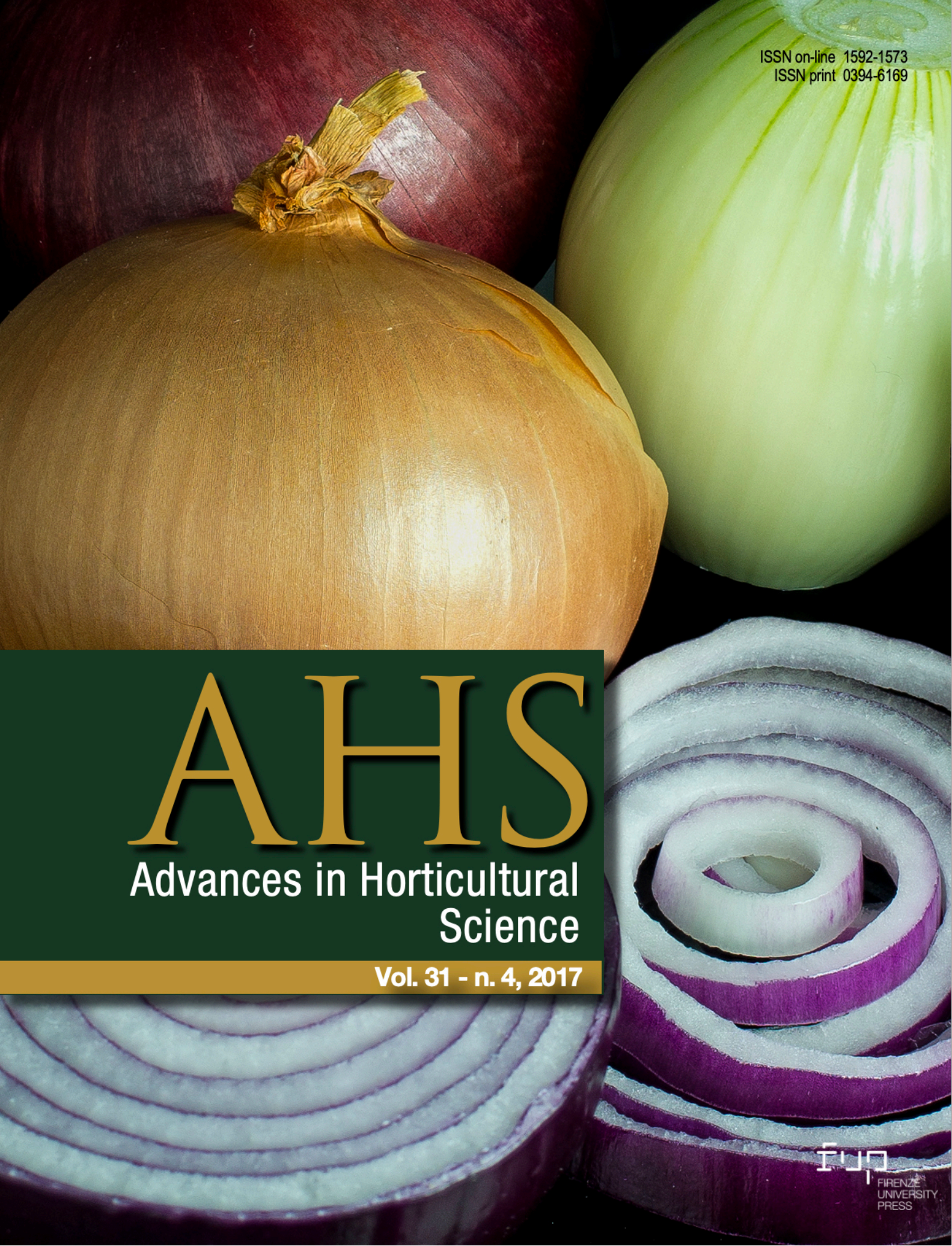

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Detection of not allowed food-coloring additives (copper chlorophyllin, copper-sulphate) in green table olives sold on the Italian market

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Abstract: Table olives are a common and well-known food in the whole Mediterranean area, produced and consumed in great quantities. Many deep-green olives can be found on sale in the South of Italy. Sometimes a deep color could be the result of the fraudulent addition of a coloring agent (E141ii, copper chlorophyllins) during the pickling process, in spite of the European Union legislation that does not allow the addition of any colorant to fruits included table olives. The objectives of this study were to use a relatively simple method of detection of E141ii added to table olives, to verify the presence on the Italian market of artificially colored table olives, and to show that also $CuSO_4$ can be employed for table olive re-greening. Compounds with chromatographic and spectral characteristics similar to the ones from the E141ii (Cu chlorin e_6, Cu isochlorin e_4, Cu pyropheophorbide a) were found in 8 samples out of 16. These results show that the fraudulent addition of colorant to table olives is a quite common practice. More pressing controls and analysis are required to ensure the complete food safety and the compliance with the current law.

1. Introduction

Table olives are a common and well-known food in the whole Mediterranean area, produced and consumed in great quantities especially in Southern Italy. Manufacturing techniques have been refined along the years, in order to optimize the quality and attractiveness of the final product and to cover different market niches. Table olives found on market's shelves exhibit different colors and shapes, depending on cultivar type, ripening stage or processing method. There are green olives, spotted and fully ripened ones. In Southern Italy the Greek method and the Spanish method are commonly used to produce green table olives, while the Castelvetrano method covers a smaller but increasing market.

With the Greek method, olives are washed with water and then stored in brine (5-8%). The addition of salt promotes the product preservation

and the development of fermentation-capable microorganisms. During this stage of treatment, olives slowly lose their bitterness (due to oleuropein's enzymatic degradation) and acquires their final taste and properties. The development of lactic fermentation is favoured (olives average final pH of 5.2), in order to improve both the food safety and its organoleptic characteristics (Piga *et al*., 2001).

The Spanish method, the most used worldwide, requires the olives to be treated with a solution of sodium-hydroxide and water in order to hydrolyze most of the oleuropeinic glucosides. Afterwards, olives are washed repeatedly with water and then stored in brine (5-6%) in which they naturally undergo a complete lactic fermentation, reaching an average pH value of 4 (Garrido-Fernandez *et al*., 1997).

Olives treated with the Castelvetrano method are plunged for 10 to 15 days in a sodium-hydroxide solution which concentration depends on olive caliber and their ripening stage. Later on, marine salt (NaCl) will be slowly added to the solution. After such treatment drupes will undergo several washings with water (Salvo *et al*., 1995) so that, in absence of a lactic fermentation, pH never goes below 6.5 (Fodale *et al*., 2007). Anyway, Castelvetrano type olives appears brilliant-green and with more compact pulp (Owen *et al*., 2003), so that they are preferred to green table olives prepared with the Greek or Spanish methods. As a matter of fact, treatment and storage in acid solutions are known to be the main responsables of chlorophylls degradation in table olives (Minguez Mosquera *et al*., 1989). The loss of the magnesium from the chlorophylls causes to turn into the corresponding pheophytins, shifting their color from green to brown (Scotter *et al*., 2005). At the end of the various pickling processes, therefore, olives had lost most part of their original chlorophylls (and of their original dye) becoming yellowish-green; in spite of that, many deep-green olives can be found on sale in the South of Italy, especially in the Bari Province.

Their sometimes unnatural colour could be the result of the fraudulent addition of a colouring agent during the pickling process, in spite of the European Union legislation does not allow the addition of any colorant to fruit and table olives (EU, Reg CE 94/36/CE, 1994).

Among the most used green food colorants, there are the chlorophyll-derived ones; Copper complexes of chlorophyillins, particularly, play a major role within this group. Copper chlorophyllins are manufactured by chlorophyll saponification which, in turn, is extracted by means of organic solvents from edible plant, such as alfalfa (*Medicago sativa* L.) and nettle (*Urtica dioica* L.) (Mortensen, 2006). The resulting chlorophyll-based salts are marketed as E141 colorant and are available in two forms: E141i (liposoluble) and E141ii (hydrophilic). These salts are widely used as food colorants (e.g. ice creams, snacks, food decorations) but their addition to table olives and other fruits is expressly forbidden. In spite of that, E141ii (due to its hydrophilic properties and color stability) is sometimes fraudulently added to table olives during the pickling process as re-greening agent.

The objectives of this study were to confirm the presence on the market of artificially colored table olives through the detect fraudulent color adulteration with E141ii, and to verify if the addition of Copper-sulphate during the pickling process could allow a re-greening of table olives as result of its interaction with Chlorophylls, leading to the formation of Cu-chlorophyllins. Nowadays, Copper-sulphate is not allowed and not even mentioned in food legislation, but it is known that it has been widely utilized by food manufacturers because of its re-greening properties on preserved vegetables (Cerutti, 2006) but its use is very dangerous because copper is toxic to the liver. Continuous consumption of olives treated with this compound could result in damage to the consumer due to its accumulation in the body (Stern, 2010). All this to generate a warning for consumers and public authorities.

2. Materials and Methods

Chemicals

All reagents were analytical or HPLC grade: Hexane and Water were supplied from Romil Ltd. (Cambrdge, UK); Methanol from Carlo Erba Reagents (Rodano, Italy); Acetic acid and *tert*-butyl metyl ether from J.T. Baker (Deventer, Netherlands). Chlorophyll a and b, pheophorbide a and copper sulfate were purchased from Sigma-Aldrich Co. (Saint Louis, USA). Sample of E-141ii colorant were supplied by Chimica D'agostino (Bari, Italy).

Plant material

Obviously deep-green table olives may have been fraudulently colored, while table olives appearing from pale-green to mustard-yellow are to be considered not colored. Therefore, 16 table olives samples were purchased from local markets (Bari, Brindisi and Lecce): 9 olives were with a brilliant-green or deep-

green color, while drupes from the remaining 7 samples were pale-green or mustard-yellow. pH values of the brine were recorded with a PC 650 probe (Eutech instruments, Singapore). Due to the lack of information reported on the labels, we could not assess the cultivar and origin of each olive sample (Table 1).

Table 1 - Main characteristics of the olives samples analyzed

Sample	pH	Colour	Cultivar	Origin
S1	5.4	G	n.r.	n.r.
S2	5.9	G	n.r.	Greece
S3	7.1	G	n.r.	Greece
S4	5.7	G	Nocellara del Belice	Italy (Sicily)
S5	6.1	G	n.r.	n.r.
S6	5.9	G	n.r.	Italy
S7	5.1	G	n.r.	Italy
S8	5.7	Y	n.r.	n.r.
S9	6.4	G	n.r.	n.r.
S10	4.2	Y	Bella di Cerignola	n.r.
S11	3.8	Y	n.r.	n.r.
S12	4.1	Y	n.r.	n.r.
S13	5.0	Y	n.r.	n.r.
S14	3.3	Y	n.r.	n.r.
S15	4.0	Y	n.r.	n.r.
S16	4.4	G	n.r.	n.r.

G= green; Y= pale-green or mustard-yellow.
n.r.= not reported.

Pigment extraction

All procedures were performed under dimmed green light to avoid any photo-oxidation of chlorophylls. 40 g of olive pulp were collected and homogenized with 25 ml of Methanol-water solution (80/20, v/v) using an Ultra-Turrax T25 (Janke e Kunkle, IKA-Labortechnik, Germany); the resulting paste was filtered by means of a Buckner's funnel with a paper filter (Perfecte 2 extra rapida, Superfiltro Milano, Italy) and a suction flask connected to a vacuum pump. The solid residue was collected, added to 50 ml of Methanol and stirred for 1 hour; the resulting solution underwent a second filtration step with the same procedure previously used. For same samples appearing still green, 50 ml of Methanol were added and a third stirring-filtering cycle has been performed. The filtrate was then mixed with an identical amount of hexane in a separating funnel in order to separate the lipophilic substances from the hydrophilic ones. The latter phase was recovered and evaporated under vacuum in a RE 111 rotavapor (Büchi, Flawil, Switzerland) at room temperature. The evaporation remnant was finally diluted up to 2 ml with Methanol and utilized for the HPLC/DAD analysis. This procedure was made up for extraction hydrophilic pigments preferably.

$CuSO_4$ addition tests

In order to verify the possibility of re-greening using copper sulphate during the olive production process or the marketing, samples of olives were treated with $CuSO_4$. It is known, in fact, that the addition of copper stabilizes the tetrapyrrolic ring of pheophytin, resulting a re-greening of olive drupes (NIIR, 2004).

A local food company provided us a sample of olives processed with the Castelvetrano method and stored in a Sodium-hydroxide/salt/water solution (pH 11.5). Four groups of 10 olives were taken from the sample: the first one was washed with water for 24 hours, stored in brine (6% NaCl, 0.6% Citric acid, 0.05% Ascorbic acid, pH: 2.3) for 48 hours (pH stabilized at 7.3) and then analyzed; the remaining 3 sub-samples were placed in beakers with 150 ml of their original packing solution, 1.5 g (1%), 7.5 g (5%) and 30 g (20%) of $CuSO_4$ were then added. After 3 hours of stirring, the coloring solution was discarded and the samples were washed for 24 hours with water. The samples were then stored in brine solution for 48 hours until pH stabilization (pH 5.4). After the brine storage, all the sub-samples went through the before mentioned pigments extraction procedure. Another trial was run to test the effect of $CuSO_4$ addition on olives treated with the classical Spanish method. 10 olives were selected from a sample which analysis already excluded any fraudulent colouration (S16). Drupes were first put in an alkaline solution (150 ml of 1% NaOH-water solution, pH: 12.8) until pH stabilization was reached (pH: 8.3), 30 g (20%) of $CuSO_4$ were then added to this medium. The drupes were then immersed in brine solution; after 48 hours the pH reached a stable value of 4.0. The sample was then treated until pigment extraction following the already cited procedures.

Analysis of chlorophylls compounds by HPLC/DAD

The determination of pigment products were carried out by HPLC using a Agilent 1100 liquid chromatograph fitted with a manual injector. A stainless steel column, Alltech Prontosil C30, 200 Å, 5 µ, 250× 4.6 mm I.D. was used. The column was protected by precolumn packed with the same material. Separation was performed using an elution gradient (flow rate 1 ml min^{-1}) with the mobile phases (A) Methanol: distilled water: Acetic acid (90:10:0.5 v/v/v) and (B) *tert*-butyl metyl ether: Methanol: Acetic acid (100:10:0.5 v/v). The gradient scheme was 0-50% B in 30 minutes, 50-100% B in 10 minutes, 100% B for 5 minutes and 100-0% B in 5 minutes.

Sequential detection was performed with a photodiode array detector (DAD) at 430 nm and 650 nm also the online UV-vis spectra were recorded from 250 to 800 nm. 100 µl of 1 mg/ml E141ii in methanol or 100 µl of extract was utilized for the analysis. To analyze the samples treated with $CuSO_4$ the flow rate was lowered at 0.7 ml/min and the elution of B was set to 0-45% in 30 minutes, 45-100% in 10 minutes, 100% for 5 minutes and 100-0% in 5 minutes. Data were collected and processed with a LC Agilent ChemStation (revision software B.04.02). Pigments were identified by co-chromatography with authentic samples and/or by comparison their spectral characteristics with literature or compound standard when available. All analysis were made in duplicate.

3. Results and Discussion

In order to identify and recognize the E141ii components likely to be present in olive samples, industrial copper chlorophyllin was analyzed by HPLC/DAD, injected and monitored at 650 nm. The resulting chromatogram shows 8 major peaks indicates with **a** to **h** in figure 1. The analysis of the elution times and absorption spectra of the peaks (Fig. 2) compared with literature (Inoue *et al*., 1994; Chernomorsky *et al*., 1997; Mortensen and Geppel, 2007; Roca *et al*., 2010; Aparicio-Ruiz *et al*., 2011; Gandul-Rojas *et al*., 2012) allowed the tentative identification of 7 of the 8 peaks: (**a**) Cu rhodin g7, (**b**) Cu chlorin e_6, (**c**) Cu chlorin p6, (**d**) Cu isochlorin e_4, (**e**) Cu 15^1-OH-lactone-pheophytin a (**g**) Cu rhodochlorin, (**h**) Cu pyropheophorbide a. We were unable to identify without any doubt peak **f**. The main components of our E141ii standard eluted according to Montensen and Geppel (2007) procedure which utilize a chromatographic method similar, were: Cu Rhodin g7 < Cu Chlorin e_6 < Cu Isochlorin e_4 < Cu Pyropheophorbide a.

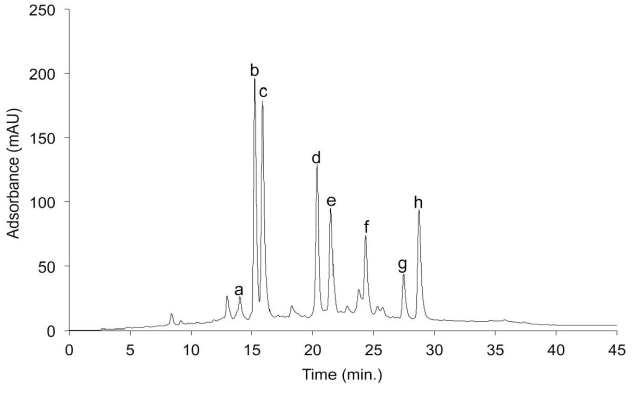

Fig. 1 - HPLC/DAD analysis of food colorant E141ii, recorded at 650 nm, showing the peaks of the main identified components: a) Cu rhodin g7; b) Cu chlorin e6; c) Cu chlorin p6; d) Cu isochlorin e4; e) 151 OH lactone pheophytin a; f) unknown; g) Cu rhodochlorin; h) Cu pyropheophorbide a.

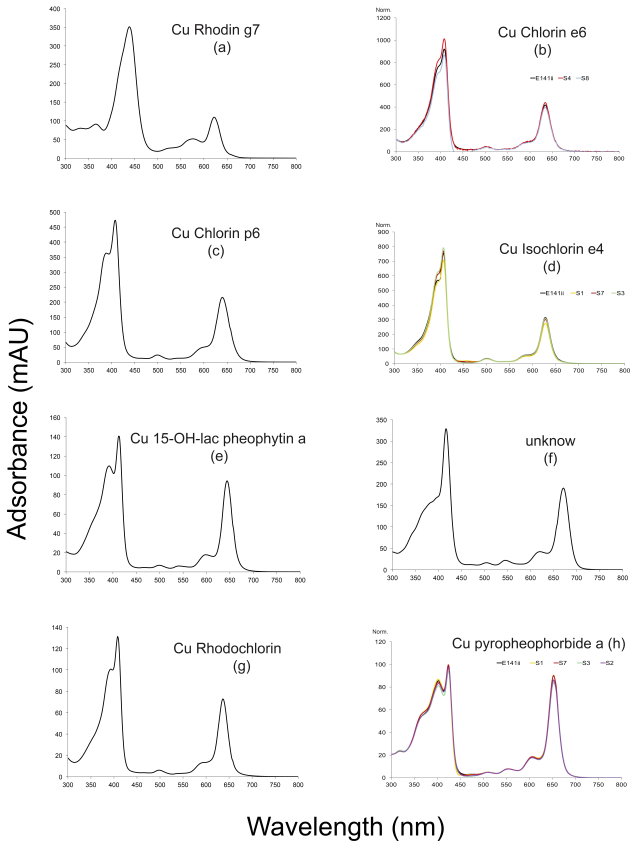

Fig. 2 - Spectra UV/Vis of the Copper chlorophyllins pigments found in the colorant E141ii and in some samples of green table olives.

Figures 3 and 4 show chromatograms resulting from the HPLC/DAD analysis of the studied samples; they have been recorded at 650 nm to facilitate the detection of chlorophylls and their derivatives, avoiding in the same time interferences from carotenoids. In 7 samples out of 16 (Fig. 3) we assessed the presence of compounds with chromatographic characteristics similar to the ones from the industrial chlorophyllin sample: Cu isochlorin e_4 (peak **d**) and Cu pyropheophorbide a (peak **h**). This finding agrees with bibliographical data, since Cu isochlorin e_4 is referred as one of the main component of commercial copper chlorophyllins (Inoue *et al*., 1994; Chernomorsky *et al*., 1997; Ferruzzi *et al*., 2002). Cu chlorin e_6 (peak **b**) were found in 2 samples. Finally, the presence of Cu rhodin g7, Cu chlorin p_6, Cu 15^1-OH-lactone-pheophytin a and Cu rhodochlorin was never observed. This result is supported by the work of Gandul-Rojas *et al*. (2012) which demonstrates

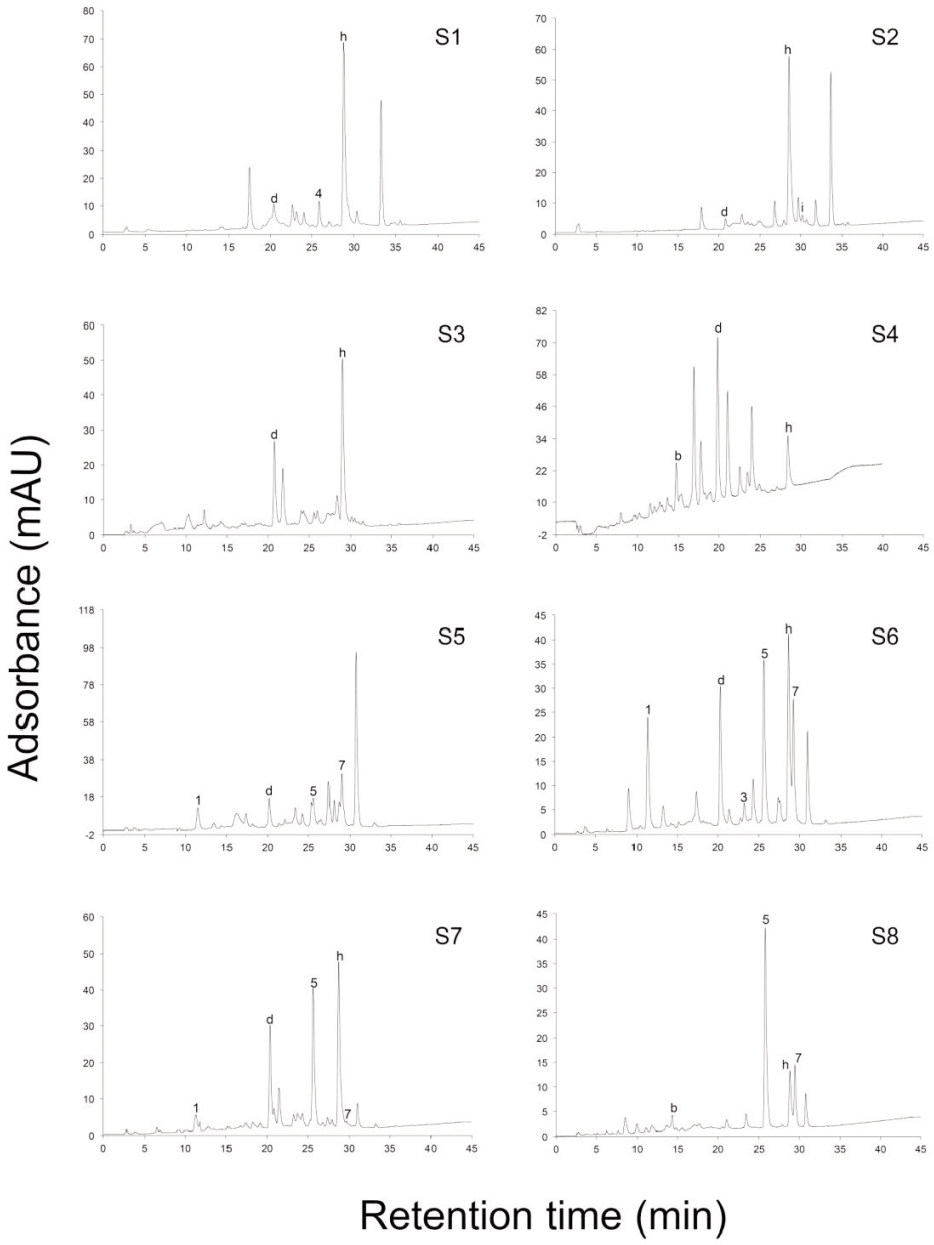

Fig. 3 - HPLC/DAD chromatograms recorded at 650 nm of the samples (S1-S8) containing Cu-components related to ones from E141ii colorant.
Peaks described in Table 2.

that the addition of E141ii to table olives led to the chromatographic identification of Cu-chlorin type compounds (mainly Cu chlorine e_6 and Cu isochlorin e_4) very different from chlorophyll derivatives usually found in green table olives. Moreover, Minguez-Mosquera *et al*. (1995) showed that under certain (still unexplained) circumstances, small amounts of Cu-chlorophyll compounds can be spontaneously synthesized within the olives, leading to localized pigment alteration on their surface (*green staining* alteration). The visual analysis of our samples led us to notice no traces of *green staining* on the drupes: Cu-compounds identified in our samples must therefore be the result of a colorant addition.

In those samples we were also able to identify several other pigments, marked with numbers, such as chlorophyll b (peak **4**; Fig. 3-S1); pheophitin a (peak **5**; Fig. 3-S6 to 3-S8) and isochlorin e_4 (peak **7**; Fig. 3-S5 to 3-S8).

Cu-compounds were never found in 8 of the analyzed samples (Fig. 4). On the other hand, those samples contained pigments identified as chlorophylls (e.g chlorophyll a; peak **6**; Fig. 4-S9, 4S11, 4-S13, 4S16) and chlorophyll derivatives (e.g. pheophorbide a; peak **2**; Fig. 4-S13, 4-S15, 4-S16, pheophytin a; peak **5**; Fig. 4-S10 to 4-S16).

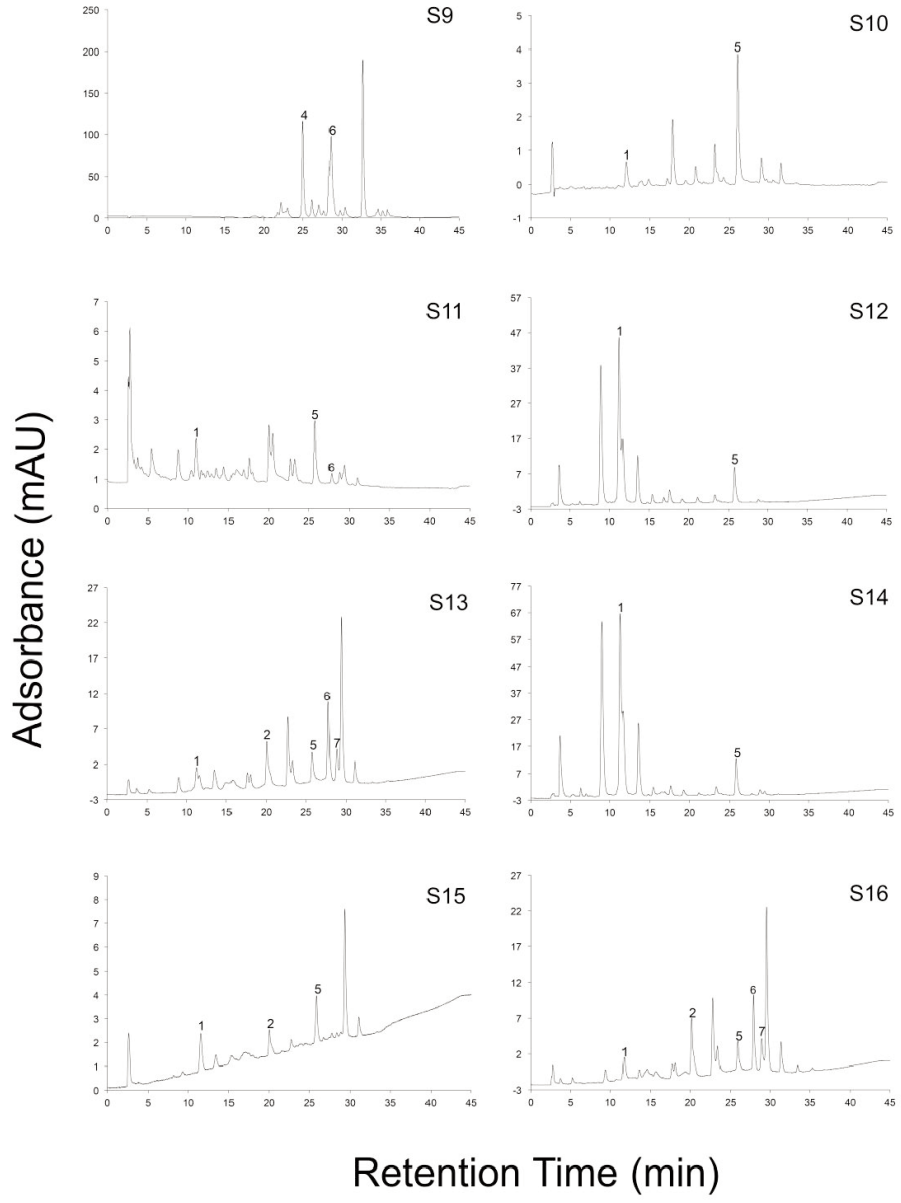

Fig. 4 - HPLC/DAD chromatograms recorded at 650 nm of the samples (S9-S16) do not contain Cu-components related to ones from E141ii colorant. Peaks described in Table 2.

Gandul-Rojas *et al*. (2012) show that chromatograms of olives treated with the Spanish method exhibit a series of major peaks belonging to Mg-free chlorophyll derivatives (mainly phaeophytins), while chromatograms of samples treated with the Castelvetrano method show peaks from chlorophylls or degraded chlorophylls with Mg (e.g. Glycoxylic acid chlorophylls; Formyl chlorophylls) in addition to the first ones.

Table 2 indicates the chromatographic, spectral characteristics and origin of the chlorophyll derivates pigments identified in the commercial E141ii and in the olive samples. The results obtained are in agreement with the literature (Hynninen 1973; Inoue *et al*., 1994; Chernomorsky *et al*., 1997; Mortensen and Geppel, 2007; Roca *et al*., 2010; Aparicio-Ruiz *et al*., 2011; Gandul-Rojas *et al*., 2012). The pigments identified are Cu chlorophyllins (peaks **a**-**h**), chlorophylls and derivatives (peaks **1**-**7**).

The analysis of the 9 samples initially suspected of have been fraudulently colored (indicated as G in Table 1), largely confirmed our hypothesys: 8 samples contains Cu compounds related to the E141ii colorant (Fig. 3). On the other hand, 6 out of the 7 samples chosen accordingly to their pale-green or mustad-yellow dyes, do not contain compounds related to the food coloring agent, underlining that the addition of E141ii during the pickling process could be the

Table 2 - The chromatographic and spectroscopic characteristics of the chlorophyll derivates pigments present in the E141ii dye and in the analyzed green table olives

Pigment	Peak [z]	Kc	Soret	Spectral data in HPLC eluent												Sample (and/or E141ii dye) where the pigment is present	References
				I		II		III		IV		V		VI			
				M	R	M	R	M	R	M	R	M	R	M	R		
Chlorine e6	1	8.83	402	-	-	-	-	500	18.02	528	25.66	(642)	9.45	662	5.47	S5 to S7, S10, S11, S13 to S16	[4]
Cu rhodin g7	a	11.21	436	366	3.27	-	-	-	-	577	6.85	-	-	623	3.11	E141ii	[3]
Cu chlorin e6	b	12.42	408	(395)	1.21	-	-	-	-	502	8.02	(588)	4.80	634	2.19	E141ii, S4, S8, S18 to S21	[2] [3] [4] [6]
Cu chlorin p6	c	13.48	407	388	1.29	-	-	-	-	500	19.52	(597)	9.76	640	2.22	E141ii	[4]
Pheophorbide a	2	17.77	408	(380)	1.53	(400)	1.10	507	9.33	537	9.34	609	9.76	666	2.01	S13, S15, S16	[2] [7] std
Cu isochlorin e4	d	17.96	406	(394)	1.16	-	-	-	-	501	18.71	(585)	11.65	627	2.20	E141ii, S1 to S7, S18 to S20	[2] [4]
Cu 15[1]-OH-lactone-pheophytin a	e	19.07	412	392	1.27	-	-	498	19.33	540	23.22	598	7.96	644	1.53	E141ii	[5]
Pheophytin b	3	20.75	436	-	-	416	2.58	526	8.03	560	19.84	600	16.01	656	4.43	S6	[2]
unknown	f	21.93	417	(397)	1.86	-	-	505	19.50	545	14.31	623	6.78	671	1.67	E141ii	-
Chlorophyll b	4	22.62	466	-	-	-	-	-	-	(550)	17.46	600	8.45	650	2.49	S1, S9	[2] std
Pheophytin a	5	23.31	410	(380)	1.52	(400)	1.11	507	9.44	538	10.11	610	10.62	667	2.12	S5 to S7, S11, S13 to S16	[2]
Cu rhodochlorin	g	25.04	408	393	1.29	-	-	-	-	498	21.50	(593)	9.67	636	1.83	E141ii	[4]
Chlorophyll a	6	26.16	430	(386)	1.70	(416)	1.12	-	-	(580)	9.80	618	5.10	665	1.13	S9, S11, S16	[2] std
Cu pyropheophorbide a	h	26.31	424	(366)	1.78	403	1.15	510	19.60	554		606	5.44	652	1.13	E141ii, S1 to S4, S6, to S8, S18 to S20	[1] [4] [5]
Isochlorin e4	7	28.55	400	-	-	500	11.87	530	31.62	560	74.80	609	31.60	665	3.06	S5 to S8, S13, S16	[1]

Compounds derived from copper-free chlorophylls are indicated by numbers; the other, containing copper, present in the E141ii dye and in some samples, with letters.
Retention factor. Kc = (tR - tM)/tM where tR is the retention time of the pigment peak and tM is the retention time of an unretained component.
M = maximun absorbance (nm); R = quotient of absorbance at Soret band divided by absorbance at wavelength indicated.
The values in parentheses indicate inflection points in the absorption spectrum.
[1] Aparicio *et al.,* 2011, [2] Gangul-Rojas *et al.*, 2012; [3] Inoue *et al*., 1994; [4] Mortensen *et al.,* 2007; [5] Roca *et al.,* 2010, [6] Chernomorsky *et al*., 1997, [7] Hynninen, 1973, std = chemical standard.

main responsible of the bright or deep-green dye of the analyzed olives.

Concerning the addition of Copper-sulphate, the re-greening made at alkaline pH was more pronounced in samples treated with the addition of 5 and 20% $CuSO_4$ in comparison respect to olives treated with 1% $CuSO_4$. In fact, untreated samples do not show peaks referable to Cu-chlorophyll compounds (Fig. 5-S17), while samples treated with 1, 5 and 20% of $CuSO_4$ show well defined peaks in their chromatograms: 2 of them were identified as Cu chlorin e_6, (peak **b**) and Cu pyropheophorbide a (peak **h**) (Fig. 5-S18; 5-S19; 5-S20).

The sample previously processed with Spanish method (therefore at acid pH) and treated with $CuSO_4$, shows a lighter colour change with respect to the other three samples. This is supported by the very low adsorbance values of Cu chlorin e_6 (peak **b**) pointed out in its chromatogram (Fig. 5-S21).

The Copper of $CuSO_4$ seems therefore to react only at alkaline pH (before the final acidification) with the degraded chlorophylls within the olives the same way it reacts with saponificated chlorophylls during industrial colorant production, leading to the synthesis of Cu-chlorophyll derivatives similar to the ones identified in the E141ii reference sample.

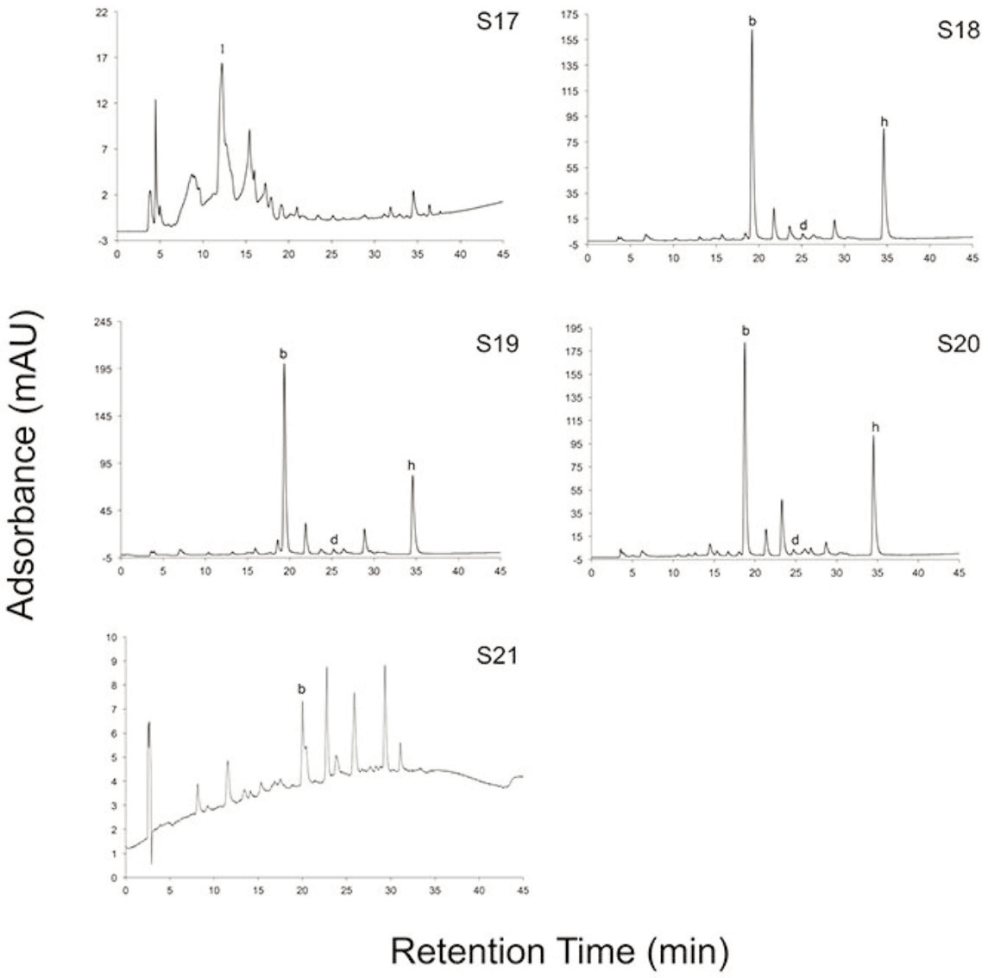

Fig. 5 - HPLC/DAD chromatograms, recorded at 650 nm, of samples treated with various percentages of CuSO4: (S17) without CuSO4 addition; (S18) CuSO4 1%; (S19) CuSO4 5%; (S20) CuSO4 20%. (S21) classical Spanish method and CuSO4 20%. Peaks described in Table 2.

4. Conclusions

The use of a relatively simple method of detection of E141ii added to table olives shows that the fraudulent addition of colorant (or copper sulfate) to table olives is a quite common practice. In fact, the 50% of the analyzed samples possess copper chlorophyll pigments, most likely due to the addition of colorant E141ii, or, worse still, of copper sulphate during the production process. Our work has shown that in these samples there is often the presence of Cu chlorin e_6, Cu isochlorin e_4 and Cu pyropheophorbide to which can therefore be considered markers for these adulterations. More controls and analysis are required to ensure the complete food safety and the compliance with the current law. The finding that even Copper-sulphate can be used to modify olive dye before marketing them, makes this need even more pressing, especially in relation to the severe liver damages it may cause.

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The Influence of synthetic strigolactones and plant extracts on the morphological parameters of onion (*Allium cepa*)

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Abstract: In recent years there has been frequent reference to the significance of strigolactones as a new group of hormones which might have a significant effect on horticultural production. The aim of this work was to find an ideal combination of stable synthetic strigolactones and plant extracts with potential effects on onion plants. The synthetic strigolactone Fenyl 7 (dihydro-3-[[2,5-dihydro-4-methyl-5-oxo-2-furanyl)oxy]-methylene]-5-phenyl-2(3H)-furanone) was tested in a carrot macerate, with citric acid and with salicylic acid. From the results it was confirmed that increasing the pH of the preparation leads to improving the stability of Fenyl 7. Evaluation has repeatedly confirmed the effect of the preparation, combining synthetic strigolactone and a macerate of carrot in a mixture of surfactants with added citric acid. In all the experiments this combination showed a statistically demonstrable influence on leaf weight (increased by 12-31%) and length (increased by 6-13%) in comparison with the controls.

1. Introduction

In several countries fertiliser use on agricultural soil results in serious problems associated with the accumulation of residues in the food chain and groundwater pollution (Hegazi *et al*., 2010). The increased costs of chemical fertilisers and the massive input of non-renewable resources have led to promote the research in the field of alternative preparations (Board, 2004). Several technological innovations have been proposed in order to enhance the sustainability of production systems through a significant reduction in the use of chemicals. An effective tool would be the use of biostimulants (Hamza and Suggars, 2001) a group of compounds that act neither as fertilisers nor as pesticides, but have a positive impact on plant performance when applied in small quantities (Calvo *et al*., 2014).

Carotenoids are essential photosynthetic pigments also identified as

precursors of different signalling molecules and hormones such as strigolactones. Strigolactones, recently recognised as a new class of plant hormones, play a key role in different developmental processes such as plant architecture, the number of shoot branches and nutrient availability (Al-Babili and Bouwmeester, 2015). Several studies have reported the ability of these molecules to stimulate root hair elongation (Kapulnik *et al*., 2011) and root growth (Arite *et al*., 2012), but also to inhibit adventitious root formation (Rasmussen *et al*., 2013; Urquhart *et al*., 2015). Furthermore, strigolactones increase stem thickness by stimulating secondary growth in interaction with auxin (Agusti *et al*., 2012), positively regulate internode length (De Saint Germain *et al*., 2013) and accelerate leaf senescence (Yamada *et al*., 2014).

Strigolactones are also signalling molecules, which participate in communication with mycorrhizal fungi, initiating the arbuscular mycorrhiza formation (García-Garrido *et al*., 2009). Through mycorrhiza strigolactones are also involved in plant nutrition increasing the reduction of the phosphates in soils (García-Garrido *et al*., 2009).

Moreover, studies have shown the role of this plant hormone on seed germination (Toh *et al*., 2012) and early seedling development (Tsuchiya *et al*., 2010). Strigolactones stimulate the germination of parasitic plants, for example witchweed or broomrape, which frequently survive for years in a dormant state. These seeds can be stimulated by preparations containing strigolactones obtained from plant roots showing effects already in extremely low concentrations: for example, a concentration less than 10^{-10} mol l^{-1} is sufficient to stimulate the germination of parasitic plants (Humphrey *et al*., 2006).

Plant extracts are mostly rather complex mixtures, and therefore individual components can function antagonistically but also synergistically. The assumption that plant extracts stimulate the growth with similar effects to strigolactones was tested in experiments with the germination of the *Striga hermonthica* plants. This test is a known method for ascertaining the presence of strigolactones and is standardised for the effects of the synthetic strigolactone GR24 (Sato *et al*., 2005; Yoneyama *et al*., 2008).

Strigolactones are now known to modify positively many plant traits important for crop yield and quality (Mason, 2013) and many commercial opportunities could arise (wood production, shoot architecture, root development, nutrient acquisition and more). Already it is possible to identify certain strigolactones that affect one process and not another.

However, one challenge is to identify stable strigolactones or methods of delivery that enhance their stability (Beveridge, 2014).

The aim of this work was to find a suitable combination of stable synthetic strigolactone and plant extracts with a positive effect on onion growth parameters. In the experiment the stability of the synthetic strigolactone Fenyl 7 was evaluated and the application of several types of bio-additive in onion seedlings and the influence on morphological parameters was followed.

2. Materials and Methods

Fenyl 7 (dihydro-3-[[2,5-dihydro-4-methyl-5-oxo-2-furanyl)oxy]-methylene]-5-phenyl-2(3H)-furanone) is a synthetic strigolactone protected as utility model No. 29064 by Industrial Property Office and produced in VUOS (Research Institute for Organic Synthesis, Rybitvi) by synthesis from 3-methyl-2(5H)-furanone and phenylbutyrolactone. The obtained compound, which occurs in two stereoisomers, was purified with the aid of column chromatography. The compound has a similar structure to other synthetic strigolactones (e.g. GR5, GR7 and GR24), but is simpler and cheaper to produce. Fractions of individual isomers were obtained and also a fraction with a mix of isomers in a concentration of 10^{-2} mol l^{-1} was used for additive preparation.

Given the instability of Fenyl 7 in water a method was sought to increase the stability of this substance in solution. Aside from using surfactants a possible means of increasing stability is to change the pH of the application solution. Natural strigolactones arise from the biosynthesis of carotenoids, and so for this purpose carrot macerate (pH = 5.2) was used, which presents its positive effects (López-Ráez *et al*., 2010). To further reduce pH and also to conserve the macerate, citric or salicylic acid were added (pH = 3.6). Fenyl 7 was dissolved in the mixture of surfactants or in the carrot macerate and converted with the aid of a vacuum evaporator to a 3 g l^{-1} mixture of surfactant. The resulting concentration for application on plants was 10^{-5} M. The carrot macerate was prepared by the maceration of carrot roots for 3-5 days in ethyl acetate. For the experiment a 1:1 mixture of MDGE (diethylene glycol monomethyl ether) and PEGSHO (poly(ethylene glycol)sorbitol hexaoleate) was used.

First the stability of Fenyl 7 in buffers (MDGE and PEGSHO) of pH 4, 7 and 9, according to OECD (2004),

was followed. These solutions were left at a temperature of 4°C and the decline in Fenyl 7 was steadily monitored over a period of 30 days or until the drop was greater than 90%. Liquid chromatography diode-array detection (HPLC-DAD) was used to determine the Fenyl 7. The determination was carried out on a LiChrospher RP-18 column of length 125 mm and internal diameter of 4 mm. The average particle size in the column packing was 5 µm. The column temperature was set at 30°C. A wavelength of 239 nm was used for the detection of measurements. A mixture of 40% MeOH (component A) and 100% MeOH (component B) was used as a mobile phase. The flow of the mobile phase was set at 0.9 ml min^{-1}. Analysis of one sample with a volume of 10 µl last 15 min. The gradient of the mobile phase was set at: 0 min 0% component B, 10 - 18 min 50% component B and 21 min 0% component B.

The influence of preparations selected according to previous laboratory research (Table 1) on the morphological parameters of onion seedlings (*Allium cepa*) was then followed. The experiment was carried out in three periods during two years. The experiments labelled 1, 2 and 3 were evaluated on the dates 12/5/2014, 17/7/2014 and 13/5/2015. The variety Lusy (SEMO, CZ) was sown in the substrate Klasmann TS3 Fine 416, the pH was 6.0 and the electrical conductivity of the substrate was 30 mS m^{-1}. The nutrient content of the substrate was as follows: 100 mg l^{-1} N, 70-150 mg l^{-1} P_2O_5, 140-300 mg l^{-1} K_2O and 60-100 mg l^{-1} Mg (PASIC, CZ). The experiment took place in a greenhouse in type 96 trays (cell volume 27 ml). 100 ml per tray (dilution of 2 ml l^{-1} water), which corresponds to a dose of 2 ml per plant, was applied as a foliar spray. Only water was used to spray the control treatment in the same period. For treatments, the variants given in Table 1 were selected in two applications (the first in the phase of the first true leaves, and the second after 14 days). The determination of fresh leaf weight (g), leaf length (m) and their number (pcs) was carried out 8 weeks from sowing on 25 plants.

Table 1 - Composition of bio-additives used and labelling of experiments

Label	Composition of mixture used
BIOA-1	Fenyl 7 (isomer 1) in mixture of surfactants
BIOA-3	Fenyl 7 (mix of isomers) in mixture of surfactants
BIOA-7	Fenyl 7 (mix of isomers) in mixture of surfactants with added salicylic acid (20 g l^{-1})
BIOA-10	Fenyl 7 (isomer 1) and carrot macerate in mixture of surfactants
BIOA-11	Fenyl 7 (isomer 1) and carrot macerate in mixture of surfactants with added citric acid (20 g l^{-1})

Data were analysed by analysis of variance and evaluated by using the LSD test at a probability of p = 0.05 (Statistica 12.0, StafSoft).

3. Results and Discussion

Given the instability of Fenyl 7 in water, a means was sought for increasing the stability of this compound in water. Accordingly, aside from the use of surfactants the pH of the environment was modified. The assumption that making the pH more acidic would lead to greater stability of Fenyl 7, as stated by (Babiker *et al*., 1988) in the case of GR7 in soil, was confirmed in this experiment. In fact, as clearly reported in figure 1, in the case of a solution with pH 9 there was a degradation of Fenyl 7 by more than 90% in three days. With reduced pH there was a reduction in the degradation of Fenyl 7. The lowest level of degradation was determined in the variant with a pH 4, where the reduction in the concentration was only by 8.5%. The ascertained state corresponds to the summary from Zwanenburg and Pospíšil (2013), that a lower pH does not lead to the hydrolysis of the tested synthetic strigolactones (GR7, GR24).

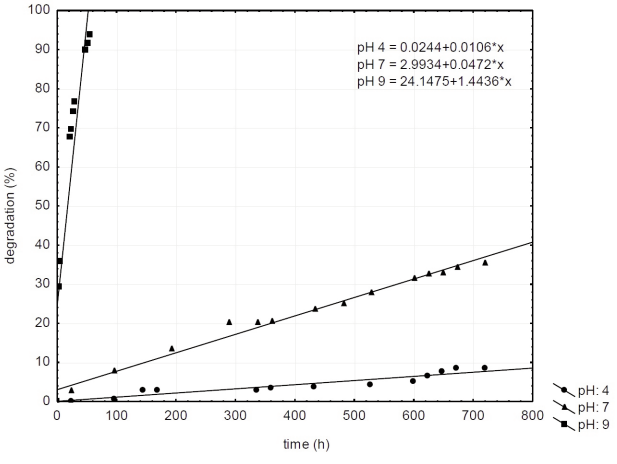

Fig. 1 - Degradation of Fenyl 7 in dependence on pH.

In the tested bio-additives (Table 1) it is possible to conclude the presence of an influence on the evaluation of morphological parameters as it is shown in

Table 2. All the treatments had a positive effect which however was not manifested in repeated experiments with all variants.

The number of leaves in onion was positively influenced only in experiment No. 2 (summer 2014) in variants BIOA-1, 3, 7 and 10 while a reduction in number of leaves was found in experiment No. 1 (spring 2014) only in variant BIOA-3. On the contrary, in the experiment No. 3 (spring 2015) the number of leaves was not influenced in comparison to control conditions. From the results obtained it is not possible to conclude that there was a positive influence of the bio-additive used on the number of leaves in onion plants.

tion it follows that the total sunlight hours for March to May was 7% higher in 2014 than in 2015. Similarly, the average temperature for the same period was 11% higher in 2014 than in 2015.

The positive effect of the external application of strigolactones on leaf length might be related to the report of (Pasare *et al*., 2013) who stated that plants with significantly reduced strigolactone content showed, among others, decreased in plant height.

Although the pea plants supplied with 3 µM of synthetic strigolactone (GR24) via hydroponics for 16 days did not significantly increase the internode length (De Saint Germain *et al*., 2013) the effect on onion leaf weight and length has been proven with

Table 2 - Average values of the monitored morphological parameters

Experiment number	Evaluated factor	Treatment					
		Control	BIOA-1	BIOA-3	BIOA-7	BIOA-10	BIOA-11
1 (spring 2014)	leaf mass (g)	2.61 a	2.77 a	2.68 a	3.61 b	2.68 a	3.43 b
	leaf length (cm)	32.92 a	34.27 ab	33.20 ab	36.80 c	32.86 a	34.73 b
	Number of leaves (pcs)	3.80 b	3.73 b	3.25 a	3.52 ab	3.55 ab	3.55 ab
2 (summer 2014)	leaf mass (g)	1.08 ab	1.56 d	1.02 a	1.24 bc	1.29 c	1.35 cd
	leaf length (cm)	23.88 a	28.12 d	25.70 bc	25.20 ab	26.40 bc	27.06 cd
	Number of leaves (pcs)	2.47 a	3.23 d	2.77 bc	3.00 cd	2.85 bc	2.60 ab
3 (spring 2015)	leaf mass (g)	1.27 ab	1.19 a	1.39 bc	1.27 ab	N/A	1.43 c
	leaf length (cm)	28.87 a	29.73 ab	30.33 bc	30.60 bc	N/A	31.27 c
	Number of leaves (pcs)	3.10 a	3.10 a	3.20 a	3.00 a	N/A	3.10 a

The letter after the value indicates the demonstrable difference according to the LSD test at p=0.05 (different letters in rows indicate significant differences between treatment variants).
N/A= not evaluated.

From the evaluation over more replicates however it is possible to confirm the positive effect of preparation BIOA-11. In all the experiments this combination showed a statistically demonstrable influence on the leaf weight and length, which attained higher values compared to the controls, as is apparent from Table 2. The recorded increase in the leaf weight was of 12-31% and in the leaf length was of 6-13% within all the evaluations. From the overall evaluation of the all tested bio-additives there is an evident tendency for the more frequent manifestation of a positive influence in the later (summer) sowings compared to the spring sowing. The recorded increase in all the evaluated parameters in the spring period of 2014 in comparison with the same period in 2015 can be assigned to the higher sunlight and higher average temperature recorded, which could have had the effect in accelerating the plant growth. According to the meteorological data from the sta-

10 µM of Fenyl 7.

From the experiments carried out we obtained the following findings. Essentially the use of one single isomer or a mixture (BIOA-1 *vs.* BIOA-3) does not have an influence on the evaluation of the morphological parameters. From the results it is not possible to conclude that there is an unequivocal difference between these variants. Also in itself the use of the carrot macerate does not have a fundamental influence on some of the morphological parameters (BIOA-1 *vs.* BIOA-10). Against this, clearly better results are achieved when the pH was modified with the aid of one of the acids (BIOA-7 and BIOA-11). While BIOA-7 was positively evaluated in two out of the three experiments, BIOA-11 was positively evaluated in all the experiments carried out. It can thus be concluded that acidification with citric acid and the presence of carrot macerate ensures the repeated effectiveness of Fenyl 7 on the morphological para-

meters in onion plants.

4. Conclusions

From the experiments it was confirmed that changing the pH of the solution into acidic territory leads to higher stability of Fenyl 7. In the case of a solution with a pH 9 the degradation of Fenyl 7 was more than 90%. With a drop down in pH there was a reduction in the degradation of Fenyl 7. The least degradation was recorded in the variant with a pH 4, where there was a reduction in concentration by 8.5%. It is also possible to summarise the positive effect of preparation BIOA-11 (Fenyl 7 and carrot macerate in a mixture of surfactant with added citric acid) on the growth parameters of onion seedlings. In repeated tests an increase in leaf weight (by 12-31%) and leaf length (by 5-8%) was verified in comparison with the control variant. Accelerated production through an appropriate bio-additive, leading to a reduction in the pre-cultivation period could have, especially in the spring period, marked economic benefits for growers. It would be of benefit in future experiments to verify to what extent the effects on seedlings will be manifested in the ensuing vegetation.

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Genetic variability and correlation studies in grapes (*Vitis vinifera* L.) in Leh District of Jammu and Kashmir

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All relevant data are within the paper and its Supporting Information files.

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Abstract: The present investigations on genetic variability and correlation in wild grape accessions were carried out in the most favourable regions of Leh district of Jammu and Kashmir. Fifty wild grape accessions from five different villages of the district were marked and evaluated for important morphological traits i.e. yield and quality parameters. Data on various vegetative, fruit physical and fruit chemical characters were taken. Significant variations were observed for all the characters studied except number of seeds per berry. High genotypic coefficient of variance (GCV) and phenotypic coefficient of variance (PCV) were recorded for yield (21.73 and 22.81), yield efficiency (41.19 and 52.73), bunch weight (21.07 and 21.23), number of berries per bunch (22.31 and 25.28), berry length (21.69 and 22.07), berry weight (21.13 and 21.28) and total sugars (22.69 and 22.70). Out of twenty studied characters, thirteen characters registered high heritability estimates, while five recorded moderate heritability estimates and number of seeds per berry and acidity showed low heritability estimates. However, cane length (59.40), leaf area (31.43), bunch weight (43.16), number of berries per bunch (27.18) and TSS/acid ratio (25.81) exhibited high genetic advance as percentage of mean indicating additive gene effect. Fruit or berry yield was positively and significantly correlated with bunch length (0.652), bunch breadth (0.584), bunch weight (0.946), number of berries per bunch (0.672), berry breadth (0.363) and number of seeds per berry (0.612). This study revealed that the characters such as yield, bunch length, bunch weight, number of bunch per vine, berry weight, number of berries per bunch, total soluble solids and total sugars are the most important traits for selecting best grape accessions.

1. Introduction

Grape (*Vitis vinifera* L.) is an economically important and widely cultivated fruit crop in the world and is the first fruit crop to be cultivated by man to produce table fruits, dry fruits, juice and wine (Frederique *et al*., 2010). It is a fairly good source of minerals like calcium, phosphorus, iron and vitamins like B_1 and B_2. The area under grape cultivation in India is 118.700 hectares with production of 2585.300 kg (NHB, 2015). In Jammu and Kashmir, grapes are grown over an area of 315 ha with annual production of 1299 MT (Department of Horticulture, 2016). The grapes are

quite heterozygous and seedling off-springs exhibit wide genetic variability not only in fruit quality but also in vegetative vigour. Because of these variations, seeds are not used for propagation of vines meant for commercial purpose. More than 9,600 grape cultivars exist around the world (Galet, 2000) and as per international variety catalogue almost 16,000 prime names appear in the genus *Vitis* (Maul and Eibach, 2003). To preserve the current genetic pool and to use it judiciously, it is necessary to evaluate the extent of this diversity by identification and distinction of grape accessions, as well as the determination of genetic relationship between local cultivars and wild relatives (Negrul, 1973). Wild grapevines (*Vitis vinifera* L.) are heavily threatened in their natural habitats and high priority is given to the collection and preservation of this germplasm (Forneck *et al*., 2003). Indeed, the preservation of wild populations is considered essential for the maintenance of genetic variability and the resistance to genetic erosion (Cunha *et al*., 2009).

Grape is grown under a variety of soil and climatic conditions in three distinct agro-climatic zones namely mild humid tropical region, sub-tropical region and dry temperate region. Under dry temperate region of Jammu and Kashmir the maximum area is in lower belt of Leh district (Angchok *et al.*, 2009). During the early period, when Ladakh was the transit point on the Central Asian trade route, the traders, nomads and invaders from Yarkand, Baltistan, Punjab, Kashmir, China and Tibet used to pass through this region (Jolden, 2012) and the grape vines got disseminated or introduced in the lower belt of Leh district. The cultivation of grapes in Leh offers a greatest advantage by being ready for harvesting during the month of August-September which is an off season for the rest of the country and can therefore fetch higher remuneration. Keeping in view the economic importance of grapes and to boost its cultivation in the Leh District, the present investigation was carried out to generate the vital information on the existing germplasm of grape vine in the Leh district and selection of elite clones for further multiplication and distribution among the farmers.

2. Materials and Methods

Experimental material and area

Thorough surveys of different areas of Ladakh region was carried out during 2014 and out of a large population of wild grape only fifty accessions were selected from five different villages [Warseedo (4), Achinathang (21), Yokmathang (7), Hanuthang (5) and Dha (13)] of Leh district. The vines were marked on the basis of health, vigour, bearing habit and desirable berry characters. All the marked vines were of seedling origin ranging an age of between 20 to 60 years. The weather and geographical features of the studied location are represented in figure 1.

Fig. 1 - Weather and geographical features of the area surveyed.

Observations recorded

Observations were recorded on vegetative, foliage, fruit physical and chemical characters. Data was recorded on the various characters such as cane length (m), cane diameter (cm), internodal length (cm), leaf area (cm^2), yield (kg/vine). Yield efficiency was calculated as per Westwood (1993).

$$\text{Yield efficiency (kg/cm}^2\text{)} \frac{\text{Yield}}{\text{Trunk cross selectional area}}$$

Where, Trunk cross sectional area (TCSA) = $\text{girth}^2/4\ \pi$. With respect to bunch and berry physical characters i.e. bunch length (cm), bunch breadth (cm), berry length (cm) and berry breadth (cm) were recorded with the help of vernier caliper. For these characters randomly ten bunches and ten berries were taken from the vines. Randomly selected samples were subjected to bunch weight (g) and berry weight (g) with the help of digital weighing balance. All bunches from the vine were counted and number of bunches per vine were observed while for number of berries per bunch randomly ten bunches were taken and counted. Number of seeds per berry was also counted. Berry chemical characters i.e. TSS (°B), acidity (%), total sugars (%), TSS/acid ratio, and juice content (%) were observed as per the standard procedure as given in AOAC (1998).

Statistically analysis

Data collected on various parameters were statistically analyzed as per the procedure given by Snedecor and Cochran (1994). The genotypic coefficients of variation and heritability (in broad sense) were calculated (Singh and Chaudhary, 1979) while genetic advances were estimated as per the procedure of Johnson *et al*. (1955).

3. Results and Discussion

Wide range of variation was observed in all the studied characters among different accessions. The extent of variability was measured in terms of range, mean, standard deviation, coefficient of variation, PCV, GCV, heritability, genetic advance and genetic gain (Table 1). Cane length and cane diameter varied from 115.82 cm (GL-49) to 228.43 cm (GL-27) and 1.21 cm (GL-2) to 2.70 cm (GL-13) with a mean value of 156.27 cm and 1.67 cm, respectively. The standard deviation and coefficient of variation for cane length was 29.34 and 18.78 per cent whereas for cane diameter was 0.29 and 16.87 per cent. Maximum and minimum values for intermodal length were recorded in accessions GL-1 (29.93 cm) and GL-23 (10.50 cm) with a mean value of 23.76 cm. Accession GL-31 (124.67 cm^2) recorded minimum leaf area while GL-14 (184.00 cm^2) recorded maximum leaf area. Average mean value for leaf area recorded was 148.62 cm^2 and coefficient of variation was 10.89 per cent. Kadu *et al*. (2007) also reported variation in leaf area as well as in vine vigour among fifteen grape cultivars. Maximum yield was observed in accession GL-15 (23.16 kg/vine) whereas accession GL-39 (11.02 kg/vine) scored minimum yield with a average mean value of 13.33 kg/vine. Thakur *et al*. (2008) and Joshi *et al*. (2015) also reported similar results with respect to yield. Yield efficiency varied from 0.02 kg/cm^2 (GL-9, GL-42 and GL-43) to 0.19 kg/cm^2 (GL-33) however mean value for yield efficiency was 0.08 kg/cm^2 (Table 1 and Fig. 2). Standard deviation and coefficient of variation for yield and yield efficiency was 2.94 and 22.07 per cent and 0.04 and 42.85 per cent, respectively. The high coefficient of variation obtained in case of yield and yield efficiency may be due to variation in the age of vine and other yield attributes such as height of the vine and vine girth.

With respect to bunch and berry characters all the accessions had wide variation. Maximum bunch length (23.50 cm) and bunch breadth (13.07 cm) was registered in GL-1 accession whereas minimum bunch length was recorded in GL-9 (11.58 cm) and minimum bunch breadth in GL-32 (7.03 cm) (Table 1). Average mean, standard deviation and coefficient of variation for bunch length was 18.30 cm, 2.84 and 15.52 per cent, respectively and for bunch breadth these values were 9.68 cm, 1.70 and 17.56 per cent, respectively. Accession GL-37 recorded minimum (76.70 g) bunch weight whereas maximum bunch weight was recorded in GL-15 (155.50 g). Average mean value and coefficient of variation for bunch

Table 1 - Genetic variability components for major characters in various grape accessions selected in Leh district

Characters	Range	Mean	SD	CoV	Coefficient of variance (%)		Heritability (%)	Genetic advance	Genetic gain (%)
					GCV	PCV			
Cane length (cm)	115.82-228.43	156.27	29.34	18.78	18.69	18.94	97.4	59.4	38.01
Cane diameter (cm)	1.21-2.70	1.67	0.29	16.87	17.02	17.47	94.9	0.57	34.13
Internodal length (cm)	10.50-29.93	23.76	4.76	20	19.92	20.24	96.9	9.59	40.36
Leaf area (cm^2)	124.67-184.00	148.62	16.2	10.89	10.73	11.22	91.5	31.43	21.15
Yield (kg/vine)	11.02-23.16	13.33	2.94	22.07	21.73	22.81	90.8	5.68	42.61
Yield efficiency (kg/cm^2)	0.02-0.19	0.08	0.04	42.85	41.19	52.73	61	0.05	62.5
Bunch length (cm)	11.58-23.50	18.3	2.84	15.52	15.16	15.95	90.3	5.43	29.67
Bunch breadth (cm)	7.03-13.07	9.68	1.7	17.56	16.93	18.77	81.3	3.05	31.51
Bunch weight (g)	76.70-155.50	100.19	21.16	21.11	21.07	21.23	98.5	43.16	43.08
No. of bunches/vine	113.00-152.00	134.38	9.89	7.35	6.94	8.14	72.8	16.39	12.2
No. of berries/bunch	38.00-110.00	67.02	15.64	23.33	22.31	25.28	77.9	27.18	40.56
Berry length (cm)	0.81-2.06	1.12	0.24	21.42	21.69	22.07	96.5	0.49	43.75
Berry breadth (cm)	0.80-1.64	1.05	0.17	15.23	15.96	16.31	95.7	0.34	32.38
Berry weight (g)	0.92-1.92	1.37	0.29	20.59	21.13	21.28	98.6	0.59	43.07
Number of seeds/ berry	1-feb	1.21	0.3	21.7	19.83	32.74	36.7	0.3	24.79
TSS (^oB)	13.70-23.10	20.15	2.03	10.01	9.95	10.25	94.2	4.01	19.9
Acidity (%)	0.15-0.27	0.21	0.03	9.52	11	16.25	45.9	0.03	14.29
Total sugars (%)	5.81-15.62	10.93	2.48	22.7	22.69	22.7	99.9	5.11	46.75
TSS/acid ratio	62.48-137.33	97.35	17.75	17.97	16.53	21.23	60.6	25.81	26.51
Juice content (%)	60.00-93.33	77.7	8.7	11.18	11.09	11.4	94.7	17.27	22.23

weight was 100.19 g and 21.11 per cent. Havinal *et al.* (2008) and Mukhtar *et al*. (2011) also reported similar results for bunch weight while studying twelve grape cultivars, however Kamiloglu and Polat (2009) observed much higher bunch weight which might be due to the age and cultural practices adopted. Number of bunches per vine and number of berries per bunch ranged between 113 (GL-10) and 152 (GL-1) and 38 (GL-18) and 110 (GL-1) with a mean value of 134.38 and 67.02, respectively. Coefficient of variation for number of bunches per vine and number of berries per bunch was 7.35 and 23.33 per cent, respectively. The difference in the yield per vine in different grape cultivars might be due to differences in weight of the bunch, number of bunches, weight of the berries and age of the vines besides their successful adoption to the varying agro-climatic conditions under which they are cultivated (Havinal *et al*., 2008).

GL-11 recorded maximum berry length (2.06 cm) whereas GL-32 and GL-33 recorded minimum berry length (0.81 cm) with a mean value of 1.12 cm and coefficient of variation was 21.42 per cent. GL-29 registered maximum berry breadth (1.64 cm) however minimum was registered in GL-32 (0.80 cm) with average mean value and coefficient of variation of 1.05 cm and 15.23 per cent. Berry weight varied from 0.92 g (GL-4 and GL-33) to 1.92 g (GL-45) (Table 1 and Fig. 2). Average mean value and coefficient of variation for berry weight was 1.37 g and 20.59 per cent. Similar values for berry characters were also reported by Al-Shawish (2010). Number of seeds per berry ranged between 1 and 2 with mean value of 1.21. Out of fifty studied accessions, forty accessions were with one seed and only ten accessions had two seeds. Coefficient of variation for number of seeds per berry was 21.70 per cent.

Total soluble solids ranged between 13.70°B (GL-41) to 23.10°B (GL-43) whereas acidity varied from 0.15 (GL-40) to 0.27 per cent (GL-18). Average mean value for total soluble solids and acidity was 20.15°B and 0.21 per cent whereas coefficient of variation was 10.01 per cent and 9.52 per cent (Table 1). Thakur *et al*. (2008) and Jiang *et al*. (2012) also reported similar range for TSS in the commercial cultivars of grape. Maximum total sugar was recorded in GL-30 and GL-31 (15.62%) and minimum was recorded in GL-42 (5.81%) with a average mean and coefficient of variation of 10.93 per cent and 22.70 per cent. GL-41 (62.48) recorded minimum TSS/acid ratio whereas GL-13 (137.33) recorded maximum TSS/acid ratio. Juice content ranged between 60 per cent (GL-32) to 93.33 per cent (GL-1, GL-12 and GL-29). Mean values and coefficient of variation for TSS/acid ratio was 97.35 per cent and 17.97 per cent whereas these values for juice content was 77.70 per cent and 11.18 per cent. Sharma and Bist (1993) and Ghosh *et al.* (2005) also reported similar results for TSS, total sugars and juice content while evaluating eight local accessions of dry temperate areas of Himachal Pradesh.

A perusal of the data revealed that the magnitude of the PCV was higher than GCV for all the characters. The estimates of PCV and GCV were high for yield (21.73 and 22.81), yield efficiency (41.19 and 52.73), bunch weight (21.07 and 21.23), number of berries per bunch (22.31 and 25.28), berry length (21.69 and 22.07), berry weight (21.13 and 21.28) and total sugar (22.69 and 22.70) indicated the presence of adequate genetic variation among the genotypes and suitability of these attributes for further improvement by selection. Bist and Sharma (1995) and Gupta *et al*. (2015) also reported high phenotypic and genotypic coefficient of variance for bunch weight, berry weight and yield characters. PCV was high and GCV was moderate for number of seeds per

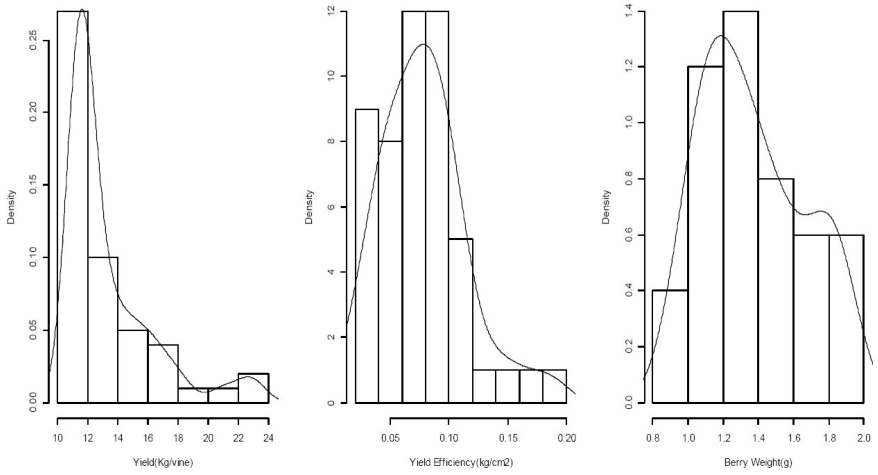

Fig. 2 - Yield, yield efficiency and berry weight in various grape accessions selected in Leh district.

berry. GCV in general, were lower than the PCV (Table 1) which indicated close association between genotype and phenotype. High value of PCV and GCV indicated the existence of substantial variability, ensuring ample scope for their improvement through selection. These results further confirmed with the findings of earlier researchers for Kumar *et al*. (2002) and Wei *et al*. (2003). Low GCV and PCV were recorded for leaf area (10.73 and 11.22), number of bunch per vine (6.94 and 8.14) and total soluble solids (9.95 and 10.25). The estimates of PCV and GCV for leaf area, number of bunch per vine and total soluble solids were low in magnitude yet these were close to each other indicating little effect of environment in the inheritance of these traits.

Heritability is a parameter of huge importance to the breeders as its magnitude indicates the reliability with which a genotype can be recognized through its phenotype expression (Table 1). Most of the characters studied had high heritability estimates, however they are moderate for yield efficiency (61.0%), number of bunches per vine (72.8%), number of berries per bunch (77.9%), and TSS/acid ratio (60.6%) and low for number of seeds per berry (36.7%) and acidity (45.9%). The heritability of highest magnitude was noticed for total sugar (99.9%) closely followed by berry weight (98.6%) and bunch weight (98.5%). High estimates of heritability in broad sense indicate that substantial improvement can be made using standard selection procedures. High heritability estimates for yield, bunch weight, berry weight, TSS were observed by Kumar *et al*. (2002), Wei *et al.* 2003 and Gupta *et al*. (2015) and are in consonance with the present study. Heritability estimates alone are not an ideal parameter for predicting the effect of selecting the desired individual. Heritability estimates along with genetic advance are more useful than heritability value alone in predicting the selection of best individuals. In the present investigations cane length (97.4 and 59.40), leaf area (91.5 and 31.43) and bunch weight (98.5 and 43.16) exhibited high genetic advance as a percentage of mean along with high heritability. These results indicated the influence of additive gene action and hence these characters are likely to respond to selection. High heritability and low genetic advance were observed for yield (90.8 and 5.68), berry length (96.5 and 0.49), berry breadth (95.7 and 0.34), berry weight (98.6 and 0.59) and TSS (94.2 and 4.01) which may be attributed to the non additive gene effects and these traits can be improved through hybridization and use for hybrid vigour. Number of seeds per berry (36.7 and 0.30) and acidity (45.9 and 0.03) showed low heritability associated with genetic advance indicating the role of non additive gene for these traits suggesting thereby that their improvement could be achieved through heterosis breeding.

A highly significant positive correlation was recorded between bunch length and bunch breadth (0.953), bunch weight (0.727) and number of berries per bunch (0.421) resulting in getting high yields (Table 2). Cane length was highly significant and positive correlated with yield (0.356), bunch length (0.356) and bunch weight (0.447). Yield registered

Table 2 - Correlation coefficients (Genotypic) in different characters in various grape accessions selected in Leh district

Characters	Cane lenght (cm)	Cane diamter (cm)	Inter-nodal lenght (cm)	Leaf area (cm^2)	Yield (kg/ vine)	Yield effi-ciecency (kg/cm^2)	Bunch lenght (cm)	Bunch breadth (cm)	Bunch weight (g)	No. of bunches/ vine	No. of berries/ bunch	Berry lenght (cm)	Berry breadth (cm)	Berry weight (g)	No. of seeds/ berry	TSS (°B)	Acidity (%)	Total sugar (%)	TSS/ acid ratio	Juice content (%)
Cane lenght (cm)	1.000	0.272	0.122	0.244	0.356**	-0.520**	0.356**	0.254	0.447**	-0.174	0.156	0.202	0.287*	0.274*	0.224	-0.01	0.268	-0.224	-0.225	0.409**
Cane diameter (cm)		1.000	0.001	0.389**	0.353*	-0.336*	0.135	0.052	0.359**	0.163	0.318*	0.262	0.266	0.023	0.428**	-0.067	-0.144	-0.226	0.099	0.465**
Internodal lenght (cm)			1.000	0.309*	0.333*	-0.052	0.328*	0.353*	0.315*	0.092	0.177	0.219	0.227	0.13	0.336*	0.027	0.056	0.154	-0.04	0.12
Leaf area (cm2)				1.000	0.315*	-0.106	0.286*	0.197	0.326*	0.071	0.172	0.222	0.198	0.137	0.26	-0.025	0.04	-0.288*	0.008	0.326*
Yield (kg/vine)					1.000	0.049	0.652**	0.584**	0.946**	0.289*	0.672**	0.337*	0.363**	0.173	0.612**	-0.024	0.052	-0.148	-0.058	0.535**
Yield efficiency (kg/cm^2)						1.000	-0.025	-0.011	-0.094	0.377**	0.202	-0.201	-0.278*	-0.308*	-0.233	-0.07	-0.101	0.115	0.039	-0.284*
Bunch lenght (cm)							1.000	0.953**	0.727**	-0.196	0.421**	0.144	0.219	0.254	0.342*	0.159	-0.012	-0.255	0.096	0.435**
Bunch breadth (cm)								1.000	0.639**	-0.206	0.294*	0.196	0.299*	0.309*	0.404**	0.163	0.151	-0.248	-0.035	0.408**
Bunch weight (g)									1.000	-0.018	0.647**	0.383**	0.430**	0.263	0.648**	0.021	0.013	-0.293*	-0.004	0.583**
Number of bunches/vine										1.000	0.209	0.173	0.035	-0.26	0.091	-0.302*	0.048	0.234	-0.23	-0.066
Number of berries/bunch											1.000	0.261	0.301*	-0.603**	0.530**	-0.129	0.031	-0.129	-0.11	0.282*
Berry lenght (cm)												1.000	0.945**	0.064	0.389**	0.008	0.144	-0.24	-0.109	0.396**
Berry breadth (cm)													1.000	0.08	0.465**	0.031	0.147	-0.242	-0.107	0.456**
Berry weight (g)														1.000	0.089	0.162	-0.055	-0.156	0.141	0.264
Number of seeds/berry															1.000	0.13	0.274*	-0.214	-0.166	0.566**
TSS (°B)																1.000	-0.102	0.323*	0.665**	-0.086
Acidity (%)																	1.000	-0.097	-0.808**	0.266
Total sugar (%)																		1.000	0.255	-0.305*
TSS/acid ratio																			1.000	-0.22
Juice content (%)																				1.000

highly significant and positive correlation with bunch length (0.652), bunch breadth (0.584), bunch weight (0.946), number of berries per bunch (0.672), berry breadth (0.363) and number of seeds per berry (0.612) while showed simple positive correlation with number of bunches per vine (0.289) and berry length (0.337) depicting that all these characters are yield contributing characters. Kliewer and Dokoozlia (2000) and Gupta *et al*. (2015) also reported positive and significant correlation between yield and bunch length, bunch breadth, bunch weight. Bunch weight showed positive and highly significant correlation with number of berries per bunch (0.647), berry length (0.383), berry breadth (0.430) and number of seeds per berry (0.648). A highly significant but negative correlation was observed between number of berries per bunch and berry weight (-0.603). Berry length and berry breadth showed highly significant and positive correlation with number of seeds per berry (0.389 and 0.465) and juice content (0.396 and 0.456). Acidity was highly but negatively correlated with TSS/acid ratio (-0.808). These results are in conformity with those reported by Kumar *et al*. (2002) and Gupta *et al*. (2015) who advocated that the importance should be given to bunch length, bunch breadth, number of bunches per vine, number of berries per vine, berry weight during selection process because these characters contribute towards the yield.

4. Conclusions

Most of the traits under study showed significant variations from low to high magnitude to heritability and genetic advance. These can facilitate selecting and utilizing the most preferred traits of interest and also hint the potential of grape for further improvement. Some traits with high phenotypic and genotypic coefficient of variation those are detrimental which make possible grape improvement. A significant positive correlation of economic traits like bunch length, bunch weight, number of berries per bunch, berry breadth, number of seeds per berry with yield was recorded suggesting that selection for these characters would lead to crop improvement.

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'Superior Seedless' grafted on three selected grapevine rootstocks grown on calcareous soil under diluted brackish water irrigation. I. Growth performances

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Key words: alternate irrigation, chlorophyll content, 41B, leaf area, P1103, R110, salinity, shoot length.

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Abstract: Mixing brackish water with conventional quality water for irrigation in ratios to maintain satisfactory vigor of grapevines might be a feasible management practice. The objective of this study was to evaluate the performance of three grape rootstocks that are used worldwide and locally; R110, 41B and P1103, irrigated with three salinity levels: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. A randomized complete block design was used with three blocks of 12 pots each. 'Superior Seedless' grafted on P1103 showed better performance regarding chlorophyll content, stem length and number of young leaves and even growth after bud break. It does seem that grapevine rootstocks that have either *V. rupestris* or *V. berlandieri* in their parentage are good candidates for salinity tolerance. It can be concluded that irrigation with diluted brackish water can be practiced for a certain period of time (two months from April to June); according to our findings under conditions of the experiment, to be followed by irrigation with good quality water in order to flush excessive salts out of the root zone.

1. Introduction

Worldwide, during the last two decades, effluent and saline water reuse for irrigation purposes has been becoming an increasingly common practice (Bustan *et al.*, 2005; Paranychianakis and Angelakis, 2008). However, effluent and saline water irrigation is also associated with potential disadvantages such as, spreading of infectious diseases, nutrients release to the environment and salt accumulation (Bustan *et al.*,

2005; Niu *et al.*, 2008). Salt accumulation in the soil profile causes adverse effects on many crop yields; mainly by causing osmotic stress. Thus, salt tolerant crops, cultivars or genotypes must be chosen to ensure that salinity-induced damage and/or yield reduction are minimal. Mixing low quality water (e.g. brackish water) with conventional quality irrigation water in ratios to keep the salinity of the irrigated soils below the threshold of the target crop might be an acceptable management practice and was reported by many researchers (e.g. Abdel Gawad and Ghaibeh, 2001).

Considerable yields were obtained using saline irrigation water (4-12 dS m^{-1}) in crops that had been previously defined as moderately sensitive to salt stress (Bustan *et al.*, 2004). In some crops (e.g., tomato) the reduction in the fresh yield was compensated by an increase in fruit dry weight and other quality parameters (Mizrahi *et al.*, 1988). Bustan *et al.* (2005) reported that the combination of fresh irrigation water (1.2 dS m^{-1}) and brackish water (7 dS m^{-1}) increased the yield level of melon in comparison to that of fresh water plants. In addition to the effects of salinity on crop yield, growth, leaf chlorophyll and mineral content are also affected. In this regard, vines that were grafted showed less Cl^- in the leaves compared to vines on their own roots (Walker *et al.*, 2004). Among the rootstocks studied, P1103 was the best chloride excluder based upon the results of leaf chloride concentrations. On the other hand, despite that own rooted 'Sultana' vines accumulated more Cl^- and Na^+ in the leaves, they were considered to better tolerate salinity conditions compared to those grafted based upon accumulating more dry matter (Fisarakis *et al.*, 2001).

There is great debate regarding the tolerance of grapevines to salinity. Moreover, contradictions can be found in the literature in terms of the salt tolerance of grapevine rootstocks implying that different factors are involved, which eventually determine grapevine response to salt stress. Southey and Jooste (1991) found that American hybrids performed poorly in response to salinity when used as rootstocks for the cultivar 'Colombard'. In addition, Cavagnaro *et al.* (2006) concluded that Argentinean cultivars performed better than European cultivars in an *in vitro* salinity evaluation study. Regarding differences in ranking rootstocks, Dardeniz *et al.* (2006) indicated that 41B was the most salt resistant rootstock, followed by 140Ru and P1103, and the least resistant was 5 BB. On the other hand, a previous study showed that the highest salt resistance was obtained when P1003 was used as a rootstock (Walker *et al.*, 2002). Our hypothesis is that alternate irrigation with diluted brackish water followed by irrigation with good quality water could result in, on one hand, saving fresh water for other uses and, on the other hand, establishing good vineyards. In addition, American rootstocks could perform differently under diluted brackish irrigation water. Therefore, the objective of this study is to evaluate the vigor performance of 'Superior Seedless' cultivar grafted on three pot grown rootstocks: P1103, R110 and 41B under different diluted brackish water irrigation levels to detect a suitable irrigation period with diluted brackish water, without showing adverse effects on growth.

2. Materials and Methods

Plant and soil material

Three grape rootstocks were evaluated in this study: R110 (*Vitis berlandieri* x *V. rupestris*), 41B (*V. berlandieri* x *V. vinifera*) and P1103 (*V. berlandieri* x *V. rupestris*). The rootstocks were purchased from Les Pépiniéristes du Comtat, Sarrians, France. After being imported, *Vitis vinifera* 'Superior Seedless' was grafted on the rootstocks in a local nursery; (Al-Bushra Nurseries, May, 2013). Grafted plant materials were planted in polyethylene bags filled with peatmoss.

The one year old grafted grapevine rootstocks were grown for several months to allow for the formation of a well-developed root system before applying treatments. Fertilizers and fungicides were applied as necessary.

The soil was brought from the southern Jordan Valley. The soil was relatively saline with an EC of 3.88 dS m^{-1}. Such soils are common in the Jordan Valley under the agricultural practices applied by farmers. Soil was crushed and sieved through 1 cm sieve and plastic pots (the working volume of the pots was 44 L) were filled with 50 kg each in order to roughly have a bulk density of 1.14 g cm^{-3}. A soil sample was taken to be analyzed for texture and some chemical properties (Table 1). The pots were placed in a controlled greenhouse. Grafted grapevines were transplanted in February and the growth was unified based on the number of buds and root length. The root system was cut back to 15 cm in length and the vegetative system was cut back to eight buds.

Table 1 - Chemical properties of the soil brought from the southern Jordan Valley

Soil property	Value
Soil texture	Clay
pH	8.25
EC 1:1	3.88 dS m^{-1}
Organic matter	1.99%
Ca	761.52 mg kg^{-1} soil
Mg	121.52 mg kg^{-1} soil
$CaCO_3$	450.00 g kg^{-1} soil
Soluble K	29.00 mg kg^{-1} soil
Soluble Na	85.00 mg kg^{-1} soil
Olsen-P	5.99 mg kg^{-1} soil
SO_4	27.73 mg kg^{-1} soil
Cl	1489.66 mg kg^{-1} soil

Irrigation treatments and experimental design

Brackish water was brought from Al-Karameh dam located in the Jordan Valley and stored in a galvanized tank. A water sample was taken to be analyzed for some chemical properties (Table 2). Three levels of irrigation water salinity, in terms of electrical conductivity (EC), were applied: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. The treatments were prepared by mixing the dam water with tap water. A portable conductivity meter (Model Cond 3210, WTW, Germany) was used to measure the EC and to obtain the determined salinity levels. A randomized complete block design was used with three blocks of 12 pots each. The grafted grapevines started to break the dormancy period during spring. Composite fertilizer (20: 20: 20), urea and ammonium sulfate were applied to the grapevines and growth was again unified before applying the assigned treatments. Irrigation with the assigned treatments started in May. All pots received the same amount of water whenever irrigation was applied. Each pot received a total amount of irrigation water equal to 446 mm. Irrigation was scheduled according to evaporation readings from free water surface (in mm) taken every 48 hours and corrected using proper grapevine crop coefficient of 0.30 (according to Food and Agriculture Organization of the United Nations- FAO).

Table 2 - Chemical analysis of brackish water used for irrigation in the current research

Water properties	Value
pH	8.62
EC	15.43 dS m^{-1}
Cl	6098.00 mg l^{-1}
Ca	801.60 mg l^{-1}
Mg	710.53 mg l^{-1}
K	144.45 mg l^{-1}
Na	2335.00 mg l^{-1}

Chlorophyll content and growth parameters

A SPAD-502 purchased from Minolta CO., LTD, Japan, was used to measure the chlorophyll content of fully expanded matured leaves before irrigation with brackish water on April, 2014 and after irrigation with water on June, 2014 and November, 2014. Shoot length, leaf area and number of newly formed leaves were recorded three times during the growing season of 2014 on June, August and October.

Leaf sampling and analysis

Fully expanded mature leaf samples were taken in June, August and October, 2014. Leaf fresh and dry weights were determined. Leaf water percentage was determined gravimetrically. After being dried, the leaf samples (5 leaves/plant) were analyzed for K, P, Na, Mg and Ca. Leaf area was also determined in November, 2014 by using a leaf area meter (AM300, Bioscientific Ltd., UK). A Cintra 5 spectrophotometer (GBC Scientific Equipment, Australia) was used for analyzing P. Flame photometer (Jenway, Germany) was used for analyzing K and Na. Ca and Mg were analyzed by titration with EDTA. Chloride was analyzed by titration with $AgNO_3$. Digestion and analysis methods followed the procedures of Estefan *et al.* (2013).

Soil analysis

The soil was analyzed for P, K, Ca, Mg, Na, Cl, pH and EC (1:1 soil: water extract) using the same previously mentioned instruments and procedures.

Statistical analysis

All statistical analyses were performed using SAS/STAT Version 9.2 and Analysis of Variance (ANOVA) was conducted by the PROC GLIMMIX procedure.

3. Results and Discussion

Soil and water analysis

One-year old grapevine rootstocks were grown on a calcareous clayey soil (45% $CaCO_3$). Soil chemical properties are presented in Table 1. The soil used in the current study is saline with relatively high organic matter. Such soils are common under agricultural practices applied in the Jordan Valley. In the Jordan Valley, farmers used to annually add organic materials, a practice that contributed to elevated salinity level. Plants were regularly irrigated with diluted

brackish water at three salinity levels. The water chemical properties of the brackish water are presented in Table (2).

Brackish water used in the current study is extremely saline with total dissolved solids of approximately 9875 mg l^{-1}. Chloride formed approximately 60% of the ionic composition of the brackish water (before dilution) followed by sodium, which formed approximately 23%. Such high EC and Cl and Na concentrations justified the use of diluted brackish water obtained by diluting the latter with tap water in order to prepare irrigation water with three salinity levels as previously mentioned. The maximum salinity level of irrigation water was 5 dS m^{-1} electrical conductivity to avoid the buildup of salts in the growth medium far beyond the threshold EC of grapevines, which is approximately 2-3 dS m^{-1}.

SPAD chlorophyll reading

The analysis of variance of the chlorophyll content of fully expanded mature leaves showed no interaction effect among rootstock, salinity and time. Meanwhile, a significant rootstock by salinity interaction at ($P \leq 0.05$) was detected only in November as well as an expected salinity by time interaction ($P \leq 0.05$). Diluted brackish water effects on 'Superior Seedless' chlorophyll content were clearly observed in June and November (Table 3). In April, no interaction was noticed among rootstock-salinity combinations due to the fact that the soil used is originally saline and no considerable salt build up was expected yet as a result of irrigation. If non-saline soil was used, findings would not be of practical significance. In addition, brackish water was diluted before being used for irrigation. Salt build up could be expected if irrigation was practiced for longer period of time. However, salt build up is not favorable because it will adversely affect grape yield, which was not measured under the conditions of our experiment.

Table 3 - The effect of brackish water treatments on the average 'Superior Seedless' chlorophyll content measured in June and November for the three rootstocks

Treatment	June	November
R1 C	28.5±1.0	27±1.0
R2 C	27.6±1.2	27.6±1.1
R3 C	25.8±1.9	24.6±1.5
R1 S1	25.7±1.2	24.1±1.3
R2 S1	26.8±1.3	25.8±1.3
R3 S1	26.6±2.2	23.7±0.6
R1 S2	23.1±1.8 (3.3) [z]	22.9±1.6 (3.5)
R2 S2	23±1.3 (4.7)	22.5±1.0 (5.2)
R3 S2	25.1±1.1 (3.9)	22.2±1.2 (6.8)
R1 S3	20.5±0.7 (6.7)	18.8±0.7 (8.4)
R2 S3	23.8±3.2 (4.9)	12.9±3.6 (15.8)
R3 S3	23±2.7 (7.4)	18.1±0.5 (12.3)

R1 = P1103, R2 = 41B, R3 = R110.
C = Control 0.8 dS m^{-1}, S1 = 1.5 dS m^{-1}, S2 = 3.0 dS m^{-1}, S3 = 5.0 dS m^{-1}.
[z] = The number in parenthesis indicates the reduction in the SPAD reading compared to the reading before treatment in April.

As expected, chlorophyll content levels decreased for all three rootstocks (Table 3). However, 'Superior Seedless' showed higher chlorophyll content when grafted on P1103 compared to R110 and 41B in November at salinity level of 5.0 dS m^{-1} (Table 3). The highest reduction in 'Superior Seedless' chlorophyll content was observed when grafted on rootstock 41B, followed by R110 and the least reduction was observed for 'Superior Seedless' grafted on P1103.

The influence of salinity levels varied with time and rootstock (Table 3). Chlorophyll content of R1 (P1103) and R2 (41B) was adversely affected in June particularly at S2 and S3. In November, the adverse effect of salinity levels on R1 and R2 at S2 and S3 was even more obvious. However, R3 (R110) was mainly affected by S3 particularly in November. This might suggest that diluted brackish water can be used for irrigation for a short period of time (i.e. from April to June) followed by irrigation with better quality irrigation water. This can be true mainly for R3 (R110) followed by R2 (41B) and for a longer period for R1 (P1103).

Additional supply of Ca^{+2} can prevent the toxic effects of Na^{+} on leaf photosynthesis (Montesano and Van Iersel, 2007). It was indicated that the effect of salinity on leaf chlorophyll content is ion-specific and not due to a decrease in the osmotic potential of the nutrient solution. In our study, 'Superior Seedless' showed higher calcium ion content in both plant tissues and soil when grafted on P1103 compared to R110 and 41B. The mean 'Superior Seedless' leaf Ca content (mg g^{-1} dry weight) grafted on the three rootstocks at 5.0 dS m^{-1} measured in October was 29.40 when grafted on P1103; whereas it was 26.00 and 19.80 when grafted onto 41B and R110, respectively (Table 4). Meanwhile, the soil Ca content (mg g^{-1} soil) for the three rootstocks at 5.0 dS m^{-1} measured in October were as follow: 5.2, 4.7 and 4.5 when grafted on P1103, 41B and R110, respectively. This might explain the relatively better performance of P1103 under the conditions of the current study.

Moreover, 'Superior Seedless' grafted onto 41B showed a higher leaf Mg content in October, however, the reduction magnitude in leaf Mg content in October compared to June was the least in 'Superior

Table 4 - The effect of brackish water treatments on the average 'Superior Seedless' Ca leaf content (mg g^{-1} dry weight) grafted on the three rootstocks measured in April, June and November

Treatment	June	August	October
R1 C	40.6±2.9	50.0±6.7	25.2±4.5
R2 C	54.2±10.0	67.8±3.8	27.9±1.9
R3 C	43.0±4.1	46.8±3.3	19.5±2.0
R1 S1	42.5±1.5	42.5±5.0	24.2±1.3
R2 S1	55.8±4.9	56.4±5.1	21.8±5.0
R3 S1	36.1±0.0	41.7±3.7	24.0±1.4
R1 S2	47.8±6.2	58.3±16.0	21.8±4.0
R2 S2	52.9±2.9	74.5±0.8	23.5±3.4
R3 S2	40.9±3.0	46.5±3.4	23.4±3.0
R1 S3	38.2±6.4	59.9±12.9	29.4±9.7
R2 S3	44.9±12.7	72.2±9.4	26.0±5.0
R3 S3	42.5±1.0	56.9±4.0	19.8±1.7

R1 = P1103, R2 = 41B, R3 = R110.
C = Control 0.8 dS m^{-1}, S1 = 1.5 dS m^{-1}, S2 = 3.0 dS m^{-1}, S3 = 5.0 dS m^{-1}.

Seedless' grafted onto P1103 in comparison with R110 and 41B (Table 5), which might also explain the relatively better performance of P1103.

In addition, 'Superior Seedless' grafted onto P1103 showed a higher Mg leaf content at salinity level (S2) in October compared to June. Meanwhile, 'Superior Seedless' leaf Mg content decreased when grafted on 41B and R110 at the same level of salinity.

Stem length and number of leaves

The analysis of variance of stem length after irrigation with brackish water measured in June, August and October detected no interaction effect. However, a salinity effect was observed slightly in October. At the highest salinity level, 'Superior' showed longer stem length when grafted on P1103 compared to R110 and 41B (Table 6). However, the longer 'Superior Seedless' stem was not significantly different when grafted onto R110 but were significantly different when grafted onto 41B.

Table 5 - The effect of brackish water treatments on the average 'Superior Seedless' Mg leaf content (mg g^{-1} dry weight) grafted on the three rootstocks measured in April, June and November

Treatment	June	August	October
R1 C	18.1±3.0	19.3±1.2	7.6±1.9
R2 C	11.5±0.5	13.5±1.4	6.0±2.5
R3 C	11.5±1.9	18.1±3.5	7.9±0.8
R1 S1	12.8±1.1	12.8±4.9	7.6±2.6
R2 S1	13.6±3.3	12.5±1.1	7.5±0.9
R3 S1	14.6±2.2	14.8±0.5	7.9±1.1
R1 S2	11.5±0.2	13.6±1.4	13.6±3.2
R2 S2	12.5±1.6	11.7±0.7	10.2±0.8
R3 S2	10.2±2.2	15.4±3.8	7.8±0.9
R1 S3	11.0±1.0	14.8±1.9	9.2±2.3 (1.8)
R2 S3	15.4±0.4	16.0±0.7	12.8±1.6 (2.6)
R3 S3	11.2±1.8	17.3±2.1	8.4±1.1 (2.8)

R1 = P1103, R2 = 41B, R3 = R110.
C = Control 0.8 dS m^{-1}, S1 = 1.5 dS m^{-1}, S2 = 3.0 dS m^{-1}, S3 = 5.0 dS m^{-1}.
(z) = The number in parenthesis indicates the reduction in the SPAD reading compared to the reading before treatment in April.

Table 6 - The effect 5.0 dS m^{-1} on average stem length (cm) for the three rootstocks measured in October

Rootstock	Stem length (cm)
P1103	110±6.0 a
41B	89±5.0 b
R110	100±10.0 ab

Different letters in a column indicate significant differences at P≤0.05 according to Fisher's Protected LSD.

This indicates that 'Superior Seedless' bud grafted on P1103 showed more growth compared when grafted onto 41B. Another indication of the vigorosity and tolerance of P1103 was the total number of leaves counted in June, August and October. According to the analysis of variance, no interaction effect was detected. However, a main rootstock and salinity effect were detected in June, August and October.

Generally, in the months of August and October and at the levels of S2 (3.0 dS m^{-1}) and S3 (5.0 dS m^{-1}), 'Superior Seedless' bud showed more number of leaves when grafted onto P1103 followed by R110 and the least number was when grafted onto 41B (Table 7). This also supports the vigor and indication of tolerance of P1103. In addition, the results of the total counting coincide well with the shoot length

Table 7 - Effect of treatments on average number of 'Superior Seedless' leaves grafted on the three rootstocks counted in June, August and October

Treatment	June	August	October
R1 C	23.7±3.6	31±3.2	60.3±5.2
R1 S1	17.3±1.5	26.3±0.4	49±5.3
R1 S2	17±0.9	24.3±2.7	36.3±3.3
R1 S3	14.7±1.2	21.7±2.7	31.3±2.5
R2 C	22.3±0.7	27±1.4	51.3±3.6
R2 S1	18.3±1.0	23±1.2	41±5.6
R2 S2	16.7±0.8	20.3±1.8	31±3.9
R2 S3	14.3±0.4	18±0.7	30.7±2.5
R3 C	25.3±3.9	35.3±3.3	62.3±5.7
R3 S1	22.7±2.5	26.7±2.5	49.3±7.8
R3 S2	19.7±1.8	24±2.1	36±3.7
R3 S3	17± 1.2	20.7±1.5	33.7±2.9

R1 = P1103, R2 = 41B, R3 = R110.
C = Control 0.8 dS m^{-1}, S1 = 1.5 dS m^{-1}, S2 = 3.0 dS m^{-1}, S3 = 5.0 dS m^{-1}.

results presented in Table 6.

Leaf area

Regarding the 'Superior Seedless' leaf area, the analysis of variance revealed no interaction or rootstock effect but a salinity effect. The reduction of leaf area by water stress (Gomez-del-Campo *et al.*, 2002) and by salinity treatments is very well documented on various crops such as tomatoes (Montesano and Van Iersel, 2007), olives (Al-Absi *et al.*, 2003) and chrysanthemums (Lee and Van Iersel, 2008). The previous studies indicated the effect of salinity on leaf area is mainly through the osmotic effect of the solution which mainly depends on the total amounts of salts in the nutrient solution. Our findings are consistent with the previous mentioned studies. Leaf area decreased with increasing salinity levels (Fig. 1). However, relatively larger 'Superior Seedless' leaves were noticeable when grafted on P1103 compared to 41B and R110. It is worth mentioning that the reduction of chlorophyll SPAD readings is attributed to the salinity effects, not to the leaf area since both parameters, chlorophyll SPAD and leaf area, were reduced with increasing the salinity levels.

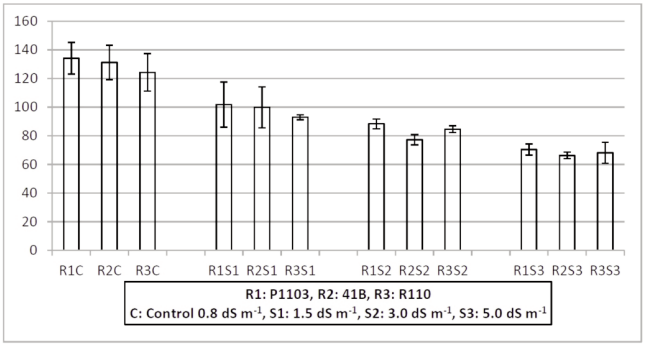

Fig. 1 - The effect of treatments on average 'Superior Seedless' leaf area (cm^2) grafted on the three rootstocks measured on November, 2014.

The vigor of P1103 and higher chlorophyll content, longer shoot and more leaves did not significantly increase the leaf fresh and dry weights (Figs. 2 and 3). Our findings also are in agreement with Walker *et al.* (1997) who found that leaf relative water contents were not affected by rootstock or salinity treatments.

Figure 3 illustrates the effect of the three rootstocks and salinity levels on 'Superior Seedless' leaf water content. Except for salinity level 1 (1.5 dS m^{-1}), 'Superior Seedless' leaves when grafted on P1103 showed relatively higher water content compared when grafted on 41B and R110.

The P1103 rootstock seems to activate a certain mechanism at high soil salinity levels (particularly at

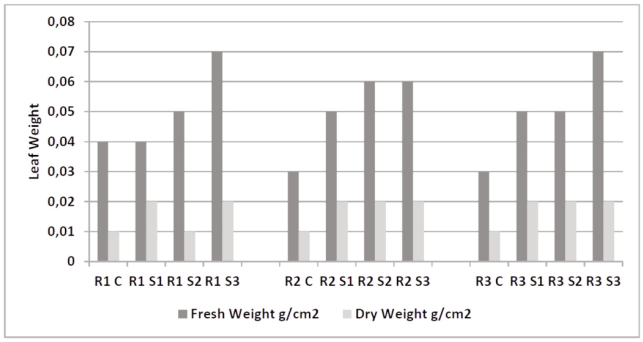

Fig. 2 - The effect of the three rootstocks and salinity on the ratio of 'Superior' leaf fresh and dry weights (g) to leaf area (cm^2).

S2 and S3). On the other side, 41B and R110 were not adversely affected by the salinity levels. Our findings are similar to those of Walker *et al.* (1997) who found that the relative water contents were not affected by rootstock or salinity treatments.

A correlation analysis has been conducted to figure out the major ions that had the greatest effect on soil salinity and consequently on rootstock performance. Results showed that the correlation between soil EC and the concentration of Cl, Na, Ca and Mg ions were 0.97, 0.95, 0.83 and 0.5; respectively. This clearly proved that Cl, Na and Ca were the major ions that influenced the response of rootstocks to soil salinity levels. Such results reflected well the chemical composition of water used for irrigation (Table 2). Many researchers focused on Cl exclusion, such as Walker *et al.* (2004) who reported that P1103 was the best chloride excluder. Our focus will be on Na exclusion by reporting the leaf K: Na ratio.

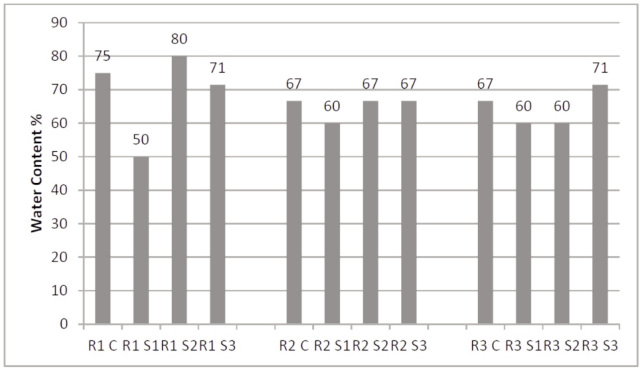

Fig. 3 - The effect of the three rootstocks and salinity levels on 'Superior Seedless' leaf water content.

Concerning the leaf K:Na ratio, as shown in figure 4, it can be seen that in August, the order of the rootstocks is as follows: R110>41B>P1103. However, Na concentration in leaves became significantly higher than that of K (K:Na ratio 1) for R110 and 41B, but particularly for 41B. On the other hand, in October,

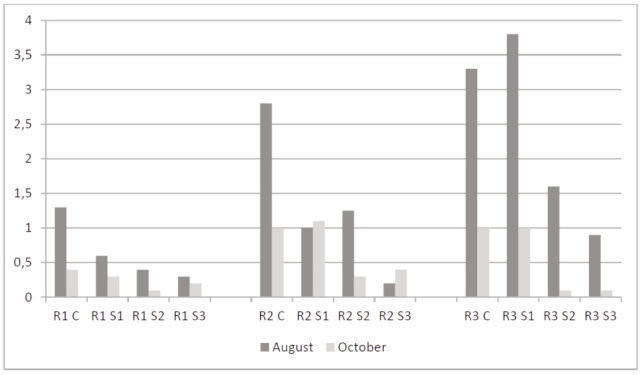

Fig. 4 - 'Superior Seedless' leaf K:Na ratio grafted on the three rootstocks at the different salinity levels measured in August and October.

41B and R110 rootstocks particularly at salinity levels S2 (3.0 dS m^{-1}) and S3 (5.0 dS m^{-1}) showed higher Na concentration in leaves than that of K (very low K:Na ratio). This might indicate that none of the investigated rootstocks is Na excluder; particularly P1103. P1103, which seems to be Na include, took up Na in greater amounts than the other rootstocks starting from earlier stages of treatment applications. This mechanism (Na uptake) could help the grapevine to relief the osmotic stress, by maintaining the water potential gradient and consequently increase water uptake. Jogaiah *et al.* (2014) found that the maximum osmotic potential was recorded on P1103 rootstock. This might explain the relatively higher leaf water content observed in P1103 as mentioned above.

Growth after bud break

In order to obtain data regarding cumulative effects of the treatments on number of 'Superior Seedless' leaves after bud break, total numbers of leaves/vine were counted on April of the following year. Analysis of variance showed that there was no interaction effect however; rootstocks did show such an effect ($P \leq 0.05$). P1103 shows vigorous growth in terms of leaves compared to the other two rootstocks (Fig. 5). Except for the control, there were significant differences between P1103 and the two other rootstocks at the three salinity levels (Fig. 5). This might indicate that P1103 was less affected by diluted brackish water irrigation.

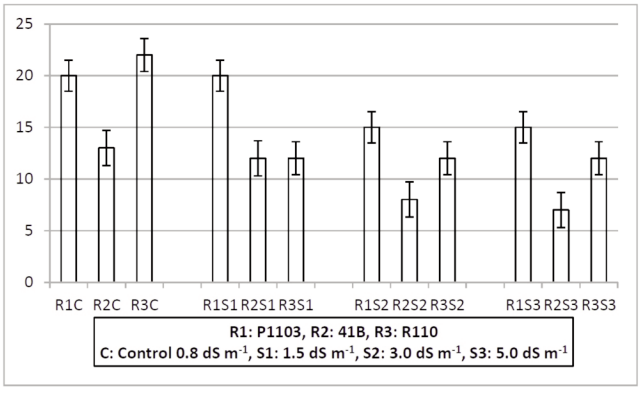

Fig. 5 - The effect of the salinity level treatments on the total number of leaves per grapevine counted in April of the following year for the three rootstocks.

4. Conclusions

Scion is dependent on the rootstock for all things coming from the soil and there could be a considerable effect of rootstock on the vine performance (Creasy and Creasy, 2009). Therefore, rootstock choice should be taken with careful consideration. P1103 (*V. berlandieri* x *V. rupestris*) rootstock seems to be a suitable choice for irrigating with diluted brackish water. Irrigating 'Superior Seedless' vines grafted on P1103 with diluted brackish water, up to 3.0 dS m^{-1}, from April until June could be a practical procedure to be adopted by grape growers in the Jordan Valley. However, irrigation should be followed by irrigation with fresh water especially that by the end of the experiment the soil EC reached approximately 6.0 dS m^{-1}. In addition, our findings coincide well with the findings of many researchers such as (Troncoso *et al.*, 1999; Walker *et al.*, 2002). It does seem that grapevine rootstocks that have either *V. rupestris* or *V. berlandieri* in their parentage are good candidates for salinity tolerance.

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Solar radiation levels modify the growth traits and bromatological composition of *Cichorium intybus*

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Key words: chicory, growth rate, radiation use efficiency, shading screens.

Abstract: Shading greenhouse may be an effective method to achieve a suitable environment for crop growth and to enhance crop yield and quality in places or seasons where there is high light intensity. Therefore, solar radiation levels may modify the biomass accumulation and bromatological composition. Different solar radiation levels (100%, 70% and 50% of available solar radiation) were simulated in order to determine crop responses to these factors in chicory (*Cichorium intybus* L. var. *foliosum*). A hydroponic experiment was conducted in an experimental greenhouse in the city of Frederico Westphalen, Rio Grande do Sul, Brazil. Plants grown in lower solar radiation levels are more efficient in converting solar radiation into dry matter, had a higher lipid content, increased chlorophyll indices *a*, *b* and total, as well as reduced leaf thickness, acid detergent fiber, cellulose, and lignin content, presenting more attractive bromatological features for commercial production. In this study it was demonstrated that the use of shading screens is an effective method to attenuate the solar radiation, this is especially relevant in places or seasons where there is high light intensity, which contribute to achieve better characteristics of the chicory produced.

1. Introduction

Growth rate, dry matter production and radiation use efficiency are considered important variables when analyzing yield-limiting factors and their interactions in different production environments. Dry matter accumulation under non-limiting conditions is directly related to the amount

of intercepted photosynthetically active radiation (Caron *et al.,* 2012). Therefore, variation in total biomass due to limiting biotic and abiotic factors may be attributed to changes in the solar radiation levels.

Changes in the solar radiation level of a given environment may affect the photosynthetic apparatus of plants. Lower solar radiation level reduces the photosynthetic rate and hence plant growth. When radiation levels is above the light saturation point, the reaction center of the PSII is inactivated and frequently damaged, constituing a phenomenon called photoinhibition (Taiz *et al.*, 2017). Due it, studies have identified a reduction of quantum efficiency, photosynthetic rate, and possible damage to the photosynthetic apparatus (Zhang *et al.,* 2008; Murchie *et al.,* 2009) beyond changes on the yield, bromatological and nutritional aspects, when plants are exposed both to suboptimal or super optimal radiation levels (Dario *et al.,* 2015; Bianculli *et al.,* 2016).

Achieving an appropriate environment in greenhouses in subtropical regions has become one of the main challenges for chicory producers, due to a large amount of solar radiation transmitted into the greenhouse and then converted into sensible and latent heat. Thus, greenhouse cooling methods and their impact present a considerable problem that requires a solution (Abdel-Ghany and Al-Helal, 2010). A strategy used in greenhouses in order to provide an appropriate environment for plant growth and to increase crop yield is the shading screens (Sethi and Sharma, 2007; Ganguly and Ghosh, 2011). Shading is one of the most inexpensive ways to reduce heat accumulation and to modify the greenhouse environment (Sethi and Sharma, 2007; Holcman and Sentelhas, 2012), besides promoting higher rates of diffuse radiation.

Solar radiation use efficiency (εb) of crops is determined by the slope of the linear regression between produced dry matter and photosynthetic active radiation (PAR) intercepted by the leaves (Monteith, 1965, 1972, 1977; Van Heerden *et al.,* 2010). Reduced solar radiation availability within the greenhouse environment may influence various traits of chicory, especially the leaf area index, the thickness of leaves and chlorophyll content, because cultivation in shaded environments often results in thinner leaves (Wherley *et al.,* 2005; Liu *et al.,* 2016). These leaves contain a greater total chlorophyll content per unit of fresh weight when compared to leaves grown under full sunlight. Chlorophyll content of each leaf, per unit area, may be smaller in environments with reduced incident solar radiation (Wild and Wolf, 1980; Taiz *et al.*, 2017).

Research showing the change of yield, and bromatological and nutritional traits as a function of incident solar radiation level, both as suboptimal and optimal levels were shown by Bianculli *et al.* (2016), Dario *et al.* (2015), and Kataria and Guruprasad (2015). To the authors' knowledge, this study is the first to investigate the impact of different solar radiation levels on plant growth, radiation use efficiency and biomass partitioning in chicory.

Information that reveals the impact of solar radiation levels on chicory as well as the bromatological composition are relevant in order to improve some management in a greenhouse environment. In addressing this lack of information, the following hypotheses were investigated: (i) solar radiation use efficiency is reduced in the plants grown under high solar radiation level; and (ii) shading screens provide an appropriate environment for plant growth, resulting in plants with better bromatological composition. These hypotheses justify the following aims: (i) to determine the influence of different solar radiation level on plant traits; and (ii) to evaluate the bromatological compositon of chicory plants under different solar radiation levels.

2. Materials and Methods

Study area

The study was conducted in an experimental greenhouse in the city of Frederico Westphalen, Rio Grande do Sul, Brazil. A hydroponic experiment was performed between the months of March 2016 to June 2016. The geographical location of the experiment was 27°23' S, 53°25' W, 490 m asl. According to the Köppen climate classification, the climate is Cfa, i.e., humid subtropical with mean annual temperatures of 19.1°C, and varying maximum and minimum temperatures of 38 and 0°C, respectively (Alvares *et al.,* 2013).

Management of hydroponic system

Chicory (*Chicory intybus* L. var. *foliosum*) seeds were inserted into phenolic foam board on 25 March 2016. Seedlings were transplanted into a system called "seedling tray" on 3 April 2016, when they reached two to three true leaves; seedling tray had 40 mm-hydroponic channels (3-cm deep, spaced by 7 cm between channels and 10 cm between plants in the channels), with 3% declivity. Seedlings remained

in this system until they reach a developmental level of five true leaves. After the seedling tray stage, the final transplant to the growth tray was performed on 11 April 2016. Each final growth tray was formed by 11 hydroponic channels (6-m long, 0.10-m wide and 0.05-m deep), subjected to a 4% declivity. The spacing was 0.20 m between plants in the channels and 0.20 m between channels. Thus, three hydroponic benches had 33 channels, with 242 plants per bench, 726 plants throughout the experiment composed the system.

A 1-HP pump coupled to a fiberglass tank with a capacity of 1000 liters powered the hydroponic system. The nutrient solution used was prepared with 400 mg l^{-1} of a commercial Hidrogood® Fert (Hidrogood Modern Horticulture, Brazil); these values were considered to be a full dose. Nutrient solution was pumped inside the hydroponic channels and was collected at the end of each channel by a system of closed system gutters. The nutrient solution used was prepared with 400 mg l^{-1} of a commercial Hidrogood® Fert (Hidrogood Modern Horticulture, Brazil); these values were considered to be a full dose. Irrigation was performed in an on-and-off system in periods of 15 minutes throughout the day (6 AM - 7 PM.); and 15 minutes each two hours during the night (7 PM - 6 AM). Potential of hydrogen (pH) and electric conductivity of the nutritive solution were assessed daily with a digital pH meter (pH-009IA model), and with a conductivity meter (Az-8301 model), respectively. The pH of the nutrient solution was kept at 6.0 (±0.5) using sulfuric acid (10% concentration of H_2SO_4) or very low sodium hydroxide (2% of NaOH). Nutrients were replaced when the electrical conductivity of the nutritive solution reached 50% of its initial concentration.

Solar radiation level and experimental design

The experimental design was a randomized complete block, arranged in a factorial arrangement (solar radiation level x evaluation periods) with four replications. Different solar radiation level (SRL) were simulated using black polyethylene meshes, fixed 1.0 m above the hydroponic benches. The following treatments were applied: 100% of available SRL (without mesh over the plants), 70% of available SRL (30% transmissivity mesh), and 50% of the available SRL (50% transmissivity mesh). Each simulated radiation level treatment composed a different hydroponic bench and the meteorological conditions in all treatment were the same, differing only the solar radiation levels in each hydroponic bench.

For the growth traits analysis, the chicory plants were collected from the central hydroponic channels of each treatment, beginning seven days after the transfer to the final growth tray. The evaluations were performed weekly until the average fresh mass of experimental plants hit 250 g (harvest point). Destructive evaluation consisted of two whole plants in each replication, totaling eight plants per treatment in each period. In laboratory the sectioning plants was performed, including the preparation of leaf discs in order to determine the leaf area and dry matter partitioning. The total dry matter (TDM) of the plants was determined from the sum components (root, stem and leaves). Each component was gathered and placed into pre-identified individual paper sacks. The sacks were then kept in a forced circulation oven at 60°C until a consistent mass was obtained. The samples were later weighed on a precision balance in order to obtain the dry mass of each component, which together resulted in the TDM.

Growth rates and bromatological analysis

In each evaluation period, the following variables were determined using the average values of dry matter (DM) and leaf area index (LAI): specific leaf area (SLA), leaf area ratio (LAR), leaf weight ratio (LWR), absolute growth rate (AGR), relative growth rate (RGR) and net assimilation rate (NAR). For more details about the used metrics of evaluation and the determination of these variables, see Thornley (1976) and Gardner *et al.* (1985).

The chlorophyll index *a*, *b*, total and *a*/*b* ratio were determined with a CFL 1030 chlorophyll meter. We selected, in the last evaluation period, fully expanded leaves from the upper third of 10 plants in each replication. All collected dry matter samples were properly prepared and subjected to bromatological analysis; the following traits were determined: ash (ASH, % of DM), lipids (LIP, % of DM), crude protein (CP, % of DM), neutral detergent fiber (NDF, % of DM), acid detergent fiber (ADF, % of DM), lignin (LN, % of DM), hemicellulose (HC, % of DM), cellulose (CEL, % of DM) and soluble carbohydrates in neutral detergent (SCND, % of DM).

For the ash content determination, the method AOAC 923.03 (1995) was used, which consider a temperature of 550°C. Lipid content was quantified according to the method proposed by Bligh and Dyer (1959). The values of NDF, ADF and LN were determined, followed by the calculations for estimating HC, CEL and CSND as proposed by Senger *et al.* (2008), and the crude protein (N x 6.25) was determined by micro-Kjeldahl method (Method 960.52) of AOAC (1995).

Radiation use efficiency

Production of dry matter was based on the model proposed by Monteith (1977), where dry matter production was calculated from intercepted photosynthetically active radiation (*iPAR*) multiplied by the use efficiency (*εb*). The *εb* was calculated by the ratio between the average production of accumulated TDM and the *iPAR* involved in the production of biomass according to the following expression:

$$DM = \varepsilon b * iPAR$$

Where *DM* = dry matter, in g; = conversion efficiency of in biomass produced, in g MJ^{-1} and = intercepted photosynthetically active radiation, in MJ m^{-2}.

Estimation of accumulated photosynthetically active radiation was determined according to the expression proposed by Varlet-Grancher *et al.* (1989):

$$*iPAR = 0.95*(inPAR)*(1-e^{(-k*LAI)})$$

Where *iPAR* = intercepted photosynthetically active radiation, in MJ m^{-2}; *inPAR* = incident photosynthetically active radiation, in MJ m^{-2}; *k* = light extinction coefficient. The *k* value calculated in the current study for the chicory plants was 0.13. LAI = leaf area index.

The light extinction coefficient (*k*) was calculated using the following equation:

$$k = \frac{-\ln(Rn/Rt)}{LAI}$$

Where *k*= light extinction coefficient, *Rn*= solar radiation measured under the plant canopy (MJ m^{-2}); *Rt*= radiation above the plant canopy (MJ m^{-2}); *LAI*= leaf area index.

Leaf area index was calculated by the following expression:

$$LAI = LA/UA$$

Where *LAI* = leaf area index; *LA* = leaf area, in cm^2; *UA* = useful area per plant, in cm^2.

The fraction of photosynthetically active radiation was considered 47% of the incident solar global radiation found in Rio Grande do Sul (Assis and Mendez, 1989). The estimation of accumulated photosynthetically active radiation was based on Monteith (1977) and Varlet-Grancher *et al.* (1989).

The *PAR* transmissivity of greenhouse cover was calculated based on an assessment using a quantum sensor, 50 cm height from the ground level, measuring 40 random points inside and outside of the greenhouse weekly during the trial period. To calculate transmissivity, the following equation was used:

$$T = (100*PARi)/PARo$$

Where *T*= transmissivity, in %; *PARi*= photosynthetically active radiation inside of the greenhouse; *PARo*= photosynthetically active radiation outside of the greenhouse.

With the transmissivity data of greenhouse and polyethylene meshes, *PARi* was estimated for each treatment, according to the following expressions:

$$PAR_{100\%} = PARi$$
$$PAR_{70\%} = PARi*0.7$$
$$PAR_{50\%} = PARi*0.5$$

Where photosynthetically active radiation inside of the greenhouse; environment with 70% of photosynthetically active radiation available and environment with 50% of photosynthetically active radiation available.

The values of incident global solar radiation during the study were obtained with the Automatic Climatological Station of the National Institute of Meteorology, located at 300 m from the study site (27°39' S and 53°43' W).

Chicory growth and nutritional composition variables were statistically analyzed with the software SAS 9.0 (SAS Institute 2002). Data were initially examined for homogeneity of variance and then subjected to analysis of variance. Tukey test (p>0.05) was used to compare the difference between the treatments.

3. Results

Radiation use efficiency in Chicory

The radiation use efficiency and photosynthetically active radiation accumulated values in the different solar radiation level during the conduct of the study are shown in figure 1. It was observed that the

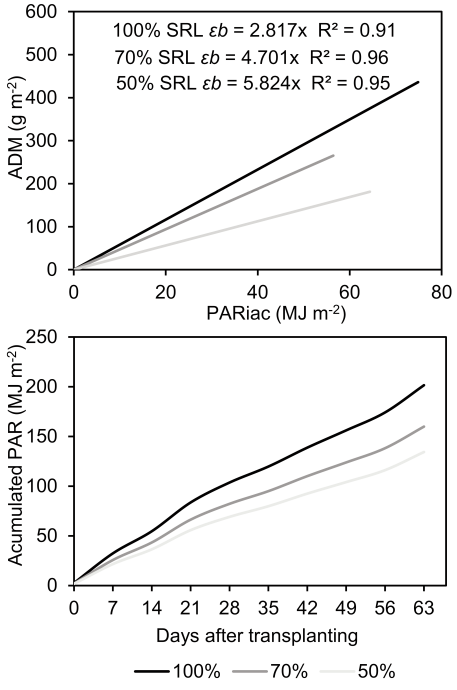

Fig. 1 - Radiation use efficiency (εb) in g MJ^{-1} and photosynthetically active radiation levels (PAR) accumulated over *Cichorium intybus* cycle. ADM = accumulated dry matter, PARiac = photosynthetically active radiation intercepted accumulated.

plants growing under reduced radiation availability (50% and 70%) presented respectively 51.6% and 40.1% higher radiation use efficiency than those growing under 100% of solar radiation level. Plants grown in low levels of photosynthetic active radiation were more efficient in converting the radiant energy into accumulated dry matter.

Growth rates and leaf traits

The absolute growth rate values were greater in the higher level of solar radiation from 42 DAT up to the crop harvest (Fig. 2A). The relative growth rate showed changes depending on the age of the plants, but for the solar radiation levels was observed difference only at 14 and 21 DAT (Fig. 2B). In relation to the net assimilation rate was not observed changes in the values in function of the solar radiation level, however, the largest net assimilation rate values were observed at 21 DAT (Fig. 2C).

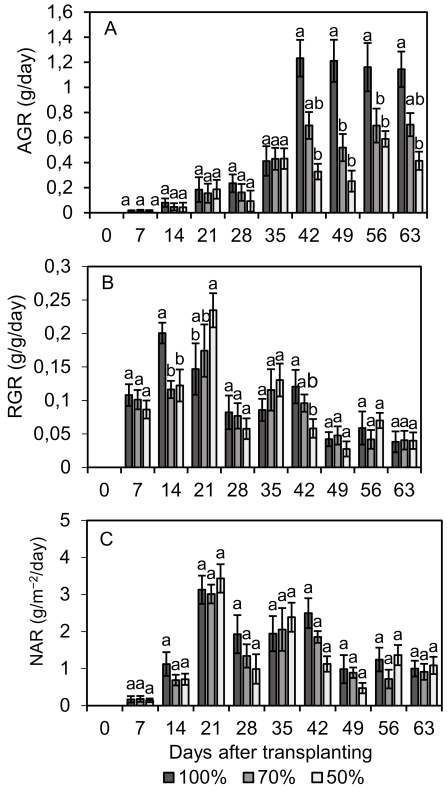

Fig. 2 - Absolute growth rate (AGR), relative growth rate (RGR) and net assimilation rate (NAR) in *Cichorium intybus* grown under different solar radiation levels. Lowercase letters denote differences within each day after transplanting by Tukey test (p<0.05). Bars represent average values ± SE (n=8).

The attenuation of 50% and 70% of solar radiation reduced the leaf area index of the plants by 51.8% and 19.8% on average when compared with the plants growing under 100% of solar radiation level from 42 DAT up to the crop harvest (Fig. 3A). For the specific leaf area, significant differences can be observed from transplanting up to 21 DAT, where the plants under lower radiation levels showed the higher specific leaf area (Fig. 3B). In relation to the leaf area ratio, it was identified higher value only at 7 DAT for the plants under 100% of solar radiation level (Fig. 3C). Additionally, for the leaf weight ratio was not verified difference in the different shading levels (Fig. 3D).

Plants grown in lower radiation levels showed higher chlorophyll index *a*, *b*, and total, which were 19.4, 36.4 and 25.3% higher than those observed for the 100% of solar radiation level, respectively.

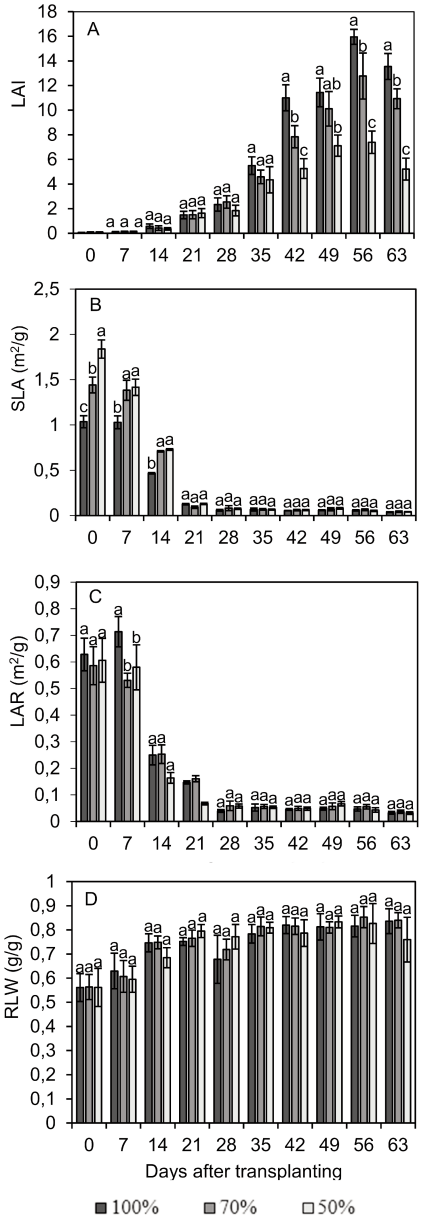

Fig. 3 - Leaf area index (LAI), specific leaf area (SLA), leaf area ratio (LAR) and leaf weight ratio (RLW) in *Cichorium intybus* grown under different global solar radiation levels. Lowercase letters denote differences within each day after transplanting by Tukey test (p<0.05). Bars represent average values ± SE (n=8).

However, plants without light restriction showed higher chlorophyll-*a*/*b* ratio (Fig. 4).

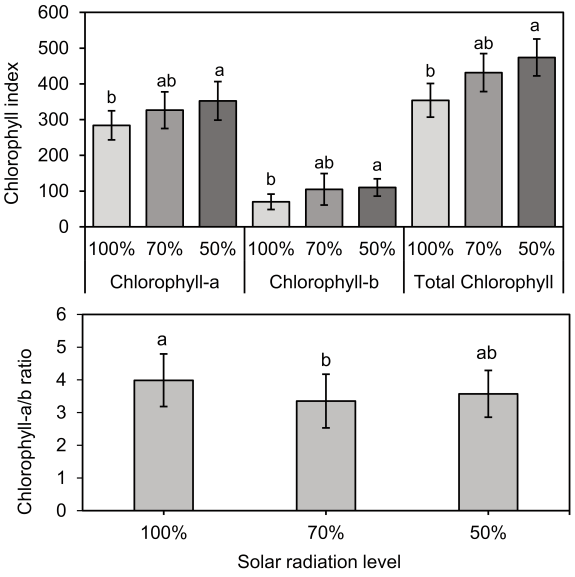

Fig. 4 - Chlorophyll-a, chlorophyll-b, and total chlorophyll index and chlorophyll-a/b ratio in *Cichorium intybus* grown under different solar radiation levels. Lowercase letters denote differences between solar global radiation levels by Tukey test ($p<0.05$). Bars represent average values ± SE (n=8).

Biomass partitioning and bromatological composition

The pattern of dry matter accumulation in the leaves, roots and stem of the chicory plants was similar for all solar radiation levels; however, there was observed an increase in the proportion of leaves during the crop cycle, with a decrease in the percentage of roots and stem of the plants. This pattern of partitioning was observed for all solar radiation levels (Fig. 5).

Reduced levels of solar radiation increased the lipids and ASH contents and decreased the neutral detergent fiber, acid detergent fiber, lignin, hemicellulose and cellulose values that characterized less rigid leaves. The crude protein content and soluble carbohydrates in neutral detergent were not affected by shading levels (Fig. 6). In overall, the bromatological traits of chicory plants were affected by the solar radiation levels.

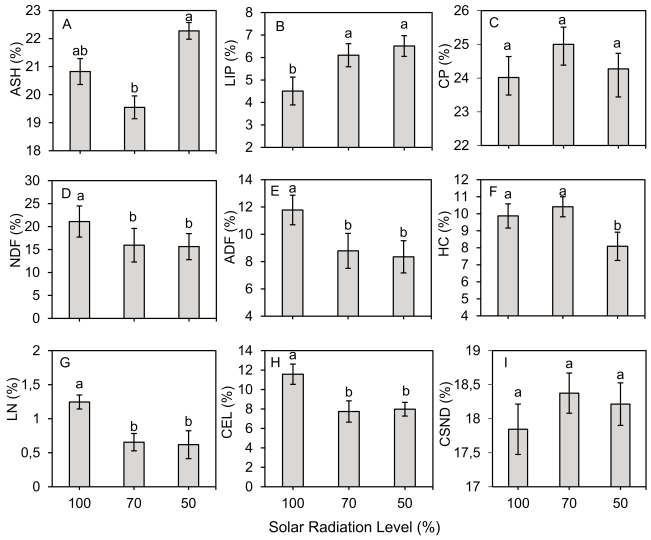

Fig. 6 - Ash percentage (ASH), Lipids (LIP), Crude protein (CP), Neutral detergent fiber (NDF), Acid detergent fiber (ADF), Lignin (LN), Hemicellulose (HM), Cellulose (CEL), Soluble carbohydrates in neutral detergent (CSND) in *Cichorium intybus* leaves growing in different solar radiation levels. Lowercase letters denote differences between solar global radiation levels by Tukey test ($p<0.05$). Bars represent average values ± SE (n=8).

4. Discussion and Conclusions

Solar radiation levels modify the radiation use efficiency and growth rates

The results showed that growing conditions have a striking effect on the radiation use efficiency in relation to its capacity to convert solar radiation into dry biomass. The efficiency of plants to convert solar energy into biomass was higher in low solar radiation

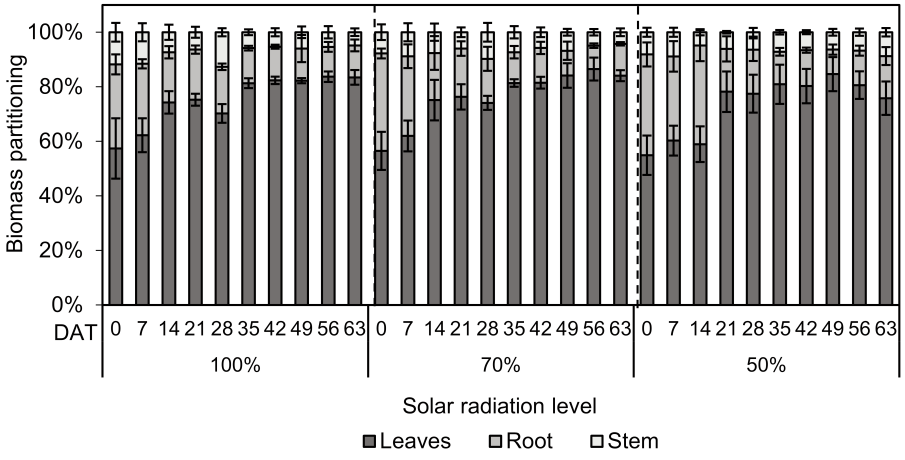

Fig. 5 - Biomass partitioning (%) in each days after transplanting (DAT) among leaves, roots and stem in *Cichorium intybus* grown under different solar radiation levels. Bars represent average values ± SE (n=8).

level. The higher efficiency of chicory plants was not enough to compensate the limitations of solar radiation. This is justified by the higher leaf area index observed under high levels of radiation.

The highest leaf area index observed for plants growing under 100% of solar radiation may be related to the higher rate of photosynthesis, since there were no limitations on the availability of solar radiation. According to Taiz *et al.* (2017), sun leaves increase CO_2 assimilation having more availability of rubisco, and can dissipate excess of light energy due to a large pool of components in the xanthophyll cycle. In this sense, it is important to emphasize that plants with higher conversion efficiency are not always the ones that result in higher yield. This affirmation was confirmed in this study, where the chicory plants grown under reduced solar radiation levels were more efficient into convert solar radiation into biomass, however, showed lower leaf area index. This response can be explained due the morphological changes occurred in function of the shading conditions.

The shading effect provided by the solar radiation level of 50% significantly increased the specific leaf area of the plants mainly up to 14 days after transplantation. The decrease in leaf thickness of plants grown at higher levels of shading maybe linked to the fact that plant prefers to spend photoassimilated for the expansion of the leaf area (Cooper and Qualls, 1967; Carvalho *et al.,* 2006; Gondim *et al.,* 2008; Lenhard *et al.,* 2013), thus ensuring itself a greater possibility of intercepting solar radiation.

In chicory, the attenuation of solar radiation above the plants with the use of shading screens resulted in an alteration in leaf thickness which was responsible for changes in the chlorophyll content and bromatological composition (Sims and Pearcy, 1992; Terashima *et al.,* 2001; Oguchi *et al.,* 2003). This plant response is correlated, where a reduction in the leaf thickness can imply in changes in the chlorophyll content and bromatological composition depending on the intensity of leaf thickness alteration in function of the level of shading. The morphological changes were considered positive, as the leaves of these plants were more tender. This characteristic may be considered in the market commercialization due to a better acceptability of the consumer's for products with greater bromatological features.

Reduced solar radiation levels improve morphological and bromatological traits of chicory

The current study confirmed the difference in the bromatological quality of chicory plants grown in different solar radiation level. In overall, a higher lipids content and lesser fiber, lignin, hemicellulose and cellulose components were observed under reduced solar radiation levels. This result reveals an important bromatological change, which confers more quality to the chicory produced.

The reduction of lipid content in chicory plants under high solar radiation levels may be associated with the occurrence of light saturation and photoinhibition. According to Taiz *et al.* (2017), the light saturation in the leaves of most species occurs between 500 and 1000 µmol m^{-2} s^{-1}, whereas the full solar radiation can provide around 2000 µmol m^{-2} s^{-1}. Excessive light causes damage to chloroplasts, one of the locations of fatty acids synthesis (Halliwell, 1987; Mittler, 2002; Gill and Tuteja, 2010).

The higher content of lipids in chicory plants produced under reduced solar radiation level is an important finding that can help people health, once lipids deliver energy and essential fatty acids, being essential for fat-soluble vitamin absorption, and may contain natural antioxidants (Lindley, 1998). Considering the consumer's point of view, the higher content of lipids and reduction of fiber, lighin, hemicellulose and cellulose components are favorable, since the leaves will present better characteristics for consumption.

Such effects produced by the attenuation of solar radiation can be explained due to the fact that shading reduces the availability of assimilates used for the development of secondary cell walls (Kephart and Buxton, 1993). Additionally, this fact is relevant because the lignin and cellulose components are important substances against abiotic stress, including UV-B radiation (Rozema *et al.,* 1997). In *Phalaenopsis orchids*, e.g., an increase in the lignin content in the leaves and roots of the plants where observed when the light intensity was increased, being this result associated with the induction of PAL, CAD and POD activities (Copur and Tozluoglu, 2008).

Regarding the morphological and physiological changes, the highest content of chlorophyll-*a*, *b* and total observed for the plants grown under 50% of solar radiation level is related to the need to increase the utilization of the available radiation for the plants. The current study confirmed the ability of plants to compensate the low level of radiation by increasing the number of photosynthetic pigments, which resulted in a higher radiation use efficiency by plants (Fig. 1).

Higher chlorophyll content in plants grown under

low solar radiation level is consistent with the findings of Minotta and Pinzauti (1996), Cardillo and Bernal (2006), and Hazrati *et al.* (2016). A lower chlorophyll *a*/*b* ratio in the lowest light intensity treatment is due to chlorophyll-*b* being degraded more slowly in shaded environments (Lee *et al.,* 2000).

Information generated in the current study is relevant, as it provides valuable information to vegetable's producers, assisting in the strategy to minimize the effect of the high solar radiation inside of the greenhouses. We proved the attenuation of solar radiation is an effective method to improve morphological and bromatological aspects of chicory. By using this simple method, both researchers and farmers might have a competitive advantage in planning their cropping systems, mainly in sites or seasons where the high light intensity is a limiting factor for chicory cropping.

Chicory plants grown in lower solar radiation levels are more efficient in converting solar radiation into dry matter, have a higher lipid content, chlorophyll index *a*, *b* and total, reduced leaf thickness, acid detergent fiber, cellulose, and lignin content, which confirm the hypothesis tested. In this study it was demonstrated that the use of shading screens is an effective method to attenuate the solar radiation; this is especially relevant in places or seasons where there is high light intensity, which contributes to achieve better characteristics of the cichory produced.

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Gamma rays induced variations in seed germination, growth and phenotypic characteristics of *Zinnia elegans* var. Dreamland

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Abstract: *Zinnia elegans* is a herbaceous annual with diverse flower colours, flower types and plant height. *Zinnia elegans* are popular as pot plants and also for landscape gardening. The commercial value of *Zinnia* can be increased with novel traits such as flower colour and form. One of the techniques to develop plant varieties with superior traits is to induce mutations using gamma radiation. Hence, three doses of gamma radiation (75Gy, 100Gy and 125Gy) were utilised to obtain new and novel varieties of *Zinnia elegans* var. Dreamland and to study the effect of gamma rays on germination of seeds, growth and survival of the seedlings, height of the plants. All the three gamma ray doses were found to decrease the germination and survival of seeds of *Zinnia elegans*. The higher doses of gamma rays were found to be detrimental for the germination and survival of seeds and height of the seedlings. Phenotypical variations such as plant height, the number of flowers and flower diameter of the third generation mutants were highly significant as compared to the control. Eight floral variations could be obtained with novel form and colour.

1. Introduction

In recent years floriculture has become a flourishing industry. One of the requirements of the floricultural industry is diversity in order to introduce new ornamental plants in the market. *Zinnias* are important ornamental plants on the world floral market. *Zinnia* has been reported to be the first flower to be grown in space stations (Loff, 2016). *Zinnia elegans* offers a wide range of flower forms and colours which have an immense ornamental value. However there is a continuous demand for ornamental

cultivars with new/novel forms and colours in modern and industrialized horticulture (Yunus *et al.,* 2013). Induced mutations offer a possibility of obtaining ornamentals with novel forms and colours to meet this demand (Tiwari and Kumar, 2011). More than 2,200 mutant varieties of crop plants have been released using induced mutagenesis and among them, 566 represent ornamental plants (Jain, 2005; Barakat and El-Sammak*,* 2011). 76 new mutant ornamental varieties with changed flower colour/shape and chlorophyll variegation in leaves have been developed using gamma rays and released (Datta *et al.*, 2009; Barakat and El-Sammak, 2011). But, no reports of registration of mutant varieties of *Zinnia* under mutant variety database of International Atomic Energy Agency are found. There is only one report of production of four new varieties in *Zinnia* by Venkatachalam and Jayabalan (1997). Radiation with Gamma rays has been reported to give rise to a large number of novel mutants in several ornamental species (Chrysanthemums, orchids, rose, pelargonium, canna, and carnations). The present study was carried out to utilize the mutagenic effect of gamma rays on *Zinnia elegans* to obtain dwarf varieties and flowers with novel architecture and colours.

2. Materials and Methods

Plant materials

Seeds of *Zinnia elegans* var. Dreamland with pink flowers which were certified to be pure breeding were procured from the Indo American Hybrid Seeds, Bangalore.

Gamma irradiation

Zinnia elegans var. Dreamland seeds were treated with gamma radiation at the Bhabha Atomic Research Center, Mumbai, India. Three gamma ray doses (75GY, 100GY and 125GY) were selected after studying the radiosensitivity and lethal dose (LD50) of *Zinnia* seeds (Pallavi *et al*., 2017). The gamma ray doses were given at the rate of 1.7Gy/min using ^{60}Co source.

Evaluation of germination of seeds, survivability and growth of seedlings

For the evaluation of germination of the irradiated seeds, survivability and growth of the seedlings, a total of 90 seeds were treated in triplicates. 30 non-irradiated seeds were established as controls. The irradiated seeds were maintained as axenic cultures. The germination of the seeds was recorded for the first seven days and the percentage of survival of seedlings on the 15th day after planting. The seedling growth parameters such as plant height and root length were recorded at an interval of 5 days for 15 days.

The data obtained was used to calculate the percentage of germination and the percentage of survival for each treatment as follows:

Germination % = No. of germinated seeds × 100/No. of irradiated seeds planted;

Survival % = No. of survived seedlings × 100/No. of irradiated seeds.

Evaluation of phenotypic variations observed in M_3 generation

To determine the effect of gamma rays on phenotypic characteristics, 500 seeds were irradiated with 75Gy, 100Gy and 125Gy doses of gamma radiation in each treatment. 500 non irradiated seeds were established as control. The irradiated seeds were sown in the green house of St. Aloysius College, Mangalore and the seedlings were grown up to the flowering stage and self-pollinated. The seeds of self-pollinated flowers were collected and sown and the procedure was repeated till the third mutant generation. The irradiated populations were screened for mutants at the M_3 generation. The plant height was recorded after 60 days of germination. The floral characters such as flower diameter, the number of flowers and flower colour were recorded at the flowering stage.

Statistical analysis

The data was recorded and statistically analysed using IBM SPSS 20 software. One-way ANOVA test was performed to determine significant differences between the variations. Tuckey HSD was used to ascertain significant differences among treatments at p= 0.05.

3. Results

Effect of gamma rays on seed germination, survivability and seedling growth

The seeds started germinating within 3 to 4 days of sowing in all the treatments and the control. The percentage of germination of control seeds was found to be 68.89±2.22. The percentage of germination of seeds irradiated with 75Gy (55.55±2.22) was not significantly different from the control. Irradiation with 100Gy and 125Gy gamma ray doses significantly decreased the germination percentage

of seeds as compared to the control (Fig. 1). Furthermore, the percentage of survival of seedlings obtained from seeds irradiated with 75Gy, 100Gy and 125Gy was significantly lower as compared to the control (Fig. 1). No significant difference was observed in the percentage of survival of seedlings obtained from seeds irradiated at 100Gy and 125Gy. There was no significant variation in plant height and root length of the seedlings up to 10 days as compared to the control. From the 10th day onwards there was a significant decrease in plant height and root length in seedlings obtained from seeds irradiated at 100Gy and 125Gy as compared to the control (Figs. 2 and 3).

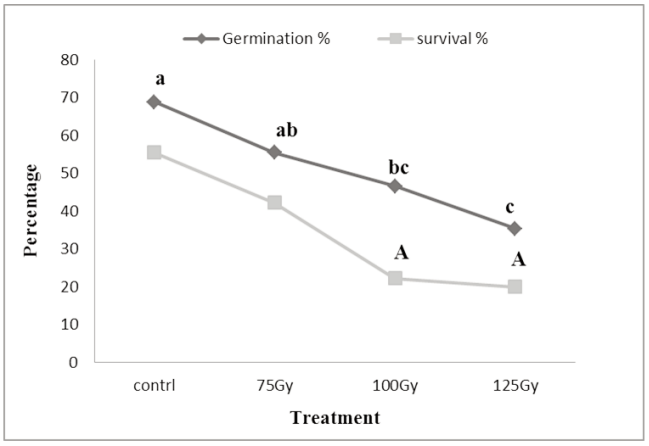

Fig. 1 - Effect of gamma radiation on percentage of seed germination and percentage of plant survival in *Zinnia elegans* var. Dreamland. The mean values with same alphabets do not differ significantly at P<0.05 level.

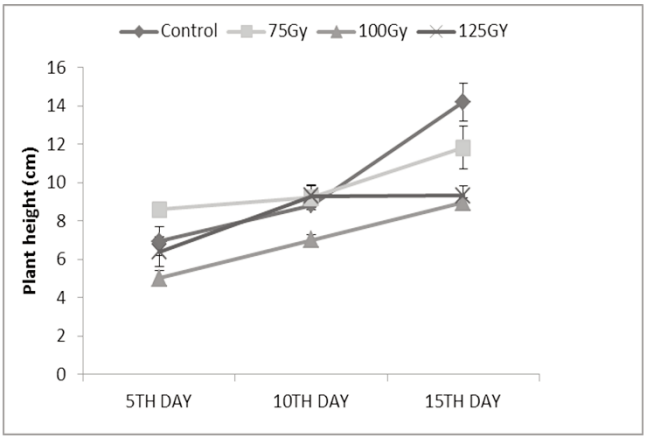

Fig. 2 - Effect of gamma radiation on plant height of the seedlings at 5 days interval.

Mutants obtained at M$_3$ generation

Variation in plant height. Variation in plant height was observed in third generation plants obtained from seeds irradiated at all three levels (Fig. 4). The lowest plant height was seen in variant V1 (18.33±2.028 cm) obtained from seeds irradiated at the 75Gy dose. The variant V3 obtained from seeds irradiated at 100Gy dose had the highest mean value of plant height (55.33±1.85 cm).

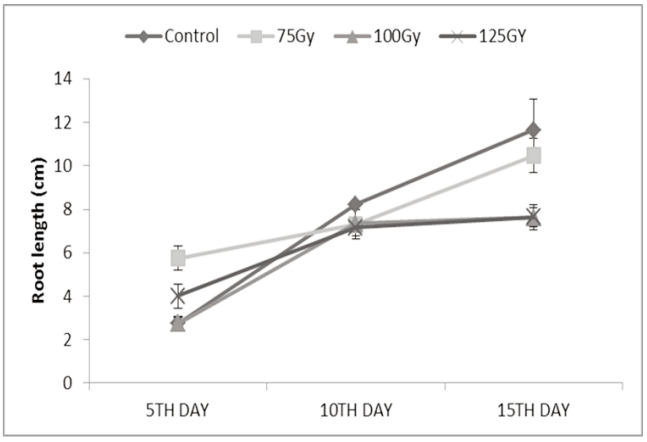

Fig. 3 - Effect of gamma radiation on the root length of seedlings at 5 days interval.

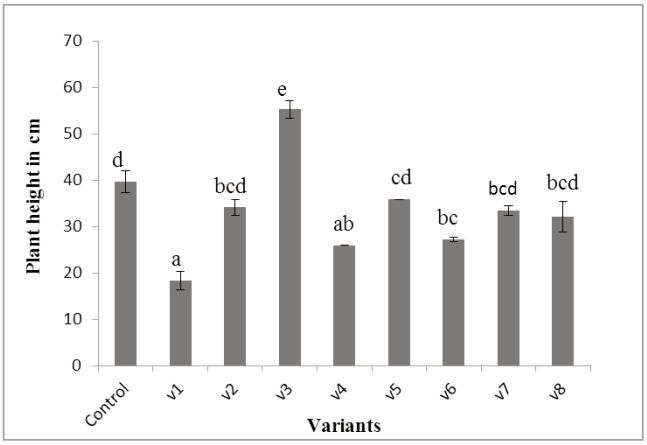

Fig. 4 - Comparison of plant heights of the variants and control plants of *Zinnia elegans* var. Dreamland. The mean values with same alphabets do not differ significantly at P<0.05 level.

Variation in number of flowers. The number of flowers found in the variants is shown in figure 5. The variant V7 gave the highest mean number of flowers

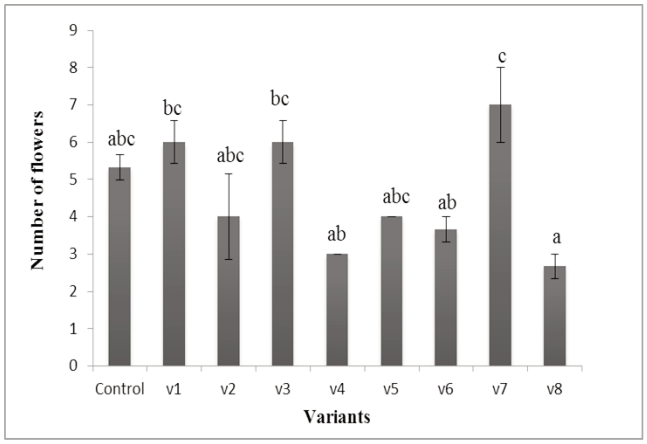

Fig. 5 - Comparison of number of flowers in control and variants of *Zinnia elegans* var. Dreamland. The mean values with same alphabets do not differ significantly at P<0.05 level.

(7±0.57) at 100Gy dose. The lowest number of flowers was given by variant V8 (2.66±0.33) at 125Gy as compared to the control (5.33±0.33). The variation found in the number of flowers in all the variants is statistically highly significant.

Variation in flower diameter. Variation in the diameter was found in the flowers of plants of seeds radiated at all three doses (Fig. 6). Except variant V3 most of the flowers were smaller than the control. The variations in the smaller flowers are not significant except for the variant V7.

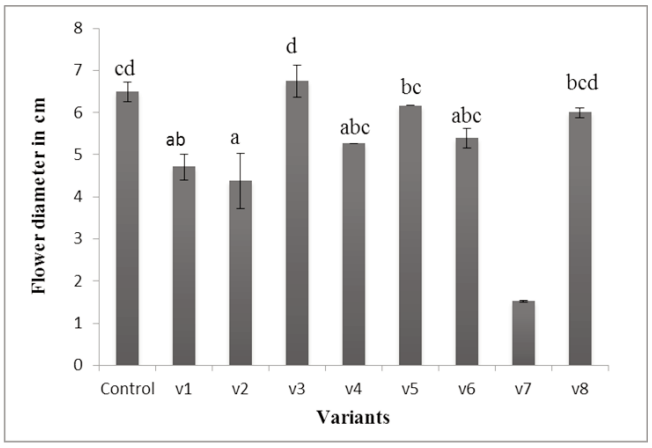

Fig. 6 - Comparison of flower diameter in control and variants of *Zinnia elegans* var. Dreamland. The mean values with same alphabets do not differ significantly at P<0.05 level.

Variation in form and colour of flowers. Variations in flower form and colour were observed after treatment with gamma rays of different doses as shown in Table 1 and figure 7. In plants irradiated with 75GY, two variants V1 with white coloured flowers and V2 with single whorled light yellow coloured flowers were obtained. In plants of seeds irradiated with 100GY, 5 flower colour variants were observed. V4 and V5 plants showed light yellow coloured ray florets with pink coloured tips. V3 showed single whorl pink flowers, V6 plants had orange yellow coloured flowers. The V7 had only yellowish disc florets without any ray florets. In plants irradiated with 125GY, only one variant with yellow flowers was obtained.

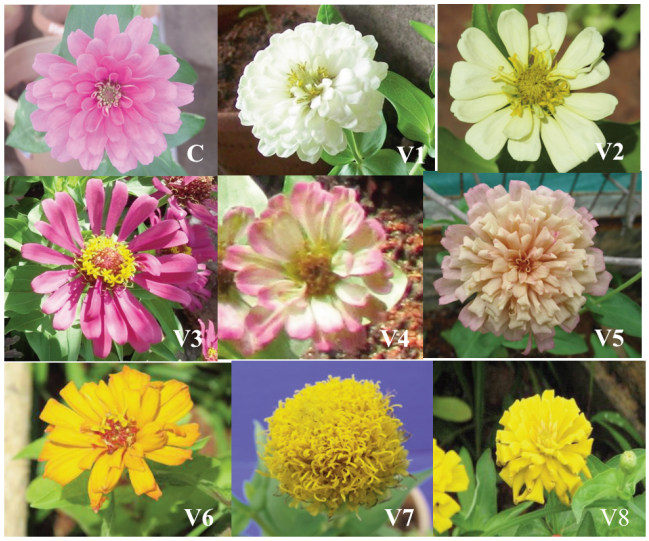

Fig. 7 - Effect of gamma radiation on flower colour and form of *Zinnia elegans* var. Dreamland. C= control, V1-V8 = Variants.

4. Discussion and Conclusions

Higher doses of gamma radiation were found in our study to reduce seed germination and survival of seedlings. This is confirmed by Hanafiah *et al.* (2010), who found that higher doses of radiation have an adverse effect on seed germination and survival of soybean seedlings. Similarly, Kumari *et al*. (2013) reported that Gamma rays significantly reduced plant survival and growth of *Chrysanthemum morifolium* variety 'otome pink'; reduction in survival increased with increase in dose. Significant reduction in survival of plants obtained from seeds irradiated with gamma rays was also observed by Jala and Bodhipadma (2011) in *Celosia argentea* var. cristata. According to

Table 1 - Effect of gamma radiation on flower colour and form

Plant variants	Gamma ray doses (Gy)	Flower characteristics
Control	-	Pink, double whorled
V1	75	White, double whorled
V2	75	Light yellow, single whorled
V3	100	Pink, single whorled
V4	100	Light yellow, double whorled ray florets with pink shaded tips
V5	100	Light yellow, double whorled ray florets with light pink shaded tips
V6	100	Orange yellow, double whorled
V7	100	No ray florets
V8	125	Yellow, double whorled

Datta and Gupta (1982), Banerji and Datta (2002), and Khan (2003), the decreased survival percentage of plants obtained from seeds treated with higher gamma radiation doses is due to chromosomal aberrations and gene mutation after irradiation treatment. According to Tiwari and Kumar (2011) many mutations can be lethal due to the inhibition of cell division and induction of cell death. In our study higher doses of radiation were found to reduce height of the plants in *Zinnia*. Significant reduction in plant height is also reported with higher doses of radiation in *Chrysanthemum morifolium* variety 'otome pink' (Kumari *et al*., 2013). The decrease in plant height and root length observed in our study has also been reported in a number of other crops (Thimmaiah *et al*., 1998; Yaqoob and Ahmad, 2003; Al-Salhi *et al.,* 2004; Toker *et al.,* 2005; Kon *et al*., 2007; El Sheriff *et al*., 2011). The plant height and root length of seedlings from the seeds treated with 100Gy and 125Gy was found to be similar to the control seedlings until 10th day in our study but both decreased after the 10th day. Similar results have been reported by Khalil *et al*. (1986) in barley and El Sheriff *et al*. (2011) in *Hibiscus sabdariffa*. According to them the probable cause for the reduction in plant height and root length after the 10th day, could be the decrease in activity of mitotic division of meristematic tissues and decrease in the moisture content of the seeds. Induction of mutations based on ionising radiations has played a major role in the development of many new and novel flower colour and shape mutants in ornamentals (Datta *et al.,* 2005). Schum and Preil (1998) reported that 55% of the records on induced mutation in ornamental plants concerned changes in flower colour and 15% in flower morphology. Yamaguchi *et al*. (2008) obtained more than 8 types of flower colour mutants from *Chrysanthemum* plants bearing pink flowers. Datta and Chakrabarty (2009) developed four mutants with flower colour and floret shape variation by irradiating the ray florets of five decorative type *Chrysanthemums*. Venkatachalam and Jayabalan (1997) induced four types of new flower colour mutations: majenta, yellow, red and red with white spots in *Zinnia elegans* Jacq. cv. crimson red using gamma irradiation. In our study, eight *Zinnia* mutants with novel variations in form and colour of flowers were obtained with gamma rays. Studies by Datta *et al*. (2009); Zalewska *et al*. (2011) on pigment analysis of florets of chrysanthemum flower colour mutants indicated that changes in flower colour were due to qualitative and quantitative changes in the pigments as a result of mutations induced by gamma rays. Flower pigments are composed of flavonoids including anthocyanins, flavones and flavonols (Harborne and Grayer, 1988; Hattori, 1992). Mutations in the core structural genes or regulatory loci of Anthocyanine biosynthesis pathway result in changes in flower colour according to Streisfeld *et al.* (2013) and Nakatsuka *et al.* (2005). The blockage in the early steps of anthocyanin synthesis leads to the loss of floral anthocyanine pigments ultimately resulting in the formation of white flowers whereas a blockage in the later steps leads to flower colour changes from blue to red because of the accumulation of a particular anthocyanin (Mato *et al.,* 2000; Lee *et al.,* 2008; Tanaka *et al.,* 2008; Casimiro-Soriguer *et al*., 2016).

A higher frequency of flower colour mutation was observed in 100Gy. This could be a suitable dose for obtaining higher number of flower colour mutants in *Zinnia elegans* var Dreamland. Our studies have resulted in obtaining one dwarf variety (18.33±2.028 cm) and eight varieties with varying floral colours from which desirable variants can be selected and commercially exploited.

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'Superior Seedless' grapevine grafted on three rootstocks grown on calcareous soil under diluted brackish water irrigation. II. Expression of antioxidant genes

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Abstract: Grapevine rootstocks that can absorb brackish water and maintain satisfactory growth of the grapevine scion might be a feasible management practice in areas suffering scarce water resources. The objective of this study was to evaluate the expression of antioxidant genes in 'Superior Seedless' leaves grafted on R110 (*Vitis berlandieri* x *V. rupestris*), 41B (*V. berlandieri* x *V. vinifera*) and P1103 (*V. berlandieri* x *V. rupestris*) in response to diluted brackish water irrigation at three levels: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. Results revealed that after salinity exposure for two weeks, the transcript levels of *APX*, *Mn-SOD* and *MDAR* increased in 'Superior Seedless' leaves grafted on the different rootstocks. However, their expression levels in response to salinity were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* gene showed obvious enhanced level in plants grafted on P1103 in response to salt exposure. Meanwhile, the expression of *CAT* gene in 'Superior Seedless' scion grafted on 41B or R110 showed almost unchanged level in control and stressed conditions. Down-regulation of *CuZn-SOD* was recorded in leaves of 'Superior Seedless' grafted on P1103. Slight up-regulation of this gene in response to saline condition was recorded when scion was grafted on 41B or R110. The expression of *GPX* was enhanced in scion grafted on P1103 and 41B. On the other hand, scion grafted on R110 showed decreased expression of *GPX* in response to salt treatment. Grapevine rootstocks that have *V. rupestris and V. berlandieri* in their parentage are good candidates for salinity tolerance.

1. Introduction

Grapes (*Vitis* sp.) are considered as one of the world's major commercially grown fruit crops. In Jordan, grapes are ranked in second place after olives regarding the total area planted. The total area planted with grapes is 3806 ha (FAO, 2014). More than half of that area is under irrigation and Jordan is now ranked as the world's second water-poorest country.

Irrigation with low quality water during the whole growing season of the crops, even the tolerant ones, does not always produce high yield. Mixing low quality water; such as dam brackish water, with conventional quality irrigation water in ratios to keep the salinity of the irrigation water below the threshold of the target crop might be an acceptable management practice and was used by many researchers (Abdel Gawad and Ghaibeh, 2001). Alternating conventional quality water with brackish water is another management practice. Its application would be easier because it does not need containers for mixing two different sources of irrigation water. The conventional quality irrigation water can be used during the sensitive stages of plant growth and the brackish water during the non-sensitive or less sensitive stages.

Considerable yields were obtained using saline irrigation water (4-12 dS m^{-1}) in crops that had been previously defined as moderately sensitive to salt stress (Bustan *et al*., 2004). Furthermore, in some crops (e.g., tomato) the reduction in the fresh yield was compensated by an increase in fruit dry weight and other quality parameters (Mizrahi *et al.*, 1988). Bustan *et al.* (2005) reported that the combination of fresh (1.2 dS m^{-1}) and brackish (7 dS m^{-1}) irrigation water increased the yield level of melon to that of fresh water plants whereas it brought about the improvement of fruit quality typical to brackish water plants, thus providing an attractive approach to optimize late-summer melon production.

Plants subjected to saline environment use different mechanisms to overcome such abiotic stress. To avoid a disorder of ion homeostasis under saline conditions, plant cells have to maintain a low Na^+ concentration and keep a high H^+ concentration in the cytosol where enzymes for metabolism are located (Zörb *et al.*, 2005).

Usually, plants use different ways to maintain a low cytosolic sodium concentration, including restricting Na^+ influx, maintaining active Na^+ efflux, and compartmentalizing Na^+ into the vacuole, etc. (Rubio *et al.*, 1995). These mechanisms involve a number of Na^+/H^+ antiporter proteins that are localized in plant plasma and vacuolar membranes (Vasekina *et al.*, 2005). They catalyze the exchange of Na^+ for H^+ across membranes and the energy needed is generated by the H^+-adenosine triphosphatase and H^+-pyrophosphatase (Niu *et al.*, 1995; Shi and Zhu, 2002).

Plants exposed to salt stress showed enhanced formation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and singlet oxygen (Mittler, 2002). The harmful effects of these molecules are referred to as oxidative stress (Halliwell, 2006). Plants have evolved an enzymatic antioxidant system to reduce the ROS levels. This enzymatic system includes superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (Apel and Hirt, 2004). The development of salt-tolerant crops is a practical solution to sustain agricultural productivity. Because of the complexity of the trait, traditional crop-breeding programs aimed at improving tolerance to salinity have limited success. Therefore, understanding the cellular and molecular bases of salinity-tolerance mechanisms is essential for marker-assisted selection and genetic engineering of salt tolerance in economic crops. Understanding the responses of plants to the major environmental stressor salinity is an important topic for the biotechnological application of functional mechanisms of stress adaptation. Plant engineering strategies for cellular and metabolic reprogramming to increase the efficiency of plant adaptive processes may either focus on (1) conferring stress tolerance by directly reprogramming ion transport processes and primary metabolism or (2) by modulating signaling and regulatory pathways of the adaptive mechanisms. The second approach seems to be more perspective because it is likely that signaling and regulatory factors orchestrate as key signaling components the transcriptional and translational control of group (1) adaptive mechanisms (Diédhiou *et al*., 2008; Popova *et al.*, 2008).

Most knowledge on molecular mechanisms involved in plant salt responses and adaptation has been derived from analyses of the glycophytic models *Arabidopsis thaliana* and rice. Such knowledge is lacking in *Vitis* species, therefore detecting expression changes of antioxidant defense genes is the objective of this study. Since the understanding of a plants response to a stress requires an evaluation of stress induced changes in gene expression, the expression of major defense genes (superoxide dis-

mutase, ascorbate peroxidase, catalase, glutathione peroxidase, monodehydroascorbate reductase) in 'Superior Seedless' grafted on different rootstocks has been examined by RT-PCR.

2. Materials and Methods

Plant material

Three grape rootstocks were evaluated in this study: R110 (*Vitis berlandieri* x *V. rupestris*), 41B (*V. berlandieri* x *V. vinifera*) and P1103 (*V. berlandieri* x *V. rupestris*). The rootstocks were purchased from Les Pépiniéristes du Comtat, Sarrians, France.

After being imported, 'Superior Seedless' bud cultivar was grafted on the rootstocks in a local nursery; Al-Bushra Nurseries, May, 2013. Grafted plant materials were planted in polyethylene bags filled with peatmoss. The one year old grafted grapevine rootstocks were grown for several months to allow for the formation of a well developed root system before applying treatments. Fertilizers and fungicides were applied as necessary.

Soil and water

The soil was brought from the southern Jordan Valley. Soil was crushed and sieved through 1 cm sieve and plastic pots (the working volume of the pots was 44 L) were filled with 50 kg each in order to roughly have a bulk density of 1.14 g cm^{-3}. The bulk density is within the typical range of bulk densities of agricultural soils. If bulk density was higher, infiltration would be very slower and hydraulic conductivity would be slower as well. Low hydraulic conductivity would exacerbate the osmotic effect and create anoxic conditions. The pots were placed in a controlled greenhouse. Grafted grapevines were transplanted in February and the growth was unified based on the number of buds and root length. The root system was cut back to 15 cm in length and the vegetative system was cut back to eight buds.

The water was brought from Al-Karameh dam located in the Jordan Valley and stored in a galvanized tank. Three levels of irrigation water salinity; in terms of electrical conductivity (EC), were applied: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. These three levels of diluted brackish water were used to find out the salt level that would result in a tolerable "adverse" effect to design alternate irrigation that would contribute to water saving. These concentrations were selected based upon the threshold EC of grapes (i.e. EC<2 dS m^{-1}). The treatments were prepared by mixing the dam water with tap water. A portable conductivity meter (Model Cond 3210, WTW, Germany) was used to measure the EC and to obtain the determined salinity levels. The twelve treatments were arranged in a randomized complete block design with three replicates. The grafted grapevines started to break the dormancy period during spring. Composite fertilizer (20:20:20), urea and ammonium sulphate were also applied to the grapevines and growth was again unified before applying the assigned treatments. Irrigation with the assigned treatments started in May. All pots received the same amount of water whenever irrigation was applied. Each pot received a total amount of irrigation water equal to 446 mm. Irrigation was scheduled according to evaporation readings from free water surface (in mm) taken every 48 hours and corrected using proper grapevine crop coefficient of 0.30 (according to Food and Agriculture Organization).

DNA and RNA extraction

Leaves were sampled starting from February until November, 2014 (every two weeks) and frozen in liquid nitrogen for analyzing DNA and RNA using kits (iNtRON, Korea). Total RNA was extracted from frozen leaf samples with the IQeasy™ Plus Plant RNA Extraction Mini Kit (iNtRON Biotechnology, Korea) according to user's manual. RNA concentration and purity were estimated based on absorbance at 260 and 280 nm. Two microgram of RNA was used to reverse transcribe the first strand cDNA using Power cDNA Synthesis System (iNtRON Biotechnology, Korea) according to the manufacturer's protocols with oligo $(dT)_{15}$ as a primer in a reaction volume of 20 µl.

The first strand cDNAs generated for all the samples were used for semi-quantitative RT-PCR to monitor the transcript levels. The gene-specific primers for RT-PCR were designed based on the basis of the sequences published in GenBank using the software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The *EF-1α* gene was selected as a reference gene. The primer sequences are listed in Table (1). The PCR reaction was performed using iNtRON *i*-MAXTM II system (iNtRON, Korea). The same thermal profile was used for all PCR reactions; PCR was initiated with enzyme activation at 95°C for 2 min followed by 32 cycles of 40s at 95°C, 40s at 56 °C and 1 min at 72°C. Different amplification cycles were tried and the product of the 32 cycles was selected to be presented. All RT-PCR products were loaded in ethidium bromide-stained 1.5 % (w/v) agarose gel.

Table 1 - Primer pairs used in gene expression analysis

Gene	GeneBank ID	Primer pairs (5'→3')	Amplicon size (bp)
Ascorbate peroxidase (APX)	EU280159	F: GACAATGAAGCACCCAGAGGAG R: AATGGGCTTCAGCATAGTCAGC	542
CuZn-Superoxide Dismutase (CuZn-SOD)	AF056622	F: CTGCTCCATCTCGTGTCTTTCT R: ATCCACAATTGTTGCTTCAGCC	452
Mn-Superoxide Dismutase (Mn-SOD)	NP_001268135	F: AGAAAATCGCTAGGGTTAGGGC R: TACCCAGCAATGGAACCAAGTT	538
Catalase (CAT)	AF236127	F: AGGCCCAGTTCTTCTTGAGGAT R: AGGCAAGCATCTCATTCTCAGC	860
Glutathione Peroxidase (GPX)	XM_002272900	F: ATGTCGAAGCAAATACAGCAGG R: TGAGAGGGGAAGTTGTTGGGTA	472
Monodehydroascorbate reductase (MDAR)	NP_001267971	F: TCATGTTTGTGTTGGAAGCGGA R: GGACAGATCAAAGGCACGAGAG	868
Elongation factor 1α (EF-1α)	XP_002277159	F: ATTGTGGTCATTGGCCATGTTG R: CCTTCGAAACCAGAGATGGGAA	566

3. Results and Discussion

As an abiotic stress, salinity induces oxidative damage in plant cells through the increased generation of Reactive Oxygen Species (ROS) in different cell compartments (Mittler, 2002). The activation of antioxidant defense system reduces the level of ROS and minimizes the impact of oxidative stress and its associated damage. Plant cells possess antioxidant enzymes which have the ability to detoxify toxic ROS (Apel and Hirt, 2004).

Rootstock choice should be taken with careful consideration since the scion is dependent on the rootstock (Creasy and Creasy, 2009). Novel rootstocks are frequently used to confer resistance to environmental adversities in horticultural crops (Albacete *et al.*, 2015). A promising approach to improve salt tolerance of horticultural species is the use of grafting on salt-tolerant rootstocks (Colla *et al.*, 2010; Giuffrida *et al.*, 2014; Simpson *et al.*, 2015; Zrig *et al.*, 2016). Grapevine rootstock selection is a key factor and could be considered an important strategy to mitigate salinity stress. Grape rootstocks do influence the scion cultivar in many growth and physiological aspects (Gu, 2003). However, some contradictions can be found in the literature in terms of the salt tolerance of grapevine rootstocks implying that various factors are involved, which eventually determine grapevine response to salt stress. For example, Southey and Jooste (1991) found that American hybrids performed poorly in response to salinity when used as rootstocks for the cultivar 'Colombard'. In addition, Cavagnaro *et al.* (2006) concluded that Argentinean cultivars performed better than European cultivars in an *in vitro* salinity evaluation study. Regarding differences in ranking rootstocks, Dardeniz *et al.* (2006) indicated that 41B was the most salt resistant rootstock, followed by 140Ru and P1103, and the least resistant was 5 BB. On the other hand, Walker *et al.* (2002) showed that the highest salt resistance was obtained when P1003 was used as a rootstock.

To determine whether NaCl-induced stress and rootstock type were able to regulate the expression levels of genes responsible for antioxidant defense response, a semi-quantitative RT-PCR assay was performed for the analysis of six major antioxidant enzyme genes (*APX*, *CAT*, *CuZn-SOD*, *Mn-SOD*, *GPX*, and *MDAR*) (Fig. 1).

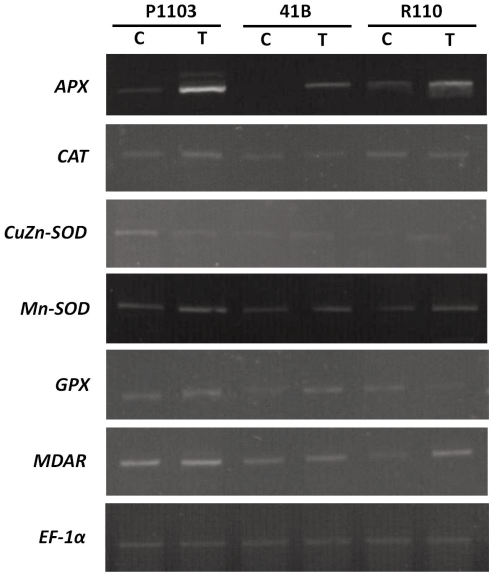

Fig. 1 - Semi-quantitative RT-PCR expression analysis of major antioxidant enzyme genes in 'Superior Seedless' grafted on different rootstocks and exposed to salinity for two weeks. The *EF-1α* gene was used as the internal control for normalization of loading.

Plants utilize antioxidant enzymes, such as: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDAR), as defense mechanisms against salinity and do have the ability to detoxify toxic ROS (Apel and Hirt, 2004).

Their expression has been studied in many crops as previously mentioned in the introduction part. However, such knowledge is lacking in *Vitis* species, specifically in *Vitis berlandieri* and *rupestris*. Meanwhile, their expression is extensively studied in both *vinifera* rootstocks and cultivars (Carvalho *et al.*, 2015).

After salinity exposure for two weeks, the transcript levels of *APX*, *Mn-SOD* and *MDAR* were increased in leaves of 'Superior Seedless' grafted on the different rootstocks. However, their expression levels in response to salinity were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* gene showed obvious enhanced level in plants grafted on P1103 in response to salt exposure. However, the expression of *CAT* gene in 'Superior Seedless' scion grafted on 41B or R110 showed almost unchanged level in control and stressed conditions.

Down-regulation of *CuZn-SOD* was recorded in leaves of 'Superior Seedless' grafted on P1103, while, slight up-regulation of this gene in response to saline condition was recorded when scion was grafted on 41B or R110. In response to salinity stress, the expression of *GPX* was enhanced in scion grafted on P1103 and 41B. On the other hand, scion grafted on R110 showed decreased expression of *GPX* in response to salt treatment.

The expression of these genes was evaluated every two weeks, and only the results after the first two weeks of the stress treatment were presented since no differences in expression were noticed at the other time points between different treatments. This indicates early differential responses to salt stress at the level of antioxidant defense genes. Such responses were previously reported in other plant species (Ellouzi *et al.*, 2014; Ranjit *et al.*, 2016).

4. Conclusions

Rootstock choice is critical in determining grapevine performance and productivity under saline conditions. Expression levels of *APX*, *Mn-SOD* and *MDAR* were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* and *GPX* genes showed obvious enhanced level in plants grafted only on P1103 in response to salt exposure. *CuZn-SOD* was down-regulated in leaves of 'Superior Seedless' grafted on P1103 and up-regulated when scion was grafted on 41B or R110. Rootstocks with enhanced expression levels of antioxidant defense genes, such as superoxide dismutase (SOD) and catalase (CAT) can possibly eliminate or reduce detrimental effects of ROS. Thus, grapevine rootstocks that possess this defense line are more preferable to be utilized for soils subjected to salinity or grapevines irrigated with diluted brackish water. Moreover, additional studies are needed to investigate the salt-stress responses of enzymatic and non-enzymatic antioxidant components in grapevine grafted on contrasting rootstocks.

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Anatomical and morphological changes in scion of some olive grafting combinations under water deficit

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Abstract: Effects of water stress deficit were studied on xylem anatomical features and some growth parameters among six olive grafting combinations; Amygdalifolia/Arbequina (Am/Ar), Amygdalifolia/Koroneiki (Am/Ko), Amygdalifolia/Zard (Am/Z), Conservallia/Koroneiki (Co/Ko), Conservallia/Zard (Co/Z) and Conservallia/Arbequina (Co/Ar) of about three-year-old olive trees (*Olea europaea* L.) under greenhouse conditions. To realize this, a factorial experiment was conducted in a completely randomized design (CRD). The results showed that rootstocks exhibited significant effects on scions xylem anatomical physiognomies, such as vessel lumen area (VLA) and vessel diameter (VD) and additionally on some growth indices including main stem length (SL), lateral shoot number (LSN) and graft union-cross sectional area (GU-CSA). Xylem anatomical characteristics including VLA, porosity, vessel frequency (VF) and VD of scions decreased when they were grafted onto Arbequina and Koroneiki rootstocks, but increased onto Zard rootstock. All growth parameters showed a decrease under drought stress, while this reduction was more pronounced for Zard rootstock than the other rootstocks. However, Co/Z showed the highest VF and the lowest vulnerability index (VI) and exhibited a better performance at the end of recovery.

1. Introduction

Olive (*Olea europaea* L.), as an evergreen tree, is often under severe drought stress conditions in summer. While withstanding a high level of drought situations (Lo Gullo and Salleo, 1988), the resistance level depends on cultivar and genetic characteristics of the plant (Bacelar *et al*., 2006; Therios, 2009). The ability of acclimation to water deficit includes morphological, physiological, biochemical and anatomical mechanisms (Bacelar *et al*., 2006; Therios, 2009) as inhibition of cell expansion, leaf area limitation, dense cuticles and trichomes, shoot growth cessation and

foliar chemistry changes (Bacelar *et al*., 2007; Guerfel *et al*., 2009).

Rootstocks can enable the scion for normal growth under water deficit (Turner, 1986). In fact, grafting of commercial cultivars on tolerant rootstocks is a specific technique of acclimation to environmental stresses as a rapid tool compared to breeding methods (Flores *et al*., 2010). Knowledge about anatomical characteristics of rootstock and scion is essential to understand their interactions under drought conditions. Some researchers suggested that vigor reduction induced by dwarfing rootstock may contribute to change water transfer at the graft union (Soumelidou *et al*., 1994; Atkinson *et al*., 2003). Other investigations have been focused on the role of tree water status, determining the vegetative structure of grafted tree (Basile *et al*., 2003; Solari *et al*., 2006). It has also been emphasized that sensitivity to drought is related to vessel dimension (Lo Gullo *et al*., 1995; Trifilo *et al*., 2007). Xylem vessels of plants subjected to drought are in danger of emboli and dysfunction. Therefore, drought-adapted trees or shrubs usually have narrower vessels with concomitant in the higher number of vessels per mm^2 compared to the drought-sensitive trees (Carlquist, 2001). In a study by Trifilo *et al.* (2007) it has been reported that dwarfing rootstocks effectively reduce grafted plant size, although they are not essentially responsible for higher tolerance to drought by scions improving tree resistance to water deficit. It is an ordinary trend to increase root, stem vessel diameter, and decrease vessel density with tree height in some fruit and forest trees (Zach *et al*., 2010). Many xylem traits such as xylem-to-phloem ratio, vessel density, and vessel size influence dramatically the rootstock growth ability (Meland *et al*., 2007; Trifilo *et al*., 2007), playing an important role in hydraulic conductance of root and stem (Tombesi *et al*., 2010; Zach *et al*., 2010). Thus, number and diameter of vessels are two main characteristics determining hydraulic conductance (Tyree and Ewers, 1991). Smaller and fewer vessels in the scion and graft union of cherry trees could be related to hydraulic resistance and a decreased growth of scion (Olmstead *et al*., 2006). Anatomical parameters such as vessel frequency, vessel lumen area and percentage of vessels on wood cross section are reliable to preselect tree vigor. It has been widely accepted that reduced plant growth via increased hydraulic resistance derives from graft union and xylem conduit structure (Goncalves *et al*., 2007; Tombesi *et al*., 2010).

A comprehensive understanding of grafted trees vulnerability to drought derived from different scion/rootstock combinations will be useful in orchards situated in semi-arid regions experiencing drought stress. In fact, olive trees grow in Mediterranean basin with low summer rainfall while irrigation is not a popular practice. Using cloned rootstocks that potentially control scion vigor is a very necessary factor for establishing new olive orchard as an innovative cultivation method (Baldoni and Fontanazza, 1989; Rugini *et al*., 1996). Information on xylem anatomical responses of grafted olives under drought stress conditions are few. It has been assumed that different olive rootstocks may induce various anatomical and morphological modifications in the scions (Therios, 2009). Hence, the aim of the present study was to investigate xylem anatomical changes of scions' stems and morphological alterations of some olive grafting combinations under water deficit conditions.

2. Materials and Methods

Plant materials and experiment location

Olive (*Olea europaea* L.) current-season-growth shoots as scion (cvs. Amygdalifolia and Conservallia) were cleft grafted onto three rootstocks of two years old rooted cuttings (cvs. Koroneiki, Arbequina and Zard) in winter 2015. These grafted plants were transplanted in 12 L plastic pots containing a substrate mixture of field soil, sieved sand, and humus in a 1:1:1 (V: V: V) proportions and was placed in a greenhouse 1100 m above sea level; with a latitude 35°56' N, and longitude of 50°58' E and temperature of 28±3°C during the day and 23±3°C at night. The physical and chemical properties of pots soil mixture are presented in Table 1.

Treatments

All grafted olive trees were irrigated at field

Table 1 - Physical and chemical properties of the soil used in this study

Soil characteristics	Value
Physical properties	
Sand (%)	39
Silt (%)	38
Clay (%)	23
Texture	Loam
Chemical properties	
Organic carbon (%)	1.19
Electrical conductivity (dS/m)	2.68
pH	7.78
N (%)	0.12
P (mg/kg)	28.6
K (mg/kg)	360

capacity (FC) until the start of experiment. Moisture content of the substrate was calculated with Time Domain Reflectometry (TDR) in two opposite sides of the containers in each pot at a depth of 20 cm and then an average was calculated. The experiment was carried out in July 2016. The graft combinations were Amygdalifolia/Koroneiki (Am/Ko), Amygdalifolia/Zard (Am/Z), Amygdalifolia/Arbequina (Am/Ar), Conservallia/Koroneiki (Co/Ko), Conservallia/Zard (Co/Z) and Conservallia/Arbequina (Co/Ar). Then, they were divided into two groups; group one was irrigated at FC as control and the group two was subjected to water shortage by withholding irrigation for a period of 4 weeks (WS), (n=6). Three grafted plants were used for anatomical measurements (n=3) and three were subjected to re-watering for determination of viability and recovery. After three weeks of re-watering (recovery period), the stem length was measured in different graft combinations.

Xylem anatomical measurements

After 4 weeks of water deficit, stem pieces (2cm above the graft union) were collected early in the morning from experimental plants in both groups and immediately fixed in FAA (formalin, acetic acid, ethanol, 1:1:1, V:V:V) in the late summer 2016. Stem pieces were cross-sectioned using a sliding microtome (GLS1, WSL, and Switzerland). Cross sections with the thickness of ~10 µm were stained with 0.1 % (w/v) safranin (staining in red lignified cell walls) and 1% (w/v) Astra-blue (staining in blue-green cellulosic walls) and observed at different magnification under a light microscope (FLUO3, BEL Engineering, Italy) equipped with a digital camera (EUREKAM, BEL Engineering, Italy) connected to a computer. The last annual rings in microscopic sections were investigated both under bright filed and fluorescence lights. The vessel lumen area (VLA), vessel diameter (VD), vessel frequency (VF=number of vessels/mm^2), vulnerability index (VI= VD/VF), and porosity (total vessel lumen area/ total analyzed area ×100) were measured using Image J software (https://imagej.nih.gov/ij/). VI was calculated to assess vessel susceptibility to damage as a reliable indicator during the water deficit. VD was calculated using the mean value of the vessel lumen area [VLA = $(VD/2)^2 \pi$], to estimate idealized diameter.

Morphological parameters

In order to evaluate the growth indices of grafted plants under the period of water stress deficit, main stem length growth (SL) was calculated based on a difference between the stem length above graft union at the beginning and at the end of the drought period. Stem diameter (middle of the graft union) was measured with a hand caliper at the end of the drought period (4 weeks) in two opposite sides and an average was applied to calculate the graft union cross sectional area (GU-CSA) in mm^2. Lateral shoot number (LSN) was recorded by counting the shoots in the main stem (above the graft union) at the beginning and at the end of the drought period. Leaf number, above the graft union, was recorded at the beginning and at the end of the drought period and then a difference was calculated as LN (the leaves having more than 1 cm length were counted). Leaf area (LA, cm^2) was measured using leaf area meter at the end of the drought period and an average of five full-expanded leaves was used for the analysis.

Data analysis

A factorial experiment was conducted in a completely randomized design (CRD) with 9 replications. Treatments included 6 graft combinations and 2 levels of irrigation. Normal distribution of data was investigated using Shapiro-Wilk test. Data were analyzed by SPSS version 20.0 statistics software using multivariate analysis of variance (MANOVA) and means were compared by Duncan's multiple range test at probability of 5%. Pearson's correlation coefficients were tested among the analyzed characteristics, using values from 36 graft combinations.

3. Results

Xylem anatomical properties

Statistical analysis of the data showed significant effects of the rootstocks on the scion VLA, SL, LSN, GU-CSA and VD. The main effects of rootstocks showed that Koroneiki induced the highest values of VLA (514.5 µm^2) and VD (25.4 µm) in the scion, which was significantly greater than those of induced by zard rootstock. However, there was no significant difference among three rootstocks in porosity, VF, and VI (Table 2).

The interactions between graft combinations and water stress on scion xylem anatomical characteris-

Table 2 - The effects of different rootstocks on xylem anatomical properties of scion

Rootstock	Vessel lumen area (µm^2)	Porosity (%)	Vessel frequency (n/mm^2)	Vessel diameter (µm)	Vulnerability index
Arbequina	464.1 a	5.3 a	107.8 a	24.2 a	0.28 a
Koroneiki	514.5 a	6.9 a	133.4 a	25.4 a	0.27 a
Zard	371.7 b	4.5 a	119.4 a	21.2 b	0.23 a

tics showed that water shortage caused a decrease in the vessel lumen area (VLA) of both scions (Amygdalifolia and Conservallia) grafted onto the Arbequina and Koroneiki rootstocks (Fig. 1). On the contrary, it increased VLA in both scions grafted onto Zard rootstock (Fig. 1A). Arbequina and Koroneiki rootstocks decreased porosity and VF of Amygdalifolia and Conservallia scions under water shortage conditions; however, Zard rootstock increased porosity and VF of both scions (Amygdalifolia and Conservallia) (Figs. 1B and 1C). Water shortage increased vessel diameter in Am/Z and Co/Z combinations compared with the corresponding controls (Fig. 1D). The highest VI was observed in Co/Ar and Co/Ko whilst the lowest gained in Co/Z under water stress conditions (Fig. 1E).

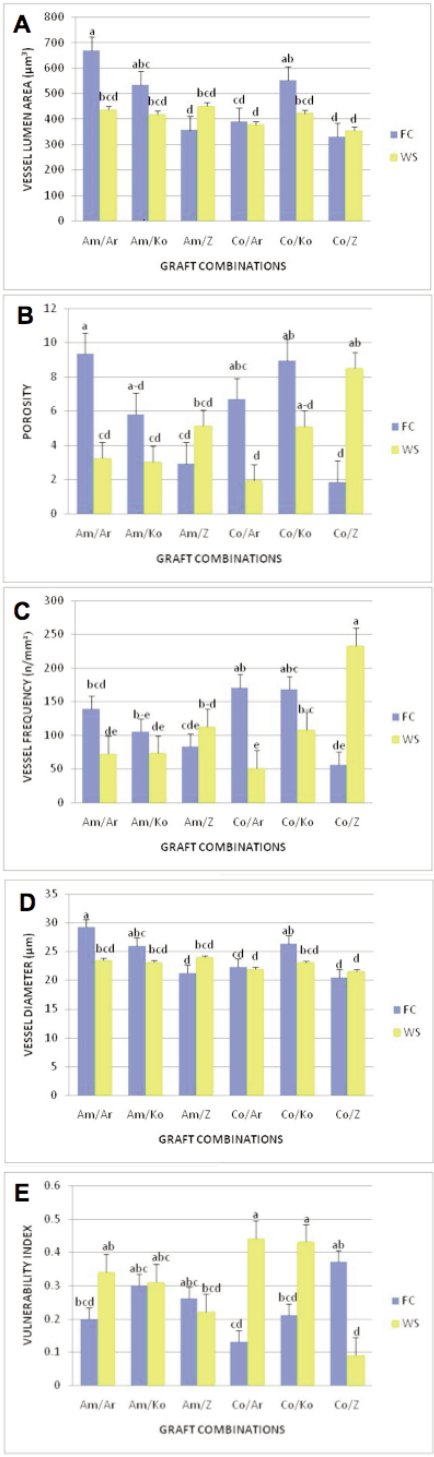

Fig. 1 - Interaction effects of graft combinations (Am/Ar, Am/Ko, Am/Z, Co/Ar, Co/Ko, Co/Z) and water stress deficit on vessel lumen area (A), porosity (B), vessel frequency (C), vessel diameter (D) and vulnerability index (E). Am (Amagdalifolia), Ar (Arbequina), Ko (Koroneiki), Co (Conservalia) and Z (Zard). Vertical bars indicate SE. Each value represents the mean ± SE of 3 replicates. Means with the same letters are not significantly different (P>0.05) using Duncan Multiple Range Test.

Scion morphological characteristics

Regardless of the scion types and irrigation levels, rootstocks affected some growth characteristics of scion; Koroneiki rootstock exhibited the highest value of scion stem length (SL), which was significantly greater than that of Zard rootstock (Table 3). This rootstock had the lowest graft union cross sectional area (GU-CSA). Arbequina presented the greatest GU-CSA and lateral shoot number (LSN). Koroneiki showed the highest leaf area (4.86 cm^2) and leaf number (24.38), however, they were not significantly different from the other rootstocks (Arbequina and Zard) (Table 3).

Table 3 - The effects of different rootstocks on xylem anatomical properties of scion

Rootstock	Leaf number	Leaf area (cm^2)	Lateral shoot number	Stem lenght (cm)	Graft union cross sectional area (mm^2)
Arbequina	18.33 a	4.10 a	1.00 a	3.95 ab	55.74 a
Koroneiki	24.38 a	4.86 a	0.71 ab	5.80 a	44.45 b
Zard	18.42 a	3.92 a	0.25 b	1.85 b	47.13 ab

In all graft combinations, water stress deficit significantly decreased the number of leaves and lateral shoot number; however, these reductions were not significant in Co/Ko combination (Figs. 2A and 2C). Drought stress also decreased leaf area in all graft combinations, although this reduction was only significant in Co/Ar combination (Fig. 2D). Water stress deficit declined scion stem length (SL) in all graft combinations such that there was no growth in Am/Z and Co/Z and stem length reduction was not significant in Co/Ko combination compared to the control (Fig. 2B). Drought stress significantly reduced GU-CSA in Am/Ar and Am/Ko while had not significant effect on other graft combinations (Fig. 2E*)*. All grafted plants continued to grow as a result of re-watering on scions stem length growth. However, the resulting SL were significantly lower than the controls except in the case of Co/Z. Am and Co scions, exhibiting no SL growth under water stress deficit conditions onto Zard rootstock, showed a SL elongation which were not significantly different from the

corresponding controls (Fig. 3). Fluorescence microscopy helped us to distinguish tree ring boundaries since the intensity of emitted light by these specimens diminished from early wood to late wood (Figs. 4C and 5A) or were different between these two sub-annual areas (Fig. 5C). The fluorescence

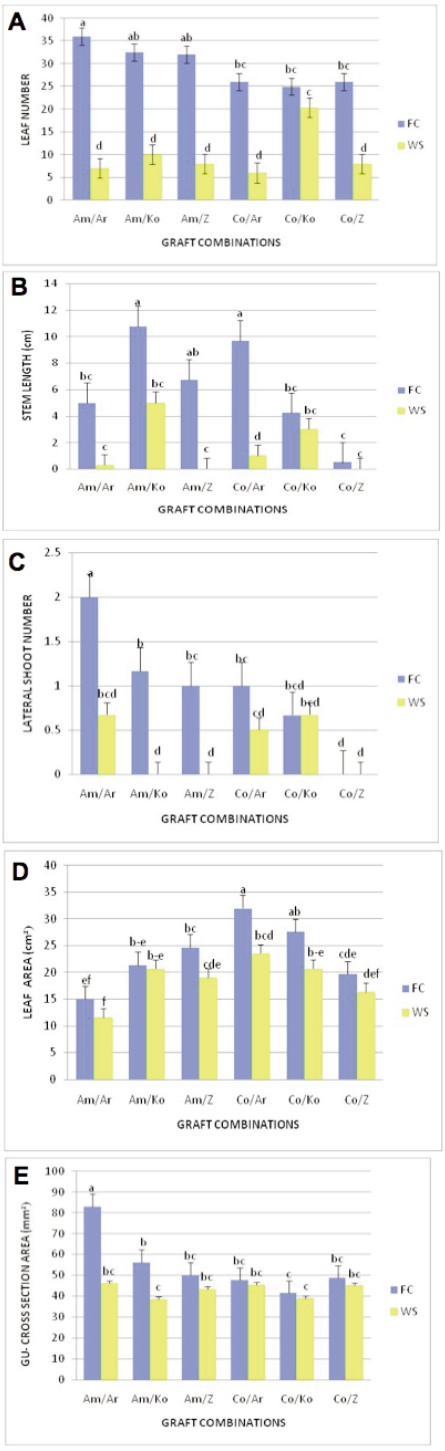

Fig. 2 - Interaction of graft combinations (Am/Ar, Am/Ko, Am/Z, Co/Ar, Co/Ko, Co/Z) and water stress deficit on leaf number (A), stem length (B), lateral shoot number (C), leaf area (D) and gu-cross section area (E). Am (Amygdalifolia), Ar (Arbequina), Ko (Koroneiki), Co (Conservallia) and Z (Zard). Vertical bars indicate SE. Each value represents the mean ± SE of 3 replicates. Means with the same letters are not significantly different (P>0.05) using Duncan Multiple Range Test.

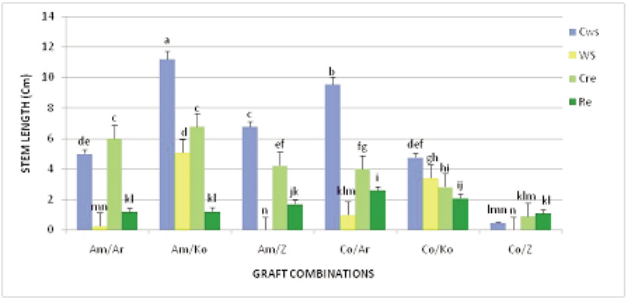

Fig. 3 - Stem length of graft combinations (Am/Ar, Am/Ko, Am/Z, Co/Ar, Co/Ko, Co/Z) under water stress deficit (WS) and Recovery (Re) and their controls (Cws), (Cre), respectively. Am (Amygdalifolia), Ar (Arbequina), Ko (Koroneiki), Co (Conservallia) and Z (Zard). Vertical bars indicate SE. Each value represents the mean ± SE of three replicates. Means with the same letters are not significantly different (P>0.05) using Duncan Multiple Range Test.

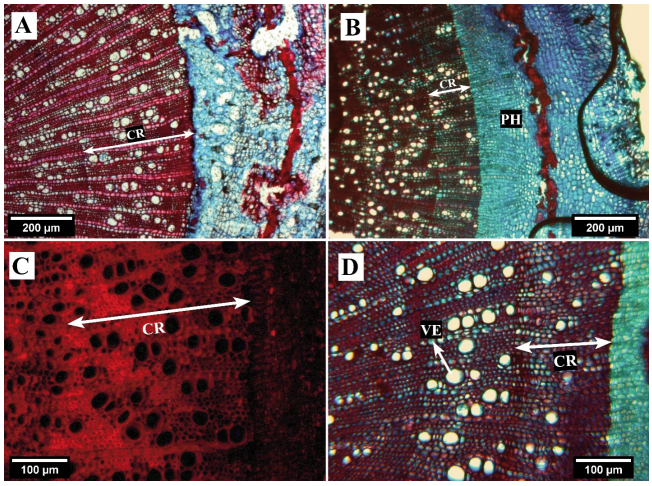

Fig. 4 - Micrographs of Am/Ko (Amygdalifolia/Koroneiki) combination of olive stem (2 cm above graft union) under bright field (A, B, D) and blue fluorescence light (D). Water-stressed samples (B and D) formed narrower rings with smaller vessels comparing with the control (A and C) (CR= current annual ring, VE= vessel, PH= phloem).

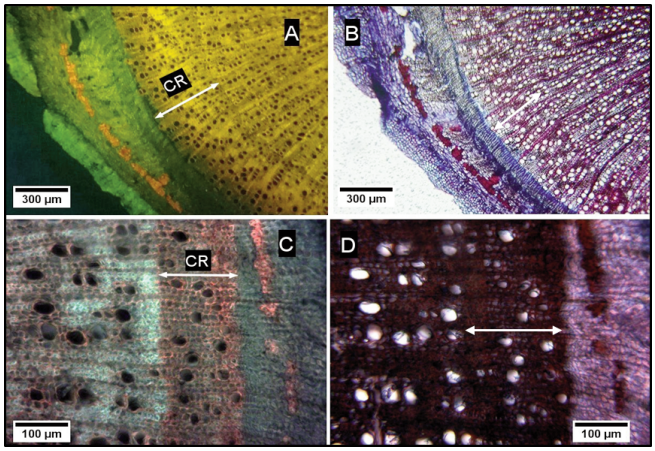

Fig. 5 - Micrographs of Co/Ar (Conservallia/Arbequina) combination of olive stem (2 cm above graft union) under irrigation (A and B) and water deficit conditions (C and D). The right-hand photos (B and D) were taken from samples under bright field light while A and C are the corresponding samples, respectively, under UV and blue fluorescence light, CR= current annual ring.

emission light in the late wood of control samples was different in intensity and/or color compared with those of under stress (Fig. 5). It seemed that the late ed fibers in water stressed samples are chemically different from control samples.

4. Discussion and Conclusions

Xylem anatomical properties

Vessel number and VD are considered as the main factors determining the hydraulic conductance (Tyree and Ewers, 1991). In addition, it has been hypothesized that VD and VF may affect drought tolerance. Thus, the highest VF value in Zard might explain its better performance under drought stress in comparison with the two other rootstocks analyzed in this study (Fig. 1C). These results are consistent with the anatomical analysis in cherry rootstocks (Goncalves *et al*., 2007; Meland *et al*., 2007; Zoric *et al*., 2012), while dispute the results on apple tree (Bauerle *et al*., 2011) due to genetic-dependent responses of different tree species. Since all plants in this study were grown at the same conditions, xylem anatomical differences among the control rootstocks may have a genetic basis; as it has been supposed that low vigor rootstocks may hereditary produce smaller vessels (Beakbane and Thompson, 1947). Correlation of an increase of VD and a decrease in VF with tree height demonstrated in many other investigations (Trifilo *et al*., 2007; Zach *et al*., 2010). Cloned rootstocks are less vigorous, having changes in the anatomy of xylem vessel, which may clarify their effects on shoot behavior (Atkinson *et al*., 2003). In the current research, Zard induced a narrower vessel formation compared with Arbequina and Koroneiki, confirming the results of a previous study on olive tree (Trifilo *et al*., 2007). Interestingly, Zard caused Conservallia to produce the highest number of xylem vessels (VF) under water stress in comparison with the scions grafted onto other rootstocks (Fig. 1C). Zard rootstock also increased VF in Am scion in lesser degree than Co. Vessel frequency (VF) is often increased by drought (Sterck *et al*., 2008; Fichot *et al*., 2011) through improving the hydraulic conductance (Scoffoni *et al*., 2012). A decreased VF has also been reported under drought stress (Corcuera *et al*., 2004), which is in line with the current results on the scions grafted onto Arbequina and Koroneiki rootstocks, suggesting drought may contradictorily influence olive different rootstocks. The drought stress increased the VI in Am/Ar, Co/Ar, Co/Ko and Am/Ko combinations, while decreased in two other combinations (Am/Z and Co/Z). It is generally accepted that cultivars with smaller and frequent vessels exhibit low VI (Carlquist, 1977), a rationale for a better water transport by Co/Z. This finding is consistent with that of Salvia (Hargrave *et al*., 1994), and suggests wider vessels (greater VD) might be more sensitive to abnormal function performance compared with vessels having smaller diameter. Additionally, it is supposed that scions may be more susceptible to show embolism onto invigorating rootstocks during water stress (Hargrave *et al*., 1994).

Scion morphological properties

Leaf expansion is the most sensitive characteristic to water deficits, because turgor reduction is the earliest significant biophysical effect of water stress, and leaf expansion is a turgor-dependent activity (Taiz and Zaiger, 2003). Limited leaf area resulted in a low photosynthesis and consequently decreased the shoot length (Marron *et al*., 2002), the number of leaves and the number of lateral shoots. Although SL reduction is expected under drought stress, differences among graft combinations can be originated from genetic elements of rootstocks (Goncalves *et al*., 2007). Vessel diameter reduction may cause a reduced growth in the scions grafted onto dwarf rootstocks and ultimately result in diminishing stomatal conductance and photosynthesis. The differences in scions growth can be caused by root hydraulic, which plays an important role in the control of olive plant growth (Nardini *et al*., 2006). Thus, it can be suggested that plant height and leaf area reduction may be correlated to a low cell development under water deficit in some species, implying the lowest values of SL and LA by Zard rootstock in this study. Although Zard rootstock stopped SL under water deficit conditions, however, it increased porosity and VF. It might be concluded that this rootstock employed assimilation for changing the pattern of xylem vessel as an alternative to elongating SL. Drought reduces the leaf number per plant. In fact, leaf area extension depends to water status, temperature, and assimilation supplied for growth, which may be affected by drought in a plant. LA reduction in the current study is in consistent with results in *Populus* and *Ziziphus* (Suther and Patel, 1992; Thakur and Sood, 2005).

In relation to correlation between measured traits, VLA and VF didn't show any correlation (r= 0.19). On the other hand, Porosity (%), is positively affected by both of these variables. Usually, when

VLA is decreased in an angiosperm plant, a decrease in water flow capacity is compensated by increasing the number of produced vessels. Hence, VLA and VF are usually negatively correlated (Oladi *et al*., 2014). However, in this research, the water deficiency resulted in a simultaneous decrease of both features (except for Zard rootstock), suggesting a different strategy employed by olive tree. Positive correlation between VD and GU-CSA demonstrated that vigorous rootstocks with higher GU-CSA had larger vessels, facilitating transfer of more water to aerial parts. A correlation value of 0.31 between LN and VD suggests that larger vessels might result in higher number of leaves in grafted trees. There is a relatively high correlation between LN and SL (r= 0.71), exhibiting a longer SL probably due to more leaf numbers on different rootstocks under irrigation and deficit conditions. Recent studies have shown that differences among rootstocks in either root size or scion extension influence soil-water-plant relationship (Clearwater *et al*., 2007; Cohen *et al*., 2007; Rodriguez-Gamir *et al*., 2010). Xylem vessel traits may determine hydraulic conductance extent and subsequently affect the current season vegetative growth. It seems VF is the main anatomical difference among the studied rootstocks. Several investigations suggested smaller and fewer vessels in graft union can contribute to water flow resistance, resulting in growth reduction (Olmstead *et al*., 2006; Goncalves *et al*., 2007) as observed in the case of Zard rootstock.

In this research, water stress deficit decreased the growth indices of all grafting combinations and among them, Zard rootstock inhibited any growth. Considering anatomical analysis, Zard rootstock induced higher porosity and VF in both scions (Am and Co) and exhibited the lowest VI under water stress conditions. In contrast, although Koroneiki and Arbequina showed some growth under water stress, however, induced high VI in both scions (Am and Co) and they did not attain the same performance compared to the control at the end of recovery. However, Co/Z combination showed the best performance compared to the control at the end of recovery.

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Short note

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Opuntia ficus-indica (L.) Mill. growing in soil and containers for urban agriculture in developing areas

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Abstract: Urbanization and poverty have brought to worse life conditions in towns of many developing countries, including difficult availability of food, especially fresh. Urban agriculture and horticulture can contribute to the availability of fresh foods, officinal and medicinal plants, but the little availability of irrigation and surface to destine for cropping suggest the convenience of little water consuming species, with little needs of soil fertility and that can be eaten entirely. *Opuntia fiscus-indica* (L.) Mill. corresponds at all these requirements, and it is a very promising strategic species that can be eaten completely (green parts, fruits and even flowers), it has good nutritional values and also interesting medical properties. A trial has been done to compare the initial productivity of cladodes multiplied in pots, car tires and open field. Our results suggest that the prickly pear can be cropped better in large exhausted tires than in small plots also saving money for the materials.

1. Introduction

Urbanization and poverty cause food insufficiency in many tropical and subtropical countries and this problem is enhanced in towns and urban peripheries where land plots to crop are few and small and there is irregular availability of foods in local markets. A few examples: Accra (Ghana) is a growing town that loses about 2.600 hectares of farm land every year to buildings and consequently reduces agricultural land availability nearby and into the town. The peripheral agriculture in Hanoi (Vietnam) produces at least 150.000 tons of fruit and vegetables per year. In Cuba urban and peripheral agriculture gives around 60 percent of horticultural produce consumption in large towns. Still in Cuba due to the constraints caused by trade embargo, a growing percentage of the agricultural production is provided by urban agriculture: in 2002 more than 14.000 ha of urban yards produced 3.100.000 tons of food, and 90%

of the Habana fresh produce comes from local urban farms. In 2003 more than 200.000 Cubans worked in the expanding urban agriculture sector (Cuban Ministry of Agriculture, 2013). In Kinshasa (Democratic Republic of the Congo), urban agriculture is producing an estimated 75.000 to 85.000 tons of vegetables per year that is 65% of the town supply.

Agriculture is being pushed far from many towns, with increasing costs of food transport, packing and conservation enhanced by the bad conditions of rural roads, and heavy losses in transit.

In these areas, growing fruit and vegetables in and around cities increases the supply of fresh produce and improves the economic access to food for poor people (FAO, 2015) that spend 60-80% of their income on food. Urban families may actually grow crops or raise small animals, and so produce some of their own food (Cohen and Garret, 2009). Moreover many foods available in towns and peripheries of developing Countries are introduced with international aid and consequently they are a constraint to food sovereignty.

Opuntia ficus-indica is a wild plant and a cultivated crop, whose value is largely underestimated. Its green parts are commonly used in environmental protection against soil erosion and as forage (Mulas and Mulas, 2004), the biomass is used for biogas (Rosato, 2014), industrial sectors use prickly pear fruits and leaves as raw materials for cosmetics, drinks and food additives (Saenz *et al*., 2013). Its flowers are useful to honey bees, but all its above ground parts are also good meals in form of fruits, fresh and cooked salads, soups, refreshing beverages, flour for breads and pastas. Fruits and cladodes are very nutritional (Rodriguez *et al*., 1996; Saenz *et al*., 2013), and their use a human functional food dates back to pre-columbian times (Ramírez *et al*., 2010). Especially young cladodes (*nopalitos* in Latin America) conserve water that is very useful in hot dry areas for both humans and livestock, around 9% crude protein, low fats (just around 1%), several minerals especially potassium (220 mg per 100 grams), calcium (16-33 mg), phosphorus (13-28 mg), and vitamins especially C vitamin (ascorbic acid 40 mg per 100 grams), with the best nutritive values being in the young cladodes (Retamal *et al*., 1987). The seeds are commonly considered a waste of the food industry however the extraction of oil is under study for applications in food, pharmaceutical and cosmetic industries (De Wit *et al*., 2017). Calories content vary within 25 and 50 kcal per 100 grams dry which is comparable to the most common fruits, moreover it has important pharmaceutical effects including antioxidants and also anti-cancerogenic effects (Livrea and Tesoriere, 2006). For all this uses the prickly pear has to be considered a multipurpose species quite useful also in many agroforestry systems.

Opuntia ficus-indica is a species of the *Cactaceae* with CAM metabolism that favors water conservation, even if young cladodes have a C_3 metabolic pathway (Mulas and Mulas, 2004) that causes water recall from lower-older cladodes during the day reducing water availability for the whole plant on benefit of the growth of younger parts (Wang *et al*., 1997).

Opuntia ficus-indica is a wild plant native of Mexican deserts, with very little needs of soil fertility and capable of growth even without any management. Few parasites treat this plant. All this makes this crop possible in most tropical-subtropical areas with enhanced hot and dry season, with poor soils and little management. It can be cropped also in permaculture and with this techniques it becomes an additional strategic plant useful as disasters relief crop beside short cycle cereals, beans and vegetables, after people displacement due to earthquakes (see in Haiti capital town), hurricanes (as common in Caribbean islands like Cuba), or after war situations (like recently happened in Somaliland, Iraq, Libya) when the whole national or regional agro-food system has to be restarted.

One of the problems related to prickly pear food harvesting and processing is related to the spines or glochids (hichy hair) that can be removed by immersion in water and mechanical treatment or fire burning. However there are spineless varieties (var. *inermis*) commonly used for forage, and also varieties almost without glochids that can be handled safely and make easy harvesting and processing, even if the absence of defensive parts make the plants more sensible to animal predation.

Due to the good nutritional values, the little water and soil needs and the easy management, prickly pears can be advised as a strategic crop for urban and peripheral areas with difficult climate or poor soil, as a green fence and also cropped in pots and exhausted tires as common in many towns of tropical developing countries.

The productivity of prickly pear has been studied for field production or in greenhouse but still oriented to later in field transplanting. Researches have been done about how cladode size, their position at

planting can influence initial growth and production (Bakali *et al*., 2016) and has been suggested that horizontal planting is the best technique in arid regions, and that cladode size influence early dry matter production. The same authors proved that cladode orientation to the sun, and the planting depth had not significant effects on early production. However Singh and Singh (2003) suggest that planting vertical is generally the best technique together with using cladodes 12 months old, and planting in spring. The effects of planting cladode parts instead of whole has been studied by Stambouli-Essasi *et al*. (2015), that suggest the possibility to use just parts in order to save planting material. Concerning plant densities in open field, Ruiz-Espinosa *et al*. (2008), suggest plantations of 60.000 plants per hectare. Concerning different clones, several authors have got diversified results in different environments, thus suggesting a relationship clone-environment (Flores, 1992; Flores-Hernandez *et al*., 2004; Ruiz-Espinosa *et al*., 2008).

Although there are few data available on *Opuntia* cultivation in containers, these are not intended for food production. Our research is oriented to the production of a strategic food plant and has compared the productivity of prickly pear cladodes grown in soil (as the case of green fencing) or in pots (the case of urban agriculture in balconies) and in exhausted tires (as common in backyards, small yards or balconies of urban peripheries of many developing countries).

2. Materials and Methods

The trial was done on a spineless cultivar of *Opuntia ficus-indica* (L.) Mill. without glochids imported from Cuba in the winter 2011, 'Milpa Alta' that is the most cultivated in Mexico (Gallegos-Vasquez and Mondragon-Jacobo, 2011), it is an erected cultivar with elliptic cladodes, spineless and practically without glochids, with yellow fruits. The introduced parts have been multiplied for four years in order to have the necessary number of green parts, and then planted for the trial in Central Italy (Florence hills, 250 m asl, exposed to south) on the first of May 2015 and then again on May 2016. The location has 750 mm average annual rainfall with 159 during the 90 summer days, 14.5°C average annual temperature, in the period 1971-2000 there was an average of 62 days with maximum temperatures above 30°C. The soils of the trial location are mainly clayey (45% clay).

The initial plantation has been done using only young cladodes produced by mother plants the summer before (August 2014), planted after winter on 1st May 2015, thus 8 months old. A second plantation was done in 2016 after winter on 1st May, starting from cladodes produced by mother plants in the summer before (August 2015), thus also these 8 months old.

All the cladodes used for first and second planting had similar size, 5-7 cm width and 20-25 cm length in order to have very similar conditions of all plants at the beginning.

Each cladode was planted in a plastic pot or in a tire or straight in the soil. In extensive soil they were planted in three single lines at distances of 50 cm on the line, simulating a soil area available similar to what they have in plots or tires.

Pots were 20 cm large x 30 cm deep that is a size quite common in local canned food tins that are used as flower pots, exhausted tires were borrowed all the same size 70 cm diameter (45 inner) x 23 cm width that is a 4WD type very common in developing Countries. One pot has been got from each tire. Tires were put on cemented area to avoid rooting in external soil. The tires were brought back to the giver after the end of the trial for ecological elimination by existing regulations. Pots and tires were filled with the same soil where cladodes were also planted straight.

Pots and tires were disposed in three lines alternated with the three lines of plants planted in the soil, and mixed in a randomized block design.

Treatments were not irrigated, in the attempt to reproduce conditions of absence or minimal management, common in strategic crops in harsh areas including home yards, balconies and peripheral areas where the water available serves mainly for human needs. Thus we got three treatments: 1) extensive soil, 2) plastic pots, 3) tires. Each treatment was implemented with 8 initial cladodes (8 repetitions), for a total of 24 plants per year.

Measurements were done at the end of the growing season (30 September 2015 and 30 September 2016 for all treatments) on:

1) total number of new cladodes originated from each planted (counting);

2) fresh weight of new cladodes (harvesting all new cladodes and weighing fresh with scale), the fresh weight has been considered because this food is eaten fresh (like pineapple or papaya, as examples);

3) dry weight (after fresh weighting, all new cladodes were split in two halves to favor dehydration and

oven dried for 5 days at 80°C, then weighted again to calculate the percentage of dry matter).

Fruits cannot be produced in the trial location during one growth season because it is too short.

Statistical analysis was done with LSD at P= 0.05.

3. Results and Discussion

Number of new cladodes

The number of new cladodes (Table 1) was the highest in extensive soil (6 in the average of the two years) and the least in pots (2.5 in the average). Obviously roots explore a larger area of extensive soil than in pots or tires and consequently have higher water and nutrients availability, moreover soil temperature in pots and tires (that are black) rises more than in the soil and this in turn causes higher evaporation and reduces water availability. This somehow contrasts with the ability of a container to conserve water better than extensive soil, but this doesn't happen in full summer when high temperatures cause strong evaporation.

Table 1 - Number of new cladodes in the three treatments in the two years of trial and average

Treatment	Number of new cladodes		
	2015	2016	*Average*
Extensive soil	5 a	7 a	*6.0*
Pot	2 c	3 c	*2.5*
Tire	4 b	5 b	*4.5*
Average	*3.7*	*5.0*	*4.3*

Tires are larger than pots and probably have given intermediate yields because there is more soil than in pots, small plots have brought the plants to stressed conditions very rapidly whilst tires have provided better conditions for some longer time.

The higher number of cladodes in the second year (5.0 in the average of treatments, in comparison to 3.7 of the first year) can be due to warmer temperatures that persisted up to the end of the summer 2016 whilst several cool and some rainy days happened in the year before.

Fresh weight of new cladodes

The fresh weight (Table 2) was higher in the extensive soil (778 grams in the average of two years) than in tires (508.5 grams) and in pots (only 172 grams). The higher yield in 2016 (554.7 grams in the average of all treatments) than in 2015 (417.7) is probably due to warmer summer temperatures of 2016 in comparison to 2015.

Table 2 - Fresh weight (grams) of new cladodes in the three treatments in the two years of trial and average

Treatment	Fresh weight of new cladodes (grams)		
	2015	2016	*Average*
Extensive soil	679 a	877 a	*778.0*
Pot	150 c	194 c	*172.0*
Tire	424 b	593 b	*508.5*
Average	*417.7*	*554.7*	*482.2*

Different letters show significantly different values at P=0.05.

Dry weight of new cladodes and percentage of dry matter

The percentage of dry matter (Table 3) was higher in 2016 (14% in the average of all treatments) than in 2015 (12%) probably because the warmer temperatures of 2016 favored evaporation whilst the cooler days and some rains increased water content in 2015.

The total dry matter yield was similarly higher in 2016 (75.4 grams in the average of all treatments) than in the year before (50.9 grams).

The highest yield was got in 2016 in extensive soil (94.4 grams in the average of the two years, with a maximum of 114 grams in 2016) and the lowest was got in pots (24.3 grams in the average of the two years, with a minimum of 19.5 grams in 2015).

Table 3 - Dry weight (DW, grams) and dry matter percentage (DM, %) in new cladodes in the three trial treatments in the two years of trial and average

Treatment	Dry weight of new cladodes					
	2015		2016		*Average*	
	DW (g)	DM (%)	DW (g)	DM (%)	DW (g)	DM (%)
Extensive soil	74.7 a	11.0 b	114.0 a	13 b	94.4	12.0
Pot	19.5 c	13.0 a	29.1 c	15 a	24.3	14.0
Tire	50.9 b	12.0ab	83.0 b	14 ab	67.0	13.0
Average	*48.4*	*12.0*	*75.4*	*14.0*	*61.9*	*13.0*

Different letters show significantly different values at P=0.05.

4. Conclusions

Most households in towns, especially in developing countries, have not soil available and must rely on small and cheap containers for gardening, under this point of view, tires not only are cheaper then pots and they are easily available in many tropical towns, but they also provide better growing conditions for

Opuntia ficus-indica than commercial plastic pots. Of course extensive soil is a better condition for growing prickly pears than into containers, at least referring to water needs and taking into account that in extensive soil problems can rise from weeds competition more than in pots or tires.

The production in our trial in Italy has not been much because of the short warm season (4 months, with only three having summer temperatures) and because we planted cladodes not yet rooted. We can reasonably consider that production would be much higher in the tropics and subtropics where temperatures are good all the year through, using, already rooted plants. The production could be increased much if some waste water can be used in the household for irrigation.

The yield of new cladodes can integrate the diet and be a useful supply for a small family during the worst part of a dry season of 3-4 months, can be also a source of herbal medicine extremely useful to families.

Although the limiting climatic conditions of our trial, the results maintains their value of comparison within treatments also for tropical countries.

A couple of issues to be investigated are whether food production in tires is economically more convenient than transforming them into handmade items such as sandals (it is a common practice), and check food quality for eventual absorption of toxic contaminants from exhausted tires into edible plants.

Finally, a next trial on the use of prickly pear as a strategic plant, could be to start growing cladodes in tires or pots and when grown use some of these to plant green fences. The use of prickly pear for green fences can also be advised in order to shift the common use of planting the poisonous *Euphorbia* spp. (usually *Euphorbia trigona*) that has been much diffused in many and large areas because its spines makes a barrier to uncontrolled livestock. Clearly the use of *Opuntia* as a green fence should be preceded by start of some livestock control, but will also provide food instead of an invasive, useless and poisonous weed.

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Review paper

AHS
Advances in Horticultural Science

A mini-review of essential oils in the South Pacific and their insecticidal properties

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Key words: essential oils, insecticidal activities, traditional medicinal plants.

Abstract: Studies on traditional medicinal plants (TMPs) found in the South Pacific that holds potential for the insect controls have been reviewed. Few TMPs are known to have insecticidal properties, however many of those are still unknown in the South Pacific. The information on plants were collected using online databases such as Science Direct, PubMed, Google Scholar, Scopus and Springer Open in order to confirm the studies that support the insecticidal properties of plants present in the South Pacific. The following study confirmed that there is a potential for the selected TMPs suggesting enough evidence for their usage in the insecticidal activities. These plants would represent an alternative in crop protection due to its novel, safe and eco-friendly substitutes for its effective insecticidal properties.

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1. Introduction

Agricultural and animal origin stored products are destroyed by more than 600 species of beetle pests, 70 species of moths and about 355 species of mites (Rajendran and Sriranjini, 2008). These insect pests have greatly affected the food commodities and resulted in one of major problem to the food industries (Isman, 2006). There are many concerns raised with the usage of synthetic chemicals for pest control. According to the Food and Agriculture Organization of the United Nations (FAO, 2015), the consequences of high usage of synthetic pesticides in the Pacific Island Countries (PIC) has led to threats to human health and the environment. Chemical pollution is a major concern to the environment and human body through food chains, which results in severe physiological disorders and diseases (Oliva *et al.*, 2001; Baldi *et al.*, 2003; Briggs, 2003; Saiyed *et al.*, 2003; Lemaire *et al.*, 2004).

The investigation in the area of natural resources have dramatically increased when it comes to public concern for the long term health and environmental effect of synthetic chemicals (Coats, 1994; Regnault-Roger and Hamraoui, 1995; Lee *et al.*, 1997; Akhtar and Isman, 2004; Ukeh and Umoetok, 2011; Khani and Heydarian, 2014; Pandey *et al.*, 2014). For

example, the massive use of chemical compound phosphine has led to environmental issues due to its insect resistance/ineffectiveness in the agricultural fields of some countries (Opit *et al.*, 2012). Likewise, the use of methyl bromide for the fumigation has been reported as ozone-depleting substance and therefore removed completely from its use in some countries (Rajendran and Sriranjini, 2008). In view of the problems with current synthetic chemicals, there is a global interest in the search of alternative strategies and among them is the use of plant extracts.

Traditional aromatic plants have a wide impact on the agriculture, since plant derivatives are considered an integral source of pesticides. It represents a total of US $700.00 million market value with a total production of 45000 tons (Tripathi *et al.*, 2009). The science of natural products has advanced significantly in recent years benefiting humankind in the form of food, clothing, shelter, tools, medicines and crop protectant agents (Copping and Duke, 2007). The TMPs are the mainstay for treatment of illness in the Pacific for years. According to Dasilva *et al.* (2004), traditional medicines hold a natural treasury that clearly depicts that Pacific is rich in plant biodiversity. However, many plants in the Pacific are yet to be exploited for their right purpose. Hence, the present paper emphasizes on the insecticidal properties of essential oils from potential medicinal plants found in the South Pacific.

2. Overview of essential oils

Essential oils are diverse groups of natural products which are mainly produced by plants for defence, signalling or derive from their secondary metabolism (Charles and Simon, 1990; Bakkali *et al.*, 2008). These oils are volatile liquids which have a lower density than water (Bakkali *et al.*, 2008). Essential oils are also known as 'essence' that are strong-smelling liquid components found in aromatic plants, grasses and trees (Ríos, 2016). Essential oils are mostly formed in plants such as from flowers, leaves, buds, fruits, seeds, bark and roots (Isman, 2000; Ríos, 2016). The synthesised essential oils are mostly kept in secondary cell cavities, epidermal cells, canals or glandular trichomes (Nazzaro *et al.*, 2013).

The extraction of essential oils can be divided into conventional and recently developed methods. The conventional methods include; hydro-or steam distillation, solvent extraction and cold pressing. Hydrodistillation being one of the oldest methods, dating back to 5000 years. While the recent methods for extracting essential oils include; supercritical fluid extraction and microwave-assisted extraction. The quality and quantity of chemical compounds are depended on different extraction method (Fig. 1).

Essential oils containing between 20-60 components at different concentrations are considered to be very complex natural mixtures (Pandey *et al.*, 2014). Essential oils are characterized by two or more major compounds with few trace compounds. For instance, the GC-MS analysis of *Ocimum tenuiflorum* L. essential oils showed eugenol (58.20%), germacrene D (11.68%), *cis*-β-ocimene (10.79%) and β-caryophyllene (4.31%) as major compounds and terpinen-4-ol (1.01%), α-copaene (1.98%), δ-cadinene (1.44%) and few others as trace compounds (Chand *et al.*, 2016). The percentage composition of essential oils may vary with plants, environmental conditions, soil types and nutrients (Masotti *et al.*, 2003; Erbil *et al.*, 2015).

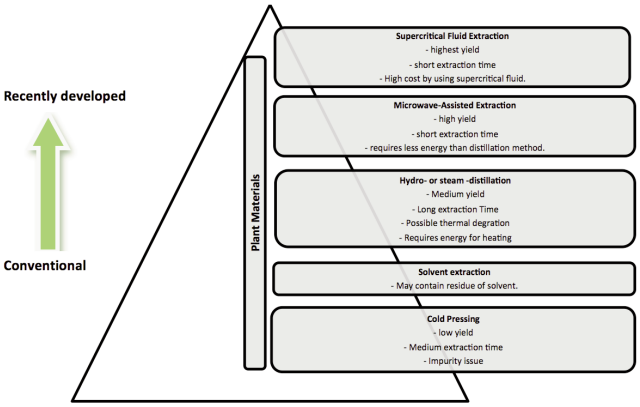

Fig. 1 - Overview of essential oil extraction methods (Park and Tak, 2015).

Formation of essential oils

Essential oils mostly have a high constituent of terpenes (Farag *et al.*, 1989). The other composition of essential oils include aromatic and aliphatic constituents that have different function to perform in relation to plants and animals (Bakkali *et al.*, 2008; Chamorro *et al.*, 2012; Hossain *et al.*, 2012; Hrckova and Velebny, 2012; Tongnuanchan and Benjakul, 2014). For instance, monoterpenes are used by plants for defence against pathogens, aid in seed dispersal and allelochemical functions, while alcohol groups have bactericidal, anti-infective and repellent properties (Table 1).

Terpenes are usually formed using mevalonate pathways. Mevalonate pathway is also known as isoprenoid pathway which occurs in all higher eukaryotes (Corsini *et al.*, 1993). This biosynthetic pathway

Table 1 - Composition of essential oils with their general function in plants and animals

Group	Sub-group	General functions in relation to plants and animals	Reference
Terpene Hydrocarbon	Monoterpenes ($C_{10}H_{16}$)	Producing defense against pathogens, help in the pollination, seed dispersal and allelochemical functions between plants and herbivores	(Lee *et al.*, 1997; Choi *et al.*, 2006; Ibanez *et al.*, 2012)
Terpene Hydrocarbon	Sesquiterpenes ($C_{15}H_{24}$)	Contact irritant effects on insects	(Gonzalez-Coloma *et al.*, 2013)
Terpene Hydrocarbon	Sesquiterpenes ($C_{15}H_{24}$)	Also used as analgesic, spasmolytic agents, calming, slight hypotension and anti-inflammatory	(Chaichana, 2009)
Terpene Hydrocarbon	Diterpenes ($C_{20}H_{22}$)	Are known to have insecticidal, antimicrobial and anti-inflammatory properties	(de Oliveira *et al.*, 2008; Gonzalez-Coloma *et al.*, 2013)
Terpene Hydrocarbon	Triterpenes ($C_{20}H_{22}$)	Components of the surface waxes that accumulate in the intra-cuticle layers of stems and leaf surface for protection against dehydrations and herbivores	(Thimmappa *et al.*, 2014)
Terpene Hydrocarbon	Triterpenes ($C_{20}H_{22}$)	Wide ranges of application of these compounds are in food, health, and industrial biotechnology sector	(Thimmappa *et al.*, 2014; Hadjimbei *et al.*, 2015).
Oxygenated Compounds	Alcohols	These compounds have bactericidal, anti-infective and repellent properties	(Ukeh and Umoetok, 2011)
Oxygenated Compounds	Phenols	Have strong toxic effects, antiseptic and insecticidal properties	(Akhtar and Isman, 2004; Romero *et al.*, 2013, cited in Pinheiro *et al.*, 2015)
Ethers	-	Severely affects the speed of germination, seedling growth and chlorophyll content	(He *et al.*, 2009)
Aldehydes	-	Used for antiviral, anti-inflammatory, hypotensive, vasodilators and antipyretic activities	(Dorman and Deans, 2000; Djilani and Dicko, 2012)
Ketones	-	Toxic effects to a number of pests	(Kordali *et al.*, 2007)
Ketones	-	Other uses of these compounds include anticoagulant, anti-inflammatory and digestant	(Peixoto *et al.*, 2015).
Organic acids and esters	-	Special properties such as anti-fungal, anti-inflammatory and antispasmodic	(Chaichana, 2009)
Organic acids and esters	-	Have potential antimicrobial properties	
Oxides	-	Used in aromatherapy, pharmaceuticals and agriculture	(Chaichana, 2009)

is used to produce dimethyl allyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). These two compounds serve as the basis for the biosynthesis of molecules in diverse processes of terpene synthesis, protein prenylation, cell membrane maintenance, hormones, *N*-glycosylation and protein anchoring (Chaichana, 2009; Cooper and Nicola, 2014).

Terpene biosynthesis involves addition of isopentenyl diphosphate (IPP; C_5) to its isomer dimethylallyl diphosphate (DMAPP; C_5 - can also form hemiterpenes) synthesizing geranyl diphosphate (GPP; C_{10}) which is a precursor for synthesis of monoterpenes. GPP and FPP form monoterpenes and sesquiterpenes skeleton respectively. Further condensation of enzyme-bound geranyl diphosphate (GPP; C_{10}) with addition of IPP units forms farnasyl diphosphate (FPP; C_{15}). Geranylgeranyl diphosphate (GGPP; C_{20}), that goes through series of reactions such as cyclization, rearrangement or coupling to form diterpenes and polyterpenes (Figure 2 shows the parental precursors to synthesise terpenes).

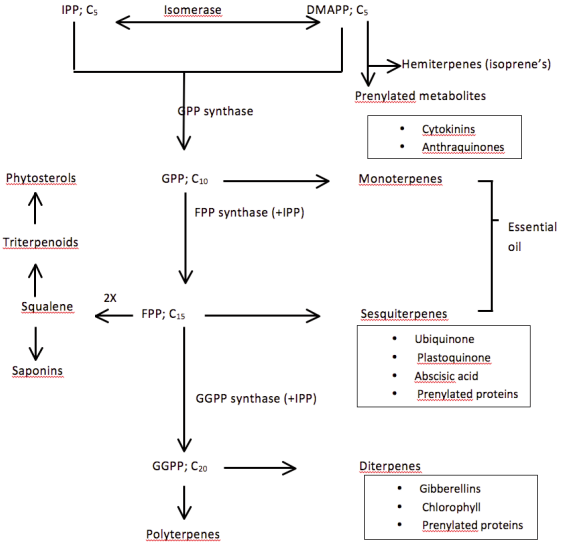

Fig. 2 - Synthesis of different classes of terpenes in plants. DMAPP - Dimethylallyl diphosphate; IPP - Isopentenyl diphosphate; FPP - Farnesyl diphosphate; GPP - Geranyl diphosphate; GGPP - Geranylgeranyl diphosphate.

Medicinal plants and their insecticidal properties

Insect control using plant materials is an ancient

practice all over the world (Gonzalez-Coloma *et al.*, 2013). This review is focused on nineteen different families of TMPs commonly found in the South Pacific that are known to have essential oils (World Health Organization, 1998).

These selected plants exhibit insecticidal properties that are traditionally used in form of medicines in the South Pacific (Table 2). The general characteris-

Table 2 - Selected medicinal plants reported for its efficiency against the insects

Family	Scientific names	Common English name	Plant part used	*Traditional Uses in the South Pacific (Treatment)	Active Constituents/Compounds	Efficiency against insects	References
Lamiaceae	*Ocimum tenuiflorum* L.,	Holy or sacred basil	Essential oils from leaves	Earache, nasal infections, cough, colds, stomach ache, hair lice, gastric, ulcer, flu, fevers, sore throat, and filariasis	-	Fumigant and repellent toxicity against the *Aleurodicus Dispersus* Russell (Spiralling whiteflies)	(Chand *et al.*, 2016)
Lamiaceae	*Ocimum basilicum* Linn. var. pilosum (willd)-Benth	Holy or sacred basil	Leaf extract	Earache, nasal infections, cough, colds, stomach ache, hair lice, gastric, ulcer, flu, fevers, sore throat, and filariasis	4h-1-Benzopyran-4-one, 5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-, catechol and Monoacetin	Repellency against the 3N7H and 3Q8I of *Anopheles gambiae* (African malaria mosquito)	(Gaddaguti *et al.*, 2016)
Lamiaceae	*Ocimum tenuiflorum* var. CIM AYU	Holy or sacred basil	Leaf extract	Earache, nasal infections, cough, colds, stomach ache, hair lice, gastric, ulcer, flu, fevers, sore throat, and filariasis	2-hexadecen-1-ol, phytol, DL-alpha-tocopherol, phenol-2-methoxy-3-(2-propenyl)-lycopersin, gamma-sitosterol, benzene, 1, 2-dimethoxy-4-(2-Propenyl)	Repellency against the 3N7H and 3Q8I of *Anopheles gambiae* (African malaria mosquito)	(Gaddaguti *et al.*, 2016)
Mimosaceae	*Adenanthera pavonina* L.	Holy or sacred basil	Seed extract	Leprosy	Trypsin inhibitor (ApTI)	Inhibitory activity of papain by trypsin inhibitor (ApTI) in *Callosobruchus maculatus* (Cowpea weevil)	(Macedo *et al.*, 2004)
Mimosaceae	*Adenanthera pavonina* L.	Holy or sacred basil	Seed extract	Leprosy	Trypsin inhibitor (ApTI)	Inhibitory activity of papain by trypsin inhibitor (ApTI) in *Diatraea saccharalis* (Sugarcane borer)	(da Silva *et al.*, 2012)
Asteraceae	*Ageratum conyzoides* L.	Goat weed	Canopy of plant species (above ground plant parts)	Infective hepatitis, eczyma, epilepsy, dizziness, diarrhoea, dysentery, sore, eyes, fever, headaches, intestinal worms, filariasis, vomiting, nausea, wounds and cuts	5, 6, 7, 8, 3', 4', 5'-Heptamethoxyflavone and coumarin	Insecticidal activity of hexane extracts against the *Rhyzopertha dominica* (F.) (Lesser grain borer)	(Moreira *et al.*, 2007)
Asteraceae	*Ageratum conyzoides* L.	Goat weed	Crude hexane extract of aerial parts of *A. conyzoides*	Infective hepatitis, eczyma, epilepsy, dizziness, diarrhoea, dysentery, sore, eyes, fever, headaches, intestinal worms, filariasis, vomiting, nausea, wounds and cuts	-	Repellent, antifeedant and toxic effects against *Helicovepra armigera* (Hübner) (Cotton bollworm)	(Ragesh *et al.*, 2016)
Asteraceae	*Ageratum conyzoides* L.	Goat weed	Crude petroleum ether extract aerial parts of *A. conyzoides*	Infective hepatitis, eczyma, epilepsy, dizziness, diarrhoea, dysentery, sore, eyes, fever, headaches, intestinal worms, filariasis, vomiting, nausea, wounds and cuts	Chromene precocene II, two flavonoids: eupalestin and lucidin dimethyl ether	Insecticidal activity against *Musca domestica* (housefly-third instar larvae), *Cynthia carye* third, (butterfly-fourth and fifth instar larvae) and *Acanthoscelides obtectus* (Bean weevil)	(Calle *et al.*, 1990)
Agavaceae	*Aloe vera* L.	Aloe, aloe vera	Leaf extract	Treat wounds and burns, sun burns, rashes, x-ray burns and stomach ache	-	Larvicidal activity on first to fourth instars larvae of *Aedes aegypti* (Yellow fever mosquito)	(Subramaniam *et al.*, 2012)
Agavaceae	*Aloe vera* L.	Aloe, aloe vera	Leaf extract	Treat wounds and burns, sun burns, rashes, x-ray burns and stomach ache		Mosquitocidal activity against the *Anopheles stephensi* (Malaria vector)	(Dinesh *et al.*, 2015)

To be continued

Table 2 - Selected medicinal plants reported for its efficiency against the insects (continued)

Family	Scientific names	Common English name	Plant part used	*Traditional Uses in the South Pacific (Treatment)	Active Constituents/Compounds	Efficiency against insects	References
Agavaceae	*Aloe vera* L.	Aloe, aloe vera	Acetone, ethyl acetate, water, and ethanol extracts	Treat wounds and burns, sun burns, rashes, x-ray burns and stomach ache	-	Acaricidal activity against female adults of *Tetranychus cinnabarinus* (Carmine spider mite)	(Wei *et al.*, 2011)
Annonaceae	*Annona muricata* L.	Soursop, custard apple	Crude ethanoic seed extract	Treating stomach ailments	-	Insecticidal activity against the *Spodoptera litura* (leafworm moth) and *Trichoplusia ni* larvae (Cabbage looper)	(Leatemia and Isman, 2004)
Annonaceae	*Annona muricata* L.	Soursop, custard apple	Fruit (pericarp) extract	Treating stomach ailments	Acetogenins -annonacin, annonacin A and annomuricin A.	Cytotoxicity towards the cell line U 937 (model cell line used in biomedical research)	(Jaramillo *et al.*, 2000)
Annonaceae	*Annona muricata* L.	Soursop, custard apple	Ethanoic seed extract	Treating stomach ailments	-	Insecticidal activity against the *Trichoplusia ni* (cabbage looper) and *Myzus persicae* (Green peach aphid)	(Ribeiro *et al.*, 2014)
Meliaceae	*Azadirachta indica* A. Juss.	Margosa, neem, Indian Lilac	Seed water extract	For diabetes, skin diseases, asthma, syphilis and used as insecticide	-	Insecticidal activity against the *Trogodarma granariun* (Khapra beetle)	(Satti *et al.*, 2010)
Meliaceae	*Azadirachta indica* A. Juss.	Margosa, neem, Indian Lilac	Neem oil from seeds	For diabetes, skin diseases, asthma, syphilis and used as insecticide	-	Insecticidal activity against the *Maruca testulalis* Geyer (Mung moth)	(Jackai and Oyediran, 1991)
Meliaceae	*Azadirachta indica* A. Juss.	Margosa, neem, Indian Lilac	Crude ethanol extracts of leaves	For diabetes, skin diseases, asthma, syphilis and used as insecticide	-	Insecticidal activity to adult *Tribolium confusum* (Flour beetle)	(Williams and Mansingh, 1993)
Annonaceae	*Cananga odorata* (Lam.) Hook. F. & Thoms.	Ylang-ylang, Kenanga	Essential oil extracts from flowers	Earaches, toothaches, headaches, stomach aches, boils, skin irritation, coughs and dizziness	-	Fumigant and Repellent toxicity against the *Aleurodicus Dispersus* Russell (Spiralling whiteflies)	(Chand *et al.*, 2016)
Annonaceae	*Cananga odorata* (Lam.) Hook. F. & Thoms.	Ylang-ylang, Kenanga	Essential oil extracts from the leaves	Earaches, toothaches, headaches, stomach aches, boils, skin irritation, coughs and dizziness	-	Insecticidal activity (contact and fumigant toxicity) to *Sitophilus zeamais* (Greater grain weevil)	(Cheng *et al.*, 2012)
Annonaceae	*Cananga odorata* (Lam.) Hook. F. & Thoms.	Ylang-ylang, Kenanga	Essential oil extracts from the leaves	Earaches, toothaches, headaches, stomach aches, boils, skin irritation, coughs and dizziness	-	Insecticidal activity against larvae of *Aedes aegypti* (Yellow fever mosquito)	(Vera *et al.*, 2014)
Solanaceae	*Capsicum frutescens* L.	Chili pepper, red pepper, paprika	Methanol extract of fruits and leaves	Skin tuberculosis, mild conjunctivitis and jaundice, boils and cough	-	Insecticidal activity to 2nd and 3rd instar larvae of *Aedes aegypti* (Yellow fever mosquito)	(Vinayaka *et al.*, 2010)
Solanaceae	*Capsicum frutescens* L.	Chili pepper, red pepper, paprika	Powdered fruits	Skin tuberculosis, mild conjunctivitis and jaundice, boils and cough	-	Discouraging oviposition and minimising damage to leaves of cowpea seeds	(Onu and Aliyu, 1995)

To be continued

Table 2 - Selected medicinal plants reported for its efficiency against the insects (continued)

Family	Scientific names	Common English name	Plant part used	*Traditional Uses in the South Pacific (Treatment)	Active Constituents/Compounds	Efficiency against insects	References
Solanaceae	*Capsicum frutescens* L.	Chili pepper, red pepper, paprika	Ethanolic extract of fruit	Skin tuberculosis, mild conjunctivitis and jaundice, boils and cough	-	Larvicidal activites against *Aedes aegypti* (Yellow fever mosquito) and *Aedes albopictus* (Asian tiger mosquito)	(Alvarez *et al.*, 2015)
Caricacea	*Carica papaya* L.	Papaya, Pawpaw	Hexanic, acetonic and methanolic extracts of seed	Sores, high blood pressure and treat diarrhea	-	Insecticidal activity against the *Spodoptera frugiperda* (Fall armyworm)	(Figueroa-Brito *et al.*, 2011)
Caricacea	*Carica papaya* L.	Papaya, Pawpaw	Leaf extract	Sores, high blood pressure and treat diarrhea	-	Insecticidal toxicity against the *Lipaphis Erysimi* Kal. (Mustard aphids)	(Ujjan *et al.*, 2014)
Caricacea	*Carica papaya* L.	Papaya, Pawpaw	Chloroform seed extract	Sores, high blood pressure and treat diarrhea	Palmitic acid, oleic acid, or stearic acid	Insecticidal and insectistatic activities against the *Spodoptera frugiperda* (Fall armyworm)	(Pérez-Gutiérrez *et al.*, 2011)
Caricacea	*Carica papaya* L.	Papaya, Pawpaw	Chloroform seed extract	Sores, high blood pressure and treat diarrhea	Palmitic acid, oleic acid, or stearic acid	Insecticidal and insectistatic activities against the *Spodoptera frugiperda* (Fall armyworm)	(Pérez-Gutiérrez *et al.*, 2011)
Caricacea	*Carica papaya* L.	Papaya, Pawpaw	-	-	-	Larvicdial and pupicidal activity to the Chikungunya vector, *Aedes aegypti* (Yellow fever mosquito)	(Kovendan *et al.*, 2012)
Fabaceae (Caesalpiniaceae)	*Cassia alata* L (Senna alata)	Ringworm bush, roman candle tree	Ethanoic extracts of leaves	Skin diarrhoea, worms, purifies blood and scabies	-	Acaricidal activity to *Rhipicephalus (Boophilus) annulatus* (Blue cattle tick)	(Ravindran *et al.*, 2012)
Fabaceae (Caesalpiniaceae)	*Cassia alata* L (Senna alata)	Ringworm bush, roman candle tree	Solvent extract of fruits	Skin diarrhoea, worms, purifies blood and scabies	-	Toxic effects against the *Callosobruchus chinensis* L. (Adzuki bean weevil)	(Upadhyay *et al.*, 2011)
Fabaceae (Caesalpiniaceae)	*Cassia alata* L (Senna alata)	Ringworm bush, roman candle tree	Leaf and stem extract	Skin diarrhoea, worms, purifies blood and scabies	-	Larvicidal effect on *Anopheles gambiae* (African malaria mosquito), *Culex quinquefasciatus* (Southern house mosquito) and *Aedes aegypt* (Yellow fever mosquito)	(Edwin *et al.*, 2013)
Apiaceae	*Centella asiatica* (L.) Urban	Indian pennywort, Asiatic pennywort	Leaf extract	Dysentery, fever, headache, diarrhea, pimples, rashes, itchy lumps, Fractures, migraines and boils	-	Larvicidal and Adult emergence Inhibition Effect against Mosquito *Culex quinquefasciatus* Say (Southern house mosquito)	(Rajkumar and Jebanesan, 2005)
Apiaceae	*Centella asiatica* (L.) Urban	Indian pennywort, Asiatic pennywort	Leaf extract	Dysentery, fever, headache, diarrhea, pimples, rashes, itchy lumps, Fractures, migraines and boils	-	Larvicidal and adulticidal activities against the Malarial Vector - *Anopheles stephensi* (Asian malaria mosquito)	(Senthilkumar *et al.*, 2009)
Apiaceae	*Centella asiatica* (L.) Urban	Indian pennywort, Asiatic pennywort	Leaf extract (hexane, diethyl ether, dichloromethane, and methanol)		-	Larvicidal activities against different strains of *Aedes aegypti* (Yellow fever mosquito) and *Anopheles stephensi* (Asian malaria mosquito)	(Nair *et al.*, 2014)

To be continued

Table 2 - Selected medicinal plants reported for its efficiency against the insects (continued)

Family	Scientific names	Common English name	Plant part used	*Traditional Uses in the South Pacific (Treatment)	Active Constituents/Compounds	Efficiency against insects	References
Rutaceae	*Citrus aurantium* L.	Seville or sour orang	Fruit extract	Headache, abdominal pain and urinary tract infections	-	Insecticidal activity against the adult *Bactrocera oleae* (Gmelin) (Olive fruit fly)	(Siskos *et al.*, 2007)
Rutaceae	*Citrus aurantium* L.	Seville or sour orang	Leaf extract	Headache, abdominal pain and urinary tract infections	-	Insecticidal activity against the adult *Bactrocera oleae* (Gmelin) (Olive fruit fly)	(Siskos *et al.*, 2007)
Rutaceae	*Citrus aurantium* L.	Seville or sour orang	Shoot extract	Headache, abdominal pain and urinary tract infections	-	Insecticidal activity against the adult *Bactrocera oleae* (Gmelin) (Olive fruit fly)	(Siskos *et al.*, 2007)
Rutaceae	*Citrus sinensis* (L.) Osbeck	orange, sweet orange	Essential oils from fruits	Sickness, abdominal pains and remedies for internal ailments	D-limonene	Larvicidal and pupicidal activities against *Musca domestica* L. (Housefly)	(Kovendan *et al.*, 2012)
Rutaceae	*Citrus sinensis* (L.) Osbeck	orange, sweet orange	Peels from fresh oranges	Sickness, abdominal pains and remedies for internal ailments	-	Insecticidal activity against mosquito, cockroach and housefly	(Ezeonu *et al.*, 2001)
Rutaceae	*Citrus sinensis* (L.) Osbeck	orange, sweet orange	Essential oils from the seeds	Sickness, abdominal pains and remedies for internal ailments	-	Insecticidal activity against the *Tribolium Castaneum (Herbst)* (Red flour beetle)	(Hussain *et al.*, 2013)
Zingiberaceae	*Curcuma longa* L.	turmeric	Leaf essential oils	Painful skin, sores and rashes in infant, sprains, bruises, eye diseases and open wounds, Colds and runny nose, dysentery and infected puncture wounds	-	Contact and fumigant toxicity against *Rhyzopertha dominica* F. (Lesser grain borer), *Sitophilus oryzae* L. (Rice weevil), and *Tribolium castaneum* Herbst (Red flour beetle)	(Tripathi *et al.*, 2002)
Zingiberaceae	*Curcuma longa* L.	turmeric	Turmeric rhizome oils	Painful skin, sores and rashes in infant, sprains, bruises, eye diseases and open wounds, Colds and runny nose, dysentery and infected puncture wounds	-	Repellency and feeding deterrent effects of Turmeric oils against the *Rhyzopertha dominica* (F.) (Lesser grain borer)	(Jilani and Saxena, 1990)
Zingiberaceae	*Curcuma longa* L.	turmeric	Leaves		α-turmerone and β-turmerome	Larvicidal activity on *Anopheles gambiae* (African malaria mosquito)	(Ajaiyeoba *et al.*, 2008)
Zingiberaceae	*Curcuma longa* L.	turmeric	Rhizomes			Larvicidal activity on *Anopheles gambiae* (African malaria mosquito)	(Ajaiyeoba *et al.*, 2008)
Fabaceae	*Erythrina variegata* L.	Coral tree	Ethanoic extracts from root and bark	Filariasis, stomach ache and fever	-	Contact toxicity and antifeedant activities against the *Spodoptera exigua* (Beet armyworm)	(Feng *et al.*, 2012)
Fabaceae	*Erythrina variegata* L.	Coral tree	Leaf extract using solvents	Filariasis, stomach ache and fever	-	Antifeedant and toxicity against the *Spodoptera litura* (Fab) (Taro caterpillar)	(Thushimenan *et al.*, 2016)

To be continued

Table 2 - Selected medicinal plants reported for its efficiency against the insects (continued)

Family	Scientific names	Common English name	Plant part used	*Traditional Uses in the South Pacific (Treatment)	Active Constituents/Compounds	Efficiency against insects	References
Fabaceae	*Erythrina variegata L.*	Coral tree	Methanoic leaf extracts	Filariasis, stomach ache and fever	-	Larvicidal activity of *Culex quinquefasciatus* (Southern house mosquito)	(Nazar *et al.*, 2009)
Cucurbitaceae	*Momordica charantia* L.	Bitter gourd, balsam pear, balsam apple	Leaf extract	Leprosy and malignant ulcers, stomach worms, fever, hypertension, diabetes and dysentery	-	Insecticidal activities of *Sitophilus zeamais* (Greater grain weevil)	(Adesina, 2013)
Cucurbitaceae	*Momordica charantia* L.	Bitter gourd, balsam pear, balsam apple	Acetone, *n*-hexane, and methanol extract of leaves	Leprosy and malignant ulcers, stomach worms, fever, hypertension, diabetes and dysentery	-	Toxicity and repellent activity against the *Callosobruchus maculatus* (Fab.) (Cowpea weevil). The order of extract toxicity was *n*-hexane> methanol >acetone	(Ajayi, 2015)
Cucurbitaceae	*Momordica charantia* L.	Bitter gourd, balsam pear, balsam apple	Methanoic fruit extracts	Leprosy and malignant ulcers, stomach worms, fever, hypertension, diabetes and dysentery	-	Larvicidal effects on *Culex pipiens* (Northern house mosquito)	(Nagappan and Gomathinayagam, 2014)
Passifloraceae	*Passiflora foetida* (L.) var. *hispida* (DC.) Killip	Wild passion fruit	Leaves and the sterm	Improve fertility in women	-	Repellent effect against the hematophagous insects	(Obico and Ragragio, 2014)
Psilotaceae	*Psilotum nudum* (L.) P. Beauv.	Psilotum	Aerial extract	Pain relief and remedy for thrush and the spore	Psilotin [6-(4'-β glucopyranosyloxyphenyl)-5,6-dihydm-2-oxo-2H-pyran]	Feeding deterrent and growth reducer to *Ostrinia nubilalis* (European corn borer)	(Arnason *et al.*, 1986)
Verbenaceae	*Vitex trifolia* L.	Vitex	Leaf extract	Stomach pains and mouth infections	-	Larvicidal activity on *Culex quinquefasciatus* (Southern house mosquito)	(Kannathasan *et al.*, 2007)
Verbenaceae	*Vitex trifolia* L.	Vitex	Hexanic and dichlorometha-nic (DCM) extracts of leaves and stems	Stomach pains and mouth infections	-	Antifeeding activity against the insect pest *Spodoptera frugiperda* (Fall armyworm)	(Hernández *et al.*, 1999)
Verbenaceae	*Vitex trifolia* L.	Vitex	Leaves and stem bark extracts	Stomach pains and mouth infections	-	Larvicidal activity on *Anopheles gambiae* (African malaria mosquito)	(Nyamoita *et al.*, 2013)

tics of most active families are discussed below.

Lamiaceae family. Lamiaceae family is also known as mint family that has strong aromatic essential oils, tannins, saponins and organic acids (Raja, 2012). Numerous insecticidal properties on a wide range of insect species have been reported from extracts obtained from the Lamiaceae family. For instance, biological activities of *Ocimum basilicum* L., *Mentha rotundifolia* L., *Origanum vulgare* L. ssp. vulgare, *Rosmarinus officinalis* L. and *Thymus vulgaris* L. have been reported against the first instar larvae of *Tribolium castaneum* Herbst (Coleóptera, Tenebrionidae) (Clemente *et al.*, 2003). Likewise, the extracts of *Plectranthus glandulosus* against the *Callosobruchus maculatus* in cowpea showed 100% mortality at 4 g/kg, within 7 days with LC_{50} of 0.39 g/kg (Danga *et al.*, 2015). The leaf extracts from Lamiaceae family have also shown post-harvest grain protectants efficacy (Nukenine *et al.*, 2007; 2011; 2013). Similarly, Bekircan *et al.* (2014) reported the antifeedant activity of *T. transcaucasicus* , *T. pseudopulegioides*, *T. leucotrichus* and *Teucrium poli-*

um L., against *Agelastica alni* L. (Coleoptera: Chrysomelidae larvae). Overall, the Lamiaceae family has an extensive range of biological activities including cytotoxic, antimicrobial, antioxidant, anti-inflammatory, hypotensive and insecticidal properties (Božović *et al.*, 2015).

Annonaceae family. Annonaceae is the largest family in the order Magnoliales and consist of 2500 species and 130 genera (Pirie *et al.*, 2005; Westra and Maas, 2012). The Annonaceae family has drawn attention since 1980s among the terrestrial plant families as a result of acetogenins that are known for a broad range of insecticidal bioactivities (Isman and Seffrin, 2014). The species of Annonaceae family such as *Asimina triloba*, *Annona muricata*, and *A. squamosa* L. are frequently considered for insecticidal activities against *Spodoptera frugiperda*, *Plutella xylostella*, *Aedes aegypti*, and stored grain insects (Isman and Seffrin, 2014). The fruit extract of *Xylopia aethiopica* and *Dennettia tripetala* were reported to have an insecticidal effect against *Sitophilus oryzae* (Coleoptera: Curculionidae). The larvicidal, ovicidal and pupicidal properties against *Aedes aegypti* have been reported using benzene, chloroform, ethyl acetate and methanol extracts of *A. reticulata* L. Nevertheless, the leaf and stem extracts of *A. coriacea* Mart., *A. crassiflora* Mart., *Duguetia furfuracea* (A. St.-Hil.) Saff. and *Xylopia aromatica* L. were reported for their phytotoxic effects on germination of lettuce, tomato and onion seeds (Novaes *et al.*, 2016).

Rutaceae family. Murraya koenigii (L) Spreng leaf extract resulted in high mortality, population reduction with delay in development of *Tribolium castaneum* - pest of stored wheat (Gandhi *et al.*, 2010). Furthermore, as reported by Arivoli *et al.* (2015), the hexane extracts of *M. koenigii* showed not only larvicidal activitiy against the vector mosquito's i.e., *A. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* but also they demonstrated that one of the six fractions obtained from the residue of hexane extract, had an effect against the third instar larvae of *A. aegypti, C. quinquefasciatus* and *A. stephensi* with a percentage of mortality of 100.0, 97.6 and 99.2%. The methanolic leaf extracts of *Atlantia monophylla* were evaluated for pupicidal activities against *C. quinquefasciatus, A. stephensi, and A. aegypti* under laboratory conditions and the respective lethal values corresponding to LC_{50} of 0.07, 0.05, and 0.07 mg/l (Sivagnaname and Kalyanasundaram, 2004). The *Zanthoxylum rhoifolium* leaves also showed insecticidal activities in *Bemisia tabaci* populations (Christofoli *et al.*, 2015). Phytochemical survey of Rutaceae family reveals the presence of flavonoids, alkaloids, limonoids, coumarins and volatile oils, of which some are associated with insecticidal activity (Rajkumar and Jebanesan, 2008; Emam *et al.*, 2009; Supabphol and Tangjitjareonkun, 2014).

Meliaceae family. Natural products of Meliaceae family such as Limonoids have biological activities against several insects. One compound widely known and commercialised is azadirachtin reported to hold antifeedant and growth-regulating properties (Champagne *et al.*, 1989). The azadirachtin compound inhibits the feeding, growth and survival of the variegated cutworm such as *Peridroma saucia*, with an EC_{50} and LC_{50} of 0.36 and 2.7 ppm in diet (Champagne *et al.*, 1989). The fruit extracts of *Trichilia elegans* and *T. catigua* revealed insecticidal activity on *Spodoptera frugiperda* (fall armyworm) (Matos *et al.*, 2009). *Azadirachta indica* A. Juss (neem derivatives) was known to used traditionally as an insecticide in the South Pacific (World Health Organization, 1998).

Modes of action of plant extract components

Natural plant products show different mode of actions mainly due to chemical components acting differently, resulting into contact toxicity, stomach poison and systemic activities if used in soils or injected on plants (Upadhyay, 2016). For instance, different plant extracts such as armoise, clary sage, oregano, lemongrass, niaouli, spearmint, cassia especial, dalmatian sage, red thyme, bay, garlic, pennyroyal, cassia pure, white thyme, cassia redistilled, star anise, peppermint, wintergreen, and cinnamon bark oils have shown potent fumigant toxicity against the *C. corticalis* (Kim *et al.*, 2012). These volatile substances affect the insect's nervous system. The nervous system is the control center of the body that transduces the activity of nerves into behaviour. The nerve cells act upon external cues (smell, taste, touch, hearing and light) as well as internal inputs from sources such as hormones, body temperature and limb position sensors in order to create control coordination in insects behaviour (Salgado, 2013). The fine-tuned control system of these insects is disrupted by the volatile nature of plant extracts when applied.

The plants extract lead to the poisoning of insects whereby certain cells show alternation of staining properties; while some cells can breakdown (cytolysis) in tissues. Similarly, within the nucleus the chro-

matin granules result into pyknosis (clump together) and the Nissl bodies (granular substances) which dissolves the nerve cells (Tanada and Kaya, 1993; Satar *et al.*, 2008). The symptoms of nerve poisons are divided in four stages: excitation, convulsion, paralysis and death. The neurotoxic fumigant results only in three stages: excitation, paralysis and death (Tanada and Kaya, 1993). The disturbance of nervous system in the insects often affects the respiratory, muscular and circulatory systems. As a result of disturbance or malfunction in the metabolic system the insect dies. In addition, the two common potential mode of action of essential oil components are discussed below.

Acetylcholinesterase. Acetylcholine (ACh) is one of the major compounds that are responsible for transmitting nerve impulses from different nerve cells and involuntary muscles. Acetylcholine is denatured by the enzyme Acetylcholinesterase (AChE) to choline and acetate and when Ach is released from synaptic vesicles depolarises the postsynaptic cell membrane. A result of the AChE activity is the regulation of the nerve impulse across the cholinergic synapses (Siegfried and Scott, 1992; López and Pascual-Villalobos, 2010). In other words, the inhibition activity of AChE activity generates the accumulation of neurotransmitters acetylcholine in neuronal synapses which creates a state of permanent stimulation resulting into lack of coordination in the neuromuscular system followed by the subsequent death of insect (Fig. 3) (Dambolena *et al.*, 2016).

Monoterpenoids were the first inhibitors that were considered to have the anticholinesterasic properties. The inhibition of AChE in stored-product insect pests, *Sitophilus oryzae* L. (Coleoptera: Curculionidae), *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae) and *Cryptolestes pusillus* Schönherr (Coleoptera: Cucujidae) is a possible mode of action from monoterponoids such as linalool, camphor, γ-terpinene, geraniol, *S*- carvone, *E*-anethole, fenchone and estragole (López and Pascual-Villalobos, 2010). For instance, 1,8-cineole (monoterpene) is found to be the best inhibitor of Acetylcholinesterase activity (IC_{50} values 0.015 - 0.05 mg/mL) (Picollo *et al.*, 2008; Dambolena *et al.*, 2016).

Octopaminergic sites. Octopamine, phenolic analogue of noradrenaline, is also present in the nervous system of the arthropods. There is some evidence that octapamine plays a role in neuromuscular transmission or rather possess a modulating influence on the nerve-muscle interaction (Candy, 1978; Enan, 2001). Octapamine act as neurotransmitters, neurohormones and neuromodulators in nervous system of inverterbrates (Kostyukovsky *et al.*, 2002). In insects, octopamine induces hyperextension of legs and abdomen due to the increased frequency of excitatory postsynaptic potentials from abdominal motor neurons (Harris-Warrick *et al.*, 1980; Livingstone *et al.*, 1980). Octopamine is likely to be involved in the regulation of heartbeats in insects since it is released in the axon terminals of pericardial organs (Evans *et al.*, 1976).

Octopamine exerts the effects through octopamine-1 and octopamine-2 receptors throughout their union with G-protein-coupled receptors (Dambolena *et al.*, 2016). For instance, carvacrol compound was found to change the conformation of the endogenous G-protein by increasing the affinity (Dambolena *et al.*, 2016). Likewise, a blockage of octopamine receptors binding sites was noted at the lowest concentration of the eugenol, α-terpineol and cinnamic alcohol resulting in decreased biding of [^{3}H]octopamine to its receptors (Enan, 2001).

The compounds such as octopamine and acetylcholine (accumulated in the nerves) in insects have diverse biological roles. Octopamine and acetylcholine compounds function as neurotransmitters (Fig. 3). If these compounds get interrupted by any

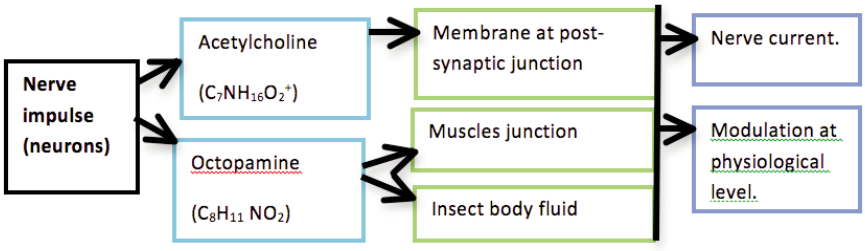

Fig. 3 - Target sites in insects as possible neurotransmitter mediated toxic action of volatile plant extracts. Adapted from: Tripathi *et al.* (2009)

chance, then it results in the damage of nervous system of the insects.

Plant extracts have long been touted as a potential alternative to synthetic insecticides presumably because of less environmental and human health impacts (Kostyukovsky *et al.*, 2002). The extracts form an impermeable film when applied on crops, which covers the insect from the air. The formation of the covering results in suffocation with the consequent death in insects (Li *et al.*, 2014). In addition, Tripathi *et al.* (2009) reported that volatile components of plant extracts such as monoterpenes have cytotoxic effects on tissues of living organisms. For example, the reduction in the intact mitochondria and golgi bodies, impairing respiration and reducing cell membrane permeability. The overall effect of plant extracts led to disruption, dissolution of cell membranes, and blockage of tracheal system of insects (Isman and Machial, 2006; Tehri and Singh, 2015).

3. Conclusions

Bio-control has been long touted as an attractive alternative over synthetic methods for the insect management. The current review has showed that out of the 19 plants selected, only *Azadirachta indica* A. Juss (neem derivatives) was known to be used traditionally as an insecticide in the South Pacific (World Health Organization, 1998).

Although essential oils are gaining momentum in market due to their environmental friendly pesticidal properties, there are few disadvantages of essential oils. Firstly, the use of essential oil in industrial farming may be not very popular mainly due to essential oils being more expensive and its less available. Secondly, the effect of separate chemical composition of essential oils is studied and trialled on insects, however, every little study concerning combined effects of essential oils is known mainly due to high level of difficulty in identifying the effectiveness (Regnault-Roger *et al.*, 2012). Thirdly, the use of essential oils for pest control is known from ancient times however only few are known to be available in commercialized market (Park and Tak, 2015).

Nevertheless, essential oils play a very important role in non-synthetic farming where the environmental safety is the primary concern (Isman, 2000). Although economically, synthetic chemicals are more often used then the plant extracts, these botanicals have the potential of providing efficient and safer approach for the environment as well as for humans (Nerio *et al.*, 2010; Pandey *et al.*, 2014).

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Investigation on rooting ability of twenty olive cultivars from Southern Italy

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Abstract: The effects of two different types of auxins (660 ppm alpha-naphthaleneacetic acid - NAA - in liquid solution or 750 ppm alpha-naphthaleneacetamide - NAD - dispersed in a talcum powder) and cuttings from three different portions of the shoots (basal, middle and apical) on the rooting ability of twenty autochthonous olive cultivars were investigated in two growing seasons (spring and autumn). The results showed that the autochthonous olive cultivars of the Campania Region are characterized by a wide variability in the potential rhizogenic ability. The two periods of cutting collection (March and September) significantly affected the rooting aptitude of the cultivars, indicating that in some cultivars the cuttings collected in autumn had a higher rooting rate than those collected in spring. The effects of NAA and NAD on rooting strongly depended on interaction with the cultivar, time of collection (autumn or spring) and type of cuttings (basal, medium or apical). Among the twenty cultivars tested, we found only eight cultivars with a satisfactory rooting ability after hormonal applications (Ortolana, Racioppella, Tenacella, Tonda, Biancolilla, Carpellese, Cornia and Pisciottana). In general, the apical and the median portions of the shoots gave the best rooting results.

1. Introduction

Among the vegetative propagation methods of olive cultivars (*Olea europaea L.*) the use of semi-hardwood cuttings is the most common, since rootings are easy to prepare and the requirement in special equipment is negligible and cheap (Cimato, 1999; Ismaili *et al.*, 2011). This is why in the Mediterranean basin olive is mainly propagated by cuttings, a propagation method that relies on the ability of the cuttings to form adventitious roots (Fabbri *et al.*, 2004). While some cultivars are easily propagated by this technique, others are difficult-to-root and this poses a challenge for their preservation and commercialization (Hartmann and Kester, 1975; Hartmann *et al.,* 1990; Carfi *et al.*, 1994; Porfírio *et al.*, 2016). Rooting aptitude of the different olive cuttings depends on both intrinsic and extrinsic factors (Wiesman and Lavee, 1994; Hechmi *et al.*,

2013), such as the genotype (Avidan and Lavee, 1978; Fabbri *et al*., 2004; Chiancone *et al.,* 2011), the age of the mother tree, the timing of cutting collection as well as the type of cuttings (Fontanazza and Jacoboni, 1975; Del Rio *et al*., 1991; Khabou and Trigui, 1999). Taking this background into consideration, the main aim of this study was to assess the rooting ability of olive cuttings obtained from twenty autochthonous olive cultivars of the Campania Region, by using different combinations of auxin treatments (NAA and NAD), times of collection of cuttings (spring and autumn) and portions of shoots to prepare the cuttings (basal, middle or apical). The cultivars considered in this study have been previously evaluated in terms of vegetative-productive characteristics and oil quality, and some of them present agronomical behaviors and quality of the oils which make them interesting for the use in new olive orchards, also for the production of oils with a strong yet greatly diverse typicality (Di Vaio *et al.*, 2013). This makes very important to know their intrinsic ability to root and the best combination of factors (hormones, time of cutting collection and portion of shoots to use) to utilize in order to obtain the best rooting results from each cultivar.

2. Materials and Methods

Experimental site and plant material

The trial was carried out in 2014/2015 at the Experimental Station "Improsta" of the Campania Region, located in Battipaglia (40°37′ 00′′ N lat, 15°03′ 23′′ E long, 72 m above sea level) Sele Plain (Salerno, South Italy). The average annual rainfall and the average minimum and maximum temperature of the area were 988 mm, 10.9°C and 21.0°C, respectively.

The experimental station hosts a germplasm collection orchard, established in 2001, which includes all the main autochthonous olive cultivars of the Region. Trees were trained at central leader and spaced 6 × 3 m.

Among the available germplasm, 20 cultivars were selected for their economic importance and potential to be used in new orchards considering their good agronomical characteristics (productivity and resistance to biotic and abiotic stresses) and high quality/typicality of the oils, since some of them also allow the production of certified oils under the Protected Designation of Origin. The cultivars were grouped according to their origin (main province of cultivation). Therefore, the selected olive cultivars were four from the province of Avellino ('Ogliarola campana', 'Ravece', 'Ritonnella' and 'Ruveia'), five from the province of Benevento ('Femminella', 'Ortice', 'Ortolana', 'Pampagliosa' and 'Racioppella'), four from the province of Caserta ('Asprinia', 'Caiazzana', 'Tonda' and 'Tenacella') and seven from the province of Salerno ('Biancolilla', 'Carpellese', 'Cornia', 'Oliva Bianca', 'Pisciottana', 'Rotondella' and 'Salella') (Di Vaio *et al.*, 2013).

Cutting collection and preparation

The cuttings were collectioned in the autumn (end of September) and in the next spring (second decade of March). For each cultivar, three trees were selected by following homogeneity criteria for developmental stage and productivity. The semi-hardwood cuttings were obtained in autumn (A) and spring (S) from 30 cm long one-year shoots/season. Each shoot was divided in three different portions (basal, middle and apical) (180 cuttings/cultivar/season). Cuttings were 10 cm long and presented 4 nodes and 2 pairs of terminal leaves.

Rhizogenic treatments

For each combination of cultivar, collection time, portion of shoot and hormonal treatment three groups of ten cuttings each were used. The trial was conducted under mist system in cold greenhouse conditions. Two different commercial formulations of auxins, alpha-naphthaleneacetic acid (NAA) in liquid formulation (hydroalcoholic solution at 30% alcohol) at the concentration of 660 ppm (T1) and alpha-naphthaleneacetamide (NAD) at the concentration of 750 ppm (T2) in talcum powder formulation (0.075/100 w.w.) were compared to untreated control (T0). These concentrations were used considering the good results obtained with NAA 500-1000 ppm in other cultivars (Denaxa *et al.*, 2011). The T1 treatment was performed by dipping 2 cm basal part of cuttings in the hydroalcoholic solution for 5 seconds, whereas the T2 treatment by dipping 2 cm basal part of cuttings in distilled water first and then in the powder. The T0 (control) was obtained by dipping 2 cm basal part of cuttings in distilled water for 5 seconds. Cuttings were then placed in perlite filled rooting benches provided with basal heating (substrate temperature 22-24°C) and with mist system to get periodically wet the cuttings avoiding their dehydration (air humidity about 90-95%).

Rooting sampling and score

Semi-hardwood cuttings were evaluated 70 days after the rooting treatments and each cutting was

scored for the rooting rate, the number of roots/cutting (primary and secondary roots), and the length of roots/cutting (the length of the different roots of each cutting was summed). The percentage of rooting was calculated as the number of rooted cuttings with respect to the total number of cuttings per treatment.

Statistical analysis

All data were statistically analyzed by three-way analysis of variance (ANOVA) using the SPSS 13 software package (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). Whenever the two-way interaction was significant, a one-way ANOVA was performed. To separate treatment means for each measured parameter, Duncan's multiple range test was performed at a significance level of $P \leq 0.05$.

3. Results and Discussion

Adventitious rooting process in cuttings is still to be unraveled under the genetic point of view, however, as referred in studies on Italian and international olive cultivars (Fontanazza and Baldoni, 1989; Chiancone *et al.,* 2011), the capability of cuttings of forming adventitious roots is mainly affected by the cultivar. Among the wide number of local and worldwide grown cultivars, the main part of them displays a poor aptitude for rooting of cuttings, whereas few cultivars are known to be well rooting cultivars. A dataset of the International Olive Council (IOC, 2005) reported the results of a screening on rooting rate of 426 cultivars: 59 cultivars showed an average rooting rate of 1.5%, 213 cultivars reached an average rate of 21.3%, whereas the rooting rate recorded in 86 cultivars was approximately 54% and only 68 (=16% of the total) showed a rate of rooting higher than 70%.

In the present study, rooting rate of the cuttings was significantly affected by the cultivar (C), by the interaction between the cultivar and the time of sampling (C × TS) and by the interaction between the hormonal treatment and the time of sampling (T × TS) (Table 1). The highest percentage of rooted cuttings, over the rooting treatment and the time of sampling, was recorded in cv. Ortolana (66%), followed by cv. Racioppella (54.6%) and cv. Biancolilla (51%) (Fig. 1). On the contrary, cv. Ogliarola campana, cv. Ortice and cv. Salella showed the lowest rooting rate (14.9%, 15.8% and 11.2%, respectively).

Timing of cutting collection, according to the literature, is the second pivotal element, following the cultivar, to be concerned about, since very relevant differences in the success of rooting process may depend on this factor (Hartmann and Loreti, 1965). Thus, the definition of the most suitable season for cutting collection from mother plants has been a critical item for researchers dealing with fruit trees species, although the fine tuning of protocols ready to use in the nurseries is still lacking, especially if single cultivars are considered. The C × TS interaction highlighted that the cultivars considered in this study responded differentially to the time of cutting sampling (Fig. 2). For instance, among the three cultivars that showed the highest rooting rate, cv. Ortolana and cv. Raciopella reached higher percentages of rooting when the cuttings were collected during spring season, whereas the rooting rate of cv. Biancolilla was significantly increased by using the cuttings collected in autumn (Fig. 2). On the contrary, the cultivars that showed the lowest rooting rate (cvs. Ogliarola campana, Ortice and Salella) did not

Table 1 - Analysis of variance for cultivars, hormonal rooting treatments, time of sampling and their interactions on rooting rate of olive cuttings

Source of variance	Rooting rate (%)
Cultivar (C)	***
Hormonal treatment (T)	NS
Time of sampling (TS)	NS
C × T	NS
C × TS	***
T × TS	*
C × T × TS	NS

NS= Non significant; *, **; ***= significant at P≤ 0.05, ≤ 0.01, 0.001, respectively.

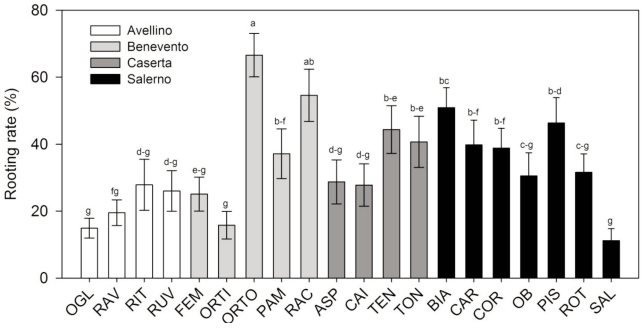

Fig. 1 - Average rooting rate (mean ± standard error) of semi-hardwood cuttings of the olive cultivars of the Campania Region. Bars with the same colour correspond to cultivars of the same province. Abbreviations of the names of the cultivars: OGL = Ogliarola campana, RAV = Ravece, RIT = Ritonnella, RUV = Ruveia, FEM = Femminella, ORTI = Ortice, ORTO = Ortolana, PAM = Pampagliosa, RAC = Racioppella, ASP = Asprinia, CAI = Caiazzana, TEN = Tenacella, TON = Tonda, BIA = Biancolilla, CAR = Carpellese, COR = Cornia, OB = Oliva Bianca, PIS = Pisciottana, ROT = Rotondella, SAL = Salella. Means followed by different letters are significantly different for P ≤ 0.001.

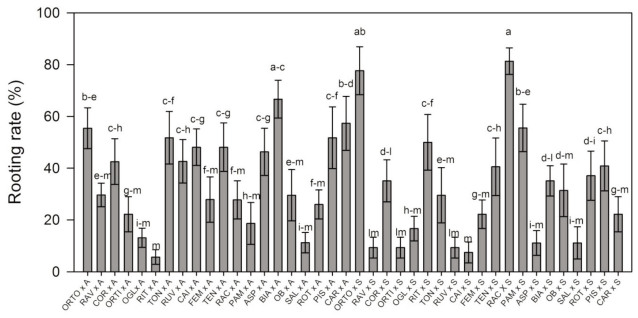

Fig. 2 - Average rooting rate (mean ± standard error) of semi-hardwood cuttings as affected by the interaction between the cultivar and the time of sampling. A= autumn; S= spring. Abbreviations of the names of the cultivars: OGL = Ogliarola campana, RAV = Ravece, RIT = Ritonnella, RUV = Ruveia, FEM = Femminella, ORTI = Ortice, ORTO = Ortolana, PAM = Pampagliosa, RAC = Racioppella, ASP = Asprinia, CAI = Caiazzana, TEN = Tenacella, TON = Tonda, BIA = Biancolilla, CAR = Carpellese, COR = Cornia, OB = Oliva Bianca, PIS = Pisciottana, ROT = Rotondella, SAL = Salella. Means followed by different letters are significantly different for P≤0.001.

perform differently comparing the two times of sampling (Fig. 2).

The overall influence of hormonal treatments (NAA or NAD) and time of sampling (T × TS) on cutting rooting rate indicated that the effect of treatments was significantly different on the cuttings collected in spring (Fig. 3). In particular, the lowest rooting rate was observed on T0 cuttings collected in spring (Fig. 3).

On the base of cutting rooting rate, the International Olive Council classified the cultivars in four ranks: 0-5%; 5-40%; 40-70% and 70-100% (IOC, 2005). According to this classification, the twenty cultivars tested in the present experiment resulted to be distributed in three different groups: twelve between 5 and 40% ('Ogliarola campana', 'Ravece', 'Ritonnella', 'Ruveia', 'Femminella', 'Ortice', 'Pampagliosa',

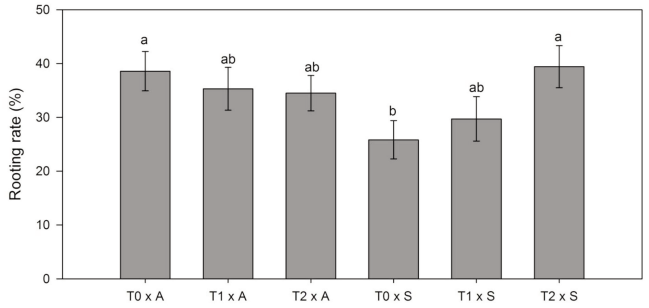

Fig. 3 - Average rooting rate (mean ± standard error) of semi-hardwood cuttings as affected by the interaction between the hormonal rhizogenic treatments (T0= control - no hormonal treatments; T1= NAA treatment; T2= NAD treatment) and the time of sampling. A= autumn; S= spring. Means followed by different letters are significantly different for P ≤ 0.05.

'Asprinia', 'Caiazzana', 'Oliva Bianca', 'Rotondella', 'Salella'); seven between 40 and 70% ('Racioppella', 'Tenacella', 'Tonda', 'Biancolilla', 'Carpellese', 'Cornia' and 'Pisciottana'); one between 70 and 100% ('Ortolana').

As far as root number and length are concerned, the effects of cultivar, hormonal treatments and portion of the shoot and their first level interactions were all significant (Table 2). The twenty cultivars of olive exhibited a high variability in the number and length of roots produced both in autumn and spring experiments. The root number and length were increased by the rhizogenic treatments. Generally, both the liquid and powdery formulations used for the rooting stimulation of the cuttings were effective in improving rooting emission and growth. With regard to the number of roots, our findings indicated that the liquid treatment with 660 ppm NAA was the most effective, showing, with respect to the control, an average increase of about 266% in cuttings from the basal, median and apical portions of the shoots. The same treatment was particularly effective in stimulating the length growth of the roots, with an average increase of 236% compared to the control. Furthermore, the root number and length of cuttings collected in autumn and spring were significantly affected by the interaction between the cultivar (C) and hormonal rooting treatment (T) (Table 2). For instance, the average root number in autumn cuttings was 2.7 roots per cutting, and the application of the rhizogenic treatment T2, compared to T0, induced a significant increase of the number of roots in nine cultivars: Ortolana (+245%), Cornia (+169%), Tonda (+160%), Ruveia (+165%), Tenacella (+192%), Asprinia (+312%), Biancolilla (+320%), Pisciottana

Table 2 - Analysis of variance for cultivars, hormonal rooting treatments, shoot portion and their interactions on number and length of roots of olive cuttings obtained in autumn or spring

Source of variance	Root number		Root length (cm/cutting)	
	Autumn	Spring	Autumn	Spring
Cultivar (C)	***	***	***	***
Hormonal treatment (T)	***	***	***	***
Portion (P)	***	**	***	***
C × T	**	***	***	**
C × P	*	***	*	***
T × P	*	NS	NS	*
C × T × P	NS	***	NS	*

NS= Non significant; *, **; ***= significant at P≤ 0.05, ≤ 0.01, 0.001, respectively.

(+610 %) and Carpellese (+236 %) (Fig. 4A).

The average root length in autumn cuttings was 4.2 cm per cutting, and, similarly to the root number, it was also increased by the rhizogenic treatment T2 in ten cultivars: Ortolana, Ravece, Cornia, Tonda, Ruveia, Tenacella, Asprinia, Biancolilla and Pisciottana (Fig. 4 B). A similar pattern was observed in the spring cuttings (Fig. 5 A, B), even though the

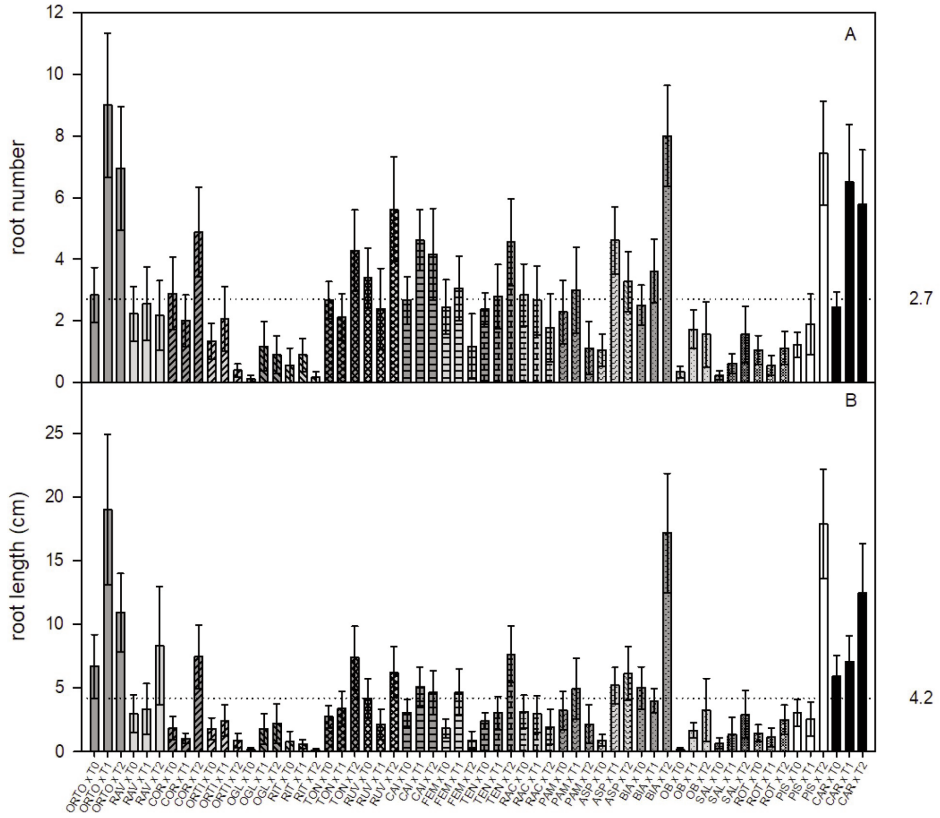

Fig. 4 - Effects of the interaction of the cultivars and the hormonal rhizogenic treatment (T0= Control - no hormonal treatments; T1= NAA treatment; T2= NAD treatment) on root number/cutting (A) and root length/cutting (B) of semi-hardwood cuttings collected in autumn. The dotted line shows the average value of all cultivars under observation. Abbreviations of the names of the cultivars: OGL = Ogliarola campana, RAV = Ravece, RIT = Ritonnella, RUV = Ruveia, FEM = Femminella, ORTI = Ortice, ORTO = Ortolana, PAM = Pampagliosa, RAC = Racioppella, ASP = Asprinia, CAI = Caiazzana, TEN = Tenacella, TON = Tonda, BIA = Biancolilla, CAR = Carpellese, COR = Cornia, OB = Oliva Bianca, PIS = Pisciottana, ROT = Rotondella, SAL = Salella.

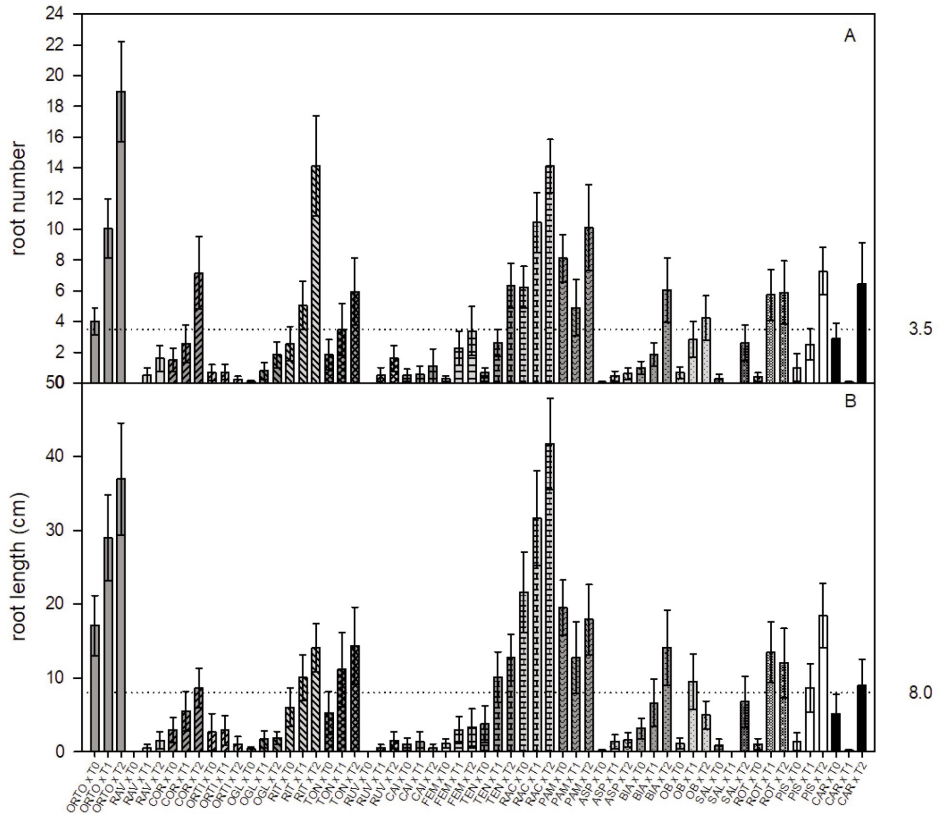

Fig. 5 - Effects of the interaction of the cultivars and the hormonal rhizogenic treatment (T0= Control - no hormonal treatments; T1= NAA treatment; T2= NAD treatment) on root number/cutting (A) and root length/cutting (B) of semi-hardwood cuttings collected in spring. The dotted line shows the average value of all cultivars under observation. Abbreviations of the names of the cultivars: OGL = Ogliarola campana, RAV = Ravece, RIT = Ritonnella, RUV = Ruveia, FEM = Femminella, ORTI = Ortice, ORTO = Ortolana, PAM = Pampagliosa, RAC = Racioppella, ASP = Asprinia, CAI = Caiazzana, TEN = Tenacella, TON = Tonda, BIA = Biancolilla, CAR = Carpellese, COR = Cornia, OB = Oliva Bianca, PIS = Pisciottana, ROT = Rotondella, SAL = Salella.

values of both the average number and length of roots were higher than those recorded in autumn.

The root number of autumn cuttings was also affected by the interaction between the hormonal treatment (T) and the shoot portion (P). Indeed, the number of roots per cutting increased from the bottom (B), to the middle (M), and then to the apical (A) portion of collected shoots with the highest value recorded in the apical cuttings treated with T1 (4.5 roots/cutting) followed by T2 treated apical and middle cuttings (3.9 and 3.4 roots/cutting, respectively) (Fig. 6 A), whereas the lowest number of roots was observed in the basal cuttings of untreated control (T0) and T1 (1.5 roots/cuttings) (Fig. 6 A). With respect to the control and T1, a significant increase of the root number was observed in the basal cutting treated with T2 (2.8 roots/cuttings). The capability of olive cuttings on forming adventitious roots is known to be related to the portion (basal, middle or apical) of the shoots, even though different cultivars can respond differentially. A general hypothesis has been formulated on the higher aptitude for rooting of sub-apical cuttings that seems to be related to a possible higher content in auxinic compounds driven basipetally from the young leaves of cuttings (Fabbri *et al*., 2004). On the other hand, only the root length of spring cuttings was significantly affected by the interaction between the hormonal treatment (T) and the shoot portion (P) (Table 2, Fig. 6 B), with the highest values (11.4 cm) recorded in the apical cuttings treated with NAA in liquid formulation (T1) and in basal, middle and apical cuttings (10.3, 10.9, and 12.2 cm, respectively) treated with NAD in powdery formulation (T2).

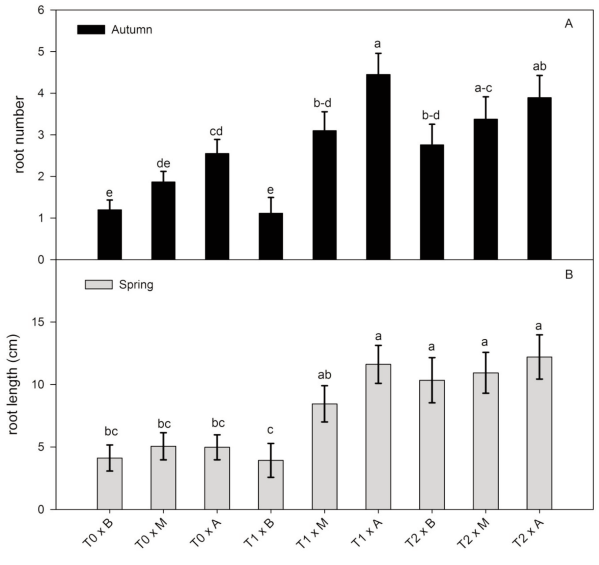

Fig. 6 - Effects of the interaction of the hormonal rhizogenic treatment (T0= Control - no hormonal treatments; T1= NAA treatment; T2= NAD treatment) and shoot portion used to make the cuttings (B= Basal; M= Medium; A= Apical) on root number/cutting (A) of semi-hardwood cuttings collected in autumn and on root length/cutting (B) of semi-hardwood cuttings collected in spring. Means followed by different letters are significantly different for P≤0.05 (root number) and P≤0.05 (root length).

4. Conclusions

This study highlighted that the olive cultivars belonging to autochthonous germplasm of the Campania Region are characterized by a wide variability in the potential rhizogenic activity. Overall the two sampling periods of cutting collection (March and September) significantly affected the rooting aptitude of several cultivars, indicating that in some cultivars the cuttings collected in autumn may have a higher rooting rate than the spring collected ones. Moreover, it was possible to evaluate the rhizogenic aptitude of the cultivars belonging to the olive Campanian germplasm under the influence of rhizogenic treatments. The results showed that the effects of NAA and NAD on rooting strongly depended on interaction with the cultivar, time of collection (autumn or spring) and type of cuttings (basal, medium or apical). In general, the apical and the median portions of shoots were confirmed to be the most suitable for improving the rooting of cuttings.

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Phenolic metabolism and antioxidant activity during endodormancy of Kiwifruit buds

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Abstract: Bud dormancy is an adaptability process in woody plants that enables them to survive in unfavorable conditions. In the present study, the phenols, antioxidant capacity, and activity of three enzymes were evaluated during endodormancy phases in two Hayward and Tomuri cultivars and two female and male Golden genotypes of kiwifruit buds. The buds were collected from ten-year-old own-rooted vines from the end of October 2015 until the end of January 2016 in the north of Iran. The results revealed that phenols, antioxidant capacity (RSA), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) activities of buds significantly increased at the beginning of endodormancy and subsequently decreased at the end of the endodormancy. The POD activity increased in Hayward and Tomuri from the onset of endodormancy and continued for two weeks after the endodormancy release. The total phenol had a positive and significant correlation with RSA and PAL enzyme activity. Furthermore, higher antioxidant capacity and phenols in both male and female Golden genotypes were attributed to the higher PAL enzyme activity in both genotypes. This study proposes that the RSA%, PAL activity, and phenol concentration could be employed as a biomarker to indicate bud dormancy phases in kiwifruit.

1. Introduction

The first axillary buds are initiated on the developing shoots of kiwifruit shortly after bud break in the first growing season (Walton *et al.,* 1997). Similar to temperate fruits, the axillary buds of kiwifruit can be induced into endodormancy by short days and low temperatures at the end of the summer or the beginning of autumn (McPherson *et al.,* 1995).

Bud endodormancy is an adaptability process in woody plants that

enables trees to survive in unfavorable conditions such as drought and extremely hot and cold weather (Arora *et al.,* 2003); in addition, more importantly, it encourages the reproductive processes such as the formation of flowers and fruit set to be accomplished in favorable condition and guarantees the reproductive growth and survival of the plants (Campoy *et al.*, 2011). Endodormancy (winter dormancy) is a true dormancy in which the bud growth is prevented by an inhibitory system within the bud (Horvath *et al.,* 2003). Overcoming endodormancy and maximum bud break and flowering in favorable environmental conditions are achieved by accumulating the minimum amount of chilling hours (McPherson *et al.,* 1997). After that endodormancy is released, the growth of buds is prevented directly by external environmental factors. This type of dormancy mainly occurs in late winter and is named ecodormancy (Horvath *et al.,* 2003).

Generally, horticultural specialists determine the time of bud break by chilling and heat units. However, this method is based on ambient temperature, varies according to the environmental conditions, and cannot express the internal situation of buds (Dennis, 2003). During bud endodormancy, there is no visible growth, but physiological changes in respiration, growth regulators, carbohydrate metabolism, the amount of water, and other compounds occur, influencing bud endodormancy control (Ben Mohamed *et al.,* 2010). A series of the changes occurring in the biochemistry of the buds appear to indicate the shift from the endodormancy stage to the ecodormancy stage (Pakish *et al.,* 2009; Szecskó *et al.,* 2002). Richardson *et al.* (2010) stated that high and stable sucrose concentrations are likely to be a good indicator of the true dormancy of the buds in kiwifruit (*Actinidia deliciosa*).

Investigating the relationship between biochemical compounds and the beginning of the endodormancy in nine varieties of apricot revealed seasonal changes in phenolic compounds and peroxidase (Laslo and Vicas, 2012). These changes are caused by the accumulation of chilling units during the endodormancy, that is necessary for the development of some phenological stages. Pakish *et al.* (2009) studied peroxidase and oxidase activities in the varieties of pistachio buds during the winter and observed seasonal variation in the November-March interval.

There is a significant difference in terms of the phenols of buds in different dormancy stages within cultivars of the same species. The total phenol of the flowering buds in peach (Szalay *et al.,* 2005) and apricot (Laslo and Vicas, 2012) increased at the beginning of endodormancy, showed a gradual increase in dormancy period, and disappeared during flowering. Large variations in the content of polyphenols can be observed in certain varieties of pistachio (*Pistacia vera* L.) in the November-March interval. The phenol of all cultivars was reduced in the swollen buds (Pakish *et al.,* 2009).

Factors controlling the onset, maintenance, and termination of endodormancy are varied and have not been studied much (Luedeling *et al.*, 2009). Moreover, the starting point of endodormancy, the end of endodormancy, and the start of ecodormancy are not clearly defined in plants; therefore, the study of changes in biochemical compounds in this field could be useful (Pakish *et al.,* 2009; Ben Mohamed *et al.,* 2010). A number of studies have been done on the changes in carbohydrates (Richardson *et al.,* 2007, 2010), nitrogen, and amino acid (Walton *et al.*, 1991) of kiwifruit in the endodormancy period; however, the activities of enzymatic and non-enzymatic antioxidants such as phenol and its metabolism have not been studied yet. Thus, the aim of this study was to inspect the changes in antioxidant capacity, total phenol, and activity of three enzymes during endodormancy and the early ecodormancy in four cultivars and genotypes of kiwifruit.

2. Materials and Methods

Plant material and sampling

Bud samples of Hayward and Tomuri kiwifruit cultivars (*Actinidia deliciosa*) and two male and female Golden genotypes (*A. chinensis*; mass selection of Golden kiwifruit cultivar seedlings) were collected from canes of ten-year-old own-rooted vines from the end of October 2015 until the end of January 2016 with a 7-day interval at 11 steps. From the first to the last sampling, the vines received 0, 222, 309, 443, 570, 740, 864, 1003, 1125, 1272, and 1450 chilling units, respectively (Richardson *et al.,* 1974). Kiwifruit vines were located at the National Citrus and Subtropical Research Institute of Iran (latitude 75.36° North and longitude 33.51° East) and were trained on a T-bar training system with a planting distance of 4×6 m. The samples were immediately frozen in liquid nitrogen and kept for subsequent analyses at -80°C. At every sampling date, 30 buds were collected from 1-year-old canes at nodes 6 to 20 starting from the basal end of canes. Ten buds

were selected randomly in three replications for further analyses of biochemical compounds (Richardson *et al.*, 2010). The activities of phenylalanine ammonia-lyase (PAL), peroxides (POD), polyphenol oxidase (PPO), antioxidant capacity (RSA), and total phenol content were determined in buds during endodormancy.

Estimation of endodormancy period

Chilling requirements and bud endodormancy period of kiwifruit cultivars and genotypes were estimated via single-node cuttings test. Simultaneously, fifteen cuttings in three replications of each cultivar and genotype were collected and the buds were taken for biochemical measurements. Cuttings from each treatment were transferred into a forcing chamber at 25°C, with 16 h of light (Wall *et al.,* 2008). The lowest mean time budburst (MTB) for half of the buds were considered as an endodormancy release (Tisne-Agostini *et al.,* 1992).

Extraction and determination of total phenol and antioxidant capacity

The total phenol of each extract was determined according to the Folin-Ciocalteu procedure reported by Meyers *et al.* (2003). Moreover, the spectrophotometric method introduced by Wettasinghe and Shahidi (2000) was employed for the chemical determination of antioxidant.

Extraction and assay of PAL

One hundred mg of kiwifruit buds powdered by liquid nitrogen were mixed with 2 ml of 1.0 mM borate buffer containing 1.0% of polyvinyl pyrrolidone. After homogenization by a homogenizer (model IKA-T8, Germany), the samples were centrifuged for 15 min at 4°C and 13,000 rpm. The supernatants were slowly transferred into the tubes by pipette. Extracts for the ensuing measurements were maintained at -80°C. PAL (EC 4.3.1.24) activity was determined according to the study carried out by Yu *et al.* (2012).

Extraction and assay of PPO and POD activities

The two hundred-mg samples of fresh buds, which were collected, were ground in liquid nitrogen and homogenated with 2 ml of potassium phosphate buffer (50 mM, pH= 7.0) containing 1% polyvinyl pyrrolidone (PVP) (W/V) and 0.05% EDTA at 4°C. The homogenate was centrifuged at 14000 rpm for 15 min at 4°C. The supernatant was used as a crude enzyme solution for assay and was maintained at -80°C for the following measurements.

The activity of PPO enzyme (EC 1.14.18.1) was quantified by the method described by In *et al.* (2007). The activity of POD enzyme (E.C 1.11.1.7) was measured by the method employed by Srivastava *et al.* (1983). The activities of these enzymes were calculated, using the Beer-Lambert law on the basis of a single enzyme unit (µmol) per mg of fresh weight according to the following formula:

$$\text{U/g FWmin} = \text{absorption changes per minute} \times \text{reaction mixtur} / \text{supernatant volume} \times \text{extinction coefficient}$$

Statistical analyses

This study was conducted as a two-factor factorial in a completely randomized design. The first factor is 11 sampling dates, and the second factor is four cultivars having three replications in the period of 2015-2016. The ANOVAs and standard errors of the mean (SE) were generated, using SAS 9. All significant means were separated, using the Duncan ($P \leq 0.01$). The correlation between the total phenol and antioxidant capacity, PAL, PPO, and POD activities was calculated via the software SPSS 22.

3. Results and Discussion

The beginning date for the chilling accumulation was considered to be when a stable chilling accumulation occurred and the temperatures causing a negative effect were infrequent (Richardson *et al.,* 1974; Guerriero *et al.,* 1990). This date corresponded with 27th October 2015. The first samples were conducted on this date. Mean time budburst was more evident on this date than on the date of the endodormancy release; thus, the kiwifruit axillary buds may inter endodormancy sooner than the end of the summer or the beginning of autumn (McPherson *et al.,* 1995). The results revealed that the maximum depth of endodormancy in this study was in late November. The duration of bud endodormancy was different in the cultivars and genotypes. The end of endodormancy was 21st December 2015 for female Golden genotype (740-unit chilling), 28th December 2015 for male Golden genotype (864-unit chilling), and 4th January 2016 for Hayward and Tomuri cultivars (1003-unit chilling) as shown in figure 1.

The antioxidant capacity indicated a significant difference between the buds of genotypes and cultivars sampled on different dates ($P \leq 0.01$). The highest value in the antioxidant capacity was observed in female Golden genotype on 28th December 2015 (Fig. 2); however, there were no significant differences in the antioxidant capacity of all buds samples

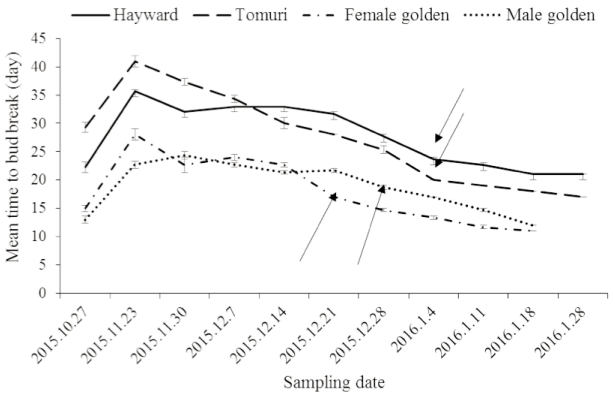

Fig. 1 - Effect of sampling date of cuttings on average of 50% bud break and endodormancy end period in four kiwifruit cultivars and genotypes during dormant season in 2015-2016. The arrows show the endodormancy end date for each cultivar and genotype.

collected in December (i.e. 07th, 14th, 21st and 28th). The lowest value in the antioxidant capacity was observed in Tomuri cultivar on 11th January after endodormancy release (Fig. 2). The antioxidant capacity of buds changed considerably at the beginning and during the maintenance and release of endodormancy (P≤0.01) (Fig. 2). The increase in antioxidant capacity from the end of October to the early November was simultaneous in four cultivars and genotypes; however, the peak period of the antioxidant capacity of buds was different between these cultivars and genotypes (Fig. 2). The antioxidant capacity showed a significant reduction (P≤0.01) in the male and female genotypes at the end of December, being simultaneous with the end of bud endodormancy of both genotypes. The reduction in the antioxidant capacity in Hayward and Tomuri cultivars occurred about two weeks later, coinciding with

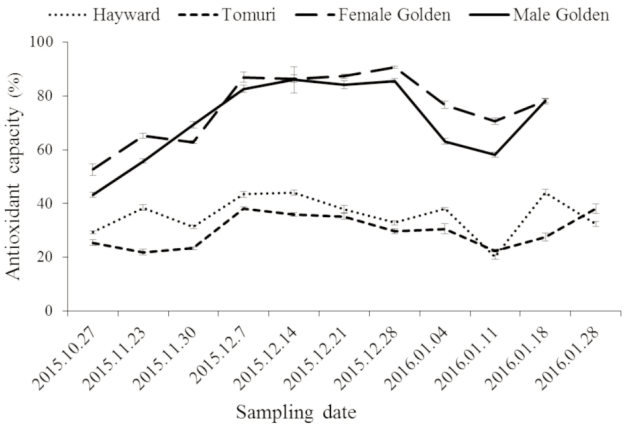

Fig. 2 - Effects of kiwifruit cultivars and different sampling dates on antioxidant capacity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

the completion of their chilling requirements and the end of endodormancy in the buds (Fig. 2).

The activity of PAL enzyme has been shown in figure 3 and demonstrated a significant difference in buds of all kiwifruit cultivars and genotypes (P≤ 0.01). The activity of this enzyme in the male and female Golden genotypes was more than that of the Hayward and Tomuri cultivars (Fig. 3). Female Golden genotype had the highest PAL activity in mid-December. The lowest activity of this enzyme was in Tomuri cultivar on 30th November 2015. PAL enzyme activity in all four kiwifruit cultivars and genotypes began an upward trend at the end of November (P≤0.01) (Fig. 3). It remained relatively stable during endodormancy in cultivars and genotypes, but was associated with a fluctuation in male and female Golden genotypes during ecodormancy. However, the enzyme activity of all cultivar showed a significant decrease (P≤0.01) at the end of endodormancy compared to the stable periods of endodormancy.

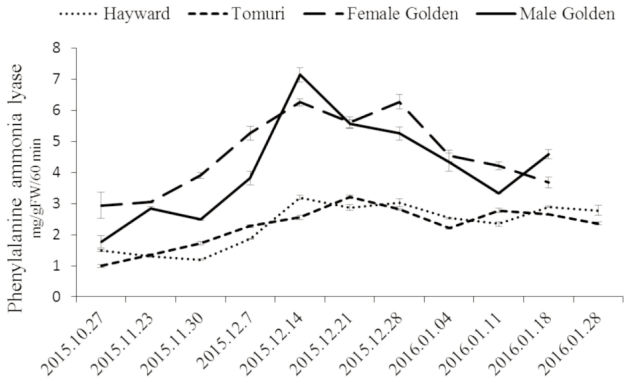

Fig. 3 - Effects of kiwifruit cultivars and different sampling dates on phenylalanine ammonia-lyase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds Moreover, ± the standard error of mean is shown on the vertical bar.

Plants release hydrogen peroxide (H_2O_2) in response to the environmental stress. Low temperature stress has also been shown to induce H_2O_2 accumulation in cells (Okane *et al.,* 1996). Hydrogen peroxide, as the second messenger of the increase in the activity of PAL enzyme, can activate PAL enzyme activity, as a key enzyme in the phenylpropanoids pathway, ultimately leading to higher total phenol and accumulation of flavonoids (Wang *et al.,* 2015). Oxidative stress caused by chilling during the endodormancy of kiwifruit buds increased the activity of this enzyme and the production of antioxidant compounds such as phenols, resulting in a higher antioxidant capacity in the buds during the endodormancy. It has been reported that cold acclimation of

plants leads to a remarkable increase in PAL activity, depending upon the range of low temperature to which the plants are subjected (Stefanowska *et al.,* 2002).

The amount of total phenol have been shown in figure 4 and varied between cultivars and genotypes of kiwifruit, and there were significant differences in their buds (P≤0.01). Male and female Golden genotypes had higher total phenol than Hayward and Tomuri cultivars (Fig. 4). Total phenol content showed substantial changes at the beginning, during the maintenance, and at the end of the endodormancy of kiwifruit buds (P≤0.01) (Fig. 4). The amount of total phenol showed an increasing trend from the late October. In the late November-early December period, coinciding with the onset of true endodormancy, the amount of total phenol reached its maximum (Fig. 4) and its value remained at a high level in the buds of cultivars and genotypes during this period. By reaching the end of endodormancy, the amount of phenol had decreased significantly at the end of December and at the beginning of January in the buds of male and female Golden genotypes and Hayward and Tomuri cultivars, respectively (P≤0.01).

Phenolic compounds are a valuable piece of evidence used in determining the differences between diverse varieties of *Myrtus communis* and *Pistacia lentiscus* and have a key role in detecting the genetic differences in biochemical methods (Tattini *et al.,* 2006). Thus, it appears that the significant differences in terms of the total phenol content between Hayward and Tomuri cultivars and male and female Golden genotypes (P≤0.01) (Fig. 4) are the result of their genetic differences.

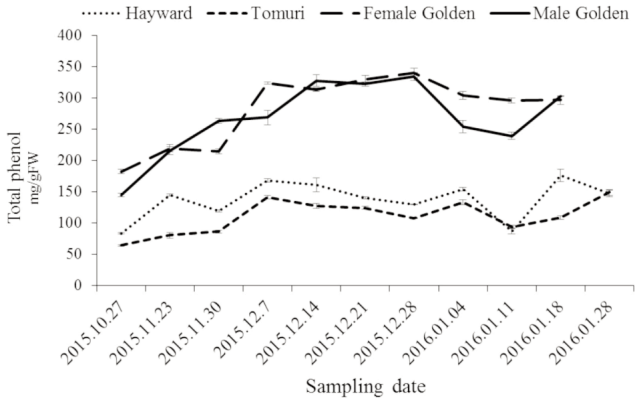

Fig. 4 - Effects of kiwifruit cultivars and different sampling dates on phenol content of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

Endodormancy is developed gradually after the cessation of growth; additionally, the severity of the endodormancy deepens in autumn and then gradually disappears by removing the physiological barriers of growth through the chilling process (Dennis, 2003). Total phenol concentration and antioxidant capacity enhance along with the development of endodormancy and reach their maximum value in the deepest stage of endodormancy (Fig. 2, 4). The changes in these two variables are similar in the establishment, maintenance, and release of endodormancy. A high total antioxidant activity in male and female Golden genotypes may be attributed to the high amount of phenol. Phenolic compounds are synthesized in plant cells in favorable environmental conditions, but environmental stresses change their levels in cells (Kliebenstein, 2004). Mid-autumn cold and the start of endodormancy period causes an increase in oxidative stress in plants. This stress results from reactive oxygen species that affect the growth of plants (Scalabrelli *et al.,* 1991; Mittler *et al*., 2004). Plants possess a protective system composed by the enzymatic antioxidant system such as peroxidase and catalase (Anderson *et al.,* 1995) and the non-enzymatic systems (Agarwal and Pandey, 2004). Phenols are non-enzymatic antioxidants and their antioxidant activities are mainly due to their redox properties which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quencher (Huda-Faujan *et al.,* 2009).

Phenols play an important role not only during cold resistance, but also during breaking the endodormancy of peach (Siller-Cepeda *et al.,* 1992) and apricot (Viti and Bartolini, 1998) buds as an antioxidant. Total phenol changes in kiwifruit buds are in agreement with the results of the studies on peach flower buds (Szalay *et al.,* 2005), apricot vegetative buds (Laslo and Vicas, 2012), and pistachio flower buds (Pakish *et al.,* 2009).

Correlation analyses showed a positive significant correlation between total phenol, antioxidant capacity and PAL activity in kiwifruit cultivars and genotypes (Table 1). Reduction in antioxidant capacity (Fig. 2) and total phenol (Fig. 4) after receiving chilling and a stable period in winter could be considered as a biomarker of endodormancy release in the cultivars which were studied.

In cultivars and genotypes of kiwifruit buds, PPO activity changed substantially (P≤0.01) as shown in figure 5. This enzyme had the highest activity in the Tomuri variety on 7th January 2016 and the lowest activity in male and female Golden genotypes at the

Table 1 - Correlation coefficient of kiwifruit cultivars and genotypes between phenol, antioxidant capacity (RSA %), phenylalanine ammonia-lyase (PAL), peroxidase (POD), and Polyphenoleoxidase (PPO)

Cultivars and genotypes		Phenol	PAL	RSA	POD	PPO
Hayward	Phenol	1	0.25 NS	0.95 **	-0.11 NS	-0.34 NS
	PAL	0.25 NS	1	0.17 NS	0.24 NS	-0.18 NS
	RSA	0.95 **	0.17 NS	1	-0.22 NS	-0.27 NS
	POD	-0.11 NS	0.24 *	-0.22 NS	1	0.44 NS
	PPO	-0.34 NS	-0.18 NS	-0.27 NS	0.44 NS	1
Tomuri	Phenol	1	0.38 NS	0.94 **	0.38 NS	0.28 NS
	PAL	0.38 NS	1	0.42 NS	0.20 NS	-0.22 NS
	RSA	0.94 **	0.42 NS	1	0.27 NS	0.28 NS
	POD	0.38 NS	0.20 NS	0.27 NS	1	0.28 NS
	PPO	0.28 NS	-0.22 NS	0.28 NS	0.28 NS	1
Female Golden	Phenol	1	0.76 **	0.93 **	0.20 NS	0.16 NS
	PAL	0.76 **	1	0.67 *	-0.19 NS	-0.16 NS
	RSA	0.93 **	0.67*	1	0.19 NS	0.14 NS
	POD	0.20 NS	-0.19 NS	0.19 NS	1	0.88 **
	PPO	0.16 NS	-0.16 NS	0.14 NS	0.88 **	1
Male Golden	Phenol	1	0.75 *	0.83 **	0.36 NS	0.26 NS
	PAL	0.75 *	1	0.74 *	0.40 NS	0.37 NS
	RSA	0.83 **	0.74 *	1	0.61 NS	0.49 NS
	POD	0.36 NS	0.40 NS	0.61 NS	1	0.95 **
	PPO	0.26 NS	0.37 NS	0.49 NS	0.95 **	1

** Correlation is significant at the 0.01 level.

first sampling date (Fig. 5). At the end of October 2015 and contemporaneous with the development of bud endodormancy, the activity of PPO enzyme, in all cultivars except Hayward, increased and reached its peak in mid-December and then decreased (P≤0.01; Fig. 5). PPO activity in the Hayward variety peaked a week earlier than the other cultivars. In Tomuri cultivar, PPO activity was stable for three weeks in the dormancy period and reduced at ecodormancy.

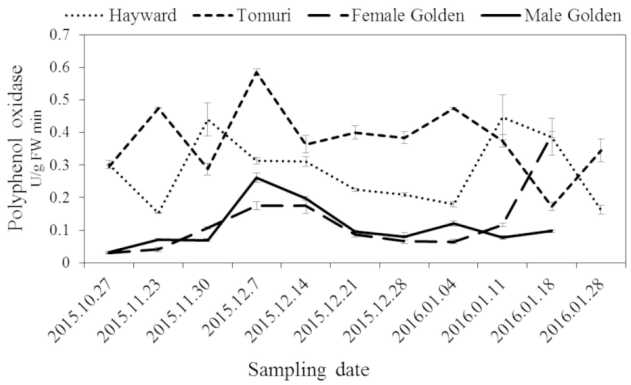

Fig. 5 - Effects of kiwifruit cultivars and different sampling dates on polyphenol oxidase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

PPO is a copper-containing enzyme which catalyzes the oxidation of phenolic compounds to quinone or quinine-like compounds in the presence of molecular oxygen. A high PPO enzyme activity after endodormancy is probably due to the removal of some growth-inhibiting phenols (Wang *et al.,* 1991), and the phenolic substances such as inhibitors or stimulants change enzyme activity (Thirugnanasambantham *et al.,* 2013).

The increased activity of the PPO enzyme at the early stage of endodormancy period and its declined activity at the end of the endodormancy of grape buds (Scalabrelli *et al.,* 1991), plums (Szecskó *et al.,* 2002), and pistachio flower buds (Pakish *et al.,* 2009) were reported, corresponding with the results of this experiment. It appears that the effect of antioxidant compounds is to inhibit free radicals and reactive oxygen species in cultivars and genotypes during stress.

POD activity was notably different in buds (P≤0.01). This enzyme had the highest activity in the Tomuri cultivar after the endodormancy release (Fig. 6). The lowest activity was observed at all cultivars and genotypes at the first sampling date (the 27th

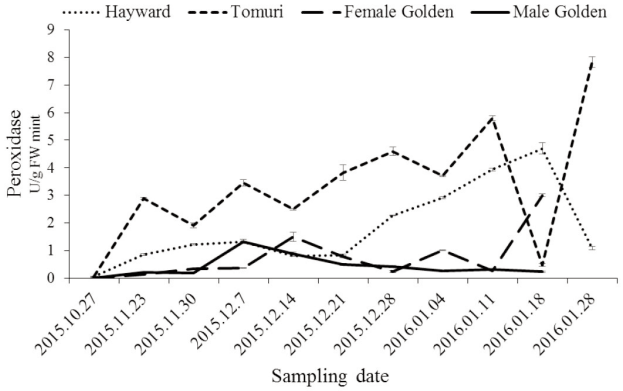

Fig. 6 - Effects of kiwifruit cultivars and different sampling dates on peroxidase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten bud. Moreover, ± the standard error of mean is shown on the vertical bar.

October). The POD activity pattern was not constant during the endodormancy of buds in the cultivars and genotypes which were studied (Fig. 6). POD activity increased in Golden genotypes later than that in the Hayward and Tomuri cultivars. The activity of this enzyme increased significantly in early December and mid-December 2015 in male and female Golden genotype buds, respectively (P≤0.01). However, POD activity decreased significantly in both genotypes at the bud endodormancy release (Fig. 6). The increased activity of POD at the end of October, in

early endodormancy of buds, for Hayward and Tomuri cultivars was in agreement with results of studies on grape buds (Scalabrelli *et al.,* 1991), onion bulbs (Benkeblia and Shiomi, 2004), and floral buds of apricots (Laslo and Vicas, 2012). The increasing activity of this enzyme continued after an endodormancy breakdown in Hayward and Tomuri cultivars; moreover, these results concurred with the antioxidant enzyme changes in pear flower buds, where POD activity increased both during and after the end of endodormancy (Hao and Feng-Wang, 2004).

There are reports on the POD activity associated with susceptibility to cold in pistachio (Pakish *et al.,* 2009) and peach (Szalay *et al.,* 2005), suggesting that the varieties resistant to cold have higher POD activity than the susceptible cultivars. Tomuri had the highest POD activity and its activity significantly increased at the ecodromancy period ($P \leq 0.01$) (Fig. 6). Therefore, Tomuri cultivar could be the most tolerant to cold, that needs to be investigated further.

A significantly positive correlation was observed between phenols, PAL, and antioxidant capacity in Golden genotypes, and between phenols and antioxidant capacity in Hayward and Tomuri cultivars (Table 1). The lowest PPO and POD activities ($P \leq 0.01$) (Figs. 5, 6) and the insignificant changes in these activities during early endodormancy could result from the higher non-enzymatic antioxidant capacity (for example, total phenol and PAL) in Golden genotypes (Fig. 2). The buds of Hayward and Tomuri cultivars showed lower PAL activity and total phenol and higher POD and PPO activities than those in male and female Golden genotypes from the early stage of endodormancy to the ecodormancy stage (Figs. 4-6). However, there was a significantly positive correlation between total phenol content and antioxidant capacity in two cultivars (Table 1). It was concluded that the antioxidant capacity in Hayward and Tomuri cultivars may be due to total phenol rather than POD and PPO activities. Gur *et al.* (1988) reported the difference in PPO enzyme activity and phenol content in apple cultivars.

4. Conclusions

Peroxidase activity increased with the onset of the buds endodormancy, but continued for two weeks after the end of the endodormancy in Hayward and Tomuri cultivars. This is probably due to the resistance of these cultivars to cold compared to the male and female genotypes and, therefore, requires further investigation. This study indicated a significant and stable increase in the PAL and RSA activities. Furthermore, we found that phenol concentration could be associated with the transition to, and maintenance of, bud in true endodormancy. Due to the positive and significant correlation between total phenol, antioxidant capacity, and PAL activity, we concluded that antioxidant capacity, in both male and female Golden genotypes, is attributed to the high phenol which was a result of high PAL enzyme activity.

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Invited paper

EVOO or not EVOO? A new precise and simple analytical tool to discriminate extra virgin olive oils

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Key words: flavors and off-flavors, Panel Test, partial least square-discriminant analysis (PLS-DA), PTR-ToF-MS, volatile organic compounds (VOCs).

Abstract: **International Olive Oil Council (IOOC) states chemical and organoleptic parameters to classify the commercial grade of olive oil. Finding tools or analytical procedures able to support the organoleptic evaluation would be helpful to streamline and facilitate the commercial classification. The aim of the present study was to evaluate a new tool and validate a procedure that allows a fast and non-invasive volatile compounds detection system, able to assign each sample to its right trade category. Moreover, we tried to test the capability of PTR-ToF-MS in grading olive oils according to their *fruity* intensity levels. A total of 273 olive oil samples collected from Argentina (21), Chile (10), Italy (191), Morocco (17), Tunisia (4) and EU (30) were analyzed and classified through: (1) Panel Test and (2) PTR-ToF-MS analysis. On the whole PTR-ToF-MS data EVOO and Not EVOO as resulted by Panel Test were clustered by PCA in two main groups and correctly classified by PLS-DA model, confirming the high confidence level (95%) in utilizing analytical spectral data for helping Panel Test and able to easy monitoring the quality formation in the oils, by a fast and cheap control from harvest until the store. The eight protonated masses detected as VIP by the model may be linked to negative olfactory notes. Finally, PCA applied on the volatile profile of 122 classified EVOO highlighted a shift of the samples distribution following the trend of the *fruity* intensity as assessed by the panelists. In conclusion, this trial confirmed the availability of a new, precise and simple analytical tool as the PTR-ToF-MS, which coupled with an appropriate multivariate data analysis, allows to classify EVOO according to their trade category and *fruity* intensity.**

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1. Introduction

The virgin olive oil is the only vegetable oil consumed without any refinement and characterized by a peculiar synthesis between taste and aroma. The importance of extra virgin olive oil (EVOO) is due to its high content of oleic acid and phenolic compounds, which act as natural

antioxidants (Bendini *et al*., 2007). Its composition makes it not only a food and dressing, but also a product able to protect the human organism from some dysfunctions and pathologies (Marone and Fiorino, 2012). As reported by Aued-Pimentel *et al*. (2013), EVOO has unique characteristics compared to other vegetable oils, such as exceptional sensory and nutritional attributes, therefore worldwide the olive oils are the most valuable ones with a price (normally 3-5 times) higher than other edible oils (Zou *et al*., 2009). As a consequence, in the last years, some adulterations of EVOO with olive oils of lower quality, or with oils of different botanical origin (Catharino *et al*., 2005; Vlachos *et al*., 2006) have been found. As defined by the International Olive Oil Council (IOOC), olive oil is split in trade categories of different quality and commercial value. Because the high commercial value, and the relatively low availability against a high consumption, some traders and bottlers are prompted to sell as EVOO inferior olive oils that does not reflect the parameters established by the IOOC. According with the IOOC rules, the trade class attribution depends not only on chemically defined parameters (i.e. free acidity and peroxides index) but also on a sensory evaluation (SE) that assesses off-flavors and *fruity* presence and intensity. Therefore the EVOO is the only traditional food that must be tested through a Panel Test. Taste and aroma are determined by the presence and the amount of peculiar volatile organic compounds (VOCs), giving to the product unique appealing proprieties. On average the olive oil contains more than 100 volatile compounds belonging to different chemical categories (Guadarrama *et al*., 2000). VOCs emission by the olive fruit and/or olive oil is mostly related to oxidative reactions (i.e. due to injuries during the fruits crushing and malaxation processes). VOCs develop according to distinctive biosynthetic pathways and, among these, the ''LipOXygenase (LOX) cascade'' determines the enzymatic splitting of polyunsaturated fatty acids (linoleic and linolenic) with the ''controlled'' production of aldehydes, ketones, alcohols, carboxylic acids, esters and other VOCs (Angerosa *et al*., 2004; Kalua *et al*., 2007).

The importance of the SE for the olive oils, is due both to its ability to identify the positive attributes and also to evaluate the defects (Peri and Rastelli, 1994). Indeed, the volatile compounds can be used: (a) to discriminate EVOO and virgin olive oil (VOO); and (b) as quality parameters, being the VOCs responsible especially for the green notes and *fruity* of high-quality EVOO oils (Gomez-Rico *et al*., 2006).

While the chemical parameters are easily evaluated through chemical analyses, the flavor and off-flavors are assessed with more subjectivity through the sensory analyses. The SE by the Panel Test is based on strict and laborious rules, and needs trained peoples; therefore, as currently planned, the Panel Test is time consuming and very expensive. Thus, while the chemical analyses guarantees objectivity, repeatability and speediness, the sensory analysis does not allow this result. Indeed, as reported by Marone *et al*. (2017) the SE presents some disadvantages such as: (1) subjectivity of the analysis which could influence the overall evaluation; (2) the need of a large number of trained panelist (8-12) to allow the statistical validation of the results; (3) a limited number of samples evaluable by each panelist a day. Moreover, the results are difficult to generalize, because a lack of a common standard shared in the world, neither easy exploitable in any situations, nor to apply at any step of chain of olive oil making before sale (i.e. processing, storage). Consequently, there is no doubt that the detection of each type of olive oil manipulation needs to be addressed to ensure a correct trade classification, quality and consumer price.

Currently, the most common used analytical techniques to detect VOCs emitted by olive oil are both chromatographic and spectrophotometric methods, as the dynamic headspace gas chromatograph (DHS-GC) (Procida *et al*., 2016), electronic nose and electronic tongue (Aparicio *et al*., 2000; Cosio *et al*., 2007) and the Proton Transfer Reaction-Time of Flight-Mass Spectrometer (PTR-ToF-MS) (Aprea *et al*., 2006; Marone *et al*., 2017).

The PTR-ToF-MS shows a high resolution coupled to a rapid screening power of samples, it is easy to handle and does not need any sample manipulation (Blake *et al*., 2009; Taiti *et al*., 2017). Moreover, this tool is applicable to any step of the olive oil production, from the processing to the market, including the product storage (Marone *et al*., 2017). Furthermore, as the PTR-ToF-MS could work at temperature near those of the tasting, it should give as output a bulk of VOCs at least similar to those perceived by panelists or consumers. A first attempt to directly link spectral data from PTR-ToF-MS as protonated masses to the olfactory sensations perceived by the panelists, to distinguish EVOO from Not EVOO, and consequently correctly classify the virgin olive oils in their trade category, was recently carried out by Marone *et al*. (2017). In this cited work, although employing a low number of samples, it was possible to build up, in a

statistically meaningful way, a color codified card highlighting some specific VOCs that seem to characterize the off flavors as perceived by the panelist. Starting from this result, the aim of the current work was to develop and to test a fast analytical method that combines efficiency, accuracy and reliability for a rapid screening and quantification of volatile compounds in olive oil samples. This analysis method should be helpful to: (1) detect the main defective odors and distinguish the olive oils trade categories; (2) understand if there is an accurate and precise correlation between the judgment provided by the Panel Test and the analysis of the volatile component by PTR-ToF-MS; (3) evaluate different quality and types of EVOO using the positive attributes (i.e. *fruity* and *green* notes).

2. Materials and Methods

Oil sampling

Analyses were carried out during 3 years of surveys (from 2015 to 2017) on the whole spectra of 273 olive oil samples, produced from 2012 to 2017 (Table 1). The olive oils came from three different continent (Africa, South America, and Europe); most samples were obtained from producers or supermarkets, both blend or monocultivar stocks; in this last case, the most of the olive oil samples were obtained at the olive mill. To enhance and enlarge the samples set variability, EVOO from supermarkets labeled as origin were acquired together with "aged" samples (certainly processed two or more years before to be analyzed). For each sample, two filled dark bottles of 250 ml were collected and quickly sent to the storage refrigerated room (17°C) until the organoleptic and VOCs analyses were carried out. Finally, for some samples, the VOCs and SE analyses were repeated during the three years of analysis.

Panel test

After the spectrometric determinations all samples were submitted to a Panel Test. All panels were organized according to the official E.U. olive oil sensory analysis Regulation (n. 2568/91 and its successive modifications) (Table 1). Each taster on the panel shall enter the intensity of the negative and positive attributes on the 10-cm scale in the profile sheet. The oil is graded by the Panel Leader in line with the median of the defects and the median for the *fruity* attribute. According to the reference ranges, an olive oil is graded as extra virgin if the median of the defects is 0 and the median of the *fruity* attribute is above 0. In the present work, all the samples that did not result EVOO were classified as Not EVOO, without any further distinction.

Volatile compounds detection

Measurements were performed with a chemical ionization mass spectrometer (PTR-MS) equipped with a Time-of-Flight (ToF) analyzer (PTR-ToF 8000 model, Ionicon Analytik, Innsbruck, Austria) in its standard mode and using H_3O^+ as ions for the chemical ionization. PTR-ToF-MS has some advantages compared to the other traditional electron ionization such as: reduced fragmentation which eases compound identification and guarantees high sensitivity with a very high time resolution and no need of sample treatments (Taiti *et al*., 2017). Previously Blake *et al*. (2009) provided a complete and detailed description of the PTR-MS technology. All the instrumental parameters used during the measurement were set as follow: a constant drift voltage of 600 V and a pressure of 2.20 ± 0.02 mbar were maintained in the reaction chamber and the instrument operated at a standard E/N value (electric field strength/gas number density) of 138 Td (1 Td = 10^{-17} cm^2 V^{-1} s^{-1}). Each sample was prepared on the basis of the following protocol: 15 g of oil (T 25°C) were introduced in apposite glass jar (750 ml), afterwards were fluxed with clean air (Zero air generator, Peak scientific) for 120 seconds and subsequently were hermetically sealed and incubated for 60 seconds at 25°C inside an incubator. Each jar was provided with inlet and outlet Teflon pipes, which connect the glass jar to the PTR-ToF-MS system and to the zero-air generator, respectively. Two replicates of each sample were analyzed and the order of samples was randomized. Besides, at the beginning of the experiment and always after three oils sample an identical empty jar was analyzed for background subtraction. Headspace concentrations of each oil sample were subsequently averaged over the two replicates and used for further statistical analysis. The range of mass spectra was recorded in the range of 20-210 m/z at 1 spectrum per 1 second, and the mass calibration was based on m/z = 21.022 (H_3O^+), m/z = 59.049 ($C_3H_7O^+$) and m/z = 137.132 ($C_{10}H_{17}^+$); the calibration was made before starting each files and, subsequently, all files were recalibrated off-line. Data were recorded with the TofDaq software (Tofwerk AG, Switzerland) and all spectra were acquired and analyzed using a procedure previously reported by Taiti *et al*., 2017. Data were expressed in ppbv following a procedure

Table 1 - Description of 273 olive oil samples in relation to provenience zone, cultivar, acquisition from producer or supermarkets, processing campaign or getting year, PTR-ToF-MS analysis year, and Panel Test judgement (EVOO/Not EVOO)

Provenience zone	Cultivar/blend	Number of samples	Get from: producer (1), supermarkets (2)	Processing campaign (A), or acquisition (B) year	PTR-ToF-MS analysis year	EVOO (0)/Not EVOO (1)
Argentina	Arbosana	4	1	2017 A	2017	1 (4)
Argentina	Blend	4	2	2012 B	2016	1 (4)
Argentina	Blend	4	1	2013 A	2016	1 (4)
Argentina	Blend	4	1	2014 A	2016	1 (4)
Argentina	Coratina	2	1	2015 A	2016	1 (2)
Argentina	Coratina	1	1	2017 A	2017	0 (1)
Argentina	Koroneiki	2	1	2017 A	2017	0 (2)
Chile	Arbequina	2	1	2012 A	2017	1 (2)
Chile	Arbosana	4	1	2012 A	2017	1 (4)
Chile	Arbosana	1	1	2013 A	2017	1 (1)
Chile	Koroneiki	1	1	2012 A	2017	1 (1)
Chile	Koroneiki	2	1	2013 A	2017	1 (2)
Italy	Arbequina	4	1	2012/13 A	2016	1 (4)
Italy	Arbequina	3	1	2013/14 A	2016	1 (3)
Italy	Arbequina	7	1	2015/16 A	2016	0 (7)
Italy	Arbequina	5	1	2016/17 A	2017	0 (5)
Italy	Arbosana	3	1	2015/16 A	2016	0 (3)
Italy	Arbosana	2	1	2016/17 A	2017	1 (2)
Italy	Blend	2	1	2012/13 A	2017	1 (2)
Italy	Blend	11	1	2015/16 A	2015	0(11)
Italy	Blend	12	1	2015/16 A	2016	0(12)
Italy	Blend	14	1	2016/17 A	2017	0(10)
						1(4)
Italy	Blend	17	2	2017 B	2017	0(13)
						1(4)
Italy	Carolea	5	1	2013/14 A	2015	1(5)
Italy	Frantoio	8	1	2013/14 A	2015	1(8)
Italy	Frantoio	2	1	2015/16 A	2015	0(2)
Italy	Gentile di Chieti	9	1	2015/16 A	2015	0(9)
Italy	Gentile di Chieti	4	1	2015/16 A	2016	0(4)
Italy	Intosso	7	1	2015/16 A	2015	0(7)
Italy	Intosso	4	1	2015/16 A	2016	0(4)
Italy	Itrana	7	1	2015/16 A	2015	0(7)
Italy	Itrana	4	1	2015/16 A	2016	0(4)
Italy	Koroneiki	3	1	2015/16 A	2016	0(3)
Italy	Koroneiki	4	1	2016/17 A	2017	0(4)
Italy	Leccino	5	1	2013/14 A	2015	1(5)
Italy	Maurino sel. Vittoria	3	1	2015/16 A	2016	0(3)
Italy	Maurino sel. Vittoria	4	1	2016/17 A	2017	0(4)
Italy	Oliana	5	1	2016/17 A	2017	1(5)
Italy	Olivastra seggianese	18	1	2015/16 A	2016	0(3)
						1(15)
Italy	Peranzana	5	1	2015/16 A	2015	0(5)
Italy	Peranzana	4	1	2015/16 A	2016	0(4)
Italy	Sikitita	5	1	2015/16 A	2016	0(5)
Italy	Sikitita	5	1	2016/16 A	2017	1(5)
Morocco	Arbequina	2	1	2012/13 A	2017	1(2)
Morocco	Picholine maroccaine	3	1	2014/15 A	2016	1(3)
Morocco	Picholine maroccaine	3	1	2014/15 A	2017	1(3)
Morocco	Picholine maroccaine	3	1	2015/16 A	2016	1(3)
Morocco	Picholine maroccaine	6	1	2015/16 A	2017	1(6)
Tunisia	Koroneiki	4	1	2012/13 A	2017	1(4)
U.E.	Blend	5	2	2016 B	2016	1(5)
U.E.	Blend	25	2	2017 B	2017	1(25)

described by Lindinger and Jordan (1998). Then, the data obtained were filtered by eliminating peaks that were lower than a threshold of 0.50 ppbv and eliminating all signals relative to ions hard to quantify precisely. After filtration, data have been sent to the statistical analysis (Fig. 1).

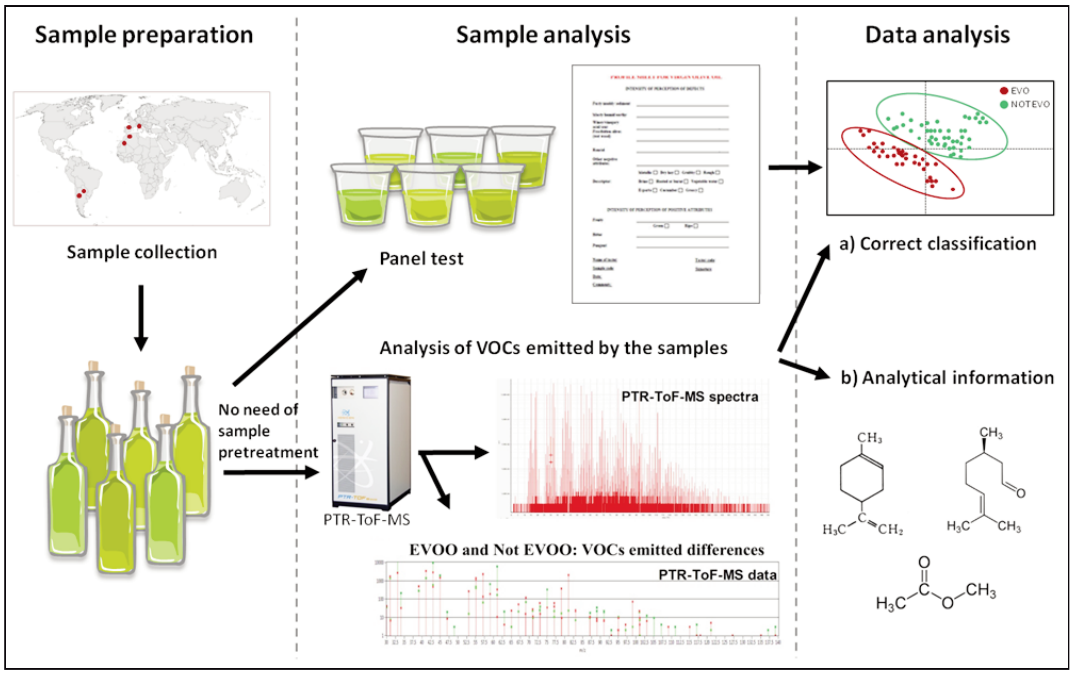


Fig. 1 - Schematic representation of oil samples analyses and classification using the PTR-ToF-MS. This technique allows rapid and non-destructive VOCs detection throughout the entire food-to-fork chain (e.g. oils) without any sample pretreatment. All data acquired by PTR-ToF-MS were used to obtain analytical information regarding the quality of product and for trade categories, varieties and geographical origin classification applying different multivariate analyses.

Multivariate data analysis

A principal component analysis (PCA, unsupervised method) was applied to the spectral data of 273 olive oil samples, submitted to a logarithmic transformation and mean centering as pre-processing. Computations were performed by PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB® R2015b (Mathworks Inc., Natick, MA, USA). A multivariate partial least squares-discriminant analysis (PLS-DA, supervised method) was applied on the spectra of the 273 olive oil samples, to develop a model for differentiating EVOO from Not EVOO. As pre-processing data, they were submitted to a logarithmic transformation and auto-scaling. The training set (85% of the samples) allowed to select the optimal number of latent variables (LVs) throughout the calibration and cross validation phases. The training and validation subsets were obtained by the Euclidean distances based on the algorithm of Kennard and Stone (1968). The test set (prediction) consisted of 15% of the samples previously removed from the dataset. As cross validation procedure, Venetian blind with 10 splits and 1 sample per split was chosen. The performances of the model were evaluated by the number of correct assignments and the root-mean-squared error of cross-validation (RMSECV), and prediction (RMSEP). The optimal number of LVs resulted associated to the minimum error and misclassification rate of the calibration dataset. The reliability of the model was tested by confusion matrices. The threshold to assign a sample to a class was chosen based on the Bayes theorem, minimizing the number of false positives and false negatives. Variable Importance in Projection (VIP) scores (p=0.01) were also calculated. A random permutation of the class labels (permutation test) was also performed (500 iterations), so to generate nonsense datasets for comparison with the true model, to evaluate the probability that the model is significantly different from one casually built up under the same conditions. PLS-DA analysis was performed by PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB® R2015b (Mathworks Inc., Natick, MA, USA). A PCA was then applied to the spectral (PTR-ToF-MS) data of the 122 samples resulting EVOO based on the Panel Test, previously submitted to a logarithmic transformation and auto-scaling.

3. Results and Discussion

EVOO or Not EVOO

VOCs emission by olive oil is characterized by the presence of different compounds belonging mainly to alcohols, esters, aldehydes, ketones, terpenes and hydrocarbons. C6 molecules are the main volatile compounds derived from polyunsaturated fatty acids

through the LipOXygenase pathway (Cecchi and Alfei, 2013), generally characterized by low molecular weight. These compounds easily come in contact with the olfactory cells and help to create flavor and sometimes off-flavor. According to Marone *et al*. (2017), there is the possibility to directly relate the volatile profile obtained by PTR-ToF-MS to distinguish EVOO from Not EVOO, and, as a consequence, to correctly classify the virgin olive oils in their trade category. To confirm the preliminary results obtained by Marone *et al*. (2017), and validate the new procedure and methodology, we used a huge number of samples that were collected and analyzed in different years. In the present work, to define their trade category, 273 olive oil samples were submitted to the SE, that classified 151 samples as Not EVOO, and 122 as EVOO. By analyzing each oil sample (Table 1), 63 volatile compounds were detected within a mass range of m/z = 20-210 (data not shown). PCA applied to the whole dataset (ppbv) allowed to get a first general overview of the data distribution. Two main groups of EVOO and Not EVOO were clearly highlighted (Fig. 2) in the bidimensional space of the first two components, despite the great variability present in the original data set, due to the great diversification in the olive oil samples. This variability is also evidenced by the need to consider the first 7 components to justify 90.17% of the total variance (respectively: 60.26%, 12.56%, 5.17%, 3.81%, 3.39%, 2.78, and 2.20%). The data ordination clearly highlights that the VOCs spectra provided by Not EVOO samples were well distinguishable from those of the EVOO, with a few partially overlapping zones in the upper right and bottom left quadrants. This behaviour indicates a different spectral distribution between flavors and off-flavors, confirming the same result obtained by the SE. Subsequently, a partial least squares discriminant analysis (PLS-DA) approach was applied to determine the trade category of the olive oil samples. A seven-component PLS-DA model, evaluated by its performances indicators (Table 2), resulted robust to discriminate the Not EVOO from the EVOO samples in the model/validation data set, and in the independent test set. The optimal number of latent variables (LVs), associated to the minimum error rate and the minimum number of not assigned samples, resulted in 7 (Table 2). The permutation test

Table 2 - PLS-DA statistics for each Y-Block (class 1 = Not EVOO; class 0 = EVOO) related to 273 olive oil samples. Sensitivity (SE); Specificity (SP); Class error, RMSEC, RMSECV, and RMSEP for Calibration (Cal), Cross Validation (CV), and Prediction (Pred), respectively. Confusion matrices for Calibration, Cross Validation, and Prediction

Statistics	LVs	SE (Cal)	SP (Cal)	SE (CV)	SP (CV)	Class. error (Cal)	Class. error (CV)	Class. error (Pred)	RMSEC	RMSECV	RMSEP
Not EVOO	7	0.972	0.968	0.954	0.960	0.029	0.043	0.042	0.206	0.261	0.226
EVOO		0.968	0.972	0.960	0.954						

Confusion matrices

			Classes		Matthew's correlation coefficient
			1-Not EVOO	0-EVOO	
Calibration results	Predicted as	1-Not EVOO	105	4	0.940
		0-EVOO	3	121	
Cross validation results		1-Not EVOO	103	5	0.914
		0-EVOO	5	120	
Prediction results		1-Not EVOO	16	2	0.903
		0-EVOO	0	22	

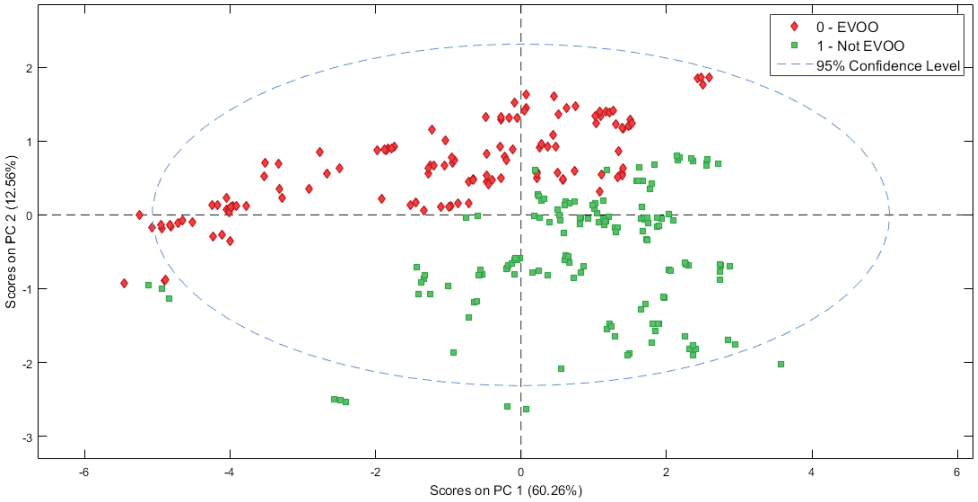


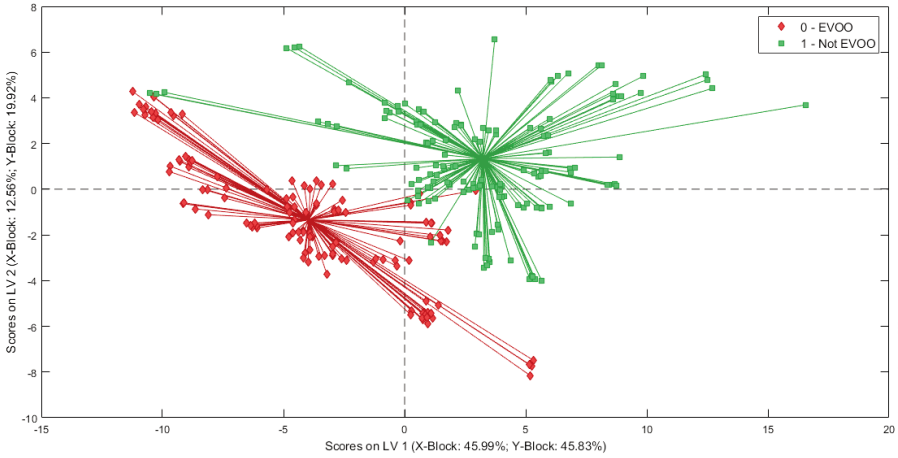
Fig. 2 - PCA ordination of 273 olive oil samples. Green = Not EVOO, red = EVOO.

indicated that the model is significant at 95% confidence level. In fact, the probability of model insignificance *vs*. permuted samples resulted 0.0 based on the Wilcoxon and Sign Test, both in Self-Prediction and Cross-Validated, and 0.005 by the Rand t-test. The model successfully classified 96.9% of samples into their trade category based on the Panel Test results in fitting, 95.5% in cross validation (internal validation) and 95% in prediction (external validation). That is, as reported in the confusion matrices (Table 2), in the calibration, on a total of 233 samples, 226 were correctly classified, while 3 resulted false positive (predicted as EVOO from the Panel Test, but classified as Not EVOO by the spectrometer) and 4 false negative (predicted as Not EVOO from the Panel Test, but classified as EVOO by the spectrometer). In the cross validation, 223 samples were correctly classified, while 5 resulted false positive, and 5 false negative. In the prediction results, on 40 samples, 38 were correctly assigned to their right class, while only 2 resulted false positive. The occurring of false positive (3 samples in prediction, judged as EVOO by the Panel Test, and classified as Not EVOO by the spectrometer), can be related to the fact that all compounds (including off-flavors) are only perceived by the human olfactory when they exceed their specific threshold values (Morales *et al*., 2013). Thus, we can assume that, below this threshold values, the presence of a given compound linked to a defect is not perceived by the human olfactory, but is inexorably detected by the spectrometer. On the other hand, only a few borderline olive oils judged as Not EVOO by the panelists but classified as EVOO by the tool (false negative) were detected. A scores plot of the first two components of the PLS-DA model for all oil samples is shown in figure 3. The PLS-DA model also allowed to evidence the significant (>1.5) VIP scores, indicating the role of the selected protonated masses to differentiate the two classes (Fig. 3). VIP scores reported in figure 3 confirm the results of our preliminary work (Marone *et al*., 2017). In particular, the masses m/z = 47.050 (Tentatively identified (TI) as: ethanol), m/z = 61.030 (TI: acetic acid), m/z = 75.040 (TI: propanoic acid) and m/z = 89.060 (TI: butanoic acid) resulted as factors able to distinguish EVOO from Not EVOO. Indeed, ethanol and acetic acid are generally considered as compounds deriving from microbial alterations due to a long time of olive storage before processing (Morales *et al*., 2000) and therefore represent a known defect. Likewise propanoic acid and butanoic acid are both considered defective compounds, that can be linked to fermentation processes in olive fruits as a long time of storage (Angerosa *et al*., 1996) or related to the sugar fermentation (Morales *et al*., 2013).

Overall, for all the samples evaluated, it is interesting to note that the procedure applied in this study to discriminate EVOO from Not EVOO is not affected by factors such as: year under analysis, harvesting year, variety and geographical origin. In fact the model resulted significant at 95% confidence level only considering as classification (variability) factor the EVOO/Not EVOO distinction.

Classification of different EVOO fruity *intensity*

EVOO are currently also labeled according to the *fruity* intensity perceptions (*robust*, *medium*, *delicate*), based on the IOOC regulation (COI/T.20/Doc. No 15/Rev. 8, 2015). Thus, we tried to evaluate the different EVOO *fruity* intensity using the dataset provided only by the VOCs profile of the 122 EVOO samples (according to the Panel Test) (Table 1) through a



Protonated *m/z*	VIP scores
47.050	1.986
53.040	1.509
59.050	1.605
61.030	2.953
73.060	1.610
75.040	1.903
87.080	1.621
89.060	1.642

Fig. 3 - Score plot (LV1, LV2) of the PLS-DA model. Green = Not EVOO, red = EVOO; VIP scores > 1.5.

PCA analysis. The first two components explained about 63% of the total variability, and the derived scatterplot (Fig. 4) showed three groups of samples that are rather well separated. It is interesting to note that linking the *fruity* score assigned by the Panel Test to each sample, the three groups distributed in the chart according to their *fruity* intensity (Fig. 4), even if the samples grouped by the tool at the bottom of the figure (Fig. 4) show VOCs profiles relatively close, while the *fruity* intensity scores attributed to the same samples by the panelists ranged from 4 to 7. According to this chemometric approach, the subjectivity of the Panel Test becomes evident at intermediate values of *fruity* intensity. PCA analysis also underlines two outliers group. In the first one, labeled with "M", the three samples belonging to cv. Maurino sel. Vittoria harvested in year 2016 are found; this can be linked to peculiar flavor notes characterizing this Tuscan clone, that showed the highest amount of terpene compounds compared to all other samples (data not shown). The second one, represented by a few samples separated from the central bulk and shifting to the right part, labeled with "S" (Fig. 4), is formed by samples with particular flavor notes (data not shown). These samples, belonging to the cv. Sikitita, as reported by García-Gonzalez *et al*. (2010), are in fact characterized by typical aromas.

Associating the *fruity* score assigned by the Panel Test to each sample, it is remarkable as the entire aromatic profile detected by the PTR-ToF-MS seems to be linked to changes in the amount of masses within the spectra rather than to the presence of specific compounds in the human olfactory perception of the *fruity* intensity.

4. Conclusions

The chemometric classification model proposed in this trial and based on the VOCs fingerprint acquired by the PTR-ToF-MS allows to distinguishing olive oil samples of different trade category. In particular, it was demonstrated that: (1) the entire volatile profile can be useful to classify oils belonging to different commercial categories (as the Panel Test), (2) the different qualities and types of EVOO can be split by using the *fruity* intensity. The accuracy of classification proposed is very high and it is more efficient than that obtained by other authors using different tools. Indeed, this tool does not require any sample pre-treatment and allows identifying compounds with low molecular weight (i.e methanol, ethanol, etc.) compared to other ones.

Given our results and the emerging need of the olive oil sector that requires the developmental analytical tools to support or integrate the Panel Test, this work opens the way for the use of PTR-ToF-MS coupled with an appropriate multivariate analysis, as a quick and cheap tool with high confidence level and

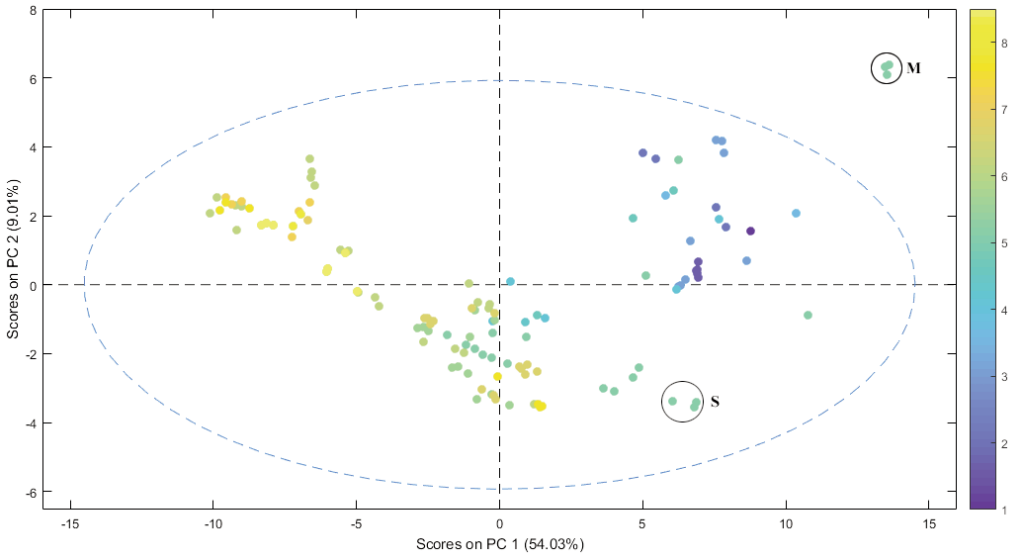


Fig. 4 - PCA ordination of 122 extra virgin olive oil samples. The objects key color indicates *fruity* intensity scores as evaluated by the Panel test increasing from blue (fruity = 1) to yellow (fruity = 8.5). Black circled samples indicate: Maurino sel. Vittoria (M) and Sikitita (S).

comparable to the Panel Test, for the olive oil quality identification.

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