On the basis of panellist's appreciation, expressed as average degree of linking (1-9), the morphological parameters of fruits (shape and size) were similarly evaluated in both 2014 and 2015 (data not shown), while the organoleptic descriptors (acid taste, aroma, texture, juiciness, sweetness) were differently assessed depending on the harvest season (Table 4). Concerning the global appreciation, the lowest score was attributed in the year (2014) characterized by exceptional rainy events over the ripening period. These conditions could have negatively influenced components linked to the sensory profile, in agreement with authors who reported that high levels of water before harvest can reduce organoleptic quality and consumer liking degree (Navarro *et al*., 2010). In fact, blind and visually impaired panellists expressed the best agreement for peaches sampled in 2015 when summer drought conditions occurred. Most of descriptors showed higher scores and, particularly in 'Mora di Dolfo', the global appreciation was associated with an increase of aroma and juiciness, as observed in other peach varieties (Di Miceli *et al*., 2010). Moreover, this variety could seem less susceptible to unfavorable climatic conditions since positively judged also in 2014 (score 6.2), although chemical attributes (TSS, TA) were similar to 'Alberta' that, instead, had a lower score (5.2). It has been established that a different sensory perception of peaches could be linked not only to basic organic components (sugars, organic acids, fibers, micro and macro elements) but in great part to the volatile compounds, which define the flavor impact (Bononi *et al*., 2012).

The correlation coefficient (Table 5) between global appreciation and some sensory and physicochemical traits of peaches showed that panel's acceptability was highly and positively correlated with aroma, sweetness and TSS. Moreover, these attributes were significantly correlated each other

Table 4 - Mean scores (acid taste, aroma, texture, juiciness, sweetness and global appreciation) recorded for peach varieties 'Alberta' and 'Mora di Dolfo', over a 2-year period

Variety/year	Ac. Taste	Aroma	Texture	Juiciness	Sweetness	Glob. appr.
2014						
'Alberta'	4.0±0.5	5.4±0.7	5.8±0.4	5.8±0.6	5.4±0.7	5.2±0.6
'Mora di Dolfo'	4.8±1.1	6.2±0.8	5.4±0.9	6.8±0.5	6.2±0.8	6.2±1.0
2015						
'Alberta'	2.8±0.5	5.8±0.6	5.0±0.5	7.0±0.4	5.7±0.7	6.0±0.4
'Mora di Dolfo'	3.3±0.6	7.7±0.2 *	5.3±0.5	8.0±0.1*	7.3±0.5*	7.3±0.3 *

Scores were based on a nine-point hedonic scale: 1= extremely dislike; 5= neither like nor dislike; and 9= extremely likeWithin years asterisk indicates significant differences between varieties by Student *t*-test ($P \leq 0.05$).

Table 5 - Coefficients of correlation between scores of global appreciation and some sensory and physicochemical traits of peach fruits

Attributes	R Spearman
Acid taste	0.1
Aroma	0.99*
Juiciness	0.8
Sweetness	0.99*
Texture	-0.4
TSS	0.99*
TSS/TA	0.4

Significant coefficients are denoted by an asterisk at $P \leq 0.05$.

(Table 6), confirming TSS as one of the most important quality indicators in determining acceptance judgments for peaches, due to its influence on the perceived sweetness (Crisosto *et al*., 2006). On the contrary, texture appeared inversely related to global appreciation. The comparison between sensory and the main chemical attributes showed significant relationships: sweetness and aroma and total soluble sugars content were directly related each other.

Table 6 - Spearman's coefficients among sensory (sweetness, juiciness, aroma, texture, acid taste) and physicochemical (TSS, TSS/TA) variables for the tested peach varieties.

Attribute	Sweetness	Juiciness	Aroma	Texture	Acid taste	TSS	TSS/TA
Sweetness	1						
Juiciness	0.8	1					
Aroma	0.99*	0.8	1				
Texture	-0.4	-0.8	-0.4	1			
Acid taste	0.1	-0.6	0.1	0.8	1		
TSS	0.99*	0.8	0.99*	-0.4	0.1	1	
TSS/TA	0.4	0.8	0.4	-0.4	-0.8	0.4	1

Significant coefficients are denoted by an asterisk at $P \leq 0.05$.

4. Conclusions

The three local peach varieties showed interesting quality traits as well as a very high antioxidant content in both studied growth-ripening seasons. The capacity to maintain the bioactive components of fruits also under unfavorable weather events confirms the importance of old genotypes to be more stable in comparison to modern ones (Donno *et al*., 2012).

The white-fleshed 'Mora di Dolfo', characterized by the highest TAC and TP content, was particularly appreciated by panellists for its excellent eating quality denoted by aroma, sweetness and well balanced sugar/acid ratio. Although fruits of this variety are perishable, like the most white-fleshed peach varieties, it could represent a valid hardiness genotype for farmers who are focused to establish local markets where consumers are obviously willing to pay a premium price for niche products. This variety, as source of appreciable attributes, could be useful for specific breeding programs to develop new peach cultivars combining quality traits, hedonic and enhanced nutritional value, actually of high relevance for the consumer.

The new sensory analysis, carried out by blind and visually impaired assessors, provided a quality evaluation linked to intrinsic rather than exterior characteristics of peaches that could prevent consumers if defects in appearance are present. This type of test could represent a valid tool to assess the interest and appreciation of fruits for a conscious consumer's choice, going beyond the appearances, for a reintroduction of interesting ancient peach varieties characterized by unconventional fruit quality traits.

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Seeds and oil polyphenol content of sunflower (*Helianthus annuus* L.) grown with different agricultural management

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Key words: organic management, polyphenol content, sunflower.

Abstract: Using a long term experiment (20 and 11 years of organic cultivation on the same soil), sunflower was cultivated under organic management and in a different part of the same farm under conventional management. Kernels, teguments and oils were analyzed for their polyphenols content. Five caffeoylquinic acids were identified. No qualitative differences were found in the three cases, while quantitative differences have been pointed out and discussed.

1. Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops. Besides soy and rapeseed oil, sunflower oil is ranking third with a worldwide production of about 44 million tons each year from 2012 to 2016, 28 million tons in the EU (Committee for the Common Organization of Agricultural Markets, 2016). Sunflowers prove to be a protein source of great interest for human nutrition; moreover, the residues originating from oil extraction are rich in phenolic antioxidants, which account for 1-4% of the total mass with chlorogenic acid being the predominant component (Weisz *et al*., 2009). Polyphenols in sunflower seeds were identified and quantified after HPLC analysis (Aramendia *et al*., 2000; Pedrosa *et al.*, 2000). The main phenolic compounds present in both the kernel and hull besides chlorogenic acid are caffeic acid and caffeoylquinic derivatives. Phenolic compounds in sunflower seeds have been shown to exert a high antioxidative potential, which might be beneficial from a biofunctional point of view and may be used as effective antioxidants for sunflower oils (De Leonardis *et al.*, 2003, 2005; Anjum *et al.*, 2012).

Organic agriculture is most widely used and its benefits concern overall the environment and the health of food, which is not contaminated with pesticides and synthetic fertilizers. However organic fertilizer of biological origin may lead in the long term to the "conventionalization of organic farming" (Darnhofer *et al.*, 2010). In order to go deeper in the organic management, the Montepaldi Long Term Experiment (MOLTE) trial in central Italy has been set up to compare three agro-ecosystems with different management: two organic (old organic since 1992 and young organic since 2001) and one conventional (Migliorini, 2014). The aim of this research is to follow the fate of polyphenols in teguments, kernels and oil from sunflower seeds grown under the above-mentioned three different conditions (one conventional and two organic) in order to assess whether such condition may affect the polyphenols profile and which compounds may be regarded as innovative parameters in order to deeper investigate the relation among soil condition, agricultural management and seed characteristics.

2. Materials and Methods

The Montepaldi Long Term Experiment (MOLTE) has been active since 1991 in the farm of the University of Florence (location Montepaldi, San

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Casciano, Val di Pesa, Long. 11°09'08'' E, Lat. 43°40'16'' N) covering a slightly sloping surface of about 15 ha at 90 m a.s.l. The MOLTE experiment has a system approach and includes the following three different micro agro-ecosystem managements:

a) "Old Organic" (OldO) with an area of 5.2 ha, divided into 4 fields under organic since 1992;
b) "YoungOrganic" (YngO) with an area of 5.2 ha, divided into 4 fields (integrated farming) from 1992 to 2000 and converted into organic management since 2001;
c) "Conventional" (Conv) area of 2.6 ha divided into 2 conventional fields, where farming techniques used were those normally used in the territory of the study area for conventional management.

On October 28, 2013, after harvesting the whole sunflower plant, seeds were put in an oven at 60°C for 48 hours and then preserved in paper bags. Seeds were manually dehulled in order to obtain kernel and tegument. Phenolic compounds were extracted for 30 min from kernels (about 2 g) and from teguments (about 1 g) with 70:30 ethanol/water (25 and 15 ml, respectively). The extracts were evaporated under vacuum at room temperature and finally dissolved in 10 ml ethanol/water (70:30). Cold-pressed seeds oils (25 ml) were extracted with 3x25 ml of 70:30 ethanol/water, adjusted to pH 2.0 with formic acid; each step involved an extraction for 30 min at room temperature. The extracts were combined and defatted with 3x50 ml hexane. The defatted extracts were evaporated to dryness under vacuum at room temperature and finally dissolved in ethanol/water (70:30) to a final volume of 4 ml.

Solvents and reagents

All the solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA). Chlorogenic acid was obtained from Extrasynthese S.A. (Lyon, Nord-Genay, France). The HPLC-grade water was obtained via double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, USA).

HPLC analysis

HPLC/DAD Analysis. The HPLC/DAD analyses were performed with an HP 1100L liquid chromatograph equipped with HP DAD (Agilent Technologies, Palo Alto, CA, USA). A Kinetex C18 column 100×2.1 mm, 5 µm (Phenomenex) operating at 30°C was used. The eluents were H_2O adjusted to pH 3.2 by formic acid and acetonitrile. A four-step linear solvent gradient was performed starting from 100% water up to 100% acetonitrile, with a flow rate of 0.2 ml/min for a 30-min period (Table 1).

Table 1 - Linear solvent gradient system used in HPLC-DAD and HPLC-MS analysis

Time (min)	A	B
0.10	100.0	0.0
5.00	80.0	20.0
7.00	80.0	20.0
13.00	70.0	30.0
18.00	70.0	30.0
22.00	30.0	70.0
26.00	30.0	70.0
30.00	0.0	100.0

Solvent A= H_2O adjusted to pH 3.2 by HCOOH. Solvent B= CH_3CN.

HPLC/ESI-MS Analysis. The HPLC-MS analyses were performed using an HP 1100L liquid chromatograph equipped with a DAD and 1100 MS detectors. The interface was an HP 1100 MSD API-electrospray (Agilent Technologies). Mass spectrometer operating conditions were the following: gas temperature 350°C at a flow rate of 10.0 l/min, nebulizer pressure 30 psi, quadrupole temperature 30°C and capillary voltage 3500 V. The mass spectrometer operated in positive and negative ionization mode at 80-120 eV, for both ionization modes.

3. Results and Discussion

In figure 1, the chromatographic profile of organic sunflower kernel, registered at 330 nm, maximum wavelength of absorbance of caffeic acid and its derivatives, is reported. Eight compounds have been identified: 3-*O*-caffeoylquinic acid, chlorogenic acid, 4-*O*-caffeoylquinic acid, three derivatives of caffeic acid, 3,5-*O*-dicaffeoylquinic acid, and 4,5-*O*-dicaf-

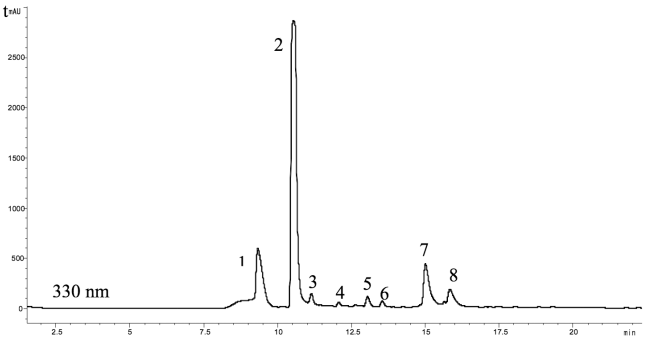

Fig. 1 - HPLC chromatogram of sunflower kernel extract recorded at 330 nm. Peaks= 1. 3-O-caffeoylquinic acid; 2. chlorogenic acid; 3. 4-O-caffeoylquinic acid; 4-6. caffeic acid derivatives; 7. 3,5- O-dicaffeoylquinic acid; 8. Ac. 4,5- O-dicaffeoylquinic acid.

feoylquinic acid. In the shells the same compounds with the exception of 4-*O*-caffeoylquinic acid and the two dicaffeoylquinic acids were identified. The same caffeoyl and dicaffeoylquinic acid derivatives were already found by Aramendia *et al*. (2000), Pedrosa *et al*. (2000), and Weisz *et al*. (2009). The qualitative pattern does not change along with the agricultural management. As concerns quantitative data, Table 2 reports the quantitative data of kernel and teguments. Total polyphenols amount is the lowest, in the case of kernel, when the plant grows on the old organic field, while it is almost the same with the other two managements; in the case of teguments, on the contrary, the lowest amount is observed in the case of conventional management. It should however be noted that in the case of teguments, polyphenols content is very low changing from about 10% to 2% with respect to kernel content. Chlorogenic acid is the main compound and it accounts for about 90% in tegument. Chlorogenic acid in our samples, both kernel and tegument, is about ten times more abundant with respect to the quantitative data reported by Aramedia *et al.* (2000) and Pedrosa *et al*. (2000). The comparison of quantitative data is however difficult since different techniques and sample treatments are involved (Weisz *et al*., 2009). Polyphenols content along with agriculture management has recently reviewed and in most vegetables grown under organic conditions, a higher content of polyphenols has been found; on the other hand a higher soil nitrogen availability decreases polyphenols content (Heimler *et al.*, 2017). Our data support previous findings that indicate how old managed organic soil was the most efficient in term of C and N storage (Migliorini *et al*., 2014). Furthermore, when the individual compounds are taken into account the relative percentages of compounds from new organic and conventional managed soils are similar, while in the case of the old managed organic soil, notwithstanding the lowest total polyphenol content, a higher dicaffeoylquinic acids content was found (17% with respect to 12%). Chlorogenic acid and dicaffeoylquinic acids derive from the phenylpropanoid pathway. Generally, this pathway is induced by biotic and abiotic stress such as wounding, UV irradiation, or pathogen attack (Moglia *et al.*, 2008). No information is available on the regulation of dicaffeoylquinic acids in any plant species, even if the same regulation of chlorogenic acid synthesis could be foreseen (Moglia *et al.*, 2008).

In Table 3, the data of sunflower oil are reported. Oil has been obtained by means of a mechanical

Table 2 - Polyphenols (mg/g, fresh weight) in sunflower kernel and tegument

Compound	Agricultural management					
	Kernel			Tegument		
	OldO	YoungO	CO	OldO	YoungO	CO
3-*O*-caffeoylquinic acid	2.51(0.37)	4.57(0.82)	4.74(0.76)	0.08(0.01)	0.08(0.01)	0.05(0.01)
chlorogenic acid	6.25(1.12)	11.39(1.94)	11.82(1.42)	0.95(0.18)	0.96(0.18)	0.41(0.07)
4-*O*-caffeoylquinic acid	0.44(0.08)	0.8(0.11)	0.83(0.13)	0.03(0.005)	traces	traces
caffeic acid derivatives	0.21(0.04)	0.38(0.04)	0.39(0.06)	traces	n.d.	traces
3,5-*O*-dicaffeoylquinic acid	1.3(0.24)	n.d.	n.d.	n.d.	n.d.	n.d.
4,5-*O*-dicaffeoylquinic acid	0.77(0.11)	2.36(0.34)	2.45(0.27)	n.d.	n.d.	n.d.
Total polyphenols	11.48	19.5	20.23	1.06	1.04	0.46

OldO= old organic; YoungO= young organic; CO= conventional (see experimental section). ND= not determined. Standard deviation within brackets.

Table 3 - Polyphenols (mg/l) in sunflower oil

Compound	Agricultural management		
	OldO	YoungO	CO
3-*O*-caffeoylquinic acid	0.232(0.192)	0.35(0.09)	0.176(0.057)
chlorogenic acid	1.96(0.393)	2.43(0.11)	1.936(0.12)
p-coumaroylquinic acid	2.36(0.318)	0.112(0.022)	0.592(0.079)
4-*O*-caffeoylquinic acid	0.048(0.005)	0.104(0.011)	0.08(0.02)
caffeic acid derivatives	2.28(0.644)	0.328(0.079)	3.216(0.415)
not identified polar compounds	0.504(0.011)	1.2(0.045)	1.456(0.429)
Total polyphenols	7.384	4.368	7.456

OldO= old organic; YoungO= young organic; CO= conventional (see experimental section). ND= not determined. Standard deviation within brackets.

press, which is the only technology that allows the maintenance of polyphenols high content (Bendini *et al.*, 2011). Oil yields are different (22.2% for old organic soil, 29.8% for young organic soil and 24.7% for conventional management) and, the young organic soil, with the highest yield, exhibits the lowest total polyphenol content. Sunflower oil-amount of 10 mg kg^{-1} has been reported (De Leonardis *et al*., 2005). Polyphenols content of the three seeds oils (Table 3) are almost in accordance with such amount. Young managed organic soil gave rise to an oil with the smallest polyphenols content notwithstanding the high polyphenols content of almond and teguments.

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Short note

Characterization of volatile compounds in *Mentha spicata* L. dried leaves

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Abstract: Gas chromatography-mass spectrometry (GC-MS) is one of the most common techniques used to measure and to characterize volatile organic compounds (VOCs). At the same time, the proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) represents a recent innovative tool that allows the on-line monitoring of VOCs providing the whole mass spectra with short response time, high mass resolution and without sample preparation. We reported as major constituents of headspace in spearmint dried leaves the monoterpenes carvone, followed by dihydrocarvone, limonene and 1,8-cineole (eucalyptol) although other monoterpenes such as *α*-pinene, 3-carene, terpineol and neodihydrocarveol, alcohols (3-octanol, 1-octen-3-ol and 3-methyl-1-butanol), esters and ketones (3-octanone) were detected in low concentrations. In this study, the analytical GC-MS and PTR-ToF-MS techniques allowed to characterize the entire volatile profile of the sample bypassing the limitations of each tool.

1. Introduction

Recently, aromatic plants have received great attention in several fields such as agroalimentary, food, perfumes, pharmaceutical and natural cosmetic products (Baatour *et al*., 2010). Among aromatic plants, mint is an herbaceous rhizomatous perennial plant belonging to the Lamiaceae family, widely used since ancient times for its characteristic flavor and aroma. Belonging to the *Mentha* genus, spearmint (*Mentha spicata* L.), cornmint (*M. arvensis* L.) and peppermint (*M. piperita* L.) are the species commonly used as spices in food preparation and nowadays represent one of the most important spices throughout the world marketed fresh or dried. The drying process is used to increase shelf-life and to prevent some biochemical reactions that may alter the organoleptic characteristics. Moreover, mint essential oil is commonly used as flavoring food agent, perfumery, cosmetic and pharmaceutical products (Díaz-Maroto *et al*., 2003). The chemical composition of spearmint essential oil depends on plant genetic structure, growth conditions and agronomic practices (Sangwan *et al*., 2001; Figueiredo *et al*., 2008). In the past, the spearmint essential oil has been intensively investigated using gas chromatography (GC) technique and the mainly compounds identified are carvone, monoterpene synthesized and stored in glandular thricomes (Gershenzon *et al*., 1989), followed by limonene and 1,8 cineole (Díaz-Maroto *et al*., 2003; Antal *et al*., 2011; Orio *et al*., 2012) while the monoterpene menthol is one of the main con-

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stituents of *M. piperita* essential oil (Orio *et al*., 2012). Surprisingly, few studies have been carried out to assess the aroma composition of fresh or dried spearmint leaves (Díaz-Maroto *et al*., 2003; Antal *et al*., 2011; Silva and Câmara, 2013).

From several years the conventional sample technique used to analyze the aroma of plant material was the headspace solid phase microextraction (HS-SPME) (Arthur and Pawliszyn, 1990) coupled to GC-MS technique. This procedure has been introduced in early 90s, as a powerful solvent-free sample preparation technique where the volatile fraction is collected by exposing a fiber, coated with single or multiple polymers, directly to the headspace above the sample. Subsequently, the fiber is introduced into the injection chamber of a gas chromatograph and high temperatures allow the release of the analytes into the chromatographic column.

Recently, a new instrument has been developed such as proton transfer reaction mass spectrometry (PTR-ToF-MS), which allows the on-line monitoring of volatile compounds and the achievement of the whole mass spectra of complex volatile matrices with short response times, high mass resolution and without sample preparation (Vita *et al*., 2015). From one side, the PTR-ToF-MS advantages include high sensitive with a rapid detection system (response time of about 100 ms) and very poor fragmentation of the volatile molecules (Taiti *et al*., 2017). By the other side, the main disadvantage is represented by the difficulty to separate volatile compounds with same chemical formula, being the unique identification of the VOCs not always possible (Taiti *et al*., 2017).

For the first time, in the present study two mass spectrometry techniques (HS-SPME/GC-MS and PTR-ToF-MS) are reported with the aim to evaluate and to characterize the aroma compounds of spearmint dried leaves.

2. Materials and Methods

Plant material

Mentha spicata seeds were germinated in fine peat in a growth chamber at 24°C, 68% humidity with a 10/14 h day/night cycle. Subsequently, seedlings were transplanted into pots (40 cm head diameter × 20 cm height), filled with sandy loam soil and transferred in greenhouse (University of Florence, DISPAA). After planting, plants were grown and maintained for 50 days in a greenhouse at day/night temperatures 26-30°C and 18-21°C, respectively and 80% humidity. The plants were irrigated every 2 days and fertilized after 10-25-40 days of growth. Forty five days after planting, midlife leaves were collected from each experimental unit and dried at 45°C for 24 h using a common heater. Subsequently, the dried leaves were finely chopped with a grinder (Mulinex AR 11, Groupe SEB, France) with a particle size about 1.0 mm.

Determination of volatile composition

PTR-ToF-MS analysis. The volatile profile of spearmint dried leaves was assess by PTR-ToF-MS (PTR TOF 8000 model, Ionicon Analytik GmbH Innsbruck, Austria) using H_3O^+ as reagent ion for the proton transfer reaction. For a detailed explanation of the PTR-ToF-MS technology see Blake *et al*. (2009). For sample preparation and experimental setup has been followed the procedure previously used by Taiti *et al*. (2016). Briefly, one-gram aliquots of chopped spearmint dried leaves were transferred into a glass jar (2/3 l) suitable for volatile analysis and provided to screw caps with two holes respectively connected with Teflon pipes to zero air generator (Peak Scientific) and PTR-ToF-MS. The employed setup allows the formation of a dynamic headspace sampling system with a constant air flow of 0.3 l min^{-1} and a constant humidity and temperature (75% UR and 24°C), which are critical parameters for VOCs determination (Mancuso *et al*., 2015). Mass spectra were recorded in the mass-to-charge (m/z) range of m/z 20-220 with a time resolution of 1 s. The tool worked with the following parameters in the drift tube: pressure 2.3 mbar, voltage 594 V, temperature 110°C, extraction voltage at the end of the drift tube (Udx) 35 V and was operated at an E/N value of 140 (1 Td = 10217 V cm^2). The inlet line, consisted of a peek capillary tube (internal diameter 0.40 mm), was maintained at 110°C in order to minimize the adsorption of chemicals onto surface (Lanza *et al*., 2015). Subsequently, the raw data and peak extraction were acquired and processed by the TofDaq software (Tofwerk AG, Switzerland), using a procedure previously reported by Taiti *et al*. (2016). In order to guarantee high mass accuracy for the post processing and for a precise conversion of time-of-flight into m/z values, the internal calibration was performed off-line, using together with known low mass ions (NO^+ peak m/z = 29.997 and $C_3H_7O^+$ peak m/z = 59.049) a known compound with high mass ion as the carvone ($C_{10}H_{15}O^+$ peak m/z = 151.075). In this way the exact masses have been assigned and determined the sum formula of all VOCs recorded with the PTR-ToF-MS.

Finally, once determined the sum formula, each peak has been tentatively assigned to a specific compound, with the help of the results obtained by GC-MS analysis, and of the available literature on volatile compounds emitted by mint products. Raw data acquired by the PTR-ToF-MS were processed and analyzed using a TofDaq software (Tofwerk AG, Switzerland) and the methodology used for data processing is reported elsewhere (Taiti *et al*., 2017).

HS-SPME/GC-MS procedure. As reported by Silva and Câmara (2013), the trivalent fiber divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) was the most effective SPME fiber able to isolate the volatile compounds from *M. piperita* and *M. spicata* fresh leaves. In our analysis, a 2 cm trivalent SPME fiber (DVB/CAR/PDMS) was used to extract the volatile fraction from the *M. spicata* dried leaves. SPME sampling device was manually inserted into the sealed vial and the fiber was exposed to headspace above the sample for 20 min at 25°C. After extraction the fiber was insert into the injector port of the GC-MS system, an Agilent 7890 a GC equipped with a 5975C MSD, where the volatile compounds where thermally desorbed at 280°C and then transferred to the analytical capillary column. The analytes separation was achieved with a Agilent DB InnoWAX, length 50 m, 0.20 µm id, 0.40 µm df column. Chromatographic conditions were: initial temperature 40°C for 0.5 min, then 6°C min^{-1} up to 260°C. This temperature was maintained for 1 min. Only the compounds with higher intensity were identified in order to support the PTR-ToF-MS analysis; the identification was based on mass spectra matching with the standard NIST08/Wiley98 libraries.

3. Results and Discussion

The aim of our research was a comparison among PTR-ToF-MS and conventional HS-SPME/GC-MS techniques performed simultaneously on spearmint dried leaves. As reported in recent literature surveys, GC-MS can be considered the main method for the analysis of volatile fractions. The HS-SPME/GC-MS analysis led to the identification of the most representative compounds from a quantitative point of view, and the typical gas chromatogram obtained is reported in figure 1A. Consequently, over fifty volatile compounds were identified (Fig. 1A). Among these, the most abundant compounds were, in order of abundance: terpenes (with carvone, dihydro-carvone, D-limonene and eucalyptol or 1,8-cineole as the most represented); alcohol (mainly 1-octen-3-ol and 3-methyl-1-butanol); ketones (e.g. 3-octanone); esters (e.g. 2-methyl-ethyl ester-butanoic acid) (Fig. 2). The same compounds were reported by Silva and Câmara (2013) in the volatile fraction of spearmint fresh leaves analyzed using the same method.

The data obtained by PTR-ToF-MS analysis were filtered following the procedure previously described by Taiti *et al*. (2017); firstly all peaks imputable to water chemistry and to interfering ions (e.g. oxygen, nitrogen monoxide) and secondly the peaks whose average concentrations were lower than 1 ppbv (parts per billion by volume) were eliminated. After filtering, a total of 54 masses were revealed and could be tentatively attributed to compounds such as aldehydes, ketones, esters, terpenes and other unidentified compounds in the range of measured masses m/z from 20 to 220 (Fig. 1B, Table 1). As

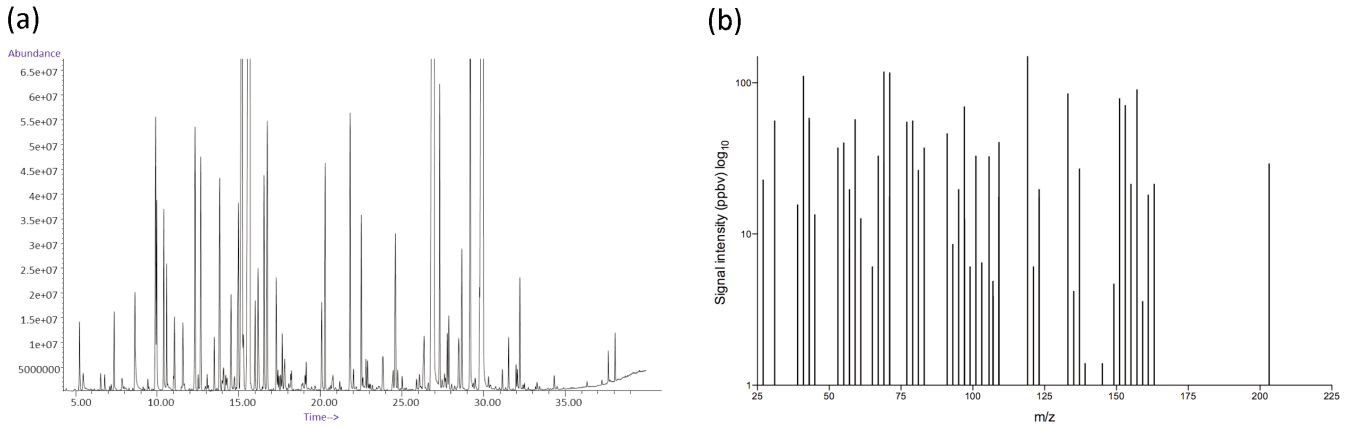

Fig. 1 - a) Typical gas chromatogram obtained by volatile fraction of *M. spicata* dried leaves obtained with HS-SPME/GC-MS using a DVB/CAR/PDMS fiber. b) Snapshot of the mass spectra of headspace of spearmint dried leaves obtained with PTR-ToF-MS.

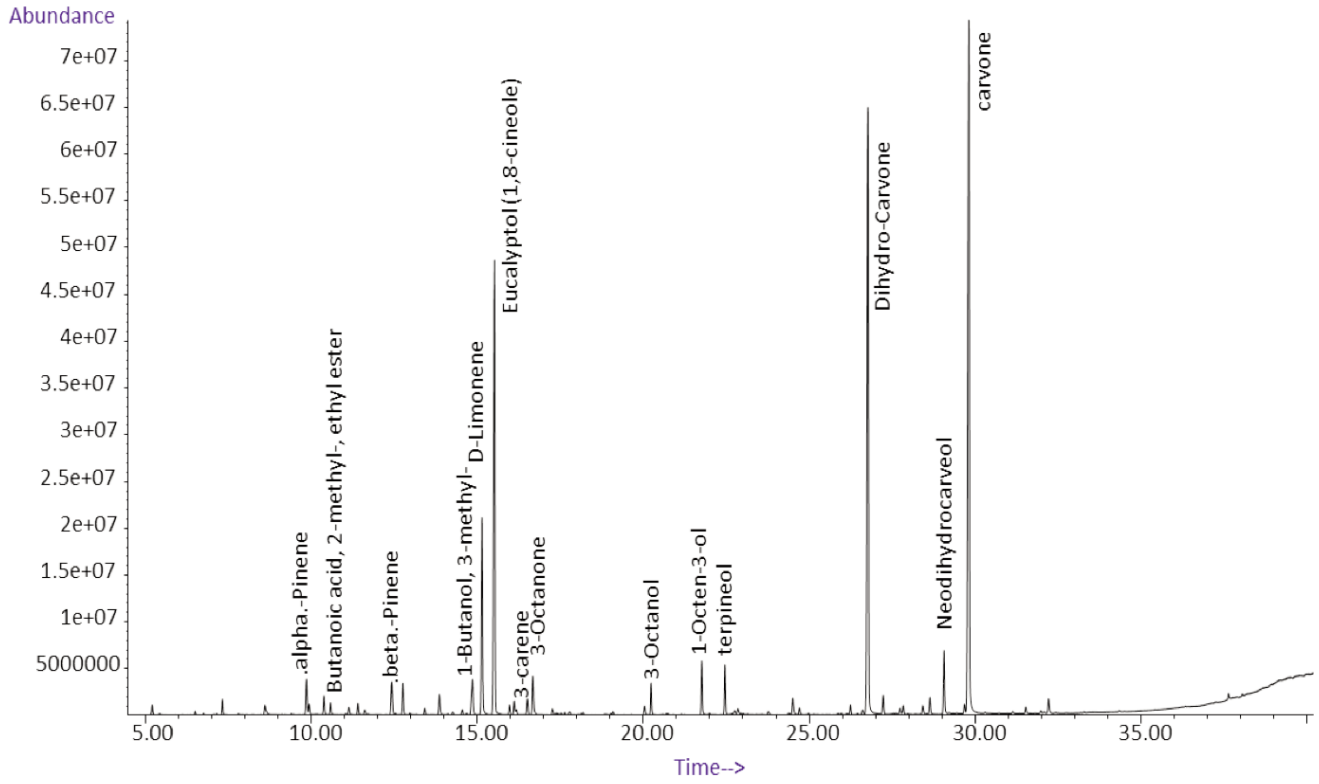

Fig. 2 - Mass spectra enlargement of widest class compounds identified by HS-SPME/GC-MS.

reported in Materials and Methods section, the detected VOCs were integrated with the fragmentation patterns of each compound (Maleknia *et al*., 2007; Kim *et al*., 2009; Brilli *et al*., 2011) and with the results of the GC-MS-based profile. Indeed, previous researches performed on different matrices demonstrated the good agreements with gas-chromatographic peaks and PTR-MS mass spectral data (King *et al*., 2010; Aprea *et al*., 2015). Generally, the correlation between GC-MS and PTR-ToF-MS data allows the characterization of the sample entire volatile profile bypassing the limitations of each tool.

All compounds identified and their signal intensity are listed in Table 1, together with their m/z ratio, chemical name, molecular formula, percentage on the total, and additional reference for peak identification. The most abundant signals detected with PTR-ToF-MS (>3% of the total) were: alkyl fragment (m/z = 41.038), isoprene (m/z = 69.069), 3-methyl-1-butanol (m/z = 71.085), dimethyl-furan (m/z = 97.065), p-cymene fragments (m/z = 119.085), p-cymenene (m/z = 133.101), carvone (m/z = 151.075), dihydrocarvone (m/z = 153.126), neodihydrocarveol together with menthol (m/z = 157.155) (Table 1). Most terpenes volatile compounds were reported in previous articles and identified as the main constituents of spearmint aroma (Kokkini *et al*., 1995; Díaz-Maroto *et al*., 2003; Orio *et al*., 2012). Alcohols, esters and aldehydes were also detected in spearmint dried leaves even though in low content. Interestingly, 3-methyl-1-butanol (m/z 71.085) was detected using both technique while some compounds such as 3-octanone, 1-octen-3-ol and 3-octanol were identified only with HS-SPME/GC-MS (Fig. 2, Table 1).

As reported by Kokkini *et al*. (1995) spearmint aroma is mainly formed by a complex mixture of various VOCs, mainly terpenes such as carvone, limonene, and 1,8-cineole that are the most important aroma constituents due to their high percentage in the volatile fraction. Indeed, terpenes (monoterpenes and sesquiterpenes) and terpenoids are the common constituents of flavor and fragrance of some plants and in particular of spearmint aroma (Telci *et al*., 2010). Terpenes are hydrocarbons (carbon and hydrogen are the only elements present) whereas terpenoids have been denatured by oxidation and thus contain additional functional groups. Regarding monoterpenes (m/z 137 and 135) and sesquiterpenes (m/z 205) hydrocarbons, their

Table 1 - Volatile compounds identified via PTR-ToF-MS

m/z	Tentative identification	Protonated chemical formula	Signal intensity (ppbv)	Compound (% on the total)	References
27.022	Acetylene	$C_2H_3^+$	22.9 ± 6.4	1.11	Vita *et al.* (2015)
31.018	Formaldehyde	CH_3O^+	56.3 ± 5.2	2.79	Kushch *et al*. (2008)
39.022	Isoprene fragment	$C_3H_3^+$	15.7 ± 2.2	0.74	Maleknia *et al.* (2007)
41.038	Alkyl fragment	$C_3H_5^+$	111.3 ± 12.7	5.81	Vita *et al.* (2015)
43.018	Alkyl fragment	$C_2H_3O^+$	58.7 ± 21.5	2.9	Vita *et al.* (2015)
43.054	Alkyl fragment (propene)	$C_3H_7^+$	56.3 ± 5.2	2.66	Taiti *et al.* (2017)
45.033	Acetaldehyde	$C_2H_5O^+$	13.5 ± 6.8	0.77	Taiti *et al.* (2016)
53.04	Cyclobutadiene	$C_4H_5^+$	37.3 ± 0.1	1.83	Vita *et al.* (2015)
55.055	C_4 aldehydes fragment	$C_4H_7^+$	40.3 ± 5.1	1.98	Taiti *et al.* (2017)
57.033	Alkyl Fragment (2-hexenal)	$C_3H_5O^+$	8.0 ± 2.4	0.38	Brilli *et al*. (2011)
57.07	Alkyl fragment (Hexanol/valeric acid)	$C_4H_9^+$	19.8 ± 0.2	0.97	Aprea *et al.* (2015)
59.049	Propanal, acetone	$C_3H_7O^+$	57.4 ± 17.2	2.7	Taiti *et al.* (2016)
61.028	Acetates	$C_2H_5O^+$	12.7 ± 4.6	0.62	Taiti *et al.* (2016)
65.039	Alkyl fragment	$C_5H_5^+$	6.1 ± 0.2	0.3	Goacher *et al*. (2010)
67.055	Terpene fragment	$C_5H_7^+$	33.0 ± 3.0	1.62	Yener *et al*. (2015)
69.033	Furan	$C_4H_5O^+$	29.9 ± 6.4	1.47	Taiti *et al.* (2017)
69.069	Isoprene	$C_5H_9^+$	118.8 ± 24.4	6.06	Taiti *et al.* (2015)
71.049	2-butenal	$C_4H_7O^+$	17.7 ± 2.2	0.87	Vita *et al.* (2015)
71.085	3-Methyl-1-butanol/Pentenal fragment	$C_5H_{11}^+$	117.3 ± 4.6	5.81	Aprea *et al.* (2015)
77.04	Alkyl fragment	$C_6H_5^+$	55. 4 ± 36.8	2.7	Goacher *et al*. (2010)
79.054	Benzene	$C_6H_7^+$	56.3 ± 5.2	2.66	Vita *et al.* (2015)
81.069	1,3-cyclohexadiene/terpenes fragment	$C_6H_9^+$	26.5 ± 6.8	1.35	Taiti *et al.* (2016)
83.086	C_6 compounds/hexenol fragment	$C_6H_{11}^+$	37.3 ± 0.1	1.83	Maleknia *et al.* (2007)
91.054	Alkyl fragment	$C_7H_7^+$	46.3 ± 5.1	2.27	Yener *et al*. (2015)
93.068	Terpene fragments	$C_7H_9^+$	8.6 ± 2.4	0.42	Maleknia *et al.* (2007)
95.085	Terpene fragments	$C_7H_{11}^+$	19.8 ± 0.2	0.97	Aprea *et al.* (2015)
97.065	Dimethyl-furan	$C_6H_9O^+$	69.8 ± 17.2	3.91	Yener *et al*. (2015)
97.101	Alkyl fragments	$C_7H_{13}^+$	12.7 ± 4.6	0.62	Aprea *et al.* (2015)
99.08	Cyclohexanone/hexenal	$C_6H_{11}O^+$	6.1 ± 0.2	0.3	Buhr *et al*. (2002)
101.096	3-hexen-1-ol	$C_6H_{13}O^+$	33.0 ±3.0	1.7	Buhr *et al*. (2002)
103.101	2-hexanol	$C_6H_{15}O^+$	6.5 ± 1.0	0.32	Buhr *et al*. (2002)
105.069	Styrene	$C_8H_9^+$	32.7 ± 1.6	1.6	Yener *et al*. (2015)
107.049	Benzaldehyde	$C_7H_7O^+$	4.9 ± 1.2	0.24	Yener *et al*. (2015)
107.086	Ethylbenzene	$C_8H_{11}^+$	3.9 ± 0.7	0.19	De Gouw *et al*. (2003)
109.065	Phenylmethanol/benzenemethanol/p-cresol	$C_7H_9O^+$	17.6 ± 0.3	0.86	Leskinen *et al*. (2015)
109.101	Terpene fragments	$C_8H_{13}^+$	40.6 ± 0.6	1.99	Maleknia *et al.* (2007)
119.085	p-cymene fragments	$C_8H_{11}^+$	153.2 ± 10	7.68	Maleknia *et al.* (2007)
121.101	Sesquiterpene fragments	$C_9H_{13}^+$	6.1 ± 1.0	0.3	Demarcke *et al*. (2009)
123.08	4-ethylphenol	$C_8H_{11}O^+$	17.5 ± 2.7	0.86	Chatonnet *et al*. (2009)
123.116	Sesquiterpene fragments	$C_9H_{13}^+$	19.8 ± 2.1	0.97	Demarcke *et al*. (2009)
133.101	p-cymenene	$C_{10}H_{13}^+$	85.2 ± 10.7	4.18	Kus and Van Ruth (2015)
135.135	p-cymene	$C_{10}H_{15}^+$	4.2 ± 0.8	0.21	Maleknia *et al.* (2007)
137.137	Monoterpenes compounds (limonene, 3-carene)	$C_{10}H_{17}^+$	27.1 ± 9.3	1.18	Maleknia *et al.* (2007)
139.111	2-pentylfuran/nopinone	$C_9H_{15}O^+$	1.4 ± 0.2	0.07	Wisthaler *et al*. (2001)
145.135	Octanoic acid	$C_8H_{17}O_2^+$	1.4 ± 0.1	0.07	Aprea *et al.* (2015)
149.135	Sesquiterpene fragments	$C_{11}H_{17}^+$	4.7 ± 1.0	0.23	Kim *et al.* (2009)
151.075	Monoterpenes oxygenate (carvone, myrtenal, carvacrol)	$C_{10}H_{15}O^+$	79 ± 13.1	3.74	Kim *et al.* (2009)
153.126	Monoterpenes oxygenate (carveol, dihydrocarvone)	$C_{10}H_{17}O^+$	71.3 ± 1.3	3.45	Maleknia *et al.* (2007)
155.145	Alcohol monoterpenes (neodihydrocarveol/terpineol)	$C_{10}H_{19}O^+$	21.5 ± 8.8	3.07	Bourtsoukidis *et al*. (2014)
157.155	Decanol/Menthol	$C_{10}H_{21}O^+$	90.6 ± 26.8	4.55	Vita *et al.* (2015)
159.135	Nonanoic acid/C9 ester	$C_9H_{19}O_2^+$	3.6 ± 1.5	0.18	Aprea *et al.* (2015)
163.148	Sesquiterpene fragments	$C_{12}H_{19}^+$	18.2 ± 3.9	0.38	Demarcke *et al*. (2009)
203.18	Sesquiterpene compounds (trans-calamenene)	$C_{15}H_{23}^+$	21.5 ± 4.9	0.89	Taiti *et al.* (2017)
205.195	Sesquiterpenes compounds	$C_{15}H_{25}^+$	29.3 ± 7.9	1.15	Taiti *et al.* (2015)

Data are expressed in ppbv as mean ± deviation standard, n=3.

amount was similar and represented about 2.5% of total compounds detected by PTR-ToF-MS, while terpenoids (m/z 151, 153, 155, 157) were the most abundant (about 15%) (Table 1).

Finally, considering the entire dataset and following the odor descriptor (www.thegoodscentscompany.com), the spearmint aroma might be due to the interaction between compounds that give a different odors such as: minty (e.g. carveol, 1,8-cineole, carvone), citrus (e.g. limonene), spicy (e.g. carvacrol, menthol, p-cymene, terpineol), green or herbaceous (e.g. 3-hexen-1-ol, hexenal, hexenol), fruity (e.g. 3-methylbutanoate, ethyl 2-methyl butyrate) and floral notes (e.g. phenylmethanol).

4. Conclusions

PTR-ToF-MS has been shown to be a very useful tool to detect VOCs emitted by dried herbs. The results obtained in this study demonstrated that PTR-ToF-MS technique coupled with the GC-MS for the identifications of isomers, can be a usefully screening tool in order to (1) assess the volatile compounds of very complex samples such as aromatic herbs and (2) achieve a fast and simple fingerprinting. This technique is able to provide the whole mass spectra of complex trace gas mixtures with short response times, high mass resolution and without sample preparation. Moreover, this technique, when coupled with HS-SPME/GC-MS, allows the overcoming of the disadvantage related to the identification of VOCs. In summary, the spearmint aroma might be due to the interaction between compounds with minty (e.g. carveol, 1,8-cineole, carvone), citrus (e.g. limonene), spicy (e.g. carvacrol, menthol, p-cymene, terpineol), green or herbaceous (e.g. 3-hexen-1-ol, hexenal, hexenol), fruity (e.g. 3-methylbutanoate, ethyl 2-methyl butyrate) and floral notes (e.g. phenylmethanol).

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Evaluation of adaptability potential of seven Iranian pomegranate cultivars in Southern Iran, Arsenjan region

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Key words: adaptability, macro and micronutrients, *Punica granatum*, tolerance.

Abstract: Present study was carried out to compare leaf mineral composition and some physiological parameters in seven Iranian pomegranate cultivars for evaluation of their adaptability differences in Arsenjan region/Fars province/southern Iran and selecting probable more abiotic tolerant cultivars in this region. Uniform and healthy rooted plants of seven commercial pomegranate cultivars were purchased from a commercial nursery and planted in a completely randomized block design in an orchard site in Arsenjan region. After full establishment the orchard samples (fresh leaves) were taken and transferred to lab for analysis. Cultivars included: Malas Yousefkhani Saveh, Naderi Badroud, Malas Daneh Ghermez Yazd, Rabab Neiriz Fars, Shirin Shahvar Fars, Shirin Poust Daneh Ghermez and Zard Anar Arsenjan. Significant differences were found among studied pomegranate cultivars for concentrations of leaf potassium, calcium magnesium, sodium and Iron concentrations. Also parameters such as leaf total chlorophyll and carotenoids content, total sugars, relative water content and electrolyte leakage, α-tocopherol and ascorbic acid concentration were significantly different in studied cultivars. 'Zard Anar Arsenjan', an indigenous cultivar of the region, and 'Rabab Neiriz Fars' were evaluated as probable more tolerant cultivars in comparison to other cultivars. This can be attributed to their more optimized leaf mineral composition and antioxidant statues.

1. Introduction

Pomegranate *(Punica granatum* L.) is a nutrient dense and one of the most popular fruits native to Iran. With a production of 700,000 tons/year, Iran is the world's leading producer (Sarkhosh *et al*., 2009). Historical evidence reveals that the primary origin of pomegranate is Iran and that it has been spread from this region to other areas (Levin, 1996). A large number of pomegranate varieties can be found in Iran, more than 760 original, wild and decorative cultivars (Mousavinejad *et al*., 2009). Considering lack of water resources and intensification of abiotic stresses such as drought and salinity, importance of pomegranate has increased in recent years, since this species is a tolerant fruit crop and thrives well under arid and semi-arid climatic conditions (La Rue, 1980). Previous investigations indicate varied levels of tolerance to abiotic stress conditions such as drought and salinity among different pomegranate cultivars (Tabatabaei and Sarkhosh, 2006; Okhovatian-Ardakani *et al*., 2010; Ibrahim, 2016) because abiotic stress tolerance is a complex parameter and depends on both genetical and physiological properties.

Differences in adaptability potentials to prevailing environmental conditions and abiotic stresses tolerance among plant species or cultivars including pomegranate can be attributed to their varied ability for nutrients uptake and subsequent different concentration of macro and micronutrients within plant organs and tissues (Jamali *et al*., 2015). Nutrients deficiencies or imbalances exert secondary, often unpredicted influences on the growth of plants by changes in growth pattern, chemical composition, and antioxidant defense capacity of plants and particularly decrease the resistance of plants to biotic and abiotic environmental stresses (Hajiboland, 2012). Macronutrients are mainly important structural components of plants and their optimum concentrations result in improved growth (Marschner, 1995). Activity of enzymes and/or production of some metabolites involving in the plants response to their surrounding environment or even modulations in the signal transduction pathways are under micronutri-

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ents influence (Hajiboland, 2012). Cultivars with a higher capacity for macro and micronutrients absorption possess a more efficient growth and development cycle, with a better adaptability to environmental conditions (Jamali *et al*., 2015).

Previous literatures on comparison of differences in leaf mineral composition and also physiological characteristics in various Iranian pomegranate cultivars are limited (in comparison to other important crops) and more investigations for evaluation of adaptability potential in different pomegranate cultivars seem necessary. So the goal of the present study was to compare leaf mineral composition and also some physiological and biochemical parameters in seven Iranian cultivars for a deeper understanding of their adaptability differences and selecting probable more tolerant cultivars in Arsenjan region.

2. Materials and Methods

Orchard and plants

Uniform and healthy rooted plants of seven commercial Iranian pomegranate cultivars were purchased from a commercial nursery and planted in a completely randomized block design with 3 replications (each replication had 3 plants) with 3 m distance in rows and 5 m distance between rows in an orchard site in Arsenjan region (hub of pomegranate growing and production in Fars), Fars province, Iran. Average annual climate parameters in the experimental region were; precipitation: 200 mm, relative humidity (Max: 55%, Min: 23%), temperature (Max: 38, Min: 4°C). The soil of the orchard was sampled at two depths and analyzed for soil texture, mineral content, organic matter, pH and EC (Table 1). Cultivars included: Malas Yousefkhani Saveh (MYS), Naderi Badroud (NB), Malas Daneh Ghermez Yazd (MDGY), Rabab Neiriz Fars (RNF), Shirin Shahvar Fars (SSF), Shirin Poust Daneh Ghermez (SPDG) and Zard Anar Arsenjan (ZAA). After 4 years and full establishment of the collection orchard, samples (fresh leaves) were taken from the trees. Leaf samples were taken from different orientations of the trees (north, south, west and east); 25 fully expanded mature leaves from each side of all trees (100 leaves per tree as bulk samples), and transported to laboratory. Leaves were taken from shoots without terminal fruit. The leaves were of spring bloom, the middle third of the branch, at a height of between 1-1.5 m including the petiole. Leaves with abnormal symptoms such as chlorosis and mechanical lesions caused by pests or diseases were avoided. The trees were grown under drip irrigation; water quality parameters are presented in Table 2. Routine cultural practices suitable for commercial fruit production were carried out during experimental period. The following parameters were measured in studied cultivars for two consecutive years and an average was reported.

Table 2 - Analysis of quality parameters of irrigation water

Parameters	value
EC (dS m^{-1})	0.91
pH	7.1
Na (%)	34.75
Cl (mg l^{-1})	45.71
SAR	3.5
TDS (mg l^{-1})	590

Measurements

Trunk circumference. Circumference of trunks was measured 5 centimeter above ground and expressed as centimeter.

Leaf dry matter content. Three uniform leaves were selected and washed with tap and distilled water, after drying with clean towel they were weighed with digital scale and then were oven dried for 72 hours in 70°C and weighed. Leaf dry matters percentage was calculated by the following formula (Eshghi and Jamali, 2009):

Leaf dry matters (%) = [Leaf dry weight (g)/leaf fresh weight (g)] × 100

Leaf water content. Leaf relative water content (LRWC) was measured by using ten leaf discs. The leaf discs of each treatment were weighed (FW). They were then hydrated until saturation (constant

Table 1 - Analysis of soil samples in the experimental region

Soil depth (cm)	Soil texture	Soil mineral content (mg kg^{-1})							Organic carbon (%)	EC (dS m^{-1})	pH
		Nitrate	Ca	Mg	K	Fe	Zn	Mn			
0-30	Loamy clay	32	1200	150	150	8.4	1	8.7	0.75	0.82	7.6
31-60	Loamy sand	40	1150	150	140	7.32	0.95	7.1	0.65	0.71	7.6

weight) for 48 h at 5°C in darkness (TW). Leaf discs were dried in an oven (DW). Relative water content was calculated according to the following expression (Jamali and Eshghi, 2015):

$$LRWC\% = (FW-DW)/(TW-DW) \times 100$$

Leaf electrolyte leakage. Leaf electrolyte leakage (EL) was determined by recording the electrical conductivity (EC) of leaf leachates in double distilled water at 40 and 100°C. Leaf samples were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water. The test tubes were kept at 40°C for 30 min and at 100°C in boiling water bath for 15 min and their respective electric conductivities (EC1 and EC2) were measured by conductivity-meter (METROHM Conductometer 644, Switzerland) (Jamali *et al*., 2015):

$$\text{Electrolyte leakage (\%)} = (EC1/EC2) \times 100$$

Leaf total sugars. The total leaf soluble carbohydrates were determined according to Irigoyen *et al.* (1992) and glucose (0-100 mg l^{-1}, from *MERCK*) was used as a standard. Leaf samples of 0.5 g (dry weight) were homogenized in 5 ml ethanol (95%) and centrifuged at 4500 × *g* for 15 min, the supernatant was removed from the sample and the residue was resuspended in 5 ml of 70% ethanol. Then the supernatant was centrifuged again for final extraction. Both supernatants were combined. Anthrone-sulfuric acid assay was used for determination. An aliquot of 100 µl was added to 3 ml of anthrone-sulfuric acid solution and the mixture was shaken, heated in a boiling water bath for 10 min and cooled at 4°C. The absorption at 625 nm was determined by spectrophotometer.

Leaf chlorophyll and carotenoids concentration. Leaf discs of 0.5 g were extracted in 5 ml of acetone (80%), then centrifuged for 10 min in 8,000 × *g*. The supernatant was used to make a final volume of 100 ml of the leaf extract. Extraction of leaf tissue with the buffer continued until decoloration. Absorbance of the extract was read at 470, 645 and 663 nm with a spectrophotometer and 80% acetone was used as a blank. Finally, Chlorophyll and carotenoids contents was calculated according to the following equations (Lichtenthaler, 1987):

$$\text{Chl a (mg. g}^{-1}\text{ fresh weight)}: [(12.25A663 - 2.79A645) \times v / 1000 \times W]$$

$$\text{Chl b (mg. g}^{-1}\text{ fresh weight)}: [(21.50A645 - 5.10A663) \times v / 1000 \times W]$$

$$\text{Chla + Chlb (mg. g}^{-1}\text{ fresh weight)}: [(7.15A663 + 18.71A645) \times v /1000 \times W]$$

$$\text{Carotenoids (mg. g}^{-1}\text{ fresh weight)}: 1000A470 - 1.82Chla - 85.02Chlb / 198$$

where Chla = chlorophyll a; Chlb = chlorophyll b; Chla+b = total chlorophyll; A = absorbance at ƛ (nm).

Leaf anthocyanins concentration. Leaf total anthocyanins were measured spectrophotometrically by pH differential method with two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). 0.5 g leaf samples were extracted with 2 ml methanol: water: concentrated HCl solution (80:20:1 v/v/v). 0.4 ml of leaf extract was mixed with 3.6 ml of corresponding buffers and read against water as blank at 510 and 700 nm. Absorbance (A) was calculated as

$$A = (A515 - A700)\ \text{pH 1.0} - (A510 - A700)\ \text{pH 4.5}$$

Then total anthocyanins content was calculated using the equation:

$$\text{Anthocyanin (µg. g}^{-1}\text{ fresh weight)} = (A \times Mw \times DF \times 1000) / e$$

Where *A* is the absorbance of the diluted sample and DF is the dilution factor (10), Mw is molecular weight of cyanidin-3-glucoside (449.2) and e= 26,900 L/mol.cm, molar extinction coefficient of cyanidin-3-glucoside.

Leaf total polyphenols. Leaf polyphenols was determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound. In brief, 1 g of lyophilized leaf samples were placed in an eppendorf tube, with 1 ml of methanol (80%), grinded at 4°C and centrifuged at 10000 × *g* for 15 min. The extract was mixed with 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1 with water) then 1 ml of a 5% sodium carbonate solution was also added. After 30 min, absorbance was measured at 725 nm and expressed as mg on g fresh $weight^{-1}$.

Leaf α-tocopherol concentration. Leaf α-tocopherol was extracted according to Chong *et al*. (2004).Two hundred mg lyophilized sample was homogenized in 1 ml acetone with a prechilled mortar and pestle at 4°C. Following the addition of 0.5 ml hexane, the homogenate was first vortexed for 30 s, then centrifuged at 1000 × *g* for 10 min. The upper hexane layer was removed while the acetone layer containing vitamin E remained in the vial. A second aliquot of 0.5 ml hexane was added, and the extraction process was repeated at least twice. α-tocopherol was estimated by the method of Kanno and Yamauchi (1997).

A 0.4-ml aliquot of 0.1% (w/v) 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine was added to 0.2 ml of pooled extract. The volume was made up to 3 ml with absolute ethanol, 0.4 ml 0.1% (w/v) ferric chloride ($FeCl_3.6H_2O$) was added, and the content was gently mixed under dim light in a dark room to avoid photochemical reduction. After a 4 minutes reaction at room temperature, 0.2 ml 0.2 M orthophosphoric acid was added and the mixture left for another 30 min. Absorbance was determined at 554 nm spectrophotometrically and reported as µg. g fresh weight^{-1}. The blank was prepared in the same manner except that absolute ethanol was used instead of the sample.

α-tocopherol (Sigma Chemical) was used as a standard.

Leaf ascorbic acid concentration. Ascorbic acid was estimated by the method of Omaye *et al*. (1979). Briefly, to 1 g of lyophilized leaf sample, 10% ice-cold TCA was added and centrifuged for 20 min at 3500 × *g* in room temperature. One ml of the supernatant was mixed with 0.2 ml of DTC reagent and incubated for 3 h at 37°C. Then 1.5 ml of ice-cold 65% H_2SO_4 was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. The color developed was read at 520 nm spectrophotometrically and reported as µg. g fresh weight^{-1}.

Macro and micronutrients. Oven-dried leaf samples were used for determination of macro and micro-nutrients. Dried samples (0.5 g) were ground and ashed at 550°C in a porcelain crucible for 6 h. The white ash was mixed in 2 M hot HCl, filtered and finally made up to 50 mL with distilled water. Sodium (Na) and potassium (K) concentration of samples were determined using flame emission method using a Sherwood Scientific Ltd model 360 flame photometer. Atomic absorption spectrophotometer (AA 6200, double beam atomic absorption spectrophotometer, Shimadzu, Kyoto, Japan) was used to determine Ca, Mg and micronutrient element including Fe, Zn, Mn concentrations (Kalra, 1998). Nitrogen (N) concentration was measured using the Kjeldahl digestion method (Kalra, 1998). Phosphorus (P) concentration was determined colorimetrically (Kalra, 1998). Chlorine (Cl) was measured by precipitation titration with silver nitrate (Mohr's method) (Kalra, 1998).

Statistical analysis

Data were analyzed by SAS and means were compared using Duncan's multiple range test at 5% probability level.

3. Results

Leaf macronutrients and Na and Cl concentrations in studied pomegranate cultivars are indicated in Table 3. Leaf N, P and Cl concentrations were not statistically different in studied cultivars. RNF and ZAA had significantly higher leaf K concentration in comparison to SPDG and NB, other cultivars were not statistically different. The highest leaf Ca concentration was observed in RNF cultivar (2.53±0.06 mg g^{-1} dry weight), however MDGY, SSF and ZAA were not statistically different. This macronutrient was 22% lower in SPDG compared to RNF. Leaf Mg concentration was significantly higher in MYS, MDGY, RNF and ZAA in comparison to NB, SPDG and SSF. Leaf Na concentration was 41% higher in MYS in comparison to ZAA. Other cultivars were not statistically different.

Leaf Fe, Zn and Mn concentrations in studied pomegranate cultivars are presented in Table 4. Leaf Fe concentration is 32% higher in ZAA in comparison to NB. Other cultivars are not statistically different. No significant difference was found among studied cultivars for leaf Zn and Mn concentrations.

Leaf dry matters, total sugars, relative water content and electrolyte leakage are shown in Table 5.

Table 3 - Leaf macronutrient and Na and Cl concentrations in studied pomegranate cultivars

Cultivars	N (mg g^{-1} DW)	P (mg g^{-1} DW)	K (mg g^{-1} DW)	Ca (mg g^{-1} DW)	Mg (mg g^{-1} DW)	Na (mg g^{-1} DW)	Cl (mg g^{-1} DW)
Malas Yousefkhani Saveh	20.50±2.07 a	1.59±0.09 a	21.60±0.72 bc	1.99±0.12 cd	0.97±0.02 ab	3.53±0.47 a	1.56±0.23 a
Naderi Badroud	22.80±0.81 a	1.63±0.02 a	20.33±0.41 c	2.11±0.05 bcd	0.89±0.06 b	2.74±0.43 ab	1.48±0.28 a
Malas Daneh Ghermez Yazd	21.453±1.48 a	1.62±0.11 a	22.60±0.72 abc	2.38±0.09 abc	1.13±0.01 a	2.43±0.36 ab	1.89±0.13 a
Rabab Neiriz Fars	20.83±0.89 a	1.60±0.09 a	23.56±0.29 a	2.53±0.06 a	1.06±0.04 a	2.49±0.49 ab	1.96±0.10 a
Shirin Shahvar Fars	22.43±0.74 a	1.59±0.02 a	22.80±0.44 abc	2.21±0.08 abcd	0.88±0.06 b	2.76±0.40 ab	1.76±0.14 a
Shirin Poust Daneh Ghermez	22.50±1.41 a	1.53±0.09 a	20.91±1.21 c	1.97±0.02 d	0.86±0.07 b	2.28±0.35 ab	1.47±0.26 a
Zard Anar Arsenjan	21.78±0.87 a	1.65±0.06 a	23.55±0.27 ab	2.50±0.02 ab	1.13±0.3 a	2.07±0.07 b	1.44±0.30 a

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated are mean ± standard error (n = 3).

Table 4 - Leaf micronutrient concentrations in studied pomegranate cultivars

Cultivars	Fe (mg g^{-1} DW)	Zn (mg g^{-1} DW)	Mn (mg g^{-1} DW)
Malas Yousefkhani Saveh	58.66±2.02 ab	23.00±4.50 a	53.00±9.70 a
Naderi Badroud	43.00±5.85 b	25.00±3.46 a	49.00±3.51 a
Malas Daneh Ghermez Yazd	46.33±4.91 ab	29.33±2.84 a	49.33±5.81 a
Rabab Neiriz Fars	53.00±8.38 ab	22.33±4.33 a	55.00±9.23 a
Shirin Shahvar Fars	53.667±2.33 ab	22.33±3.66 a	50.66±3.38 a
Shirin Poust Daneh Ghermez	58.00±2.08 ab	23.00±3.05 a	48.66±0.88 a
Zard Anar Arsenjan	64.667±3.71 a	26.66±7.35 a	56.00±2.64 a

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated are mean ± standard error (n = 3).

RNF had significantly higher leaf dry matter compared to MYS, SSF and SPDG. ZAA, NB and MDGY were not statistically different. The highest leaf total sugars (47.63±4.99 mg g^{-1} dry weight) were detected in RNF which were significantly higher than SPDG, SSF, NB and MYS. ZAA showed the highest leaf relative water content (83.83±0.74%) however RNF, SSF and MDGY were not statistically different. This parameter was lower in MYS, NB and SPDG compared to ZAA. Leaf electrolyte leakage was significantly higher in MYS in comparison to all other cultivars.

Concentrations leaf pigments in studied pomegranate cultivars are indicated in Table 6. NB had significantly lower leaf total chlorophyll concentration in comparison to RNF, other cultivars were not statistically different. Leaf carotenoids concentration was significantly higher in ZAA, SPDG, SSF and RNF compared to NB and MYS. Leaf anthocyanins concentration was not different among studied cultivars. MYS showed lower chlorophyll a to b ratio in comparison to RNF, SSF, SPDG and ZAA.

Leaf total polyphenols concentration was statistically higher in RNF, SPDG and ZAA in comparison to MYS, NB and SSF (Fig. 1). The highest leaf α-toco-

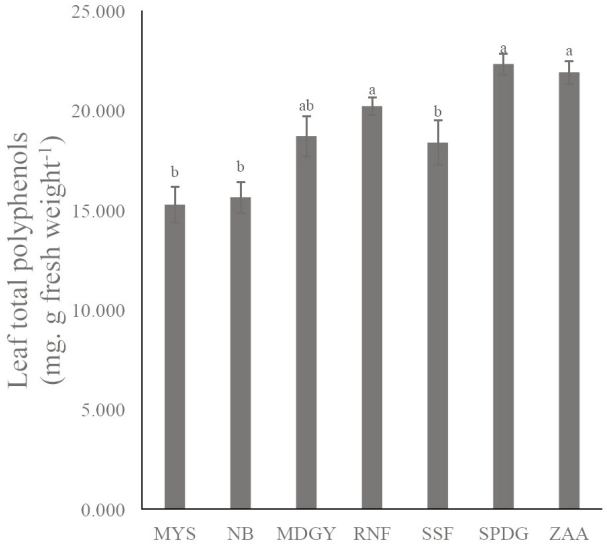

Fig. 1 - Leaf polyphenols concentration in studied cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=3).

Table 5 - Leaf dry matter, total sugars, relative water content and electrolyte leakage in studied pomegranate cultivars

Cultivars	Leaf dry matter (%)	Leaf total sugars (mg g^{-1} DW)	LRWC (%)	EL (%)
Malas Yousefkhani Saveh	24.33±1.20 d	22.44±2.26 d	79.05±0.67 c	24.80±1.77 a
Naderi Badroud	30.33±1.45 ab	29.50±1.45 cd	80.92±1.27 bc	20.08±1.28 b
Malas Daneh Ghermez Yazd	32.33±0.66 ab	39.63±2.56 ab	82.22±0.63 ab	19.20±1.51 b
Rabab Neiriz Fars	33.00±1.52 a	47.63±4.99 a	82.70±0.24 ab	18.66±0.50 b
Shirin Shahvar Fars	25.66±1.76 cd	35.72±3.29 bc	81.62±0.89 abc	18.55±1.24 b
Shirin Poust Daneh Ghermez	28.66±0.66 bc	29.66±1.15 cd	80.55±0.86 bc	17.20±0.49 b
Zard Anar Arsenjan	30.66±2.18 ab	42.41±1.22 ab	83.83±0.74 a	17.10±0.10 b

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated are mean ± standard error (n = 3).

Table 6 - Leaf pigments concentrations in studied pomegranate cultivars

Cultivars	Total chlorophyll	Carotenoids	Anthocyanins	Chlorophyll a/b ratio
Malas Yousefkhani Saveh	1.35±0.06 ab	0.15±0.01 c	0.25±0.004 a	0.88±0.04 c
Naderi Badroud	1.16±0.08 b	0.20±0.01 b	0.25±0.007 a	1.01±0.04 bc
Malas Daneh Ghermez Yazd	1.30±0.17 ab	0.25±0.01 ab	0.24±0.002 a	1.15±0.02 ab
Rabab Neiriz Fars	1.46±0.04 a	0.27±0.005 a	0.26±0.01 a	1.19±0.03 a
Shirin Shahvar Fars	1.44±0.04 ab	0.28±0.004 a	0.25±0.02 a	1.19±0.01 a
Shirin Poust Daneh Ghermez	1.40±0.07 ab	0.26±0.02 a	0.25±0.02 a	1.29±0.03 a
Zard Anar Arsenjan	1.34±0.10 ab	0.29±0.006 a	0.26±0.01 a	1.26±0.04 a

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated are mean ± standard error (n = 3).

pherol concentration was obtained from SPDG (229 µg. g fresh weight^{-1}), however it was not statistically different in comparison to ZAA, RNF and SSF. MYS, NB and MDGY had significantly lower leaf α-tocopherol concentration compared to SPDG (Fig. 2). Leaf ascorbic acid concentration in RNF was significantly higher in RNF in comparison to MDGY, other cultivars were not statistically different (Fig. 3). RNF had significantly higher trunk circumference in comparison to MYS. Other cultivars were not statistically different (Fig. 4).

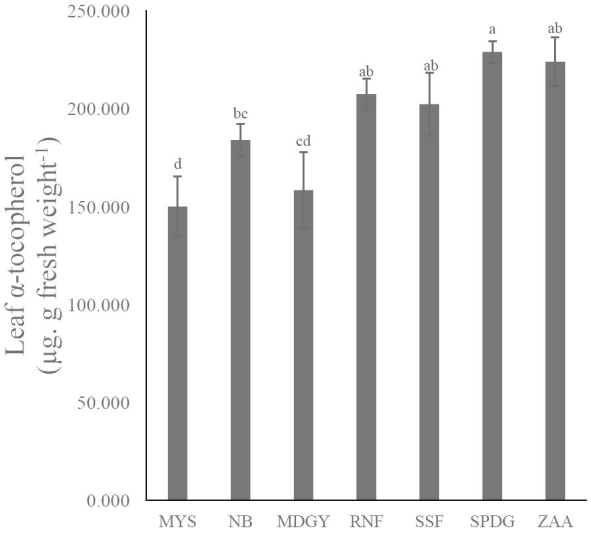

Fig. 2 - Leaf α-tocopherol concentration in studied cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=3).

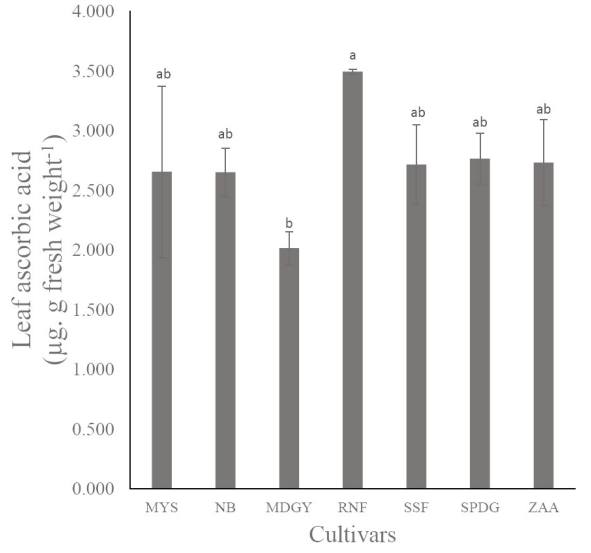

Fig. 3 - Leaf ascorbic acid concentration in studied cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=3).

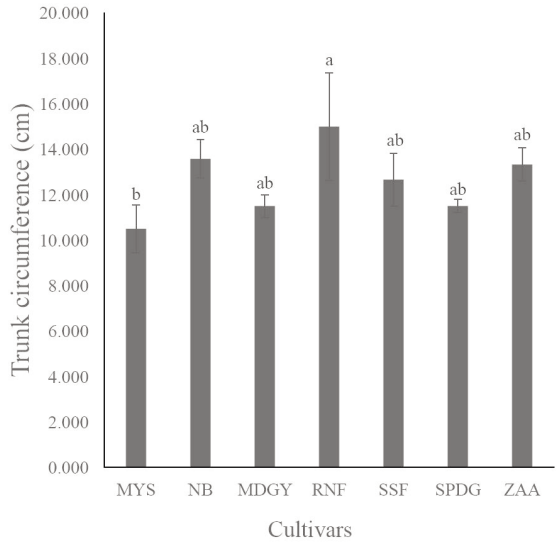

Fig. 4 - Trunk circumference in studied cultivars. Columns with the same letters are not statistically different at 5% probability using Duncan's test. Vertical bars indicate standard errors (n=3).

4. Discussion and Conclusions

In present study significant differences were found between studied pomegranate cultivars for concentrations of leaf macro and micronutrients. This was in agreement with previous studies. Giménez *et al*. (2000) compared leaf mineral composition (macro and micronutrients) in two pomegranate cultivars; MC1 (one of the most commonly grown cultivars in the south-east of Spain) and a well-known Israeli early variety. They found significant differences between these two pomegranate cultivars for concentrations of leaf macro and micronutrients. Normality ranges for N, P, K, Ca and Mg were also varied. El-Agamy *et al*. (2010), compared the salt tolerance of two important Egyptian pomegranate cultivars, Manfalouty and Nab El-Gamal *in vitro*. They found significant differences in these two cultivars under saline and non-saline conditions for absorption of N, K and Ca. Khayyat *et al*. (2014) evaluated physiological and biochemical responses of Malas Mommtaz and Shishe Kab under saline conditions; they reported with increase in salinity (from 4.61 to 7.46 dS m^{-1}) shoot Cl concentration augmented in Malas Mommtaz but decreased in Shishe Kab, also this cultivar (Shishe Kab) managed Na^+ transport into the leaves better than Malas Mommtaz. Sarafi *et al*. (2017) indicated that Wonderful and Ermioni cultivars had different ability for P, K, Ca, Mg and Zn uptake under optimum conditions. Similar differences were observed between Rabab and ShisheKab

by Hasanpour *et al*. (2015).

RNF and ZAA had higher relative water content and lower electrolyte leakage in comparison to MYS. Presence of higher K concentration in plant tissues will lead to more efficient water relationships in the leaves (Marschner, 1995). Photosynthesis, stomatal activity, transport of sugars, protein synthesis are all dependent on K (Prajapati and Modi, 2012). Ca pectates provide stability and mechanical strength to cell walls (Pilbeam and Morley, 2007; Taiz and Zeiger, 2010). Structural impairments in membrane structure are very common in Ca-deficient cells which make cell membranes very leaky and cause extensive loss of organic (*e.g*., sugars, amino acids) and inorganic electrolytes from root or leaf cells (White and Broadley, 2003). Leaf total sugars varied among studied cultivars. About half of osmotic potential in glycophte species is related to presence of sugars (Cram, 1976). Accumulation of higher total sugars has been reported to be associated with higher tolerance to salinity and/or drought in various species; grape, barley, *Zygophyllum album* and soybean (Ashraf and Harris, 2004). Five sunflower cultivars were evaluated for their salinity tolerance, more resistant lines had higher total sugars (Ashraf and Tufail, 1994). Comparison between wild populations of *Melilotus indica* (salt tolerant) and *Eruca sativa* (salt sensitive) showed that the former had higher leaf sugars (Ashraf and Harris, 2004).

Significant difference was observed between cultivars for leaf chlorophyll and carotenoids concentration in present study. In various studies the chlorophyll concentration were used as a sensitive indicator of the cellular metabolic state (Chutipaijit *et al*., 2011). Higher chlorophyll concentration is related to elevated tolerance against abiotic stresses such as drought and salinity (Hasanuzzaman *et al.*, 2013). In addition to harvesting solar energy, carotenoids play protection roles keeping integrity of photosynthesis apparatus against photo oxidative damages by scavenging free radicals (Dall'Osto *et al*., 2007; Andrade-Souza *et al*., 2011). Carotenoids are precursor of ABA which is an important phyto-hormone regulating plant responses in response to stresses. So presence of higher carotenoid concentration lead to lower photo-oxidative damage and higher potential for regulating plant growth under stress conditions (Götz *et al*., 2002; Han *et al*., 2008).

Non enzymatic antioxidants (leaf polyphenols, α-tocopherol and ascorbic acid concentration) were statistically different among studied cultivars. Varied antioxidant profile in different species and cultivars is one of the main reasons responsible for their different adaptability and abiotic tolerance potential (Munns and Tester, 2008; Jamali *et al*., 2016). Polyphenols have strong antioxidant properties and presence of an elevated level of them is associated with increased abiotic stress tolerance (Jamali *et al*., 2016). Among vitamin E family, α-tocopherol has the highest antioxidant activity (Garg and Manchanda, 2009). Several lines of evidence indicate that α-tocopherol plays a major role in keeping an adequate redox state in chloroplasts (Munne-Bosch, 2005). Deficiency of this antioxidant leads to a slightly increased susceptibility to photooxidative stress (Kanwischer *et al*., 2005). Plants have different capacity of ascorbate metabolism which is due to the variation of ascorbic acid synthesis and regeneration. Plant with higher amount of ascorbic acid content demonstrate better protection against oxidative stress. Ascorbate influences many enzyme activities, minimizing the oxidative damage through synergic function with other antioxidants (Foyer and Noctor, 2005 a, b). Ascorbic acid plays a role as a co-factor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy (Pourcel *et al*., 2007).

Trunk circumference was significantly higher in RNF compared to MYS. Difference in mineral composition and ability of nutrients uptake in these cultivars could be one of the reasons responsible for this, as discussed above. Difference in enzymatic (data not shown) and non-enzymatic antioxidant responses and also growth regulators such as GA_3, zeatin and ABA (data not shown) in these cultivars are other reasons for varied growth rate observed between pomegranate cultivars in the present study.

Pomegranate is a tolerant species, however various varieties have significantly different adaptability potential. This can be contributed to varied ability for macro and micronutrients uptake, different enzymatic and non-enzymatic antioxidant profile and endogenous plant growth regulators. However, environmental conditions play an important role in this regard and might alter the final response of plant. ZAA is an indigenous variety of Arsenjan region/Fars province/Iran. High level of leaf K, Ca, Mg and Fe, enzymatic and non-enzymatic antioxidants (data not shown) and higher level of zeatin (data not shown) are the reasons responsible for better adaptation of this cultivar. Similar responses were observed in RNF. NB and MYS showed lower adaptability to the regional conditions among all studied cultivars. Further studies with same cultivars in other pomegranate growing regions seem necessary for a com-

prehensive evaluation about their adaptability capacity.

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Mini review

Rocket salad: crop description, bioactive compounds and breeding perspectives

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Key words: *Diplotaxis tenuifolia*, *Eruca sativa*, flavonols, genetic improvement, glucosinolates, phytochemicals, rocket salad.

Abstract: **Rocket salad is a plant member of the Brassicaceae family whose name encloses species of the *Eruca* and *Diplotaxis* genera characterized by leaves with peculiar pungent taste and strong flavour. It has been originated in the Mediterranean area and nowadays is worldwide cultivated and consumed as food condiment and in ready-to-use mixed salad packages. Several other uses are recognized in cosmetics and medicine. This crop represents a valuable source of health benefits due to the presence of a range of health-promoting phytochemicals including carotenoids, vitamin C, fiber, polyphenols, and glucosinolates. These compounds are potentially linked in the prevention of certain diseases and types of cancer. Glucosinolates, represent the major class of compounds in rocket, and their hydrolysis products are responsible of the typical pungent aromas and flavours. Despite the continuous increase of the global consumption during the recent years, few efforts have been carried out in genetic improvement programs aimed to constitute new varieties due to biological and reproductive barriers. In the present article is provided a brief overview of the principal species of rocket salad used in dietary and discussed the qualitative properties as well as the potentiality and constraints for breeding.**

1. General Aspects

Rocket salad also known as arugula is an annual herbaceous plant whose name encloses several species of the Brassicaceae family characterized by leaves with peculiar pungent taste and strong flavour. The crop has been originated in the Mediterranean and Near East, with a major centre of diversity in the regions of Western Mediterranean (Hall *et al.,* 2015), which represent also the main areas of cultivation thanks to their growing conditions and climate.

Several species, mainly belonging to the genus *Eruca* and *Diplotaxis*, are widely cultivated and recognized as rocket salad. The most common are *Eruca vesicaria* (L.) Cav. and *Diplotaxis tenuifolia* (L.) Dc. *Eruca vesicaria* includes four subspecies namely subsp. *vesicaria*, subsp. sativa (Miller), subsp. *longirostris* (Uechtr.) and subsp. *pinnatifida* (Desf.) (Gomez-Campo, 2003). Among these, the subsp *sativa,* also called *Eruca sativa*, has been spreaded in different part of the world as cultivated rocket and is the most consumed and economically relevant. This species is diploid with eleven chromosomes (2n = 22) (Padulosi and Pignone, 1996) with annual life cycle flowering at begin of spring and ending with the production of seeds in late spring/early summer. Nowadays it is cultivated in all continents in both marginal areas and/or fertile soils. Plants of this species are characterized by a height of about 15.0 cm, flowers with calyx caduceus, sepals only two cucullate and corolla cream or whitish (Gomez-Campo, 2003).

Diplotaxis genus includes about 33 species (www.theplantlist.org) with great variability related to morphological traits and chromosome number (Table 1). The genus includes both annual and perennial plants with leaves of different shape, thickness,

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Table 1 - List of *Diplotaxis* species recognized and their chromosome number

Species	Chromosome number
D. acris (Forsk.) Boiss.	
var. *acris*	11
var. *duveyrieriana* (Coss.) Coss.	*na* *
D. antoniensis Rustan	*na*
D. assurgens (Del.) Thell.	9
D. berthautii Br.-Bl. and Maire	9
D. brachycarpa Godr.	9
D. brevisiliqua (Coss.) Mart.-Laborde	8
D. catholica (L.) DC.	
var. *catholica*	9
var. *rivulorum* (Br.-Bl. And Maire) Maire	*na*
D. erucoides (L.) DC.	
subsp. *Erucoides*	7
subsp. *longisiliqua* (Coss.) Gómez-Campo	7
D. glauca (J.A. Schmidt) O.E. Schulz	13
D. gorgadensis Rustan	
subsp. *gorgadensis*	*na*
subsp. *brochmanii* Rustan	*na*
D. gracilis (Webb) O.E. Schulz	13
D. griffitthii (Hook.f. and Thomps.) Boiss.	*na*
D. harra (Forsk.) Boiss.	
subsp. *crassifolia* (Rafin.) Maire	13
subsp. *harra*	13
subsp. *lagascana* (DC.) O. Bolòs and Vigo	13
subsp. *confusa* Mart.-Lab.	13
D. hirta (Chev.) Rustan and Borgen	13
D. ibicensis (Pau) Gómez-Campo	8
D. ilorcitana (Sennen) Aedo et al.	8
D. kohlaanensis A. Miller and J. Nyberg	*na*
D. muralis (L.) DC.	
subsp. *ceratophylla* (Batt.) Mart.-Laborde	*na*
subsp. *muralis*	21
D. nepalensis Hara	*na*
D. ollivierii Maire	*na*
D. pitardiana Maire	*na*
D. scaposa DC.	9
D. siettiana Maire	8
D. siifolia Kunze	
subsp. *bipinnatifida* (Coss.) Mart.-Laborde	*na*
subsp. *Siifolia*	10
subsp. *vicentina* (Samp.) Mart.-Laborde	10
D. simplex (Viv.) Sprengel	11
D. sundingii Rustan	13
D. tenuifolia (L.) DC.	
subsp. *cretacea* (Kotov) Sobr. Vesp.	11
subsp. *tenuifolia*	11
D. tenuisiliqua	
subsp. *rupestris* (J. Ball) Mart.-Laborde	*na*
subsp. *tenuisiliqua*	9
D. varia Rustan	*na*
D. villosa Boulos and Jallad	*na*
D. viminea (L.) DC. var. viminea and var. integrifo-	10
D. virgata (Cav. DC.)	
subsp. *sahariensis* Coss.	*na*
subsp. *virgata*	9
subsp. *rivulorum* (Br.-B1. and Maire) Mart.-	*na*
subsp. a*ustralis* Mart.-Lab.	*na*
D. vogelii (Webb) O.E. Schulz	*na*

* na = not available.

indentation, and flower colour (white, yellow and purple). The most common species cultivated across all continents are *Diplotaxis tenuifolia* and *Diplotaxis muralis.* Both are perennial being cultivated in the winter and producing new sprouts in the spring. This aspect, combined to the dehiscence of the siliqua and the large number of viable seeds, helps to spread these species as weeds. *Diplotaxis tenuifolia* is the most used for human consumption; plants are characterized by average height of 80 cm, a deep tap root, fleshy leaves, and oblong, lobed with pointed apexes.

Main differences occurred between *Eruca* and *Diplotaxis* genus in terms of plant architecture, leaf morphology, chromosomal number and phytochemical compound contents. *Eruca* species, being annuals, tends to have a higher growth rate, increased size of leaves and early flowering. These characteristics result in a high production of biomass, which make the system of cultivation different respect to the *Diplotaxis* spp. and requiring a lower seeding density and a lower number of harvests. Another trait discriminating the two genera is the larger seed dimension of the *Eruca* species (1.5 mm in length) with respect to *Diplotaxis* spp. (about 0.7 mm in length) (Padulosi and Pignone, 1996). A wider germination temperature range and greater speed of germination is also observed within the *Eruca* species, which is probably due to their annual nature, requiring greater energy of the plant to produce viable seeds (Hall *et al.,* 2015).

The consumption of rocket salad dates back since ancient time and included food and non food uses such as oil, deodorant, cosmetic and medical purposes (Hall *et al.,* 2012). Aphrodisiac properties and medical uses related to anti inflammatory and depurative effects (Padulosi and Pignone, 1996) were emphasized by ancient poets during Greek and Roman times. Nowadays leaves are eaten fresh in salads or as topping of many dishes (e.g. pizza) or cooked in soups. Several recipes provide the preparation of pureed, sauces and pesto. Other cosmetic uses concern the production of creams and lotions for body.

Rocket salad is worldwide cultivated and commercialized in many countries as mix salad packages. In Europe, the needing of prepared products ready to use as well as the major attention given to a well balanced and assorted diet, composed of a variety of health-promoting compounds, has facilitated its consumption. In central and northern markets, over half of the rocket comes from Italy and Spain, which,

thanks to their geographical position and mild climatic conditions, represent the main producers. *Diplotaxis* is much more cultivated, fitting better to the needs of the farmers and being better suited to commercial utilization, thanks to the possibility to perform several harvests per cycle with yield increasing after the first harvest (Hall *et al.,* 2015).

Arugula is recommended in diets, having a very low-calorie vegetables (25 calories per 100 grams of fresh leaves) and being a very good source of vitamins and minerals (Table 2). Furthermore, it contains a range of vital compounds, with important nutraceutical and anticancer properties, which are discussed in the next paragraph.

2. Bioactive Compounds

Many studies associate a highly significant reduction in the risk of cancer as well as a tumorogenesis inhibition and hepatoprotective effects with increasing consumption of *Cruciferae* (Lynn *et al.,* 2006; Juge *et al.,* 2007; Lamy *et al.,* 2008; Alqasoumi *et al.,* 2009). Rocket contains a range of health-promoting phytochemicals including carotenoids, vitamin C, fiber, polyphenols and glucosinolates (Bennett *et al.,* 2006; Heimler *et al.,* 2007).

Table 2 - Rocket salad nutritional values for 100 g of fresh leaves (USDA Nutrient Database *)

Nutrient	Unit	Value
Energy	kcal	25
Water	g	91.71
Carbohydrate	g	3.65
Protein	g	2.58
Sugars	g	2.05
Fiber	g	1.6
Lipid	g	0.66
Vitamins		
Vitamin C	mg	15
Thiamin (Vitamin B_1)	mg	0.044
Riboflavin (Vitamin B_2)	mg	0.086
Niacin (Vitamin B_3)	mg	0.305
Pyridoxine (Vitamin B_6)	mg	0.073
Folate (Vitamin B_9), DFE (z)	µg	97
Vitamin A, RAE (y)	µg	119
Vitamin A	IU	2373
Vitamin E	mg	0.43
Vitamin K	µg	108.6
Minerals		
Calcium, Ca	mg	160
Iron, Fe	mg	1.46
Magnesium, Mg	mg	47
Phosphorus, P	mg	52
Potassium, K	mg	369
Sodium, Na	mg	27
Zinc, Zn	mg	0.47

*https://ndb.nal.usda.gov/ndb/foods/show/3569?manu=&fgcd=&ds=
(z) Dietary folate equivalents.
(y) Retinol activity equivalents.

Glucosinolates (GLSs) represent the major class of compounds in rocket and their contents in this crop have been well documented in the literature (D'Antuono *et al.,* 2008; Pasini *et al.,* 2012). When glucosinolates are exposed to myrosinase (EC 3.2.1.147, thioglucoside glucohydrolase) during tissue damage, glucose and an unstable intermediate are formed. This intermediate degrades to produce a sulfate ion, and a variety of products including isothiocyanates, nitriles and, to a lesser extent, thiocyanates, epithionitriles and oxazolidines. The relative proportion of these hydrolysis products depends on the plant species studied, on the glucosinolate itself (as side chain substitution), and reaction conditions like pH, metal ions or epithiospecifier protein (Bennett *et al.,* 2007).

Both *Eruca* and *Diplotaxis* species contain similar profiles of GLSs within the leaf tissue, the most prominent of which are glucosativin (4-mercaptobutyl-GLS), glucoerucin [4-(methylthio)butyl-GLS] and glucoraphanin [4-(methylsulfinyl)butyl-GLS]. Glucosativin and glucoerucin breakdown products are thought to contribute most to pungency and flavour in rocket (Pasini *et al.,* 2012). Numerous other GLSs have also been identified within rocket tissue, for example diglucothiobeinin [4-(b-D-glucopyranosyldisulfanyl) butyl-GLS] (Kim *et al.,* 2007), 4-hydroxyglucobrassicin (4-hydroxy-3-indolymethyl-GLS) (Cataldi *et al.,* 2007) and 4-methoxyglucobrassicin (4-methoxy-3-indolymethyl-GLS) (Kim and Ishii, 2006).

Phenolics are the most abundant antioxidants in the human diet. Considerable evidence indicates that some of the protective effects of phenols in fruits and vegetables may be due to flavonoids (Clifford and Brown, 2006). Rocket species also contain large concentrations of polyglycosylated flavonol compounds, which are known to infer numerous beneficial health effects in humans and other animals. Particularly of note are their effects on the gastrointestinal tract and in cardiovascular health (Bjorkman *et al.,* 2011; Traka and Mithen, 2011). Several studies in rocket have identified and quantified polyglycosylated flavonols, which belong to three core aglycones:

isorhamnetin, kaempferol and quercetin (Bennett *et al.,* 2006).

Pasini *et al*. (2012) studied the glucosinolate and phenolic profiles of 37 rocket salad accessions (32 *Eruca sativa* and 5 *Diplotaxis tenuifolia*) obtained by liquid chromatography-mass spectrometry. The authors isolated eleven desulpho-glucosinolates (DS-GLSs) and the glucosinolate profiles did not differ between the two species. Total DS-GLS content, expressed as sinigrin equivalents (SE) revealed a certain variability, ranging from 0.76 to 2.46 mg g^{-1} dry weight (dw) but, again, the quantitative analysis did not discriminate *Eruca* from *Diplotaxis*. Moreover, the polyphenol evaluation by HPLC-DAD-MS allowed the identification of two different classes of compounds in the two rocket salad species. Qualitative differences were observed between the polyphenol profiles at specific level: quercetin derivatives were the main phenolics of *Diplotaxis*, whereas kaempferol derivatives characterised *Eruca* samples. The contents of total flavonoids determined as rutin equivalents (RE) ranged from 4.68 to 31.39 mg g^{-1} dw. Kaempferol-3,4'-diglucoside (71.4-82.2%) and isorhamnetin-3,4'-di-glucoside (7.8-18.4%) were always isolated as first and second more abundant phenolic compounds in *Eruca* samples. No marker phenolic compounds were isolated in *Diplotaxis* samples.

Durazzo *et al*. (2013) reported significant differences in the quality of conventional and integrated cultivation practices on the nutritional properties and benefits of wild rocket [*Diplotaxis tenuifolia* (L.) DC.], while no influence on biological activity was evidenced. The authors also determined the cytotoxicity and antiproliferative activity of rocket polyphenol extract on human colon carcinoma (Caco-2) cells, evidencing a significant accumulation of cells in G1 phase and a consequent reduction in the S and G2 + M phases in response to the treatment. Regarding antioxidant properties, they found FRAP (Ferric Reducing Antioxidant Power) values ranged from 4.44±0.11 mmol kg^{-1} fresh weight (fw) to 9.92±0.46 mmol kg^{-1} fw for conventional rocket and from 4.13±0.17 mmol kg^{-1} fw to 11.02±0.45 mmol kg^{-1} fw for integrated rocket.

Villatoro-Pulido *et al*. (2013) analysing four *E. sativa* accessions reported the total content of glucosinolates ranged from 6.12 to 12.33 mg g^{-1} of dw. Glucoraphanin represented up to 52% of the total glucosinolates in leaves of one accession. Accessions showed differences in the hydrolysis of glucoraphanin to the isothiocyanate sulforaphane. No correlation between these compounds was observed, which insisted differences in the myrosinase activity within accessions. The same authors highlighted that rocket leaves had variable phenolic profiles represented by quercetin-3-glucoside, rutin, myricetin, quercetin and ferulic and p-coumaric acids. A high variability was observed for the total carotenoids ranged from 16.2 to 275 μg g^{-1} with lutein as the main carotenoid. Moreover, they found glucose was the predominant sugar, representing >70% of the total soluble carbohydrates.

Bell and collaborators (2015) used Liquid chromatography mass spectrometry (LC-MS) to obtain glucosinolate and flavonol content for 35 rocket accessions and commercial varieties. They identified 13 glucosinolates and 11 flavonol compounds; semi-quantitative methods were used to estimate concentrations of both groups of compounds. Minor glucosinolate composition was found to be different between accessions; concentrations varied significantly. According to Pasini *et al*. (2012) they confirmed flavonols differentiation between genera, with *Diplotaxis* accumulating quercetin glucosides and *Eruca* accumulating kaempferol glucosides. The authors detected several compounds in each genus that have only previously been reported in the other.

Recently, we investigated the qualitative and quantitative profiles of glucosinolates and polyphenols, highlighting flavonoid glycoside compounds (flavonols), in 39 accessions of wild and cultivated rocket (Taranto *et al.,* 2016). Seven DS-GLSs were detected in rocket leaves belonging to two chemical classes: five aliphatic compounds (glucoerucin, glucoraphanin, progoitrin, glucoalyssin, and glucosativin) and two structurally related compounds containing one intermolecular disulfide linkage, 4-(β-D-glucopyranosyldisulfanyl)butyl-GLS and dimeric 4-mercaptobutyl-GLS. The species studied significantly differed for GLS content: total average concentrations being 29.61 and 19.41 mg g^{-1} dw for *E. sativa* (21 accessions) *and D. tenuifolia* (16 accessions), respectively. Total GLS content ranged from 2.10 to 40.96 mg g^{-1} dw and from 11.61 to 26.96 mg g^{-1} dw, for *Eruca* and *Diplotaxis* accessions, respectively. Additional accessions of *D. muralis* and *Erucastrum* spp. were evaluated exhibiting an average GLS content of 17.39 and 3.63 mg g^{-1} dw, respectively.

Fifteen flavonol compounds were tentatively identified in the thirty-nine accessions studied. *Diplotaxis* accessions were characterized by nine dif-

ferent flavonols mainly represented by quercetin derivatives, total average content being 7.17 mg g^{-1} dw with a range from 4.91 to 8.57 mg g^{-1} dw. The most abundant flavonol compound in *Diplotaxis* was quercetin 3,4'-diglucoside-3'-(6-sinapoylglucoside). As regards *Eruca* accessions, the more abundant flavonoid group was represented by kaempferol derivatives, in agreement with a previous report (Martínez-Sánchez *et al.,* 2007). The 21 *Eruca sativa* accessions showed a flavonol total average concentration of 8.13 mg g^{-1} dw, the lowest and the highest content being 0.82 and 10.16 mg g^{-1} dw, respectively. The most abundant flavonol was kaempferol 3,4'-diglucoside.

According to previous research, isorhamnetin 3,4'-diglucoside was the only compound common to *Diplotaxis* and *Eruca* accessions studied (Martínez-Sánchez *et al.,* 2008; Pasini *et al.,* 2012; Bell *et al.,* 2015). However, some exceptions have been observed. Specific compounds mainly detected in *Eruca* such as kaempferol 3-glucoside and kaempferol 3-diglucoside-7-glucoside have been reported also in *Diplotaxis* commercial varieties (Bell *et al.,* 2015). Other compounds specific for *Diplotaxis* (i.e., quercetin 3,4'-diglucoside-3'-(6-caffeoylglucoside) and quercetin 3,4'-diglucoside-3'-(6-sinapoylglucoside) have been also identified in *Eruca* (Bell *et al.,* 2015). These inconsistencies could be related to the genetic material used. Overall the results of the analysis of glucosinolates and flavonols evidenced how the *Eruca sativa* gene pool contains potential candidates to use in breeding programs for quality.

3. Potentiality and Perspectives for Breeding

Despite the global consumption of rocket salad has increased in the recent years, little efforts have been spent by both private and public breeding programs aimed to constitute new varieties. The importance in phytochemicals has been above discussed and novel knowledge as source of resistances have been recently described (Pane *et al.,* 2017). Nowadays, constraints are mainly caused by pathogens, nitrate accumulation, early flowering and physiological disorders due to intensive culture system. Accessions of *Eruca sativa* are reported to be late-bolting (Kenigsbuch *et al.,* 2014) and to accumulate less nitrate than *Diplotaxis tenuifolia* (Cavaiuolo and Ferrante, 2014), being good candidates for the improvement with respect to the latter. However, several limitations for the transfer of these useful traits are linked to the failure of intergeneric crosses between *Eruca* and *Diplotaxis* due to post-zygotic barriers (Tripodi unpublished) resulting in the absence of a cost effective hybridization system available for rocket. Moreover, interspecific crosses among *Diplotaxis* species are difficult due to their different chromosomes number (Table 1).

Eruca sativa x *Brassica rapa* and *Diplotaxis tenuifolia* x *Brassica rapa* hybrids are instead possible using embryo rescue (Agnihotri *et al.,* 1990; Jeong *et al.,* 2009) and somatic hybridization (Zhang *et al.,* 2008) techniques, making the two rocket salad species a good source to use for the improvement of *Brassica rapa*. The possibility of intercross has been applied in the development of cytoplasmic male sterile (CMS) *Eruca sativa* plants transferring a male sterile cytoplasm from *Brassica oleracea* or *Brassica napus* (Merete *et al.,* 2012). Two approaches have been used: one requiring the application of embryo rescue after the first cross hybridization, subsequent chromosome doubling and backcrossing of the resulting hybrid to *Eruca* sativa, another, using protoplast fusion from cytoplasmic male sterile *Brassica*, subsequent regeneration of allogenic cells and crossing of the regenerated plant with pollen from *Eruca sativa*. The same approach has been used by Hosemans and Leviell (2012) by transferring cytoplasmic male sterility from *Raphanus sativus* to *Diplotaxis tenuifolia*. *Raphanus sativus* has been also used to transfer CMS in *Eruca sativa* (Nothangel *et al.,* 2016).

Despite these achievements, breeding activities are still carried out by means of traditional selection schemes such as mass selection or single seed descent. New possibilities may be obtained by TILLING (McCallum *et al.,* 2000) in order to select mutants for gene of interest or genome wide association approaches (GWAS) (Huang and Han, 2014) for the dissection of the genetic basis of complex traits and the development of markers for breeding assisted selection. Mutagenesis mediated by ethyl methanesulfonate (EMS) is already reported with success in *Diplotaxis tenuifolia* (Kenigsbuch *et al.,* 2014), resulting in a mutant showing late flowering and delayed postharvest senescence. These approaches successful in *Brassica* species (Stephenson *et al.,* 2010; Xu *et al.,* 2016) may be also applied in rocket salad for a better exploitation of the genetic potentiality of this crop, and furthermore, to address the challenges of the modern agriculture that demands major security and quality of foods.

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Fungal colonization improved growth and modulated the expression of myrosinases in black cabbage

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Key words: *Brassica oleracea*, colonization, *Piriformospora indica*.

Abstract: The role of beneficial microorganisms, such as mycorrhizas, in improving the resistance to environmental stress of colonized plants is well-known. Plants of Brassicaceae family are of large economic importance, especially for the synthesis of anticarcinogenic compounds such as glucosinolates and their derivatives isothiocyanates. The endophyte fungus *Piriformospora indica* is able to colonize them and improves their growth and response to environmental stress. However, no information are available on the impact of colonization on glucosinolate metabolism. In this work, colonization of black cabbage (*Brassica oleracea* cv. Acephala sabellica) is reported as well as the effects on plant growth and on the expression of myrosinase encoding genes, the isothyocianate producing enzymes. Results indicate that *P. indica* successfully colonized black cabbage as validated by the expression of the marker gene Ptef1. Colonized plants showed increase of biomass weights and shoot length respect to the uncolonized plants and a decrease of myrosinase gene expression. This last finding indicates that *P. indica* might affect the resistance against biotic stress of black cabbage.

1. Introduction

Over the last 20 years, low-input and organic agriculture has increased worldwide to preserve agroecosystem functionality (Postma-Blaauw *et al.*, 2010). The main point of such an agriculture is a systemic approach to integrate sustainable yield and crop quality together with high-energy efficiency and low environmental impact (Pimentel *et al*., 2005; Moonen and Bàrberi, 2008). In the framework of this view, the natural roles of microorganisms, such as arbuscular mycorrhizas in improving soil fertility have gained a growing interest for the use of such microrganisms as ecosystem engineers and biofertilizers (Fitter *et al*., 2011). Although arbuscolar mycorrhizal fungi normally infect most species of plants, some plants taxa do not usually form generally recognisable mycorrhizas. Among them, the family of Brassicaceae have been considered to be nonmicorrhyzal plants (Lambers and Teste, 2013), probably because their roots released anti-fungal metabolites such as isothiocyanates in the surrounding environment (Tester *et al*., 1987). Isothiocyanates are produced by hydrolization of glucosinolates that are a group of secondary metabolites present in Brassicaceae (Halkier and Gershenzon, 2006).

The endophyte fungus *Piriformospora indica* (*P. indica*), a basidiomycete of the order Sebacinales, was isolated from the Indian Thar desert in 1997 (Varma *et al*., 1999). *P. indica* has received a great attention over the last few decades due to its ability to promote plant growth, protection and stress tolerance in colonized plants (Verma *et al.*, 1998; Banhara *et al*., 2015). *P. indica* is similar to arbuscular mycorrhizal fungi, but it is a facultative symbiont and can be easily grown on various synthetic media. Likewise, *P. indica* has a wide host range, colonizes the host roots, grows inter and intracellularly, and forms pear-shaped chlamydospores within the cortex, improving the growth of many plant species, enhancing nutrient uptake, enabling plants to cope with environmental conditions, and to survive under abiotic stresses. It also confers resistance to toxins, pathogenic microorganisms, and increases seed biomass yield (Oelmuller *et al.*, 2009). Among others, *P. indica* is able to colo-

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nize plants of Brassicaceae family (Sherameti *et al.*, 2005) and improves their growth and response to environmental stimuli. *P. indica* triggered local and systemic root responses in *Arabidopsis thaliana* (Pedrotti *et al*., 2013). In Chinese cabbage (*Brassica rapa*), it has been reported that *P. indica* colonization confers drought tolerance stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized Ca(2+)-sensing receptor (CAS) protein in the leaves (Sun *et al*., 2010). Black cabbage, (*Brassica oleracea* cv. acephala sabellia) a variety of kale largely used in Italian cuisine, especially in Tuscany, where has been grown for centuries (Appleman *et al*., 2008), is generally considered a nonmicorrhyzal plants (Lambers and Teste, 2013). In this work, with purpose to assess whether *P. indica* colonizes black cabbage and to study the colonization effects on this cultivar, seedlings were inoculated with *P. indica*; morphological parameters and the expression of myrosinase encoding genes were studied.

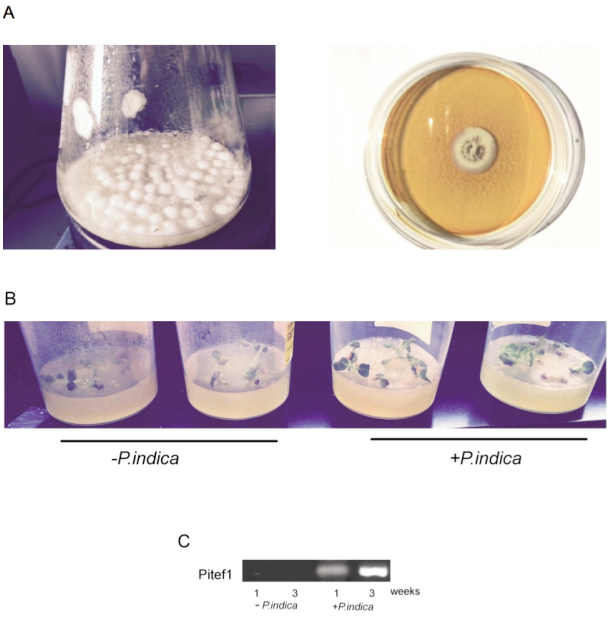

Fig. 1 - *P. indica* grown in liquid (left panel) or agar medium (right panel) (A); control (- *P. indica*) or colonized (+ P. indica) black cabbage seedlings grown on 1.5% agar (B); Pitef1 expression in *-P.indica* (left panel) or + *P.indica* plants (right panel) (C).

2. Materials and Methods

Growth conditions of plants and fungus, and estimation of plant growth

Seeds of *Brassica oleracea* L. ssp. *oleracea* convar *acephala* (DC.) Alef. var. *sabellica* L. were surface-sterilized with 75% alcohol three times for 10 min, and then placed on a Petri dish containing sterilized water. Plates were incubated at 22°C under continuous illumination (for seed germination). After 7 days, seedlings were transferred in Petri dish plates with solid (1.5% agar) complete medium (CM) (Pham *et al*., 2004). Six seedlings were used per plate.

Piriformospora indica growth conditions

P. indica cultures, DSM11827, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (Lahrmanna *et al*., 2013) were propagated at 28°C in liquid CM for two days than plated in agar CM (Fig. 1A). The amount of 200 mg of fungal mycelium were used to colonize the seeds. 0.1 ml of CM medium containing fungal mycelium were positioned 1 cm away from each seedling. The same amount of autoclaved mycelium was used as control. Plant growth was monitored day by day. Histograms report biomass weight and shoot length as mean±SD. The statistical significance of differential findings between samples was determined by ANOVA using NIA software; $p<0.05$ was considered statistically significant.

RNA extraction and genes expression Pitef1 expression

Brassica leaves were disrupted by liquid nitrogen and then suspended in the double volume of PBS. Total RNA extraction and cDNA synthesis were performed from 50 mg of lised leaves samples, modifying the protocol of the Taqman Gene Expression Cells-to-CT TM Kit (Applied Biosystems) as reported in Podda *et al.* (2014). Two µL of the cDNA were used for sqRT-PCR amplification performed with GoTaq Green Mastermix (Promega, USA). The following standard thermal profile was used for all PCRs: 94°C for 3 min; 35 cycles of 90°C for 30 s, 59°C for 40 s, and 72°C for 40 s; 72°C for 7 min as final extension. PCR products were separated by 1% agarose gel electrophoresis and stained with GelRed (Biotium). cDNA fragments were purified from gels and sequenced by BMR-Genomics (Italy). Transcript levels were measured by Scion Image program and normalized with the constitutive reference actin gene (Wang *et al*., 2016). Three independent biological replicates were used. In order to verify the colonization level, the presence of *P. indica Transcription Elongation Factor* Pitef (Butehorn *et al.*, 2000) was tested in the *P. indica* leaves before or after fungal colonization.

The following primers have been used:

	F 5'	Rew 5'
Pitef1	ATTGCCTGCAAGTTCTCCGA	CTTCGTAACCTTGCCACCCT
TGG1	TCTTAACGTGTGGGATGGCT	CCTCCTTTGTTCACTCCCCT
TGG2	AGATGTGCTGGACGAACTCA	CGGCGTAACAGGTAGGATCA
PEN2	GCATCATCATCCAACAGCGT	ACGCCTTGATCAGTTCTCCA
Actin	AATGGTACCGGAATGGTCAA	AGTTGCTCACAACACCATGC

3. Results and Discussion

P. indica growth and black cabbage colonization

In order to evaluate the effects of *P. indica* colonization in black cabbage, the protocol used by Dolatabadi and Goltapeh, (2013) has been optimized for this kale variety. The fungus *P. indica* was grown in liquid medium (Fig 1 A, left panel) and then transferred on agar complete medium (Fig 1 A, right panel). Then 7 week-old black cabbage seedlings were inoculated with *P. indica* mycelium in sterilized conditions in tubes. To validate the successfully colonization, the expression of *Pitef1* was assessed as the gene has been demonstrated to be useful for estimating the amount of active mycelium introduced in seedlings (Butehorn *et al*., 2000). A strong expression of *Pitef1* was observed in leaves of colonized seedlings of black cabbage one and three weeks after fungal inoculation whereas no transcript was observed in not-colonized seedlings (Fig. 1C).

Evaluation of black cabbage growth parameters

The effects of colonization on growth parameters, biomass weights and shoot lengths, were measured in the inoculated plants in the first three weeks of growth. Colonization by P*. indica* resulted in a rapid enhancement of about 30% of root and shoot biomass respect to the not colonized plants, just after one week from the inoculation (Fig. 2 A, B). Results are in agreement with those reported by Dolatabadi and Goltapeh, (2013) who found that *P. indica* and *Sebacina vermifera* improved the growth of *B. oleracea* and other brassicaceae plants. Satheesan et al., (2012) reported improved growth of *Centella asiatica* after inoculation by *P. indica.*

Expression of TGG1, TGG2, PEN2 in the leaves

An increase of glucosinolates was found within ten days from germination in black cabbage. Glucosinolates are secondary metabolites present in Brassicaceae (Yi *et al.*, 2015). When plants are damaged due to insect herbivore attack, glucosinolates are hydrolyzed quickly with myrosinase (β-thioglucoside glucohydrolase or thioglucosidase) resulting in production of isothiocyanates, thiocyanates, nitriles and others compounds (Bones and Rossiter, 2006; Hopkins *et al.*, 2009). No information are available in the literature on the modulation of glucosinolate by products in the leaves during fungal colonization.

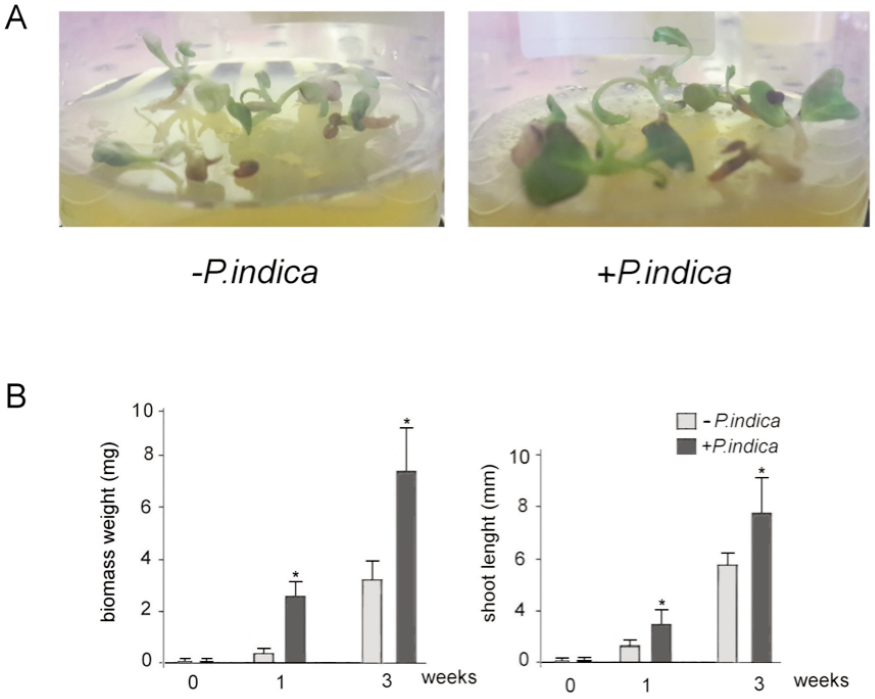

Fig. 2 - Influence of *P. indica* on black cabbage growth parameters during three weeks from the inoculation. Picture of the plants after one week from the inoculation (A); Biomass weight (mg) or shoot length (mm), at 0, 1 and 3 weeks after *P. indica* colonization (B).Values are the mean of ten independent experiments for each condition (control or inoculated) ±SD. Asterisk means significant difference at $p \leq 0.05$.

Thus, in this work the expression of TGG1 and TGG2, which encode myrosinases hydrolysing aliphatic glucosinolates or PEN2, encoding the enzymes hydrolysing indole glucosinolates was evaluated. Intriguingly, a decrease of TGG1, TGG2 and PEN2 expression was observed at three weeks of colonization (Fig. 3). Similar results have been reported by Witzel *et al*. (2015) in *Arabidopsis thaliana* infected by *Verticillium longisporum*. As the glucosinolate-myrosinase system is relevant for defence against insect-herbivore (Winde and Wittstock, 2011), the decrease of the expression of the genes relative to this pathway, is of particular importance and should be further investigated for extensive periods. Although *P. indica* colonization improves plant growth and resistance to abiotic stress (Sun *et al*., 2010), a negative impact on defence response pathway might increase the susceptibility of colonized black cabbage against biotic stress.

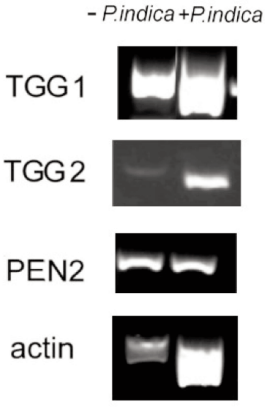

Fig. 3 - Influence of *P. indica* on black cabbage myrosinases. Analysis of TGG1, TGG2 and PEN2 expression in leaves three weeks after colonization by sqRT-PCR. Actin was used as reference gene.

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Morpho-chemical and aroma investigations on autochthonous and highly-prized sweet cherry varieties grown in Tuscany

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Key words: fruit aroma, morpho-chemical parameters, *Prunus avium* L., PTR-ToF-MS, volatile compounds.

Abstract: The morpho-chemical and aromatic characteristics of four sweet cherry cultivated varieties (*Prunus avium* L.) grown in the area of Lari (Pisa, Central Italy) were evaluated with the aim to investigate their properties, mainly concerning volatile organic compounds (VOCs). Of these, three cultivars ('Di Giardino', 'Di Nello', and 'Marchiana') represent ancient sweet cherries recovered through a private cultivation program (belonging to the group of the so-called 'Ciliegia di Lari'); their evaluation was compared with the commercial cultivar Ferrovia, highly-prized variety marketed in Italy and abroad. Morpho-chemical analyses highlighted statistical differences among the cultivars under study, mainly on total soluble solids (TSS) and tritatable acidity (TA). Aroma investigation was performed with PTR-ToF-MS (proton transfer reaction - time of flight - mass spectrometer) approach, employed here for the first time in cherry fruits. About 50 VOCs were detected; among them, those belonging to the chemical classes of aldehydes and alcohols were the most represented although with different intensities between samples. Tentative identification of some key VOCs for cherry fruit was also performed and preliminary conclusions on the characterization of ancient and wide spread Italian cultivars were given.

1. Introduction

Sweet cherry fruit (*Prunus avium* L.) is one of the most appreciated spring-summer fruit in temperate areas of Europe, especially in Mediterranean basin (Landi *et al*., 2014). Its economic importance is due to the nutritional, technological and commercial value of fruits. Fruits are rich in many antioxidants and nutrients (Ballistreri *et al*., 2013), such as phenolics, flavonoids, anthocyanins and carotenoids, and are characterized by sensory qualities highly appreciated by consumers.

In Tuscany region (Central Italy) its cultivation has a long tradition, and the hilly area in the South-South East of Pisa province, especially the area of Lari, is one of the most important and famous districts for the sweet cherry production in Tuscany (Gargani *et al*., 2013).

As with other fruit species, the introduction and diffusion of new varieties of sweet cherry in specialized crop systems has marginalized the local ones to the point that some have disappeared, while others are still present as single plants or in mixed orchards.

In those situations, autochthonous and ancient varieties have formed plant populations, resulted from the selective pressure exerted by both natural environment and human cultural practices; importantly, these populations act as a natural reservoir of genetic variability and source of useful genes for the selection of new varieties, for the improvement of the existing ones, with the global objective to guarantee the levels of sustainability and stability of production systems (Di Matteo *et al*., 2016). Concurrently, autochthonous and ancient varieties could represent source of new agro-economic systems, based on the use of crop residuals, or on an alternative use of the products.

Globally, during the last decades, worldwide biodiversity has been lost at an unprecedented rate in all the ecosystems, including agro-ecosystems. Accordingly, a number of instruments and tools that contribute to a sustainable development while

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addressing objectives and priorities related to biodiversity have been established at different levels, from global to national ones (FAO, 2010); in Italy, for example, since 2015, the law n. 194/2015 has been published with the aim to safeguard and enhance the protection of biological resources relevant to food and agriculture from the risks of extinction and genetic erosion.

In this context, many studies have been performed to explore plant biodiversity, especially through the investigation of plant and fruit metabolomics. Indeed, plants produce a wide range of metabolites, the main part being involved in secondary metabolic pathways; these are the result of different plants responses, through the course of evolution, to specific needs and stimuli. Among such metabolites, volatile organic compounds (VOCs) play a dominant role (Dicke and Loreto, 2010), being released by quite any kind of plant tissues (Peñuela and Llusia, 2001; Dudareva *et al*., 2006) as green leaf volatiles, nitrogen-containing compounds and aromatic compounds. Plants VOCs can be emitted constitutively (Holopainen and Gershenzon, 2010; Holopainen *et al*., 2010), or as a consequence of the interactions of plants with biotic and abiotic factors (Spinelli *et al*., 2011).

Therefore, VOCs can be considered as important metabolites for the characterization of biodiversity. Accordingly, the study of VOCs emitted by fruits, represents a strategic tool for discriminating varieties of the same species growing in the same environment.

To this end, the aim of the present study was to investigate the properties, mainly concerning VOCs, in fruits of a collection of autochthonous and ancient sweet cherry varieties, and to compare them with those of a commercial variety.

2. Materials and Methods

Plant Material

Fruits of four sweet cherry cultivated varieties for fresh consumption (namely: 'Di Giardino', 'Di Nello', 'Marchiana' and 'Ferrovia') used in this study were collected at fully ripe grade from ten years old *Prunus avium* L. trees grown in an experimental farm in Lari (Pisa province, Italy), in late May-early June 2017. The genotypes were planted with a spacing of 6.0×4.0 m, trained as spindle-bushes, and managed according to standard cultural practices. Yield per tree was comparable among cultivars (around 35-40 tons per hectare).

The cherry orchards were located about 130 m above sea level (lat. 43° 33' 08'' N, long. 10° 35' 21'' E). Three of these cultivars ('Di Giardino', 'Di Nello', 'Marchiana') represent ancient native sweet cherry cultivars recovered through a private cultivation program (belonging to the group of the so-called 'Ciliegia di Lari'); instead 'Ferrovia', highly-prized variety marketed in Italy and abroad, has been used as commercial cultivar in order to compare the morpho-chemical and the aromatic characteristics with the native ones.

For each cultivar, 1 kg of homogeneous and healthy fruits were harvested randomly from multiple trees at a commercial ripening stage based on color change and fruit firmness. Samples were transported to the laboratory in isothermal plastic bags within 2 h from harvesting and stored at 4°C until the analysis were performed (at least 24 h after the sampling). Subsequently, the measurements were made in the following order, according to the degree of destruction: fruit skin color, morphological parameters, volatile compounds and chemical parameters. Before the measurements, fruit samples were washed in deionized water.

Morpho-chemical parameters

Fifteen fruits from each sweet cherry cultivar were used to assess the morphological parameters and the skin color. The total weight (fruit and seed) was determined using a digital balance (Sartorius TE1502, USA) while their three linear dimensions (length, width and thickness) and the stalk length were measured using a electronic digital caliper (Stainless Hardened, sensitivity of 0.01 mm). The ripening stage of sweet cherries is characterized by fruit color changing from green to red (Diaz-Mula *et al*., 2009). The color characteristics were analyzed on the fruit surface (skin) using a Minolta CR-200 chromatometer (Minolta, Ramsey, NJ) and *L* (lightness), *a* (green to red) and *b* (blue to yellow) values were measured. Subsequently, *a* and *b* values were used to calculate the color index (*a*/*b*) since this value shows a continuous increase during sweet cherry un grirororrroriroro ripening (Díaz-Mula *et al*., 2009). Furthermore, Hue angle ($\tan^{-1}$ *b*/*a*) and the Chroma index ($\sqrt{a^2 + b^2}$) were assessed as two main parameters used to describe visual color appearance (Little, 1975; McLellan *et al*., 1995). Results are reported as mean ± deviation standard.

The chemical parameters were determined in triplicate thus each sample was represented by the pulp of three fruits. Few drops of fruit juice were used to determine the total soluble solids (TSS) with a refractometer (N1 Atago Co., Japan) and expressed

as °Brix. Subsequently, the fruit pulp was shredded and blended with 150 ml of deionized water. The obtained solution was filtered and used to measure pH with a digital pH meter (Basic20, Crison Instruments) and titratable acidity (TA) by tritation with 0.1 N NaOH up to pH 8.1 and expressed as percentage of malic acid. Furthermore, TSS/TA ratio has been assessed since this value is linked to the fruit flavor and is one of the main indicator of the fruit quality (Alonso and Alique, 2006). Data are reported as mean ± deviation standard.

PTR-ToF-MS profiling

The volatile profile of four different sweet cherry samples and the tentative identification of each detected compound was evaluated by PTR-ToF-MS (model 8000, Ionicon GmbH, Innsbruck, Austria) which guarantees high sensitivity with a very high-time resolution (Taiti *et al*., 2017).

A further description of PTR-ToF-MS is given by Lindinger *et al*. (1998). The analysis method and instrumental settings were carried out following the procedure previously used by Taiti *et al*. (2016). Briefly, each sample consisted of four freshly cut fruits (including the seed). For the analysis, each fruit was cut in 2 parts, inserted in a clear glass jar (3/4 l at 22°C, with a dynamic headspace flushing flow rate of 0.75 l per meter, lpm) equipped with two Teflon inlet and outlet tubes on opposite side, connected respectively to a zero-air generator (Peak Scientific) and the PTR-ToF-MS. Before each analysis, the jar was cleaned for 1 minute with free VOCs air and subsequently was incubated for 80 s. Blank measurements were carried out between samples to monitor background air. The analyses were performed in independent triplicates and an averaged mass spectrum per sample was calculated after background and transmission correction (N=10). The mass spectral data (m/z = 20-210) of four sweet cherry cultivars was assessed after the removal from the dataset of masses m/z = 32 (O_2^+) and m/z = 37 (water cluster ion), other interfering ions and their isotopologues. The instrument was operated at E/N value of 133 Townsend (1 Td = 10^{-17} cm^2 V^{-1} s^{-1}). The chamber ionization conditions were kept as follows: drift temperature 60°C, drift voltage 580 V and drift pressure 3.80 mbar. Each sample measurement was performed with an acquisition rate of 1 spectrum/s for 80 s.

The raw data were acquired by the TOFDAQ Viewer® software (Tofwerk AG, Thun, Switzerland) and the count losses due to the ion detector dead time were corrected off-line following the methodology based on Poisson correction as previously reported by Titzmann *et al*. (2010). Moreover, to reach a good mass accuracy (up to 0.001 Th), the instrumental calibration was based on m/z = 29.997 (NO^+), m/z = 59.049 ($C_3H_7O^+$) and m/z = 137.137 ($C_{10}H_{17}^+$) and was performed off-line. Finally, the VOCs identification was based on models of fragmentation available in the literature (Buhr *et al*., 2002; Lee *et al*., 2006; Aprea *et al*., 2007; Maleknia *et al*., 2007; Kim *et al*., 2009; Jardine *et al*., 2010; Tani, 2013) and compared with published VOCs emitted from sweet cherry fruits (Table 1).

Statistical analysis

Analysis of mean values and deviation standard, multivariate analysis of variance (ANOVA, $p \leq 0.05$), and mean separation by Tuckey's test ($p \leq 0.05$) were performed using the statistical package GraphPad Prism 5.0 software, IL, USA).

3. Results and Discussion

As known, cherry fruit quality (expressed as concentration of nutritive and bioactive compounds) is mainly affected by genotype, environment and orchard management (Predieri *et al*., 2004; Gonçalves *et al*., 2006). In this work all cherry cultivars were obtained from the same farm and all trees were grafted onto the same rootstock, therefore, the differences observed should be attributed almost exclusively to genetic characteristics.

Morpho-chemical parameters

Fruit color and size are the main parameters employed to visually evaluate the sweet cherries (Romano *et al.*, 2006). The morpho-chemical attributes of the four cherry cultivars are shown in Table 2.

Among comparative cultivars the highest average fruit weight was measured in 'Ferrovia' (7.24±0.82 g), while all other autochthonous accession showed lower values; furthermore, 'Di Giardino' (5.46±0.65 g) and 'Di Nello' (4.62±0.62 g) were also statistically different compared to 'Ferrovia' and 'Marchiana'.

As far as concerning other morphological parameters, cv. Ferrovia confirmed to be the biggest (21.54±1.24, 24.33±0.84, 21.07±0.97 mm for length, width and thickness, respectively), while cv. Marchiana was the smallest (15.59±0.99, 18.76±0.82, 15.12±0.73 mm for length, width and thickness respectively). Fruits belonging to 'Di Giardino' and 'Di Nello' showed intermediate shape. Interestingly, the stalk length was highest for 'Di Nello' which belongs to the category of varieties with 'medium stalk length', while the others (less than 39 mm) are culti-

Table 1 - List of VOCs detected in the four sweet cherry samples. Chemical classes and references, where available, are indicated

m/z	Protonated chemical formula	Chemical class (tentative identification)	References
27.022	$C_2H_3^+$	alkyl fragment	
30.046	$C_2H_6^+$	alkene	
31.018	CH_3O^+	aldehyde	
31.054	$C_2H_7^+$	alkyl fragment	
33.033	CH_5O^+	alcohol	
39.022	$C_3H_3^+$	unknown fragment	
41.038	$C_3H_5^+$	alcohol fragment/ester fragment	
43.018	$C_2H_3O^+$	ester fragment	
43.054	$C_3H_7^+$	alcohol fragment	
45.033	$C_2H_5O^+$	aldehyde	1
47.049	$C_2H_7O^+$	alcohol	2
51.038	$CH_7O_2^+$	alcohol	
53.040	$C_4H_5^+$	alkyl fragment	
55.055	$C_4H_7^+$	aldehyde fragment	
57.033	$C_3H_5O^+$	aldehyde	
59.059	$C_3H_7O^+$	aldehyde/ketone	3
61.028	$C_2H_5O^+$	acid	4
65.038	$C_5H_5^+$	alkyl fragment	
67.054	$C_5H_7^+$	alkyl fragment	
69.033	$C_4H_5O^+$	heterocyclic aromatic compound	5
69.069	$C_5H_9^+$	terpene	1
71.049	$C_4H_7O^+$	aldehyde	
71.085	$C_5H_{11}^+$	alcohol	
73.065	$C_4H_9O^+$	aldehyde/ketone	6
75.044	$C_3H_7O_2^+$	ester	
77.040	$C_6H_5^+$	alkyl fragment	
79.049	$C_6H_7^+$	alkene	
81.069	$C_6H_9^+$	terpene fragment	
83.086	$C_6H_{11}^+$	alcohol fragment	
85.065	$C_5H_9O^+$	ald	
87.044	$C_4H_7O_2^+$	ketone	
89.059	$C_4H_9O_2^+$	ester	
91.054	$C_7H_7^+$	unknown fragment	
93.069	$C_7H_9^+$	alkyl	6
95.086	$C_7H_{11}^+$	unknown fragment	
97.064	$C_6H_9O^+$	aldehyde	1
99.080	$C_6H_{11}O^+$	aldehyde	1
101.096	$C_6H_{13}O^+$	aldehyde	2
103.075	$C_5H_{11}O_2^+$	acid	4
107.049	$C_7H_7O^+$	aldehyde	2
109.101	$C_8H_{13}^+$	unknown fragment	
115.075	$C_6H_{11}O^+$	acid	4
117.091	$C_6H_{13}O_2^+$	acid	2, 7
119.101	$C_9H_{11}^+$	alkyl fragment	
121.065	$C_8H_9O^+$	aldehyde	6
127.111	$C_8H_{15}O^+$	ketone	7
137.137	$C_{10}H_{17}^+$	terpene	1

Refereces legend for data available on cherry fruit: (1) Vavoura *et al*., 2015; (2) Serradilla *et al*., 2012; (3) Mattheis *et al*., 1992; (4) Wen *et al*., 2014; (5) Zhang *et al*., 2007; (6) Bernalte *et al*., 1999; (7) Sun *et al*., 2010. Where legend is missing, the VOCs identification was based on fragmentation models available in literature (Buhr *et al*., 2002; Lee *et al*., 2006; Aprea *et al*., 2007; Maleknia *et al*., 2007; Kim *et al*., 2009; Jardine *et al*., 2010; Tani, 2013).

vars with 'short stalk length' (Roselli and Mariotti, 1999; Fajt *et al*., 2005).

Color parameters, especially *a/b*, Hue angle and Chroma indices, were comparable to those reported by Díaz-Mula *et al*. (2009) for ripe cherry fruits. In this work, based on the Hue angle values reported by Crisosto *et al*. (2002), the analyzed varieties showed fruits with color tending to: full light red for 'Marchiana' (25.29±1.22), between full light red and 50% bright red for 'Ferrovia' (22.72±1.49), between 50% bright red and full dark red for 'Di Nello' and 'Di Giardino' (Table 2; see also pictures inserted in figure 1, representing cherry samples for each cultivar under study).

The chemical parameters of sweet cherries as total soluble solids (TSS) and titratable acidity (TA), as well as indicators of the degree of ripening, are also important quality indexes for cherry cultivars evaluation (Crisosto *et al*., 2003).

The results of the chemical parameters are presented in Table 2. Significant differences were found in TSS values especially with regard to cv. Di Nello (12.94±1.08 °Brix) which showed values significantly lower than the other cultivars under study. Moreover, the highest values were detected in 'Ferrovia' (22.77±0.58 °Brix) while 'Marchiana' and 'Di Giardino' showed very similar values to each other (Table 2). Thus, excluding the cv. Di Nello, all cultivars analyzed were above the limit of 14-16°Brix, considered acceptable for marketing cherries as suggested by Crisosto *et al*. (2003).

Some differences ($p \leq 0.05$) were also found in TA among the sweet cherry cultivars (Table 2). The highest average values were found in 'Ferrovia' (0.90±0.05 as percentage of malic acid per fresh weight) followed by 'Di Giardino' and 'Marchiana' (0.58±0.05 and 0.64±0.02, respectively), and finally by 'Di Nello' cultivars that showed the lowest values (0.37±0.05). Accordingly, 'Ferrovia' fruits showed the lowest pH value, and 'Di Nello' the highest one ($p \leq 0.05$).

Moreover, since the TSS/TA ratio has being related to the consumer acceptance, giving that the sugar concentration increases while acidity remains relatively constant during the maturation or ripening process (Spayd *et al*., 1986), such index can be used as well as a quality parameter in sweet cherry (Alonso and Alique, 2006). Indeed, as it has been observed elsewhere for sweet cherry fruits (Crisosto *et al*., 2003; Garcia-Montiel *et al*., 2010) the increase in TSS/TA ratio during ripening process is due to the higher increase in TSS than the increase in TA.

Table 2 - Morpho-chemical proprieties of sweet cherry samples. Data represent mean ± deviation standard

	'Di Giardino'	'Di Nello'	'Marchiana'	'Ferrovia'
Morphological proprieties				
Fruit weight (g)	5.46±0.65 b	4.62±0.62 c	6.68±0.64 a	7.24±0.82 a
Fruit length (mm)	22.47±0.94 a	20.33±0.92 b	15.59±0.99 c	21.54±1.24 a
Fruit width (mm)	19.24±0.75 bc	19.83±0.92 b	18.76±0.82 c	24.33±0.84 a
Fruit thickness (mm)	20.16±1.01 a	18.60±1.21 b	15.12±0.73 c	21.07±0.97 a
Stalk length (mm)	30.34±3.05 b	41.26±3.92 a	30.79±4.04 b	32.12±3.82 b
Skin color				
L	31.26±1.64 b	30.68±1.36 b	33.67±1.53 a	31.09±1.34 b
a	13.66±2.85 b	13.36±2.71 b	19.72±2.06 a	13.50±2.99 b
b	4.35±2.06 b	4.70±1.79 b	9.36±1.41 a	5.73±1.59 b
a/b	2.94±0.51 a	2.69±0.45 ab	2.12±0.11 c	2.40±0.17 bc
Hue angle (deg)	18.74±2.72 c	20.10±4.22 bc	25.29±1.22 a	22.72 ±1.49 ab
Chroma	13.13±3.95 b	14.23±2.95 b	21.83±2.45 a	14.67±3.37 b
Chemical proprieties				
pH	3.68±0.10 b	4.03±0.10 a	3.47±0.01c	3.52±0.03 c
TSS (° brix)	16.34±2.57 b	12.94±1.08 c	16.10±0.65 bc	22.77±0.58 a
TA	0.58±0.05 b	0.37±0.05 c	0.64±0.02 b	0.90±0.05 a
TSS/TA ratio	29.39±1.99 ab	32.80±3.47 a	25.22±1.81 b	25.26±1.39 b

The letters after the values indicate the significant differences within the same row according to Tuckey's test (p≤0.05).

Specifically, this ratio was highest in 'Di Nello' (32.80±3.47) and lowest in 'Marchiana' and 'Ferrovia' (25.22±1.81 and 25.26±1.39, p≤0.05) (Table 2). This data suggest that also cv. Di Nello, that had reported the lowest TSS (12.94±1.08 °Brix), given the low acidity (0.37±0.05) could be considered as qualitatively comparable to the other cultivars, from the point of view of the fruit maturity.

PTR-ToF-MS profiling

Beside fruit sweetness and skin color, aroma is perhaps the most appreciated fruit characteristics (Romano *et al*., 2006). The typical aroma composition of each fruit is affected by their chemical composition (including phytonutrients) as: fatty acids, amino acids, carotenoids, phenols and terpenoids (Sun *et al*., 2010). Thus, the fruits aroma is often for-

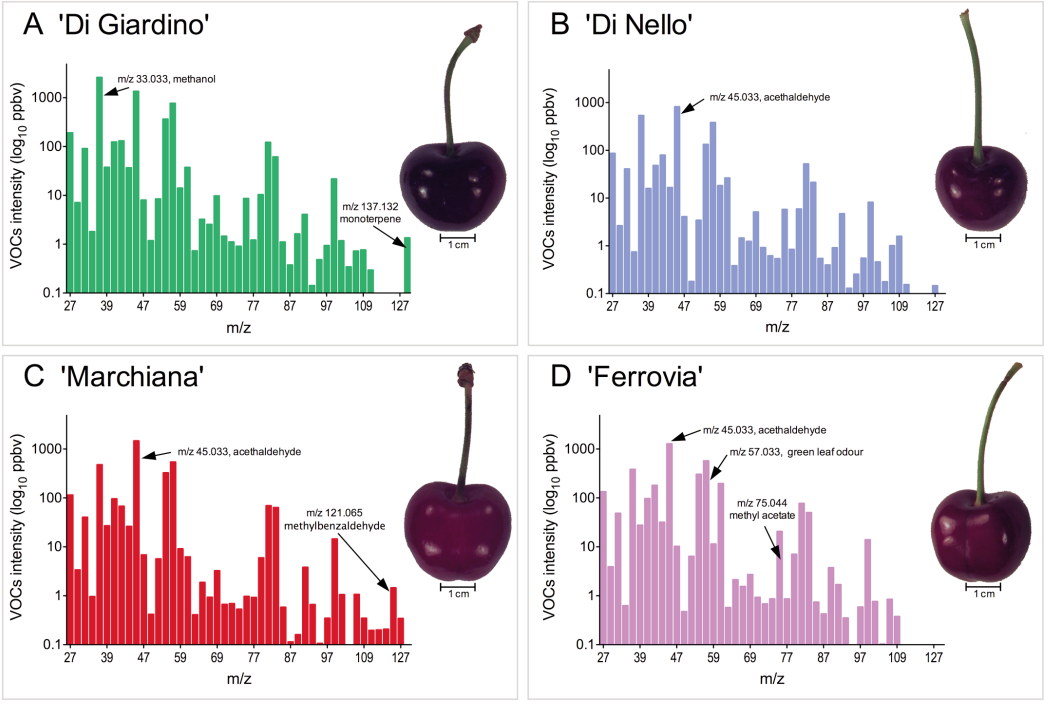

Fig. 1 - VOCs profile of the four sweet cherry cultivars under study. Representative pictures of each cultivar are also shown.

mulated by a complex mixture of VOCs, whose composition is species-specific and sometimes is variety-specific (El Hadi *et al*., 2013). It follows that, it is important to identify at first the entire volatile imprint for all cherry types and subsequently investigate which compounds contribute to create the unique aroma/flavor of each sweet cherry, as it represents a fundamental quality parameter used by the consumer.

By the PTR-ToF-MS analysis 47 peak signals were identified (Table 1); among them, a minimum of 40 peaks for 'Ferrovia' to a maximum of 45 peaks for Marchiana cultivar were detected (Fig. 1). As a general overview, cultivars under study showed highest intensities for different VOCs (Fig. 1). In particular, among all samples, the biggest peaks detected, corresponding to VOCs with highest intensity, were the following m/z: 27.022, 31.018, 33.033, 41.038, 43.018, 45.033, 57.033, 61.028, 75.044, 81.069, 83.086 (each present in percentages >1 on the total). In respect to each variety, cv. Di Giardino showed the main peak at m/z = 33.033 (2592 ppbv, on average over replicates), tentatively identified (TI) as methanol, linked to mild alcoholic odour; cv. Marchiana and Di Nello were rich in m/z = 45.033, TI as acetaldehyde (respectively 1459 ppbv and 816 ppbv, on average over replicates), linked to pungent/fruity odour (this volatile compound was well represented in all samples); cv. Ferrovia had the biggest signal at m/z = 33.033, followed by m/z = 57.033 (568 ppbv, on average over replicates), TI as fragment of hexanal and/or hexyl acetate, linked to green leaf odour (Fig. 1).

In addition, 'Di Giardino' showed high signal intensities for terpene compounds compared to the other varieties used in study (Fig. 1A). Being monoterpenes (C10) and sesquiterpenes (C15) the compounds that most affect the aromatic profile in some fruits, their presence in 'Di Giardino' determines probably a characteristic aroma. The different terpenes composition in sweet cherry varieties has been also observed by Vavoura *et al*. (2015) that showed, in five varieties, a different composition in types and amount of terpenes compounds. Interestingly, peak detected at m/z = 121, TI as methylbenzaldehyde, referred as cherry-like scent (Bernalte *et al*., 1999), was observed only in 'Marchiana' (Fig. 1C). Instead, 'Ferrovia' showed an interesting peak at m/z = 75.044, TI as methylacetate linked to ether sweet fruity odour (Fig. 1D). Concerning the cultivar Di Nello, it is worth noting that intensities of VOCs related to green leaf odour (m/z = 57, 81, 99 and 101) were inferior in respect to the other sample varieties (Fig. 1B).

As reported by Sun *et al*. (2010), the aroma compounds in sweet cherry is determined by a great number of organic components, especially aldehydes, alcohols, esters, acids and terpenes. Hence, each peak detected and tentatively identified was then clustered according to the chemical class (Table 1). This allowed to drawing some further interesting consideration and to better appreciate the differences between aroma profiles of sweet cherry fruits belonging to different cultivars (Fig. 2).

Aldehydes are the most abundant class of VOCs (in percentage) in all cherry samples (73%, 66%, and 63% respectively in 'Marchiana', 'Ferrovia' and 'Di Nello', Fig. 2B,C,D), excluding 'Di Giardino' where alcohols were the biggest class (47%, Fig. 2A). Among aldehydes, the peaks detected at m/z = 45, 57, 59, 99, 101 are linked to compounds known to be among the most important aroma compounds of sweet cherry fruit (Mattheis *et al*., 1992; Wen *et al*., 2014; Vavoura *et al*., 2015). In contrast to the results of Vavoura *et al*. (2015) but according to Serradilla *et al*. (2012), 2-propanone (m/z = 59) was not the most abundant compound identified, although it has been detected in all cherry cultivars under study. On the contrary, the compounds known as 'green leaf volatiles' such as 2-hexenal (m/z = 99.080), hexanal (m/z = 101.096) and their main fragment (m/z = 57.033) were found. All of them, even in very small quantities as hexanal, give a strong odour due to their low perception threshold (Matsui, 2006).

Besides aldehydes, alcohols revealed to be abundant in all cherry samples (Fig. 2), and in fact they represent important compounds for the aroma in sweet cherry (Sun *et al*., 2010). In this study, methanol (m/z = 33), ethanol (m/z = 47) and propanol (m/z = 61) were highlighted. Such alcohol compounds are precursors of natural aroma and appear as a result of anaerobic respiration in fruits and are linked to the normal maturation process (Taiti *et al*., 2015). In addition, both autochthonous and commercial cherries analyzed were rich in ethanol, which most probably derives from carbohydrates metabolism (glycolysis) (Mattheis *et al*., 1992). These data also suggest that the intensity of ethanol emission is at least partially associated to TSS content; in fact, the lowest intensity of ethanol was found in 'Di Nello' (4.05±1.40 ppbv), the cultivar with lowest TSS (Table 2), while the highest was found in 'Ferrovia' (10.2±1.60 ppbv), the cultivar richest in TSS (Table 2); similarly, the other two varieties, 'Di

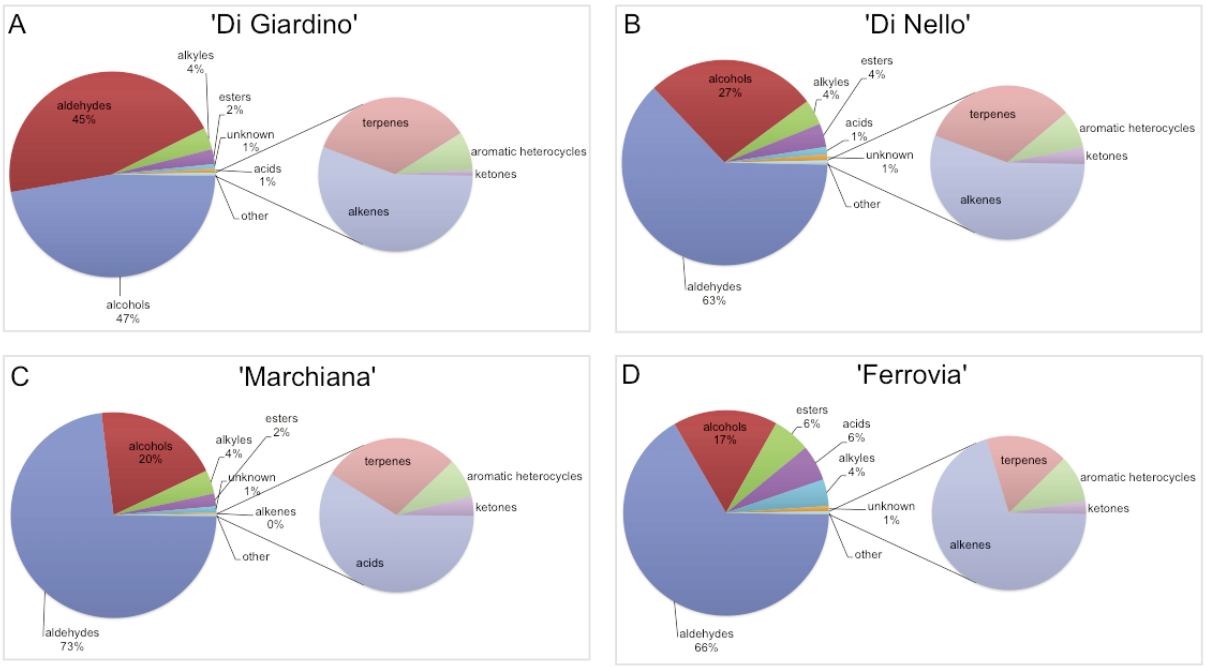

Fig. 2 - Pie charts of the VOCs emitted by the four sweet cherry cultivars under study, expressed as chemical classes according to Table 1.

Giardino' and 'Marchiana', had similar ethanol content, at intermediate intensity in respect to 'Di Nello' and 'Ferrovia', according to TSS data (Table 2).

As for the determination of acids, a total of four acids were detected, acetic (m/z = 59), 3-methylbutanoic (m/z = 103), hexenoic (m/z = 115) and (E)-2-hexanoic acid (m/z = 117), confirming results reported by Sun *et al*. (2010). In our cherry samples, 'Ferrovia' showed the highest percentage (6%) of this class of compounds (Fig. 2D).

Finally, esters were found in all cherry cultivars (Fig. 2) but in low concentrations (especially for 'Marchiana') (Fig. 2C). For example ethyl acetate (m/z 89.059), that is identified as impact volatile in sweet cherry fruits (Zhang *et al*., 2007) and is associated to pineapple aroma, showed the highest emission level in 'Ferrovia' (3.73±0.83 ppbv), followed by 'Di Giardino' (1.67±0.77 ppbv), 'Di Nello' (0.91±0.15 ppbv) and finally by 'Marchiana' (0.16±0.08) with values below 1 ppbv.

4. Conclusions

In this study, fruit properties of a collection of autochthonous and ancient sweet cherry varieties (*Prunus avium* L.) were investigated and compared to the properties of a commercial variety.

Morpho-chemical analyses showed statistical differences among the cultivars, the main ones found on fruit total soluble solids (TSS) and acidity. Concerning TSS, two of the authoctonous cultivars investigated, as well as the commercial one 'Ferrovia', resulted acceptable for the market (TSS above the limit of 14-16 °Brix, Crisosto *et al*., 2003). Fruits from the cultivar Di Nello, on the other hand, despite they turned out to be not so rich in sugars, showed the biggest TSS/TA ratio, another important index related to fruit quality and acceptance (Alonso and Alique, 2006).

Concerning the analysis of aromatic compounds, the use of PTR-ToF-MS (the first in our knowledge) allowed to distinguish each cultivar's peculiarities. Among autochthonous cultivars, 'Di Nello' was the weakest in emitting green leaf related odours; 'Di Giardino' showed to be rich in the chemical classes of terpenes, which most affect the aromatic profile in some fruits; methylbenzaldehyde (cherry scent) was detected only in 'Marchiana'. Such results would suggest potentiality interest for all these autochthonous varieties, not only for commercial purposes, but also for breeding ones.

In conclusion, this study on sweet cherry fruits provides preliminary information that can be useful tools for the enhancement and utilization of native cultivars.

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Effects of salinity stress on certain morphological traits and antioxidant enzymes of two *Carica papaya* cultivars in hydroponic culture

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Key words: antioxidant enzymes, *Carica papaya*, hydroponic, morphological factors, salinity stress.

Abstract: *Carica papaya* L. is the only species belonging to the *Carica* genus. Salinity stress in soil or water, especially in hot, arid regions, could limit plant growth and reduce its yield. This research studied six-month old seedlings of two cultivars of papaya ('Sinta' and 'Solo'), in solid, disease-free form for two weeks inside a half-dose of Hoagland solution. Results obtained from the effects of salinity stress indicated that the longest root and shoot were observed in the control treatment in 'Sinta'. Moreover, there was no significant difference between the two cultivars in terms of root length, shoot length, fresh weight of roots and fresh weight of shoots in different salinity levels. The highest dry weights of roots and shoots were found in the 'Sinta' control treatment, while the lowest was observed in 'Solo' 6 dS/m treatment. There was no significant difference between the two cultivars in terms of dry weights of roots and shoots. Finally, the interaction of salinity levels showed that increasing salinity in both cultivars led to higher peroxidase, catalase, superoxide dismutase and ascorbic peroxides activity. By increasing the salinity level, the total protein and proline greatly increased in both cultivars, where the maximum value was found in the 'Sinta' 6 dS/m salinity treatment, and this was significantly different from other treatments. A comparison of the different salinity levels showed that there was a significant difference between the 6 dS/m salinity treatment and other treatments.

1. Introduction

The ability of plants to tolerate salinity stress is facilitated by a series of biochemical pathways which maintain or absorb water, protect plants chloroplast function and sustain an ionic balance. Some of these pathways include the synthesis of active osmotic metabolites (Zhifang and Loescher, 2003). Some proteins and enzymes destroy free radicals (Mittova *et al*., 2003). *Carica papaya* L. is the only species belonging to the *Carica* genus. It can be found in tropical region of America. The cultivation of this crop is common in southern Mexico, Central America and South America, as well as most countries in the tropics. Papaya is a fast-growing tree (it produces fruit in the third year after planting), extremely sensitive to cold, and planted only in the tropics. Papaya fruit is rich in carotenoids, vitamins B, C, lycopene and mineral fibers. The skin, flesh and seeds of this product contain a number of phenolic compounds. Salinity stress in soil or water, especially in hot, arid regions, could limit plant growth and reduce its yield (Koca *et al*., 2007). Plants growing in areas with extreme salinity are divided into halophytes and glycophytes. Most glycophyte plants do not have the ability to tolerate salinity stress (Sairam and Tyagi, 2004). During salinity stress, all the main processes, including photosynthesis, lipid metabolism and energy, are affected (Sairam and Tyagi, 2004). The first response is to reduce the development rate of leaf area and then complete cease. However, growth process resumes as soon as the problem is fixed (Parida and Das, 2005). The plant either tolerates the stress, or avoids it. The former generally occurs at the cell level while the latter occurs at the plant level. During salinity stress, a plant can undergo dormancy (avoidance) or make certain cellular adjustments in order to resist drought stress (Yokoi *et al*., 2002). The

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aim of this study is to survey the effect of salinity stress on certain morphological traits and also on antioxidant enzymes of two *Carica* cultivars in hydroponic culture condition.

2. Materials and Methods

Six-month old seedlings of two cultivars of papaya ('Sinta' and 'Solo'), solid and disease-free, were placed for two weeks in a glass containing 700 ml of a half-dose Hoagland solution, and were then transferred to the hydroponic system. Afterwards, 500 ml of the half-dose Hoagland solution per seedling was added to the hydroponic system. After two weeks, the salinity treatments were initiated at pH=6 as salinity stress continued for 8 weeks. Then, the leaf samples were placed in aluminium foil and frozen at -20°C for further measurements of protein and antioxidant enzymes. Plant height was measured with a ruler. At the end of the experiment, the fresh weight of plant organs was measured and then rinsed through distilled water and finally kept in the oven at 70°C until the dry weight was stabilized. Then, shoots and roots dry weights were measured. A factorial experiment was conducted based on completely randomized design with 5 replications in the greenhouse of the Horticultural Science Department, University of Shiraz. The factors included salinity treatments: 0 (Control), 2, 4, 6, 8 and 10 dS/m NaCl which were added to half dose Hoagland solution and 2 cultivars of papaya ('Sinta' and 'Solo'). Data analysis was done using SAS (version 9.2; SAS Institute, Cary, NC, USA), mean comparisons were carried out using LSD test at 5% of probability.

Extraction for measuring the amount of protein and antioxidant enzymes

For extraction, 0.5 g of root or leaf sample was first ground in liquid nitrogen and then 2 ml of extraction buffer was added and homogenized in a porcelain mortar. Then this mixture tube was centrifuged at 13,000 rpm for 15 min at 4°C. The upper phase was isolated for the purpose of reading the protein content and enzyme activity. For the preparation of the extraction buffer (50 ml), 0.607 g of tris(hydroxylmethyl)aminomethane and 0.05 g of Polyvinylpyrrolidone (PVP) were dissolved into 45 ml of distilled water (pH 8.0).

Total protein

The Bradford assay (1976) was used to determine the protein concentration. To measure the protein concentration, 20 ml extract was diluted in 80 µl of extraction buffer; 5 ml of fresh reagent Coomassie was added, stirred for 2 min, and finally, after 5 min, the optical density was read at a wavelength of 595 nm. Besides, the extraction buffer was used as control. The concentration of protein in the sample was obtained according to the absorption, using the standard curve. Bovine serum albumin was used as the standard and total soluble protein concentrations were expressed in mg g^{-1} fresh weight.

Proline

The method of Bates *et al*. (1973) was employed to measure the concentration of proline. According to this method, 0.5 g of leaves from each sample was placed in 10 ml of an aqueous solution of sulfosalicylic acid (3%) and the mixture was completely homogenized in a porcelain mortar. Then, the homogenized mixture was filtered through paper no. 2. In the next stage, 2 ml of solution was mixed with 2 ml of a reagent, creatininedimenhydrinate and 2 ml of acetic acid was added to each tube. Then, the samples were placed in bain-marie bath for 1 h at a temperature of 100°C and were immediately transferred into an ice bath for a few minutes. Afterwards, 4 ml of toluene was added to each tube and the samples were stirred through Vortex for 15 to 20 s until they were completely homogeneous. The supernatant phase was used to determine proline concentration based on the proline standard curve in the spectrometer at a wavelength of 520 nm. Proline concentration was calculated using L-proline for the standard curve.

Guaiacol peroxidase (POD) activity

To measure the quantitative concentrations of this enzyme, the method of Chance and Mahly (1955) was used with minor modifications. Measurements were done according to the oxidation of guaiacol by that enzyme. In this method, 33 mol of extract was dissolved into 1 ml of a peroxidase solution containing 13 Mm guaiacol, 5 mM hydrogen peroxide (H_2O_2) and 50 mM potassium phosphate buffer (pH=7), and the absorbance values were read for one minute at 10-s intervals and at a wavelength of 470 nm. To prepare 1000 ml of potassium phosphate buffer, 39 ml potassium phosphate and 50 mM monohydrate were mixed with 61 ml potassium dihydrogen and 50 mM phosphate.

Ascorbic peroxidase (APX) activity

To measure the quantitative concentrations of this enzyme, the method of Nakano and Asada

(1981) was used. According to this method, 50 ml of the extract was mixed with 1 ml of ascorbic peroxidase containing 50 mM potassium phosphate buffer (pH=7), 0.1 mM EDTA, 0.5 mM ascorbic acid (ASA), and 0.15 mM peroxide hydrogen (H_2O_2). Then, the absorption at a wavelength of 290 nm was read within one minute through a spectrophotometer. An enzymatic unit of ascorbic peroxidase is equivalent to a dissolution of 1 mM ascorbic acid in a minute.

Catalase (CAT) activity

To measure the quantitative concentrations of this enzyme, the method of Nakano and Asada (1981) was used. According to this method, 50 ml of the extract was mixed with 1 ml of catalase containing 50 mM potassium phosphate buffer (pH=7) and 15 mM hydrogen peroxide (H_2O_2). Then, the absorption at a wavelength of 240 nm was read within one minute through a spectrophotometer. An enzymatic unit of catalase is equivalent to a dissolution of 1 mM hydrogen peroxide (H_2O_2) in a minute.

Superoxide dismutase (SOD) activity

To measure the quantitative concentrations of this enzyme, the method of Beauchamp and Fridovich (1971) was used. The measurement was based on the ability of SOD enzyme to stop the photochemical reduction of NBT by superoxide radicals in the presence of riboflavin in light. According to this method, 50 ml of the extract was mixed with 1 ml of superoxide dismutase containing 50 mM potassium phosphate buffer (pH 7.8), 75 mM of NBT, 13 mM of L-methionine, 0.1 mM of EDAT and 2 mM riboflavin. It should be noted that the solution was stored separately in a dark container and after the addition of soluble extract and measurement solution, superoxide dismutase was added to Qt. The mixture reacted when placed in the light chamber for 15 min. The solution was then placed in a spectrophotometer and the absorbance was measured and read at a wavelength of 560 nm.

3. Results

The interaction between salinity levels and cultivars on root length showed that the highest root length was found in the control treatment in Sinta, while the minimum value was found in the 6 dS/m treatment of both 'Sinta' and 'Solo'. Seedlings of both cultivars in the 8 and 10 dS/m treatments exhibited no salinity resistance and were dried, therefore, these two treatments were excluded from the results; results also showed no significant differences between the two cultivars, in terms of root length, at various salinity levels (Fig. 1).

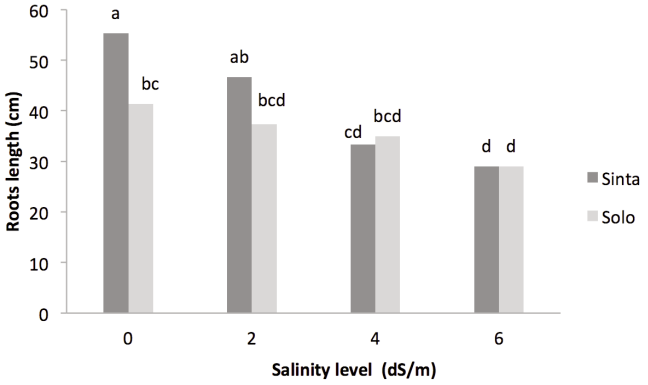

Fig. 1 - Interaction of cultivar and salinity levels on roots length. The means followed by the same letters were not significantly different at p≤0.05.

As shown in figure 2, the highest shoot length was observed in the 'Sinta' control treatment, which was significantly different from other treatments. Moreover, there was no significant difference between the two cultivars in terms of salinity levels.

The control group of both cultivars generally had a significant difference with other treatments, while there existed no significant difference between concentrations of 2, 4 and 6 dS/m.

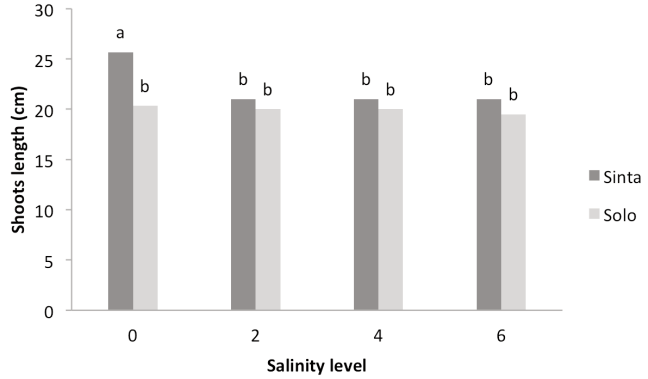

Fig. 2 - Interaction of cultivar and salinity levels on shoots length. The means followed by the same letters were not significantly different at p≤0.05.

Evaluation of salinity effect on roots fresh weight between the two cultivars showed that the highest and lowest values were observed in the control treatment and the Sinta 6 dS/m treatment, respectively. There was no significant differences between the two cultivars in terms of root fresh weight. The comparison of the various salinity levels, showed a significant difference between the control and other salinity treatments, while there was no significant difference

between treatments 2, 4 and 6 (dS/m) (Fig. 3).

In this study maximum root dry weight was observed in the 'Sinta' control (Fig. 4) while the minimum was observed in the 'Solo' 6 dS/m treatment. The comparison between the two cultivars showed a significant difference between them in terms of roots dry weight. The mean salinity concentrations indicated that the control had a significant difference with other concentrations, while there was no significant difference between concentrations of 2 and 4 dS/m.

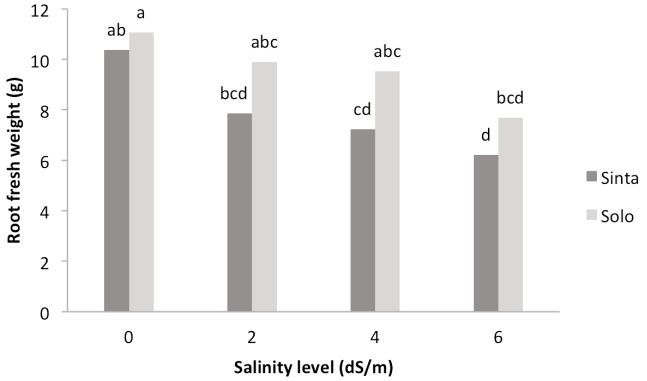

Fig. 3 - Interaction of cultivar and salinity levels on root fresh weight. The means followed by the same letters were not significantly different at p≤0.05.

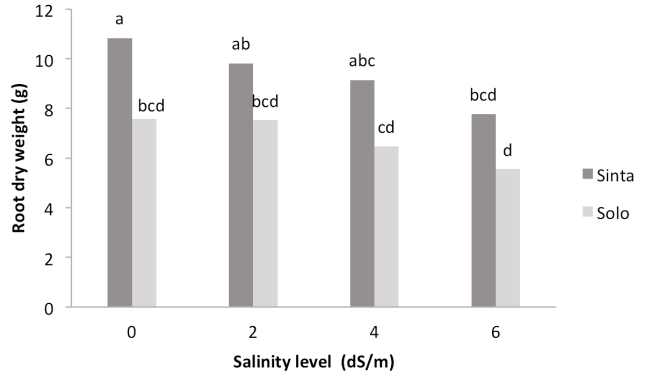

Fig. 4 - Interaction of cultivar and salinity levels on root dry weight. The means followed by the same letters were not significantly different at p≤0.05.

Evaluation of treatments applied in this study on shoots fresh weight indicated that control treatment in both 'Sinta' and 'Solo' had the highest values, while treatments 4 and 6 dS/m had the lowest values. A comparison of the two cultivars showed no significant differences in term of shoot fresh weight. In the comparison of salinity concentrations, no statistically significant difference was found between the control and 2 dS/m (Fig. 5).

According to figure 6, the findings of this study regarding the shoots dry weight showed that the highest dry weight was found in the control 'Sinta', where there was no significant difference between

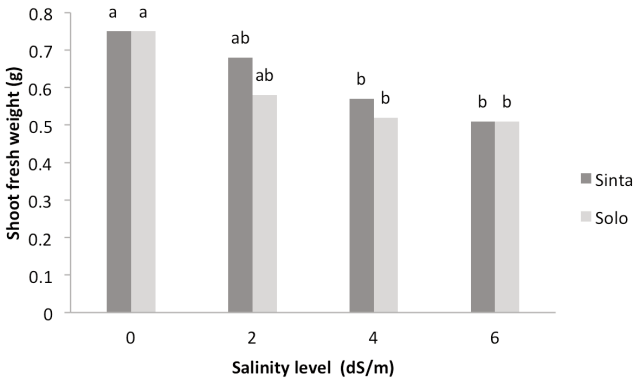

Fig. 5 - Interaction of cultivar and salinity levels on shoot fresh weight. The means followed by the same letters were not significantly different at p≤0.05.

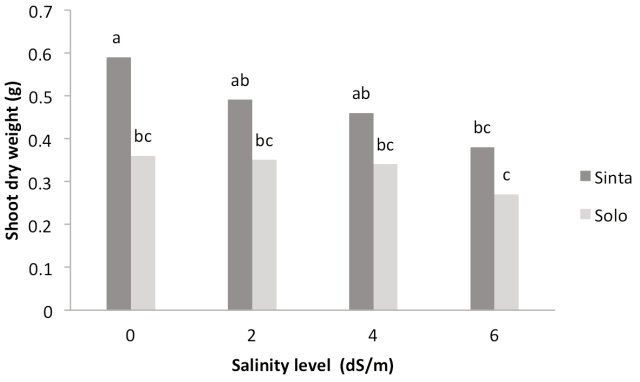

Fig. 6 - Interaction of cultivar and salinity levels on shoot dry weight. The means followed by the same letters were not significantly different at p≤0.05.

this treatment and the 2 and 4 dS/m salinity treatments. The lowest shoot dry weight was found in the 'Solo' 6 dS/m salinity treatment. A comparison of the two cultivars represented a significant difference in the shoots dry weight. Moreover, the comparison of various salinity levels showed no significant difference between the control and 2 dS/m salinity treatments for both cultivars.

The interaction effect of cultivar and salinity levels in this study showed that an increase in the salinity levels enhanced peroxidase activity in both cultivars. The highest enzyme activity was observed in 'Sinta' 6 dS/m salinity treatment while the lowest was in 'Solo' control treatment. A comparison of values between the cultivars showed a significant difference in terms of peroxidase activity. Moreover, the comparison of various salinity levels showed a significant difference between the 6 dS/m salinity treatment and other treatments for both cultivars (Fig. 7).

According to figure 8, the increased salinity levels in both cultivars enhanced catalase activity. The maximum level of this enzyme was found in the 'Sinta' 6

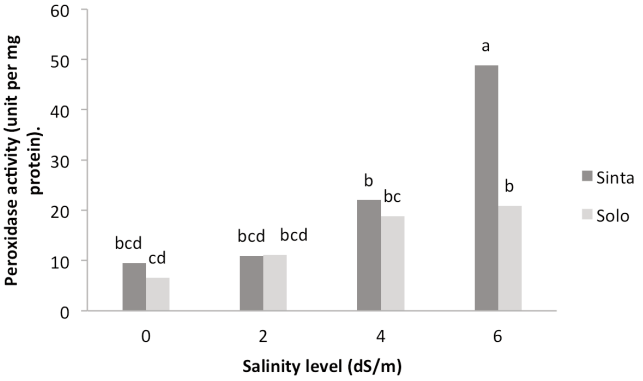

Fig. 7 - Interaction of cultivar and salinity levels on peroxidase activity. The means followed by the same letters were not significantly different at p≤0.05.

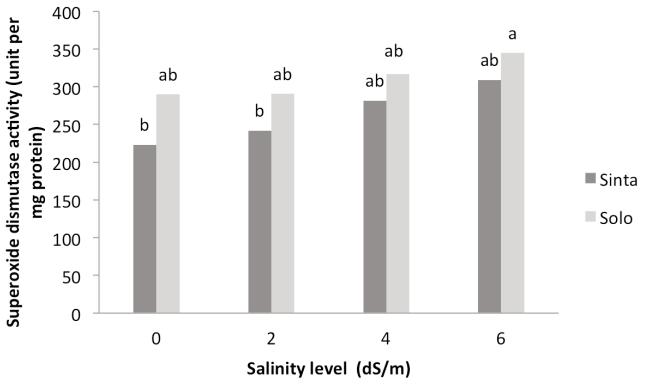

Fig. 9 - Interaction of cultivar and salinity levels on superoxide dismutase activity. The means followed by the same letters were not significantly different at p≤0.05.

dS/m salinity treatment, which showed a significant difference with other treatments, while the minimum values were observed in the control treatment of both cultivars. A comparison of the two cultivars showed no significant differences. In the showed results, there was a significant difference between the different levels of salinity in the 6 dS/m treatment and the control 2 dS/m, while there was no significant difference when compared to the 4 dS/m treatment.

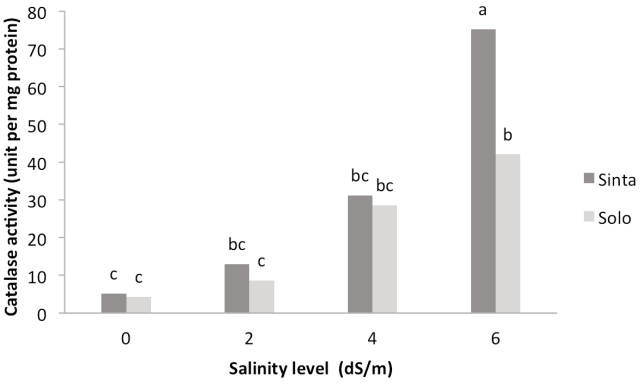

Fig. 8 - Interaction of cultivar and salinity levels on catalase activity. The means followed by the same letters were not significantly different at p≤0.05.

In this study, the interaction effect of salinity stress on superoxide dismutase activity showed an increase at higher salinity levels in both cultivars. The maximum activity of this enzyme was found in the Solo 6 dS/m salinity treatment which was not significantly different when compared with other treatments and the 'Sinta' 4 and 6 dS/m salinity treatments. The comparisons between the cultivars were significantly different. The comparison of different salinity levels showed no significant difference between the 4 and 6 dS/m treatments (Fig. 9).

Results reported in figure 10 showed that as salinity levels increased, the ascorbic peroxidase activity also increased. An evaluation of the interaction of salinity levels on the enzyme activity showed that the highest value was found in Sinta 6 dS/m treatment while there was no significant difference with the same treatment in 'Solo'. The comparison between the cultivars showed no significant difference. Moreover, a comparison of various salinity levels showed a significant difference between 6 dS/m salinity treatment and other treatments for both cultivars.

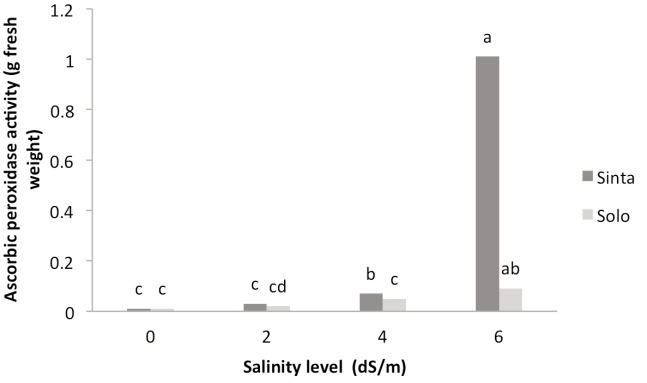

Fig. 10 - Interaction of cultivar and salinity levels on ascorbic peroxidase activity. The means followed by the same letters were not significantly different at p≤0.05.

From figure 11, the interaction effect of salinity levels on activity of total protein in this study showed that as salinity levels increased there was higher protein content in both cultivars. The maximum value was observed in 'Sinta' of 6 dS/m salinity treatment, which was significantly different from other treatments, while the lowest was in the control for the two cultivars which were not significantly different. No significant difference was found when the culti-

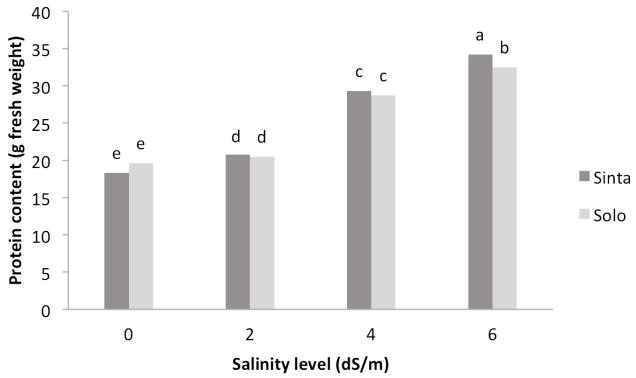

Fig. 11 - Interaction of cultivar and salinity levels on protein content. The means followed by the same letters were not significantly different at p≤0.05.

vars were compared. As for the various salinity levels, the results showed that the 6 dS/m salinity treatment was significantly different from other treatments.

Evaluation of interaction effect between salinity levels on proline showed an increase in salinity level in both cultivars, followed by higher proline. In fact, the maximum amount of proline was observed in the Sinta 6 dS/m salinity treatment which was significantly different from other treatments. There was no significant difference when both cultivars were compared. Moreover, the comparison of various salinity levels showed a significant difference between the 6 dS/m salinity treatment and other treatments for both cultivars (Fig. 12).

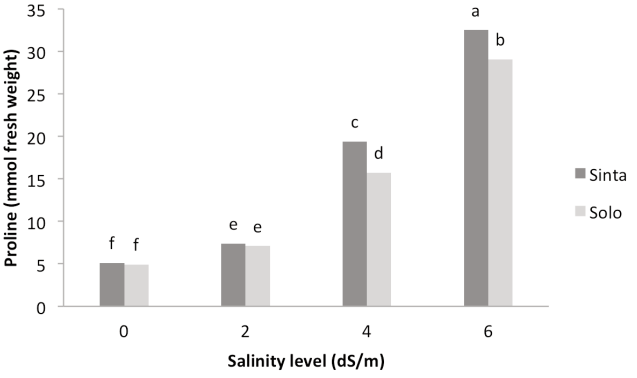

Fig. 12 - Interaction of cultivar and salinity levels on proline. The means followed by the same letters were not significantly different at p≤0.05.

4. Discussion and Conclusions

As mentioned in the results, the vegetative indicators decreased upon initiation of salinity treatments. Literature indicates that plants, especially glycophytes, are highly sensitive during their early vegetative growth, for instance chickpea (Khan *et al.,* 2016), pepper (Penella *et al.,* 2016), grapevine (Ikball *et al.,* 2014), tomato (Manai *et al*., 2014), rice (Horie *et al*., 2012), cantaloupe (Botia *et al*., 2005). Increased salt leads to Na^+ sediment into root growth area, thus reducing the ability to select K^+ versus Na^+ for root cells, which ultimately reduces their growth rate (Zhong and Lauchli, 1994). When exposed to saline conditions, plants show reduced uptake and low tissue retention of $K^{+.}$ (Chakraborty *et al.,* 2012; Gharsallah *et al.,* 2016). Accordingly, K^+ is considered as a key regulatory elements in plant metabolic process by promoting Na^+ exclusion and osmotic adjustment (Chakraborty *et al.,* 2016; Gharsallah *et al.,* 2016). Reduced shoot growth due to salinity usually appears as shoots with low growth and reduced leaf area (Lauchli and Epstein, 1990). Saline conditions reduce root growth and reduce water movement through the root with a decrease in hydraulic conductivity (Acosta-Motos *et al*., 2017). Root hydraulic conductance is expressed in terms of root dry weight. Root dry weight values which determine the root length and surface area, may vary greatly, thus affecting the water absorption (Jonathan *et al.,* 2006; Zobel *et al.,* 2007; Acosta-Motos *et al*., 2017). The results of our study are in accordance with these results. Decreasing in fresh weight or dry weight has been observed in all plant tissues subjected to salt stress especially in the aerial part (Acosta-Motos *et al*., 2017). The stem growth is also reduced by salinity conditions. One of the important reason for decreasing root and shoot growth under saline condition could be the decreasing of nitrogen uptake in response to external NaCl salinity due to antagonism between Na^+ and NH_4^+ or between Cl^- and No_3^- (Parihar *et al.*, 2015; Salachna and Piechocki, 2016). Another reason responsible for the reduction of vegetative index in the shoots, similar to what was mentioned for roots, is ion imbalance and increased ratio of Na to Ca (Neves-Piestun and Bernstein, 2005). In the current experiments, the concentration of proline, which has an important role in eliminating free radicals and enzymes, increased at higher salinity levels. Accumulation of proline under salinity conditions has been indicated to correlate with salt tolerance (Mansour and Ali, 2017).

In fact, to eliminate osmotic stress created by high salinity, plants need to synthesize compatible organic solutes such as proline in the cytosol (Gharsallah *et al*., 2016).

Besides its role as an osmolyte, proline contributes to scavenging ROS, stabilizing sub cellular structures and functioning as a signal (Szabados and

Savoure, 2010). Generally, plants under salinity conditions need to sustain their water potential (below potential of ground water) because they can continue absorbing water from the soil in order to maintain their turgor (Tester and Davenport, 2003). To maintain osmotic potential as well as ionic balance, plant cells during stress tend to accumulate any substance compatible with metabolism which does not interfere with other biochemical processes and are active in osmotic terms (Zhifang and Loescher, 2003). These substances cover a wide range of compounds such as carbohydrates, proteins and amino acids, including proline. This amino acid accumulates at a higher concentration than other amino acids in plant cells (Abraham *et al.,* 2003). It is possible that proline as a signaling molecule or regulator can activate the response as an adjustment process (Maggio *et al*., 2002). Hence, given the foregoing facts, there is a positive relationship between proline and curtailed damage of salt sensitivity, where the current study was consistent with previous works. In relation to salt damage to the overall growth of plant, the concentration of enzymes eliminating free radicals is very important. In their resistance against the damaging effects of reactive oxygen species, plants possess anti-free radical enzymes such as catalase, peroxidase, superoxide dismutase and other enzymes which eliminate reactive oxygen species and free radicals (Mittova *et al*., 2003). In our study, the concentration of eliminating free radicals enzymes such as peroxidase, catalase, superoxide dismutase and ascorbic peroxidase increased at higher salinity levels. The increases in theses enzymes activities are an adaptive trait to overcome salt damage by reducing toxic levels of H_2O_2 and provide protection against oxidative stress (Chawla *et al*., 2013; Gharsallah *et al*., 2016). Catalase, ascorbic peroxidase and glutation peroxidase have been reported as antioxidant enzymes in different plant tissues (Chawla *et al.,* 2013).

During salinity stress, the balance between production and consumption of reactive oxygen species (ROS) is disrupted, leading to the formation of condensation oxide (Spychalla and Desborough, 1990). ROS has the potential to damage cellular structures, perchloric acids, fats and proteins (Valko *et al*., 2006). In some salt-tolerant plants, increased in catalase activity have been recored after increasing NaCl, such as those described in myrtle, suggesting increased photorespiratory activity (Acosta-Motos *et al*., 2015, 2017). Catalase is often related to an enhanced tolerance to salt stress (Gao *et al.,* 2008; Gharsallah *et al*., 2016). Similarly, ascorbate peroxidase activity under salinity stress increases (Mittova *et al.,* 2004; Gharsallah *et al*., 2016). These responses to salinity were the results of differentially increased activities of ascorbate peroxidase and catalase over that of superoxide dismutase (Mittova *et al.,* 2004; Gharsallah *et al*., 2016). The results of our study were in accordance with these results.

The results of this study indicated also that as salinity levels increased, there was higher protein content in both cultivars. This result was in agreement with that of Abdel-Haleem (2007) who reported as increase in protein band which might be involved in mungbean tolerance. Plants growing in saline environments show distinct changes in the pattern of synthesis and accumulation of proteins. Salinity causes either decreased or increase in the level of soluble proteins or completely disappears in some proteins when compared to the control treatment (Win and Zaw, 2017).

Salinity is an important stress in arid and semi-arid region that reduces crops productivity, including that of the cultivars of *Carica papaya* L. here investigated ('Sinta' and 'Sola'). Shoots and roots growth were decreased under salinity conditions. There was no significant difference between the two cultivars in terms of root length, shoot length, fresh weight of roots and shoots in different salinity levels. Interestingly, on the other hand, in both cultivars increasing salinity led to higher peroxidase, catalase, superoxide dismutase and ascorbic peroxidase activity to provide protection against oxidative stress. Also the increases in those enzymes activities are an adaptative trait to overcome salt damage by reducing toxic levels of H_2O_2. Furthermore, in this experiment the concentration of proline, which has an important role in eliminating free radicals, increased at higher salinity levels in both cultivars; plants need to synthesize compatible organic solutes such as proline. Finally, in this study increasing salinity led to higher protein content in both cultivars; proteins play a major role in salt stress acclimation and plant cellular adjustment. Salinity causes either decrease or increase in the level of soluble proteins.

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Short note

HPLC/DAD, GC/MS and GC/GC/TOF analysis of Lemon balm (*Melissa officinalis* L.) sample as standardized raw material for food and nutraceutical uses

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Key words: aromatic compounds, comprehensive two-dimensional (2D) gas chromatography, HS-SPME-GC×GC-TOF fingerprint analysis, hydroxycinnamic acids, lemon balm, phenols.

Abstract: *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. Traditionally administered in infusion form, it has therapeutic properties, such as sedative, carminative and antispasmodic, but also it is used for treatment of headache, rheumatism, indigestion and hypersensitivities. Lemon balm has a complex chemical composition. The aim of this work was the comprehensive characterization of secondary metabolites of a dried Lemon balm (*Melissa officinalis* L.) sample, through HPLC/DAD, GC/MS and GC/GC/TOF analysis, as raw material for the standardized phyto-complexes production useful for food and nutraceutical application. This sample contained rosmarinic acid (caffeic acid dimer) as the main compound of phenolic fraction (32.4 mg g^{-1}). Citronellal was the most abundant compound in the volatile fraction, followed by α-citral and β-caryophyllene. The total citral amount, in terms of sum of α- and β-citral, was 149.4 mg_{citral} kg^{-1}. Comprehensive two-dimensional GC fingerprint analysis of lemon balm produced rationalized peak patterns for up to 200 volatile compounds.

1. Introduction

The Lamiaceae are a promising source of natural antioxidants due to the large amount of phenolic acids found in many species of this family (Ziaková *et al*., 2003). *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. Lemon balm is used as aromatic, culinary and medicines and is also used by food industry to flavour different products owing to its particular taste (López *et al*., 2009). The raw plant samples originating from *M. Officinalis* L. species are also used in the traditional medicine for the treatment of headache, flatulence, colic, nausea, indigestion, anaemia, nervousness, vertigo, malaise, asthma, bronchitis, syncope, amenorrhea, cardiac failure, insomnia, epilepsy, depression, psychosis, hysteria, ulcers and wounds (WHO, 2004; Karasová and Lehotay, 2005; Dastmalchi *et al*., 2008). Therefore, its aqueous and alcoholic extracts are traditionally used for their spasmolytic, nervous sedative, antiviral and antioxidant activities (López, *et al*., 2009; Atanassova *et al*., 2011; de Carvalho *et al*., 2011; Lin *et al*., 2012). All of these properties of lemon balm have been related to the high levels of phenolic acids found in this species, mainly hydroxycinnamic acid derivatives such as rosmarinic acid (Fecka and Turek, 2007). Some studies already reported other phenolic compounds in lemon balm. Heitz *et al*. (2000), isolated luteolin 3-*O*-glucuronide as the major flavonoid presented in *M. officinalis* from France. In 2002, Patora and Klimek isolated and determined the structure of six major flavonoids (apigenin and luteolin derivatives) in lemon balm from Poland based on spectral data. Lemon balm has a complex chemical composi-

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tion. It contains hydroxycinnamic acids (up to 6% of rosmarinic acid, *p*-coumaric and caffeic acids) and up to 0.37% of an essential oil composed of monoterpenes (more than 40%) and sesquiterpenes (more than 35%). Among the most significant terpenoids there are citral, citronellal, geraniol, nerol, linalool, farnesyl acetate, humulene, caryophyllene and eremophilene (ESCOP, 2003; WHO, 2004). The essential oil is considered to be a therapeutic principle usually responsible for most of the biological activities, such as spasmolytic, antimicrobial, antitumour andantioxidant ones, but also plant polyphenols, especially rosmarinic acid, are involved as well (Sadraei *et al*., 2003; de Sousa *et al*., 2004).

Phenolic acids are secondary metabolites, which create a large group of naturally occurring compounds, showing a broad spectrum of biological activities. The phenolic acids are important bioactive constituents of *M. Officinalis* L.; among them rosmarinic, caffeic, chlorogenic and ferulic acids are especially interesting. A literature screening showed that rosmarinic acid exhibits anti-inflammatory, antibacterial, and antiviral activities, it reduces atopic dermatitis and prevents Alzheimer's disease (Huang *et al*., 2009; Fujimoto and Masuda, 2012). Ferulic acid has strong antioxidant, antimicrobial, anti-inflammatory, anti-thrombotic, and anti-cancer activities (Peng *et al*., 2012). Chlorogenic acid shows anti-inflammatory, anti-bacterial, and anti-obesity properties (Sun *et al*., 2013), whereas caffeic acid has anti-inflammatory, antioxidative and immunomodulatory effects (Anwar *et al*., 2012).

Chemical quality evaluation of a herbal medicine should consist of two aspects (Jin *et al*., 2008). The first is identification and quantitation of one or more constituents that occurred in high quantities. The other is development of chemical fingerprint, which has been introduced and accepted by the WHO and other authorities as a strategy for quality assessment of herbal medicines (Chang *et al*., 2008; Li *et al.,* 2010). Among the separation methods, e.g. HPLC, HPTLC, GC and CE, which have been recognized as a rapid and reliable means for the identification and quantitation of the herbal medicine constituents, HPLC is the most popular method and is widely used in the fingerprint analysis (Kong *et al*., 2009; Peng *et al*., 2011). Up to now, coherent data is missing about the evaluation of quality consistency of the medicinal raw plant samples of *M. Officinalis* L. obtained from different manufactures.

The aim of this work is the comprehensive characterization of secondary metabolites of a dried lemon balm (*Melissa officinalis* L.) sample, through HPLC/DAD, GC/MS and the innovative GCxGC/TOF analysis, as raw material for the standardized phytocomplexes production useful for food and nutraceutical application. Therefore, in this study a combinative method is developed based on the reference HPLC, GC and GCxGC fingerprint and quantitation of selected phenolic and volatile compounds to assess the quality consistency of lemon balm.

2. Materials and Methods

Plant material

The dried vegetal tissues of *Melissa officinalis* L. were collected by Officinali Agribioenergia factory (Medicina, Bologna, Italy). The dried sample were finely chopped with a grinder (Mulinex AR 11, Groupe SEB, France) with a particle size of about 1.0 mm.

Determination of volatile composition and polyphenolic compounds

GC-MS analysis. In the first step of GC-MS analysis, HS-SPME-GC-MS was selected as the most suitable technique to recover and analyze the highest number of Volatile Organic Compounds (VOCs) in *Melissa officinalis* L. samples. Consequently, a dried foliar sample was ground and a homogenous powder was obtained. Fifty mg of the powdered sample, together with 2 g of NaCl and 5 mL of deionized water were placed into a 20-ml screw cap vial fitted with PTFE/silicone septa. After 5 min of equilibration at 60°C, VOCs were absorbed exposing a 2-cm trivalent SPME fiber (DVB/CAR/PDMS by Supelco) for 10 min into the vial headspace under orbital shaking (500 rpm) and then immediately desorbed at 280°C in a gas chromatograph injection port operating in split less mode. The chromatographic analysis was performed in a GC system coupled to quadrupole mass spectrometry using an Agilent 7890a GC equipped with a 5975C MSD. The separation of analytes was achieved by an Agilent DB InnoWAX column (length 50 m, id 0.20 µm, df 0.40 µm). Chromatographic conditions were: initial temperature 40°C, then 10°C min^{-1} up to 260°C, hold for 6.6 min. Compounds were tentatively identified by comparing calculated Kovats retention index and mass spectra of each peak with those reported in mass spectral databases, namely the standard NIST08/Wiley98 libraries. Each sample was analyzed in triplicate.

In the second step of GC-MS analysis, solid-liquid extraction followed by liquid injection was selected to

quantify the main VOCs identified by HS-SPME-GC-MS, namely citronellal, α-citral (geranial), β-citral (neral) and β-caryophyllene. To this aim, 0.5 g of powdered sample was extracted with 3 ml of heptane; the extraction was performed for 15 min in an ultrasound bath and for 24 hours in a shaker at 1000 rpm and 24°C. The mixture was than centrifuged at 6.000 rpm for 30 min and the supernatant was recovered and used for the GC-MS analysis, which was performed by the same chromatographic system used for the HS-SPME-GC-MS analysis. Each sample was analyzed in triplicate.

Two five-level calibration curves were built using β-caryophyllene standard (range 0.625-40 ppm, R^2 0.9961) and citral standard (range 1-100 ppm, R^2 0.9995). Citronellal, α-citral and β-citral was expressed as mg_{citral} kg^{-1}, and β-caryophyllene was expressed as $mg_{caryoph}$ kg^{-1}.

GCxGC-MS analysis. VOCs were absorbed exposing a 2-cm trivalent SPME fiber as described in GC-MS analysis. An Agilent 7890a GC equipped with a 5975C MSD was used and comprehensive GC×GC analyses were carried out on an Agilent GC 7890B, with an Agilent flow modulator system, coupled to an TOF-DS Markes detector. The analytes separation was achieved with a HP-5MS UI column (0.18x0.18mm, 20 min) coupled with a InnoWAX column 0.23x0.32 mm, 5 min. A tentative compounds identification was performed by comparing mass spectra of each peak with those reported in mass spectral databases.

Extraction and HPLC/DAD analysis of phenolic compounds. An aqueous extract was prepared adding 50 ml to 2.5 g of dried material. The solution was heated at 75°C and kept at that temperature for 60 min, then, the mixture was centrifuged and analyzed by HPLC/DAD.

Standards and solvents. Authentic standards of kaempferol 3-*O*-glucoside and rosmarinic acid were purchased from Extrasynthèse S.A. (Lyon, France), β-caryophyllene and citral were purchased from Sigma-Aldrich. All solvents used were of HPLC grade purity.

HPLC/DAD analysis. Analyses of flavonols and phenolic acids were carried out using an HP 1200 liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated using a 250x4.6 mm i.d., 5 mm LUNA C18 column (Phenomenex, USA). UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 250, 280, 330 and 350 nm. The samples were analyzed by gradient elution at a flow rate of 0.8 ml/min. The mobile phase was a multi-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN in 55 min.

Identification and quantification of individual phenolic compounds. The identity of phenols was ascertained using data from HPLC-DAD, by comparison with bibliographic data and combination of retention times and UV/Vis spectra with those of authentic standards. The quantification of individual phenolic compounds was performed directly by HPLC-DAD using a five-point regression curve (r≥0.998) in the range of 0-30 mg on the basis of authentic standards. In particular, flavonols were determined at 350 nm using kaempferol 3-*O*-glucoside as a reference compound, while the phenolic acid derivatives were determined at 330 nm using rosmarinic acid as reference compound. Each sample was analyzed in triplicate.

3. Results and Discussion

GC-MS is widely recognized as the most suitable analytical technique for the analysis of VOCs; in particular, the HS-SPME-GC-MS allows the identification of the most representative compounds.

We selected the DVB/CAR/PDMS fiber since it proved to be the most universal assembly for sufficient isolation of compounds with different physic-chemical properties (Cui *et al*., 2009).

Figure 1 shows the Total Ion Chromatogram (TIC)

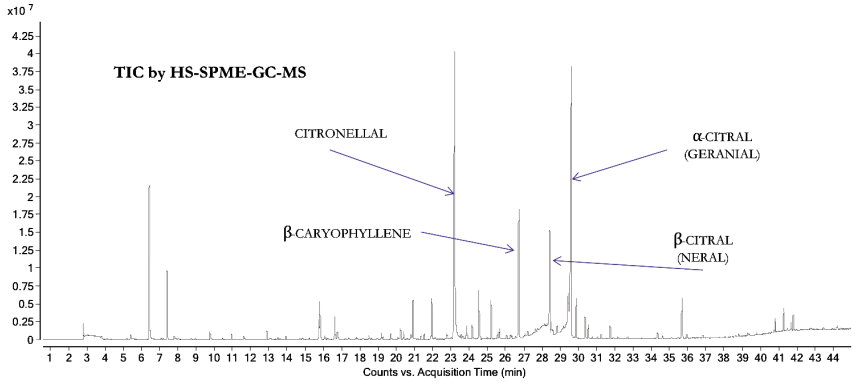

Fig. 1 - Total Ion Chromatogram (TIC) by HS-SPME-GC-MS analysis of *Melissa officinalis* L. foliar sample.

Table 1 - Relative abundance of the VOCs identified by HS-SPME-GC-MS analysis

rt (min)	Tentative identification	Abundance (% on the total)
23.2	citronellal	27.54
29.61	α-citrale (geranial)	25.00
26.73	β-caryophyllene	9.24
28.42	β-citrale (neral)	7.61
29.44	Germacrene D	3.00
24.55	linalol	2.82
35.7	caryophyllene oxide	2.70
15.81	(E)-2-hexenal	2.65
21.96	1-octen-3-ol	2.47
29.88	geranyl acetate	2.42
25.22	methyl citronellate	2.34
30.37	δ-cadinene	1.44
16.63	E-ocimene	1.35
30.55	γ-cadinene	0.91
23.87	copaene	0.88
31.75	geraniol	0.79
25.68	Isopulegol isomer B	0.64
20.24	(Z)-3-hexen-1-ol	0.57
16.77	3-octanone	0.52
34.34	β-ionone	0.48
25.58	Isopulegol isomer A	0.46
6.52	ethyl acetate	0.46
20.39	3-octanol	0.44
19.18	6-methyl-5-hepten-2-one	0.38
22.77	α-cubebene	0.36
19.7	2,6-dimethyl-5-heptenal	0.36
20.84	nonanal	0.33
11.61	hexanal	0.25
16.08	Z-ocimene	0.21
18.5	(E)-3-hexen-1-yl acetate	0.20
13.95	β-myrcene	0.17
37.78	nerol	0.15
17.86	methyl hex-2-enoate	0.10
17.81	octanal	0.10
10.44	2,5-diethyltetrahydro-furan	0.09
7.11	2-methyl-butanal	0.07
7.21	3-methyl-butanal	0.06
15.21	D-Limonene	0.05
9.85	1-penten-3-one	0.05
13.62	1-penten-3-ol	0.05
8.73	pentanal	0.05
4.40	dimethyl sulfide	0.04
15.02	3-methyl-1-butanol	0.04
18.34	(Z)-2-penten-1-ol	0.03
13.11	(E)-2-pentenal	0.03
13.37	5-methyl-hexanal	0.03
14.71	heptanal	0.03
16.41	(Z)-4-heptenal	0.02
5.22	2-methyl-propanal	0.02
8.6	2-pentanone	0.01

Data are expressed as area % on the total area of all the identified peaks.

obtained by HS-SPME-GC-MS analysis of the dried powdered foliar sample of *Melissa officinalis* L. Up to 50 Volatile Organic Compounds were identified and their abundance was reported as percentage on the total area of the identified peaks (Table 1). Terpenes were the most representative class of compounds, in terms of both number of molecules and relative abundance (15 monoterpenes, 71.91%; 8 sesquiterpenes, 19.01%). Among them, the most abundant compounds were citronellal (27.54%), α-citral (25.00%), β-caryophyllene (9.24%) and β-citral (7.61%). The other main classes of identified VOCs were 11 aldehydes (total 3.64%), 6 alcohols (3.60%), 4 ketones (0.96%) and 3 esters (0.76%).

Starting from these data, we selected the most representative VOCs from the HS-SPME-GC-MS analysis and quantified them in terms of mg kg^{-1} on dried foliar sample basis. To this aim, sample was extracted and analyzed by liquid injection GC-MS, as described in the experimental section. Table 2 summarizes the obtained results.

Table 2 - Content of the main VOCs by liquid injection GC-MS analysis

VOC	Amount
citronellal	119.2 (mg_{citral} kg^{-1})
α-citral (geranial)	109.7 (mg_{citral} kg^{-1})
β-citral (neral)	39.7 (mg_{citral} kg^{-1})
β-caryophyllene	74.3 ($mg_{caryoph}$ kg^{-1})

Data are the mean of three determinations (standard deviation <3%) expressed in mg kg^{-1} dry weight.

Citronellal was the most abundant compound in the heptanoic extract, followed by α-citral and β-caryophyllene. The total citral amount, in terms of sum of α- and β-citral, was 149.4 mg_{citral} kg^{-1}, that was more than citronellal amount.

HS-SPME and GC×GC-MS fingerprint analysis are ideal tools to analyze complex volatile matrices, and provide a sensitive method for the direct comparison and chemical visualization of plant volatile components.

GC×GC-MS is currently adopted as separation technique not only because of its high separation power and sensitivity but also for its ability to produce more widely distributed and rationalized peak patterns (Cordero *et al.*, 2008) for chemically correlated group of analytes.

HS-SPME GC×GC-TOF-MS analysis of the complex volatile fraction of lemon balm was submitted to

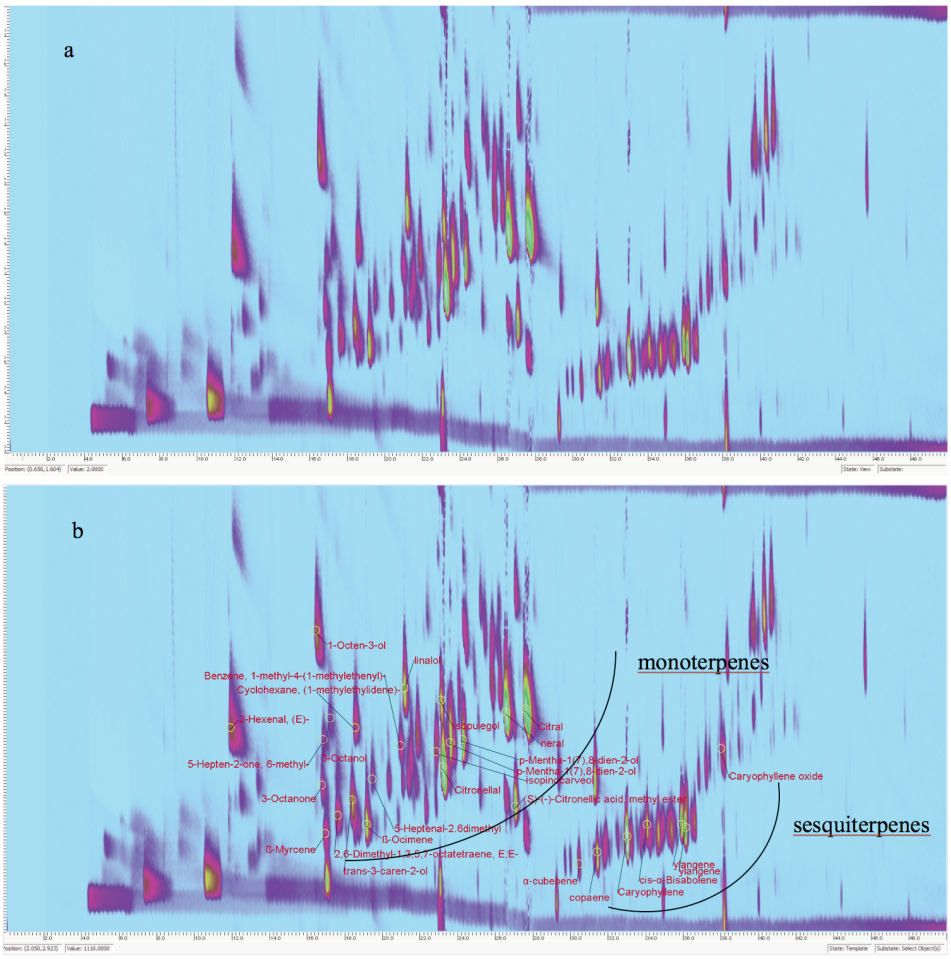

Fig. 2 - Contour plot from GC 2D-MS/TOF analysis of lemon balm. a: graphic view of the blobs/compound detected; b: sample template of the main compounds detected.

advanced fingerprinting analysis of 2D chromatographic data.

In figure 2a is reported a "contour plot" from GC 2D-MS/TOF analysis where the "blobs" correspond to a single volatile compound detected. 417 blobs were detected and, after subtracting base line blobs corresponding to fiber blending or background interferences, 203 blobs/compounds were identified.

An advanced, effective and reliable non-targeted analysis approach known as comprehensive template matching fingerprinting (Cordero *et al*., 2012) was adopted (Fig. 2b). This method considers, as comparative feature, each individual 2D peak together with its time coordinates, detector response and MS fragmentation pattern, and includes them in a sample template that is created by the analyst and can be used to compare plots from different samples directly and comprehensively. In this case a template was created for a comprehensive comparative analysis of 2D chromatographic data and to correctly interpret visual differences in further analysis.

The most intense blobs corresponded to citronellal, α-citral and β-citral as evidenced in the GC-MS analysis. Also the sesquiterpene β-caryophyllen was an intense blob. Up to 24 blobs/compounds belonging to the class of sesquiterpenes were distributed in a defined part of the contour plot (Fig. 2b). GC×GC-MS analysis produced distributed and rationalized peak patterns for sesquiterpenes, monoterpenes and oxigenated monoterpenes (Fig. 2b).

The analysis can be see also in the 3D view, showing the complex volatile fraction of lemon balm leaves (Fig. 3).

Data of the retention time and λ max in the visible region of specific standard (rosmarinic acid) obtained by HPLC-DAD analysis allowed the identification of phenolic compounds of the cultivated lemon balm sample. All these derivatives were calculated based on rosmarinic acid calibration curve (35.23 mg g^{-1}).

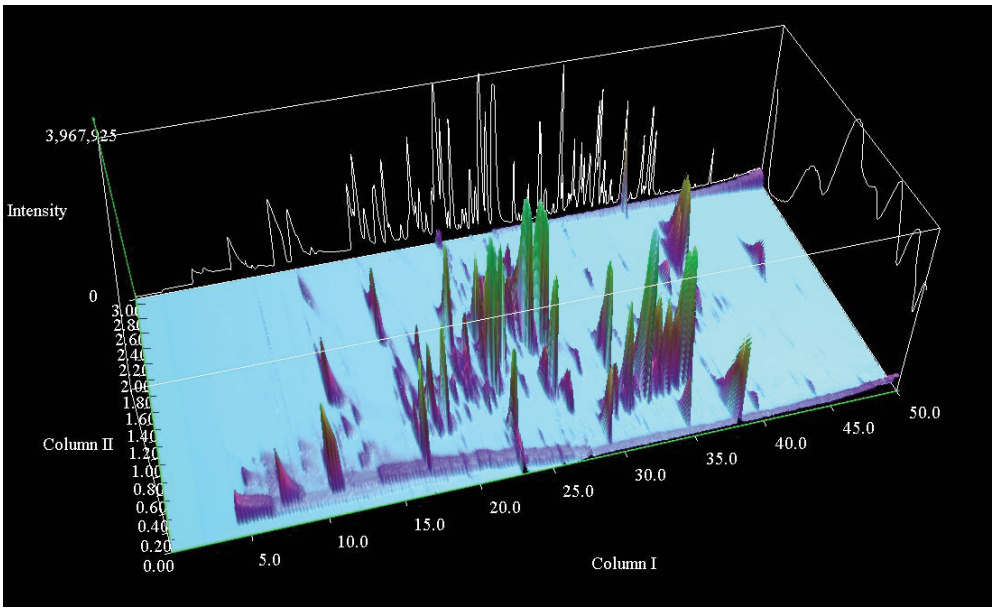

Fig. 3 - 3D view from GC 2D-MS/TOF analysis of lemon balm.

Data are the mean of three determinations (standard deviation <3%) expressed in mg g^{-1} dry weight. The lemon balm sample studied presented the rosmarinic acid (caffeic acid dimer) as the main compound. Few studies report the existence of rosmarinic acid as being the most abundant phenol in this species (Caniova and Brandsteterova, 2001; Ziaková *et al.*, 2003; Fecka and Turek, 2007; Lee, 2010).

The rosmarinic acid content was slightly high (32.4 mg g^{-1}) and in accordance with those reported by Fecka and Turek (2007) that presented values of rosmarinic acid in *M. officinalis* ranging from 32.6 to 5.1 mg g^{-1} of infusion.

It was also identified a flavone, the only flavonoid found in this sample (1.7 mg g^{-1}). This peak presented a UV spectra with λ max at 350 nm, and was tentatively identified as a luteolin derivative, described as the major flavonoid in *M. officinalis* by Heitz *et al*. (2000).

4. Conclusions

The results obtained, using integrated chromatographic techniques, allowed to evaluate the quali-quantitative content of secondary metabolites present in officinal species, such as *Melissa officinalis* L. Parameters evaluated, in particular the high-content of active ingredients, can be new values to be included in technical data sheets to define quality of raw materials for the production of standardized fraction in biomolecules content.

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