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Original article

First observations of graft combination on bergamot fruit quality, Femminello cultivar

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Summary

Introduction – **The bergamot is cultivated along the Calabrian coast. It is grafted onto sour orange that is susceptible to *Citrus tristeza* virus. Although no infections have been reported on this scion/rootstock combination, CTV is present in Calabria and it is well known that this rootstock is very susceptible to the virus. Furthermore, very few are the experimental works that have as object the food consumption of bergamot. The experiment was carried out to evaluate two alternative rootstocks to sour orange for bergamot, during the first years from planting, in the main cultivation area for bergamot essential oil production. The influence of rootstock was evaluated about bergamot fruit quality for fresh consumption, because the nutraceutical role of this citrus for food has been discovered in recent years. The quality of the essence was also evaluated concerning the most important molecules that characterize the bergamot essence, being linalyl acetate, linalool, and limonene. The yield was also evaluated.** *Materials and methods* – **The experiment was carried out to evaluate the influence of the alternative rootstock to sour orange on the quality of the bergamot for food consumption. The influence of rootstock on the fresh yield, and the yield and quality of the essential oil was also evaluated. The experiment was carried out during three years (2017/2019) in Reggio Calabria (Italy) on bergamot tree (*Citrus bergamia* Risso & Poiteau) cv. Femminello, grafted onto sour orange, Troyer citrange, and Volkamer lemon. At harvest, biometric measurements, yield per tree, organoleptic and nutraceutical parameters, gland counts, quality of essential oil, and yield of essential oil of the fruit were detected.** *Results and discussion* – **The main qualitative and nutraceutical parameters were similarly replacing sour orange with Volkamer lemon and Troyer citrange. About the production per plant and yield efficiency, the worst results were obtained with Troyer citrange. The better yield of essential oil per plant and a high quality of the essential oil were obtained with Volkamer lemon rootstock.** *Conclusion* – **Therefore, Volkamer lemon appears like an excellent alternative to sour orange.**

Keywords
essential oil, sour orange, total antioxidant capacity, *tristeza*, Troyer citrange, Volkamer lemon

Significance of this study

What is already known on this subject?
- The bergamot fruit is used both as essential oil and for fresh consumption. Sour orange is sensitive to *Citrus tristeza* virus (CTV) and it is the main rootstock used to bergamot.

What are the new findings?
- Alternative rootstocks to sour oranges have been identified. These have given excellent fruit quality and yield for many species of *Citrus*.

What is the expected impact on horticulture?
- Few studies are carried out to bergamot. Increased research to find a new optimal rootstock has a significant impact to give good production and CTV non-susceptible tree.

Introduction

In Italy, the cultivation of bergamot began around the 17th century. This citrus spread in the province of Reggio Calabria with the first planting of bergamot in 1750 (De Nava, 1910).

It is difficult to establish exactly how this citrus fruit arrived in Calabria and, above all, what the origin is. Gallesio (1811) considers bergamot a hybrid between lemon (*Citrus limon* L. Burm.) and sour orange (*Citrus aurantium* L.).

Tanaka (1936) believes that citrus fruit originated as a result of a genetic mutation of another species. Swingle (1943) believes that bergamot is a hybrid of sour orange; Chapot (1962) considers it a cross between sour orange (*Citrus aurantium* L.) and lime (*C. aurantifolia* [Christm. & Panzer] Swingle); this would also be confirmed in studies conducted by Federici *et al*. (1998, 2000) with molecular genetic techniques. Subsequent studies, carried out with the help of molecular markers, consider citron (*Citrus medica* L.) and sour orange (*Citrus aurantium* L.) as possible parents of bergamot (Deng *et al*., 1996; Nicolosi *et al*., 2000; Moore, 2001). It is known for essential oil extracted from its pericarp used in perfumery. However, in recent years, bergamot is also consumed as a fresh product and juice for its organoleptic and healthful qualities, but also in pastry (candied fruit, creams, ice creams) as already done for other species of *Citrus*.

The bergamot is an evergreen plant propagated through grafting and successfully cultivated along the Calabrian coastal strip that extends from the town of Scilla to the town of Monasterace. The Calabria region is the main bergamot producing area in the world; in fact it produces 90% of the total world production of bergamots (Navarra *et al*., 2015).

In Calabria three cultivars of bergamot are grown: ‘Femminello’, ‘Castagnaro’, and ‘Fantastico’.

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This citrus was initially propagated on lime (*C. aurantifolia* [Christm. & Panzer] Swingle) for its high affinity; only later, as a result of the gummy epidemics, the bergamot was grafted onto sour orange (*Citrus aurantium* L.). This rootstock was used because it adapts to the most diverse soil conditions, and is at the same time able to produce high-quality fruits (Wutscher, 1979; Louzada *et al*., 2008). It is the most widely used rootstock in the Mediterranean basin, specifically in Cyprus (Georgiou, 2009), Egypt (Bassal, 2009), Greece (Elena *et al*., 2006), Israel (Benjamin *et al*., 2013) and Italy (Castle, 2010).

However, the spread of the *Citrus tristeza* virus (CTV), considered the pathogen that more than others threatens citrus growing worldwide (Lee and Bar-Joseph, 2000), has imposed a review of rootstocks in the nursery sector of the citrus.

Up today bergamot is grafted onto sour orange, and no infections have been reported, but CTV is present in Calabria (Albanese *et al*., 2010) and it is well known that this rootstock is very susceptible to the virus (Carrero, 1981; Lee and Bar-Joseph, 2000).

Moreover, Cardenosa *et al*. (2015) and Bermejo and Cano (2012) reported that the potential effects of the rootstock on the scion are still unexplored for some *Citrus* species. It is the case of bergamot.

The fresh consumption of bergamot has been only discovered in recent years both to organoleptic and nutraceutical characteristics.

Therefore, the aim of this work was to test two rootstocks alternatives to sour orange, citrange Troyer and Volkamer lemon, non-sensitive to CTV, as reported by some authors (Yoshida, 1993; Deng *et al*., 2001; Mendez *et al*., 2001; Geoirgiou, 2009; Shafieizargar *et al*., 2012; Forner-Giner, 2020). The experiment was carried out during the first years from planting, in the main cultivation area for bergamot essential oil production.

The influence of the rootstock was evaluated about bergamot fruit quality for fresh consumption. The quality of the essence was also evaluated concerning the most important molecules that define the bergamot essence (linalyl acetate, linalool, and limonene). The yield was also evaluated.

Materials and methods

Orchard

The experiment was carried out during three years, 2017/2019, on young bergamot trees (six years) at the Foti farm, sited to Pellaro, Reggio Calabria city (38°01'57.2"N; 15°39'46.5"E), Italy. Scion of bergamot (*Citrus bergamia* Risso & Poiteau) 'Femminello' grafted onto sour orange (*Citrus aurantium* L.) (SO), citrange Troyer [*Poncirus trifoliata* (L.) Rat. × *Citrus sinensis* (L.) Osbecke] (TC) and Volkamer lemon (*C. volkameriana* Tan. et Pasq.) (VL) were planted in the spring of 2014 in a sandy-loam soil (sand 42.1%, silt 31.5%; clay 26.4%), with pH 8 (sub-alkaline; active lime 1.2%, soil salinity 128.3 μS cm^{-1}), high organic matter (5.1%) and 0.95 g kg^{-1} nitrogen content; therefore, the soil characteristics were suitable for all the rootstocks used. The trees were spaced 5 × 5 m apart (400 plants ha^{-1}) with a North-South orientation and trained to 'globe' shapes. The climate was Mediterranean type, with dry summers and with fresh, wet winters; the average temperature of the hottest month (August) is 28.70 °C, whereas that of the coldest month (January) is 10 °C. The rainfall concentrates in the autumn-winter period and the annual precipitation is 560 mm. The climate data, above mentioned, are the averages of the last twenty years.

The orchard was managed using the standard integrated pest management system and stable drip irrigation and fertilization system.

Experimental design

Fifteen replicate trees per graft combination were distributed in a block design with three blocks.

Tree dimension parameters were calculated using the "Analysis" tool of Adobe Photoshop® CS6 Extended software (Adobe, San Jose, CA, USA). This tool, used for image processing, allows defining, after setting the measurement scale, the variables necessary for the calculation both of the tree height and canopy volume (Barrett and Brown, 2012).

The canopy surface area was determined by analysing, with Photoshop's tool just described, an aerial frame acquired with a Parrot Bebop Drone (Parrot SA, Paris, France) equipped with a Parrot P7 dual-core processor for computing power and a quad-core graphics processor.

1. Yield performance. At the end of the experiment (at harvest, 260 days after full bloom, DAFB), the trees were harvested, and the number of fruit per tree and yield per tree (kg $tree^{-1}$) was determined. Yield efficiency was estimated as the ratio of yield per tree to canopy volume (kg m^{-3}).

At harvest (260 DAFB) for each tree, five fruits were randomly selected. They were used to measurements of the following parameters:

2. Fruit morphometric characteristics. Transversal and longitudinal diameter, rind thickness, fresh weight (FW), dry weight (DW of flavedo, albedo and pulp) were measured using respectively a digital caliper and an electronic balance (Mettler-Toledo GmbH, Greifensee, Switzerland).

The colour of skin and pulp, in terms of CIELAB L^*, a^*, b^* values, chroma and °hue were determined using a Minolta CM-700d spectrophotometer (Minolta, Osaka, Japan). Juice yield was obtained using a juice extractor; the juice content was calculated as the percentage ratio of juice volume (mL) to fruit weight (g).

3. Soluble solids and titratable acidity. On the juice were measured: soluble solids content, using a handheld digital refractometer (PR-1, Atago, Tokyo, Japan); titratable acidity using an automatic titrator (Titralab AT1000 series, HACH, Colorado, USA): 25 mL bergamot juice diluted with (1:1) and titrated to pH 8.2 with 0.1 N NaOH (mEq. NaOH 100 g fresh $fruit^{-1}$). Titratable acidity was expressed as a percentage of monohydrate citric acid.

4. Nutraceutical parameters: ascorbic acid; total antioxidant capacity, total polyphenols content, total flavonoid content, total carotenoid and chlorophyll content. The ascorbic acid (AA) content was determined using the procedure based on the reduction of the dye 2.6-dichlorophenol-indophenol by ascorbic acid (mg ascorbic acid 100 g^{-1} FW).

Total polyphenols content (TPC) and total antioxidant capacity (TAC) analyses were performed. These analyses were performed on pulp and flavedo and albedo. For each block, five fruits for graft combination were placed in polyethene bags and frozen at -80 °C, until the analysis of TAC, and TPC. The bergamot samples were homogenised using an Ultraturrax blender (20,000 rpm; T 25 Basic, IKA Werke, Germany). The TPC and TAC were analysed separately using a Lambda 35 spectrophotometer (Perkin Elmer Corporation, USA). Before measuring the TPC and TAC, standard curves were prepared for each test. The TPC (mg gallic acid equivalents g^{-1} FW) was determined using the Folin-Ciocalteu method (Slinkard and Singleton, 1997). The TAC was determined using the modified TEAC assay and expressed as μmol Trolox equivalents g^{-1} FW (Pellegrini *et al.*, 1999; Re *et al.*, 1999). The TEAC assay included both the hydrophilic and the lipophilic contributions (Scalzo *et al.*, 2005) of the bergamot samples.

Total flavonoid content (FDT) of the samples was measured using a colorimetric method (Zhishen *et al.*, 1999; Dewanto *et al.*, 2002). The methanolic extract (250 μL) was mixed respectively with 1.25 mL DI water and 75 μL of 5% $NaNO_2$ solution, then allowed to mix for 6 min. After addition of 150 μL of 10% $AlCl_3$ solution and mixing for 5 min, the reaction was initiated by adding 0.5 mL of 1 M NaOH, and the total volume was made up to 2.5 mL with DI water. Sample absorbance was read at 510 nm using a UV/Vis spectrophotometer (Lambda 35, Perkin Elmer Corporation, USA). Total flavonoid content was expressed as μg (+)-catechin equivalents (CA) g^{-1} FW.

The preparation of samples for total flavonols content (FLT) determination was done according to the aluminium chloride colorimetric technique: 0.5 mL of each extract was mixed with 0.5 mL aluminium chloride (2%), and then 1.5 mL potassium acetate (5%) was added. After 150 min, the absorbance was determined at 440 nm. The calibration curve was plotted by different concentration of quercetin equivalents (Miliauskas *et al.*, 2004).

Total Chl ($a+b$) (TChl) and carotenoid (TCar) were calculated according to Porra *et al.* (1989).

Total chlorophyll ($a+b$) and carotenoid content of exocarp from each treatment were extracted with 80% acetone and centrifuged at 20,000 rpm for 20 min at 4 °C. All pigment extraction was done in the dark with the samples kept on ice. The absorbance of the supernatant was measured using a dual-wavelength/double beam spectrophotometer (Lambda 35, Perkin Elmer Corporation, USA). The equation used for the quantification of Chlorophyll *a*, Chlorophyll *b*, and carotenoids are given below:

$\text{Chl } a\ (\mu g\ mL^{-1}) = (12.25 \times A\ 663.2) - (2.79 \times A\ 648.8)$;
$\text{Chl } b\ (\mu g\ mL^{-1}) = (21.50 \times A\ 648.8) - (5.10 \times A\ 663.2)$;
$\text{Total Chl}\ (\mu g\ mL^{-1}) = (17.67 \times A\ 648.8) + (7.12 \times A\ 663.2)$;
$\text{Carotenoids}\ (\mu g\ mL^{-1}) = [(1000 \times A\ 470) - (1.82 \times \text{Chl } a) - (85.02 \times \text{Chl } b)]/198$.

5. Glands oil count. On portions of peel, the number of gland oil (n cm^{-2}) was determined using the "Counting" tool of the Adobe Photoshop® CS6 Extended software (Adobe, San Jose, CA, USA). The images were acquired in TIFF format (Tagged Image File Format) by a single-lens reflex digital camera (Nikon D5500, Tokyo, Japan) equipped with a 24.2 effective megapixel CMOS APS-C sensor over an area of 23.2 × 15.4 mm.

6. Essential oil yield. Extraction of the essential oil was carried out in the laboratory by applying manual pressure on the peel to cause the breaking of the utricles and the release of the oil itself, which was collected on a watch glass, transferred to a test tube and centrifuged. The essential oils (1 μL) were injected into a chromatograph gas (Trace 1310, Thermo Fisher Scientific, Massachusetts, USA), coupled to a single quadrupole mass spectrometer (ISQ Lt, Thermo Fisher Scientific, Massachusetts, USA), equipped with a capillary column (TG-5MS – 30 m × 0.25 μm × 0.25 μm). Transfer line, ion source and injector were adjusted to temperatures of 250 °C, 260 °C and 200 °C, with a helium flow rate of 1 mL min^{-1} column and a split ratio of 60.

The metabolites were identified based on the retention index [calculated on the basis of a mixture of alkanes (C8–C40) injected separately] and comparing the mass spectra with those present in the spectral libraries (NIST 2005, Wiley 7.0, *etc.*). The essential oil yield per fruit was calculated as g 100 kg^{-1} fresh fruit weight.

Statistical analysis

All data were analysed using one-way ANOVA tests for means comparisons, with standard errors. Mean comparisons were discriminated according to Tukey tests and were considered significant at $P < 0.05$. Analyses were carried out using SPSS v. 22.0 (IBM Corporation, New York, USA).

Results and discussion

The results of this first observation period have shown some differences in the behaviour of the Femminello cultivar as a function of the rootstock.

The development of the canopy was strongly influenced by rootstock. As well as Roose *et al.* (1989), we have regarded size development of citrus scions onto Troyer rootstock like the standard citrus tree size compared to other grafted combinations.

The canopy volume of F/TC did not show significant differences compared to F/SO and F/VL, for this parameter (Table 1). Instead, the canopy volume was 47% higher in trees grafted onto SO compared to F/VL graft combination (Table 1). This trend was similar to the one observed by Georgiou (2002) on *Citrus clementina* Hort. ex Tan., but it was in disagreement to that reported by Shafieizargar *et al.* (2012) and Benyahia *et al.* (2017) on an orange tree. The canopy projection area was not statistically significant between F/TC (1.99 m^2) and other two graft combinations according to those observed on the same graft combinations by Yildiz *et al.* (2013). Instead, the canopy projection of F/SO was significantly higher (+63%) compared to F/VL (Table 1). The height of canopy was 12% smaller onto SO rootstock compared to the tree grafted onto TC and VL. The height of 'Femminello' onto these two last rootstocks was similar. Then, in this area, the tree grafted onto sour orange has shown a more expanded habitus as canopy projection area than the other rootstocks, whereas the VL showed the habitus significantly least expanded. No differences were recorded between TC and other rootstocks for this parameter.

Table 1. Effect of rootstock on yield and tree size of 'Femminello' bergamot trees (means of three years).

Rootstock	Yield (kg $tree^{-1}$)	±SE	Fruit number	±SE	Canopy volume (m^3)	±SE	Canopy height (m)	±SE	Canopy projection area (m^2)	±SE	Yield efficiency (kg m^{-3})	±SE
SO	30.59b	0.601	147c	3.785	0.841b	0.069	1.274a	0.022	2.411b	1986.330	38.44ab	0.578
TC	18.25a	0.568	82a	3.085	0.685ab	0.046	1.463b	0.055	1.991ab	1461.100	27.83a	0.967
VL	25.13ab	0.813	105b	4.664	0.574a	0.064	1.498b	0.041	1.499a	762.952	44.14b	1.326

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

Table 2. Effect of rootstocks on fruit weight, longitudinal and transversal diameter, relative length, rind thickness, juice content, and dry weight of 'Femminello' bergamot fruit (means of three years).

Rootstock	Fruit weight (g)	±SE	Longitudinal diameter (mm)	±SE	Transversal diameter (cm)	±SE	Relative length	±SE	Rind thickness (cm)	±SE	Juice content (%)	±SE	Dry weight (%)					
													Flavedo	±SE	Albedo	±SE	Pulp	±SE
SO	165.35a	6.11	71.14a	1.05	70.20ns	0.85	70.67a	0.85	3.34ns	0.07	41.46b	1.41	23.04ab	0.16	23.87ns	1.02	8.98ab	0.12
TC	181.25ab	4.54	73.70ab	1.09	70.18	0.55	71.94ab	0.75	3.27	0.10	41.32b	1.01	23.54b	0.64	24.02	1.29	11.17b	0.95
VL	189.55b	6.84	76.73b	1.23	70.72	0.95	73.72b	1.00	3.18	0.12	34.93a	1.53	21.34a	0.74	24.79	0.79	6.63a	0.69

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

Yield per plant was influenced by combinations of grafts. The differences between the rootstocks were stable during the years of observation. In particular, the sour orange has increased the yield of about 65% and 25% compared to respectively TC and VL rootstocks. Crop load is determined by the number of fruit per tree. The number of fruit was 44%, and 28% significantly lower, respectively, using the TC and the VL rootstocks compared to SO (147 ± 3.78) (Table 1).

In our results, F/VL gave significantly higher values of yield efficiency than other grafted combinations. The better yield efficiency of trees grafted on VL rootstocks compared to other rootstocks (including SO and TC) was also found in other orange cultivars (Zekri and Al-Jaleel, 2004; Castle *et al.*, 2010). Davies and Albrigo (1995) suggest that the yields and net profits are higher when the rootstock used is VL. The yield efficiency was lowest onto TC grafted, whereas no significant differences were observed among SO and other rootstocks (Table 1).

The fresh fruit weight ranged from 165 g to 190 g. No significant differences were observed among F/SO and F/VL compared to TC, but FFW was 15% higher in F/VL than F/SO (165.35 ± 6.11 g) tree, according to Verzera *et al.* (2003) for bergamot onto the same rootstock adopted in our experiment.

The higher number of fruits (sinks) per m^3 of the canopy, found in F onto SO (Table 1) may have affected the fruit size as an effect of the stronger competition among fruit (sinks) for assimilating compared to F/VL. Shafieizargar *et al.* (2012) reported that Queen orange grafted onto sour orange, produced smaller sized fruits, whereas on the Volkamer lemon it produced fruits of good quality (marketable sized and moderate number of fruits per tree) according to our results.

About the other fruit biometric parameters as equatorial diameter, no significant differences were recorded, whereas significant differences were found for polar diameter and H/D ratio. Therefore, the shape of the fruit produced by plants grafted on VL was similar compared to grafted onto TC, but significantly more elongated compared to grafted onto SO (Table 2).

The dry matter content is an indirect indicator of the soluble solids content. It showed differences between the different grafted combinations. The F/TC showed a dry matter content in the pulp of about 11%. This content was significantly higher compared to F/SO, and F/VL grafted combinations, where the dry matter content was 9% (± 0.12) and 6.6% (± 0.69), respectively. Mesocarp (albedo) did not show statistically significant differences between the grafted combination, around 24%; exocarp (flavedo) showed the highest content in dry matter onto TC rootstocks (23.5% ± 0.64), while it was lowest onto VL rootstocks (21.3% ± 0.74); no differences were observed between SO (23% ± 0.16) and other grafted combinations, for this parameter (Table 2). Many authors (Albrigo, 1977; Syvertsen and Graham, 1985; Vasconcellos and Castle, 1994) hypothesize that the quality of citrus fruit (non-climacteric and accumulating soluble solid) is closely related to the effect of rootstock on plant-water relations and sucrose transport.

The thickness of the peel is a parameter capable of conditioning the quality of bergamot fruit both in the pre-harvest phase and in the post-harvest phase. The rootstock can condition peel thickness in many *Citrus* species (Al-Jaleel and Zekri, 2003; Fallahi *et al.*, 1991). Georgiou (2000, 2002), and Ramin and Alirezanezhad (2005) reported that the VL causes a thicker peel.

Table 3. Effect of rootstock on fruit peel colour characteristics of 'Femminello' bergamot fruit (means of three years).

Rootstock	*L**	±SE	*a**	±SE	*b**	±SE	Chroma	±SE	°Hue	±SE
SO	56.971a	0.592	-7.073ab	0.325	32.455a	0.732	57.483a	0.590	187.45ab	0.210
TC	60.145b	0.609	-6.130b	0.367	36.953b	0.738	60.549b	0.602	186.36a	0.235
VL	58.366ab	0.509	-7.982a	0.197	34.107a	0.552	58.937ab	0.507	187.83b	0.188

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

However, we have found that the thickness of the bergamot peel wasn't influenced by the rootstock, according to observations in bergamot, orange, and grapefruit by other authors (Verzera *et al.*, 2003; Economide and Gregoriou, 1993; Shafieizargar, 2012; Georgiou and Gregoriou, 1999).

The colour of the flavedo is influenced by chlorophyll, carotenoids and anthocyanin pigments (Lancaster *et al.*, 1997). During the fruit ripening, the reduction of the daily temperature activates the enzymes that degrade chlorophyll, highlighting the carotenoids, which accumulate during maturation (Alquezar *et al.*, 2008; Fatthai *et al.*, 2011). The colorimetric parameters detected on the flavedo allowed to evaluate the influence of the rootstock on the pigmentation of the flavedo of the 'Femminello' fruit. Indeed, the lightness was highest for TC (60.15 ± 0.61) and the lowest values for SO (56.97 ± 0.60), at harvest (Table 3); onto VL, no differences were observed comparing TC and SO (Table 3).

The chromatic component *a** reached a value of -6.13 (± 0.37) for TC graft combination, and of -7.07 (± 0.325) and -7.98 (± 0.20), respectively, for SO and VL rootstocks. The hue was higher onto VL compared to the TC graft combination. No differences were observed for SO compared to other rootstocks.

The juice percentage is considered a key factor in the *Citrus* species usually used for the consumption of fresh produce (Shafieizargar *et al.*, 2012). Bergamot grafted on VL showed a yield decrease of about 15%, compared to TC and SO grafted combinations, in disagreement with what is observed in other *Citrus* species traditionally used for the production of juices (Castle and Baldwine, 2005; Benyahia *et al.*, 2017; Shafieizargar *et al.*, 2012; Emmanouilidou and Kyriacou, 2017), but according to reports by other authors (Economide and Gregoriou, 1993; Georgiou, 2000, 2002; Stuchi *et al.*, 2003; Ramin and Alirezanezhad, 2005; Mademba-Sy *et al.*, 2012).

However, in our experiment with all rootstocks, the juice percentage was above the minimum acceptable (35%) for citrus fruit used for fresh consumption (Table 2).

The SST plays the central role in defining the overall sweetness and organoleptic values in citrus juice (Kader, 2008), and it showed significant differences in the experiment. SST was 13% higher in the fruit of tree grafted onto SO compared to other rootstocks (Table 4); differences induced by different rootstocks were also found in other *Citrus* species by several authors (Georgiou and Gregoriou, 1999; Georgiou, 2002; Ramin and Alirezanezhad, 2005). Contrasting results were found by Economides and Gregoriou (1993) and Georgiou (2000) because SST did not show statistical differences in *Citrus paradisi* Macf. cv. Marsh, and in [*Citrus reticulata* Blanco × (*C. paradisi* Macf. × *C. reticulata*)], cv. Nova, grafted on the same rootstocks used in our research (Table 4).

TA value was similar to those observed for 'Femminello' by Calvarano *et al.* (1996). However, TA has shown an increase of 15% in the juice using SO rootstock, compared to the other two graft combinations. A similar increase was observed by Continella (1998) in Clementine trees grafted onto the same rootstocks.

However, the SST/AT ratio (an important index to define the internal, and therefore sensory, quality of the fruits) did not show statistically significant differences between the different graft combinations adopted (Table 4).

As for the nutraceutical aspects, the antioxidant capacity of the fruit is the result of synergy by several biomolecules present in varying amounts depending on the type of fruit. Among these biomolecules, it is certainly possible to include polyphenols, chlorophyll pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids), flavonoids, flavonols, and ascorbic acid (AA). Ascorbic acid is a compound with strong reducing power. In kiwifruit, AA accounts for about 40% of the total antioxidant capacity (Tavarini *et al.*, 2008). In citrus, AA is also present in high concentrations. There are several factors which can affect the AA content. The ripening period plays a central role in defining what will be the quality of production. In some fruit species such as *Prunus armeniaca* 'Tilton', *Prunus persica* 'Elberta', and *Carica papaya* 'Solo', the content of ascorbic acid increases with the degree of ripeness (Zubeckis,1962; Wenkam, 1979), whereas in citrus fruits the immature fruits contain the highest concentration of vitamin C, concentration that tends to decrease with maturation, as a result of the increase in the size of the fruit (Nagy, 1980).

The ascorbic acid did not show statistically significant differences between the graft combinations; the average value was 38 mg of ascorbic acid 100 mL^{-1}, according to the values reported by Ramful *et al.* (2011) (Table 5).

As about the total antioxidant capacity (TAC) for each layer of the fruit, the exocarp (flavedo) and the endocarp (pulp) did not show significant differences among grafted combinations for this parameter, whereas mesocarp (albedo) has shown the highest antioxidant capacity in the SO grafted combination (+20%), compared to the TC and VL (Table 5).

Table 4. Effect on fruit quality characteristics of 'Femminello' bergamot fruit (means of three years).

Rootstock	TA (%)	±SE	SST	±SE	SST/AT	±SE	Ascorbic acid (mg 100 mL^{-1})	±SE
SO	4.748b	0.143	8.506b	0.196	1.792ns	0.050	38.687ns	1.042
TC	4.151a	0.144	7.533a	0.122	1.824	0.039	38.164	1.222
VL	4.141a	0.092	7.794a	0.058	1.909	0.038	37.157	1.571

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

Table 5. Effect of rootstock on TAC, TPC, FDT, FLT, TChl and TCar from the flavedo, albedo and pulp of 'Femminello' bergamot fruit (means of three years).

Rootstock	TAC (µmol Trolox g^{-1} FW)			TPC (mg GAE g^{-1} FW)			FDT (mg quercetin g^{-1} FW)			FLT (mg quercetin g^{-1} FW)			TChl (µg mL^{-1})	TCar (µg mL^{-1})
	Flavedo	Albedo	Pulp	Flavedo	Albedo	Pulp	Flavedo	Albedo	Pulp	Flavedo	Albedo	Pulp	Flavedo	Flavedo
SO	8.23±0.27ns	12.18±0.56b	4.06±0.22ns	3.73±0.39ns	5.04±0.54ns	1.04±0.14ns	16.28±0.74ns	7.55±0.58ns	0.635±0.04ns	1.06±0.07ns	0.33±0.33a	0.13±0.13ns	83.43±0.75c	25.05±2.12c
TC	6.93±0.27	10.08±0.44a	3.69±0.64	3.25±0.06	4.58±0.09	1.17±0.07	14.70±0.64	6.32±0.39	0.638±0.02	1.10±0.03	0.44±0.44b	0.14±0.14	77.22±0.48b	20.34±1.01b
VL	8.07±0.62	10.80±0.11ab	3.59±0.79	3.74±0.20	4.81±0.18	1.06±0.05	16.11±0.56	7.32±0.17	0.62±0.10	1.16±1.16	0.37±0.37a	0.12±0.12	65.05±0.19a	15.28±0.05a

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

The total polyphenols did not show statistically significant differences between the layers of the fruit and between the rootstock (Table 5); also for these parameters, the values are according to those measured by Ramful *et al.* (2011).

Flavonoids are secondary metabolic compounds, with low molecular weight and localised in the cellular vacuoles. They accumulate during the development of the various organs and are responsible for both the colour of the fruits and the colour of the flowers. Furthermore, they protect the plants from biotic and abiotic stress, preserving their cellular integrity thanks to the numerous hydroxyl groups present on the benzene rings. Finally, they also play an active role in hormonal transport (Samanta *et al.*, 2011).

In the *Citrus* genus, the flavonoids are contained in high quantities in flavedo and fruit seeds (Yao *et al.*, 2004). About 60 compounds have been identified. In citrus, the most representative flavonoids are hesperidin, narirutin, naringin and eriocitrine (Schieber *et al.*, 2001). Dugo *et al.* (2005), Nogata *et al.* (2006), and Gliozzi *et al.* (2013) report that bergamot differs from other citrus fruits also in the number of flavonoids present in the different layers of the fruit.

In our experiment, the skin reported values between 14.70 (±0.64) and 16.30 (±7.55) mg quercitin g^{-1} FW, among grafted combinations (Table 5). The results obtained have shown a higher content of flavonoids in exocarp (flavedo), in according to other publications (Mandalari *et al.*, 2006; Anagnostopoulou *et al.*, 2005; Savary *et al.*, 2002; Tripoli *et al.*, 2007; Clifford, 1999). In the mesocarp (albedo), the content in total flavonoids (FDT) varied between 6.32 (±0.39) and 7.55 (±0.58) mg quercetin g^{-1} FW, whereas in the endocarp (pulp) the lowest values were recorded, between 0.62 (±0.10) and 0.638 (±0.02). In percentage, the quantity was 55% lower in mesocarp (albedo) and 96% lower in the endocarp (pulp) compared to exocarp (flavedo). About each layer, the differences between the grafted combination were not statistically significant (Table 5).

The flavonols (FLT) are biomolecules belonging to the family of flavonoids. They showed the highest increases only in the albedo layer in F/TC graft combination (Table 5); instead, the flavedo and the pulp showed similar values between the different graft combinations (Table 5).

About the total chlorophyll, the F/SO has shown an increase of 23% and 85% compared to the F/TC and F/VL respectively (Table 5). The same trend was observed for total carotenoid (Table 5).

The counting of the glands, inside which the essential oil is formed, has not shown statistically significant differences between the different treatments; however, the number is, around 120–140 cm^2, comparable with what is reported in the literature (Table 6) (Dugo and Bonaccorsi, 2013).

About the essential oil of bergamot, Sawamura *et al.* (2006) report that the essence of bergamot is widely used in the perfume industry due to its fresh and elegant aroma. It is considered one of the finest oils at the international level. It can vary in its composition, both in terms of quality and quantity, about the pedoclimatic environment, the composition of the soil, the age of the plant, and the fruit degree of ripeness as reported by Masotti *et al.* (2003) and Angioni *et al.* (2006). The composition of this oil is now known thanks to the contributions provided in this sense by some authors (Costa *et al.*, 2010; Dugo and Bonaccorsi, 2013). It consists of a volatile fraction (93–96% of the total) and a non-volatile fraction (4–7% of the total) (Navarra *et al.*, 2015).

The most important molecules that characterise the bergamot essence are linalyl acetate, linalool, and limonene.

TABLE 6. Effect of rootstock on number of glandular cavities and essential oil yield of 'Femminello' bergamot fruit (means of three years).

Rootstock	Glands (nr cm^{-2})	±SE	Essential oil yield (g 100 kg^{-1})	±SE	Essential oil yield (g plant^{-1})	±SE
SO	127.670ns	2.186	122.900a	2.456	47.24	1.23
TC	124.000	6.928	170.840b	3.671	47.54	1.85
VL	132.000	5.132	127.100a	2.843	56.10	1.93

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

TABLE 7. Percentage composition of the volatile fraction of bergamot essential oil and Lynalil acetate/Linalool ratio, in relation to the rootstock (means of three years).

	VL	±SE	SO	±SE	TC	±SE
Linalool	29.563a	6.684	45.709b	9.717	39.028ab	7.031
Lynalil acetate	9.684ns	1.636	9.648ns	2.133	9.722ns	3.278
Limonene	38.870c	3.353	23.391a	5.316	31.154b	3.802
Lynalil acetate/Linalool	0.328b	0.465	0.211a	0.371	0.249a	0.893

Different letters in the same column indicate significant difference (P = 0.05) using Tukey's test; n.s.: not significant.

The relationship between linalyl acetate and linalool is considered to be one of the most important quality indices of bergamot essential oil. In the province of Reggio Calabria the ratio, in general, reaches values close to 0.3 (Di Giacomo and Mincione, 1995; Sawamura *et al*., 2006; Bouzouita *et al*., 2010).

Monoterpenic hydrocarbon limonene (38.87 ± 3.53 in the VL thesis, 23.40 ± 5.31 in the SO thesis, 31.15 ± 3.80 in the TC thesis), sesquiterpenic linalool alcohol (29.56 ± 6.68 in the VL thesis, 45.71 ± 9.72 in the SO thesis, 39.02 ± 7.03 in the TC thesis), monoterpenic linalyl acetate ester (9.68 ± 1.63 in the VL thesis, 9.65 ± 2.13 in the SO thesis, 9.72 ± 3.28 in the TC thesis), showed the highest concentrations (Table 7).

The ratio of linalyl acetate to linalool gave the following results: 0.33 for VL, 0.21 for SO and 0.25 for TC. The monoterpenic ester linalyl acetate showed no differences between the grafted combinations to comparison, reaching concentrations slightly lower than the values reported in the bibliography (11.80–41.36) (Dugo and Bonaccorsi, 2013). Limonene, however, showed the highest values for VL, 66% and 25% higher than onto SO and TC (Table 7).

Another compound that helps to define the quality of bergamot essential oil is linalool. It showed 45.71% and 39.03% significantly higher concentrations respectively for SO and TC compared to VL (29.56%) (Table 7).

A fundamental role in the composition of the essential oil is played by the place where the fruits ripen. According to Dugo *et al*. (1987, 1991), the composition of the volatile fraction does not seem to be influenced by altitude, but by latitude and proximity to the sea. The monotropic hydrocarbons, proceeding from the Tyrrhenian coast to the Ionian coast of Calabria, show a decrease, while the total alcohols, esters and individual components of these chemical classes show an opposite trend (Di Giacomo and Mincione, 1995; Dugo and Bonaccorsi, 2013).

In the test conducted, the production of essential oil was influenced by the rootstock, in fact, the TC thesis gave yields of 170.84 g 100 kg^{-1} (±3.67), 39% and 34% higher than those recorded in the SO (122.9 g 100 kg^{-1} ±2.45) and VL (127.10 g 100 kg^{-1} ±2.84) (Table 6) grafted combinations.

Conclusion

These first three years of observation have shown that the rootstock has little influence on quality of the product for food consumption. Indeed, the main qualitative (SST/TA) and nutraceutical parameters (ascorbic acid, TAC, TP, FDT) were similar replacing sour orange with Volkamer lemon and Troyer citrange. Only the content of flavonols, chlorophyll, and carotenoids were overall lower than sour orange and Troyer citrange.

With regard to the production per plant and yield efficiency, the worst results were obtained with Troyer citrange.

If it adds the oil essential, which is currently the one that determines the economic value of the planting, the quantity produced for 100 kg of fresh fruit was higher for TC. However, if considering the essential oil per plant, the better performance was obtained with VL. Furthermore, the high quality of the essential oil obtained with the VL compared to the other rootstocks (sour orange included) suggest the VL as optimal rootstock for bergamot 'Femminello'.

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