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Effects of Altering Carbohydrate Supply to Fruit during Development on the Carpometric and Qualitative Characteristics of "Feminello Zagara Bianca" Lemon

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Abstract: In this study, the effects of altering carbohydrate supply, carried out through branch girdling, on the carpometric and qualitative characteristics of the Feminello Zagara Bianca variety lemon fruits were evaluated. Four girdling times (70, 100, 130, 160 days) were compared with a control. The results showed important effects of alteration times of the supply of carbohydrates to fruit sinks on many carpometric and qualitative characteristics of lemons. Fruits from trees with early girdled branches (70 and 100 DAFB) showed higher weights and juice contents than those with later girdled branches (130 and 160 DAFB) and from ungirdled trees. The increased availability of carbohydrates for fruit in the initial period of the cell enlargement phase allowed the lemons of the trees girdled at 70 and 100 DAFB to anticipate the degreening process of the peel and pulp and ripening process. The advancement of the ripening process determined that the fruits of trees with early girdled branches had a lower acidity content compared with those harvested after the catabolism of citric acid. The knowledge acquired with this study provides new information on factors affecting the growth and ripening of lemons, the improvement of fruit quality, and the anticipation of harvest time.

Keywords: girdling; *Citrus limon* (L.) Burm.; fruit growth; cell enlargement; peel degreening; ripening; ascorbic acid; antioxidant activity

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1. Introduction

Lemon (*Citrus limon* L. Burm.) is one of the most popular citrus species in the world after orange and mandarin [1]. The best fruit quality is usually obtained along cool coastal areas, where relatively low temperatures increase the acidity and intensity of the yellow fruit color [2]. Lemons are citrus fruits of high nutritional value and with numerous beneficial effects on human health, such as cytotoxic effects on human colorectal carcinoma [3], analgesic activity [4], antimicrobial [5] effects against human pathogens [6], and functional ingredients [7,8], and are, therefore, highly appreciated by consumers [9].

Lemon orchard efficiency, understood as obtaining products with minimum expenditures of economic, natural and human resources, is essentially based on the possibility of modifying the structure of trees so as to be able to direct the maximum of resources towards fruits. In order to achieve this goal, it becomes crucial to know in depth the physiology of this species and in particular the factors and mechanisms that control and regulate fruiting. In fact, the production and quality of citrus fruits depends on the regulation of the physiological and biochemical processes that determine the growth and ripening of the fruits [10]. In particular, the availability of carbohydrates strongly affects fruit growth and ripening [11,12]. In fact, the connection between carbohydrates and fruit growth is supported by large studies, including several on source–sink imbalances [13], defoliation [13,14], sucrose supplementation [13], shading [14], defruiting [14], girdling and fruit thinning [15].

However, most studies have been conducted on orange [16], satsuma mandarin [13,17,18] and mandarin [19], while very limited studies have been conducted on lemon, a species with a ripening mechanism quite different from other citrus fruits [20,21]. Particularly, substantial differences are found in the accumulation of carotenoids in the flavedo and juice sacs [20,21], of total polyphenol, of ferulic, sinapic, p-coumaric and caffeic acids and ascorbic acid both in peeled fruits and in their peels as well as on the total radical-trapping antioxidative potential (TRAP) [22]. Another important difference concerns the composition of the total soluble solids of the juice [23]. The chief soluble constituents of lemon juices are the organic acids; those in oranges and grapefruit are chiefly soluble carbohydrates. In lemons, the organic acids (titratable acidity) remain fairly stable with the fruit growth and then slowly decrease as the ripening progresses; in oranges and grapefruits, on the other hand, the total soluble solids (chiefly soluble carbohydrates) increase with fruit growth and maturity, while the concentration of acids decreases as the season advances.

The aim of this study was to evaluate how the change in the supply of carbohydrates in the different stages of fruit development, through the alteration of their translocation by girdling, influenced the growth and ripening of lemons. Knowing the effects that the level of carbohydrate supply to fruit has on the ripening process and the qualitative characteristics of lemons could be important not only to increase knowledge about the mechanisms that regulate the fruiting but also to identify agronomic strategies that enhance its performance.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

The study was carried out during the crop year 2019/2020 in a commercial lemon grove (14 m a.s.l., 16°36′45′′ E longitude, 40°06′38′′ N latitude) located within the production area of the Limone di Rocca Imperiale PGI in Calabria (Southern Italy). The experiment was conducted on 25-year-old "Feminello Zagara Bianca" lemon trees grafted on bitter orange (*Citrus aurantium* L.) rootstock and planted at 5 m × 5 m distances. The Feminello Zagara Bianca variety originated from a vegetative mutation of the old Italian lemon variety of Feminello. Its name comes from the fact that its flowers are white and not purplish like other lemon varieties. It is a variety highly appreciated by growers because it has good tolerance to mal secco disease, a high level of production and produces lemons with good commercial value.

The cultivation area is characterized by an average annual temperature of 17.6 °C. The average temperatures of the hottest month (July) and the coldest month (January) are, respectively, 27.3 and 9.6 °C. The average annual rainfall in the area is about 610 mm. The soil texture of the plot was loamy sand (60.5% sand, 21.9% silt, 17.6% clay). The soil had a pH of 8.5 and electrical conductivity (EC) of 0.52 mS cm^{-1}, as determined in the saturation extract. The cation exchange capacity, measured in the surface 60 cm of the profile, was 12 meq/100 g. The trees were irrigated weekly from May to October using drip irrigation. The total seasonal amount of irrigation water was about 6000 cubic meters/ha. Nitrogen (N) was applied at a rate of 250 kg N ha^{-1} (2/3 in mid-March and 1/3 during the summer period through fertigation), phosphorus (P) and potassium (K) were respectively applied at a rate of 76 kg P_2O_5 ha^{-1} and 200 K_2O ha^{-1} (in mid-December). Soil management, pruning and pest control were carried out according to the Limone di Rocca Imperiale PGI production specification. Particularly, the soil management included a shallow plowing, carried out in late winter to loosen and break up soil and bury the fertilizer scattered on the ground, and periodic cuttings of weeds during spring and summer. The pruning, carried out in April, had the aim of limiting the height of the plants to facilitate harvesting operations, allow light penetration into the canopy, prevent crowding of main scaffold branches and to remove branches that cross, remove or shorten water shoots to prevent them from becoming too dominant and balance the load of the fruiting branches. The sanitary integrity of the trees and fruits was guaranteed through continuous monitoring of the main parasites of the lemon tree, using pesticide control treatments when necessary and according to the principles of integrated pest management.

2.2. Treatments

For the study, 30 trees in excellent vegetative-productive and phytosanitary state, homogenous in terms of trunk and canopy size, number of main branches, vegetative vigor, leaf density and productive load were selected and appropriately marked. Four girdling periods were compared with a control without girdling. The girdling was carried out at 70, 100, 130 and 160 days after full bloom (DAFB). Full bloom was defined as when 60% of the flowers were open. The girdling was performed on the main branches at a distance of about 50 cm from the start of scaffold branches. Girdling was obtained by removing a ring of bark (about 3 mm in width) around each branch using a sharp knife, and the belt was renewed every 2 weeks until harvest to prevent rapid closure of the girdle (Figure 1). The experiment was carried out using a randomized full block design with six replicates for each treatment. Each replica was represented by a single tree, consisting of four main branches, all of which were girdled.

Figure 1. Phases of the girdling operations: (**a**) girdle making; (**b**) covering the cut with air-permeable but water-resistant grafting tape; (**c**) wound healing status 1 month after the conclusion of the study.

2.3. Fruit Sampling

The fruits, resulted from the principal bloom, were harvested manually with specific pruning shears in mid-February. At this time of the season, the fruits had almost reached their final size and far exceeded the minimum threshold for fruit weight as well as the peel color requirements established by the Limone di Rocca Imperiale PGI production specification for harvesting. In the area in which the study was carried out, this period also coincides with the time of harvesting the fruits intended for the market. Sixteen fruits (four fruits per main cardinal position) were collected from each selected tree for a total of 96 lemons for each treatment. The fruit harvest was performed in all tree orientations, internal and external, aiming to avoid the edge effect. The picked fruits were put into plastic bags, appropriately marked and perforated to avoid the formation of condensation. The fruit samples were then transferred to the laboratory on the same day, where they were immediately analyzed.

2.4. Carpometric Measurements

Carpometric measurements were performed by weighing and measuring the fruits individually. Fruit weight was determined using an analytical balance (mod. XB 4200C, Precisa Gravimetrics AG, Dietikon, Switzerland). Fruit size was determined by measuring width and height using a digital caliper (mod. 1651DGT, Beta Utensili s.p.a., Sovico, Italy). To determine the peel thickness, the lemon fruits were split transversely in the equator area, and subsequently, the flavedo and albedo measurements were taken at equidistant points using the same digital caliper used to determine the size. Furthermore, the number of seeds (distinguishing them as normal and aborted) and the number of segments were counted on each fruit, by means of simple visual manual counting. After these measurements, the fresh and dry weights of flavedo, albedo, pulp and seeds were determined on a sample of four fruits from each tree.

The volume of each fruit was measured using the water displacement method. Each fruit was completely submerged in a 1000 cm^3 capacity graduated beaker half-filled with water, and the volume of fruit was determined by measuring the amount of displaced water. Water temperature during the measurements was kept at 25 °C. Specific gravity of fruits was determined by weighing the fruits in air and then determining their volume in water (specific gravity of fruit = fruit weight/fruit volume).

2.5. Determination Flavedo and Pulp Color

Flavedo and pulp color were measured using a colorimeter (model CR-300, Minolta, Osaka, Japan). Regarding the determination of the flavedo color, four readings were taken in four equatorial and equidistant zones of all fruits. As for the pulp, the measurements were performed on transversely cut fruit in the equatorial area by taking four equidistant readings in the middle of the pulp. Color was evaluated according to the Commission Internationale de l'Éclairage (CIE) and expressed as L*, a* and b* color values [24,25]. These values were used to calculate the hue angle (H° = arctan (b*/a*), target color (C* = (a^{*2} + b^{*2}) 1/2) and citrus color index (CCI = a* × 1000/L* × b*).

2.6. Qualitative Characterization of Fruits

Analyses were determined on six juice samples (one sample for each block) for each treatment. The juice was carefully obtained using a commercial manual juicer. The resulting juice was then passed through a strainer. The total soluble solids (TSS), pH, titratable acidity (TA), iodometric titration of vitamin C, organic acids, individual sugars, total phenolic compounds and antioxidant activity of the juice samples were determined. The total soluble solids (TSS) content was evaluated by means of a digital refractometer (mod. PAL 1, Atago Co., Ltd., Tokyo, Japan) and expressed as °Brix. The pH was measured after diluting the juice in a ratio of 1:20 with distilled water. The acidity content was determined in the same diluted juice solution by titrating to pH 8.2 using 0.1 N NaOH, and the value was expressed as % (*w*/*v*) citric acid. L-ascorbic acid was determined by the 2,6-dichloroindophenol titration method, according to the official methods of the American Organization of Analytical Chemistry [26] as explained by Nielsen [27]. Measurements were made at constant room temperature (22 ± 2 °C). Organic acid analysis was carried out following the methods reported by Panebianco et al. [28]. The HPLC analysis was performed with an HPLC (Knauer, Berlin, Germany) equipped with a UV detector set at 210 nm, equipped with a 20 µL Rheodyne injection valve. Sugar contents were determined using a Knauer HPLC System equipped with a Refractive Index detector (RI Detector 2300, Knauer, Berlin, Germany), and the Augustin and Khor [29] method was followed (appropriately modified).

Total phenolic compounds (TPC) were measured following the method reported by Letaief et al. [30], appropriately modified. A 0.1 mL amount of lemon juice was mixed with 0.5 mL of Folin–Ciocalteu phenol reagent; after 5 min, 5 mL Na_2CO_3 solution (5%) was added. The absorbance was measured at 750 nm using a spectrophotometer (Agilent 8453UVvis, Agilent Technologies, Santa Clara, CA, USA), and the results were expressed as mg L^{-1} gallic acid equivalents (GAE).

Total antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays using the De Bruno et al. [31] method, appropriately modified. For the DPPH assay, 10 µL of diluted lemon juice were added to 6×10^{-5} mmol L^{-1} of DPPH solution to achieve a final volume of 3 mL and left in the dark for 15 min. The decrease in absorbance was determined at 515 nm using a spectrophotometer (Perkin-Elmer UV-Vis λ2, Waltham, MA, USA). For the ABTS assay, the reaction mixture was prepared by mixing ABTS and 10 µL of each sample, and the absorbance was measured after 6 min at 734 nm by means of a Perkin-Elmer spectrophotometer. For both antioxidant assays, the results were expressed as TEAC values (μmol Trolox mL^{-1} of sample).

2.7. Statistical Analysis

Significant differences in the data were evaluated by analysis of variance (ANOVA), followed by Tukey's multiple range test for $p < 0.05$. Statistical analysis was performed with the Systat statistical program (SYSTAT Software Inc., Chicago, IL, USA).

3. Results

The results of this experimentation showed important and significant effects of alteration times of the supply of carbohydrates to fruit sinks, on many carpometric and qualitative characteristics of lemons at harvest. Fruits from trees with early girdled branches (70 and 100 DAFB) showed significantly higher weights than those with later girdled branches (130 and 160 DAFB) and from ungirdled trees (Table 1).

Table 1. Effects of girdling time on the main carpometric characteristics of fruits.

Parameter	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
Fruit weight (g)	226.7 ^{a}	220.5 ^{a}	194.6 ^{b}	185.5 ^{b}	173.4 ^{b}	**
Fruit height (mm)	95.8 ^{a}	95.4 ^{a}	93.8 ^{ab}	92.8 ^{ab}	90.0 ^{b}	**
Fruit width (mm)	73.0 ^{a}	72.0 ^{a}	69.1 ^{b}	67.2 ^{bc}	65.2 ^{c}	**
Ratio of height/width	1.31 ^{c}	1.33 ^{bc}	1.36 ^{ab}	1.38 ^{a}	1.38 ^{a}	**
Specific gravity of fruit (g cm^{-3})	0.86	0.85	0.86	0.85	0.86	n.s.

Significant level: n.s. = not significant; ** = $p < 0.001$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

In particular, compared to the control, the fruits of the trees in which the phloem interruption was carried out at 70 and 130 DAFB had 31 and 27% greater weights, respectively (Table 1).

Regarding the height of the fruits, whose values ranged between a maximum value of 96 mm (girdling carried out at 70 DAFB) and a minimum of 90 mm (ungirdled control), the lemons of trees with branches girdled at 70 and 100 DAFB had statistically superior values compared to the control but not compared to those girdled at later times. On the other hand, more evident differences were found regarding the width of the fruits. The lemons of the trees girdled at 70 and 100 DAFB, compared to the control, had larger widths of 12–10%. Intermediate values were found in the lemons from the trees girdled at 130 DAFB, while no statistically significant differences were highlighted between the fruits of the later girdled trees (160 DAFB) and those of the ungirdled control. Important and significant differences between treatments were also found regarding the height/width ratio. The fruits of the trees girdled at 70 and 100 DAFB, compared to those of the control and trees girdled at 160 DAFB, had a less elongated shape. Intermediate values were, however, found in the fruits of the trees girdled at 130 DAFB. There was no significant difference in the specific gravity of fruits.

The interruption of the phloem flow in general and, more specifically, the time in which it occurred also affected the characteristics of the peel both in terms of color and thickness (Table 2).

Concerning the color, significant differences between the treatments were observed for the coordinate a*. In the fruits of the girdled trees at 70 DAFB, the a* values were significantly higher than those of the control. Despite the fairly limited range of variation, with values that ranged between a minimum of 84.3 and a maximum of 85.6, significant differences between treatments were also found with regard to hue angle (H°). Fruits of the girdled trees had significantly lower values than control. The trend of the peel of the fruits of the girdled trees to degrade the chlorophyll more rapidly than control was confirmed by the results relating to the citrus color index (CCI). Fruits from girdled trees showed CCI values of the peel significantly higher than those found in fruits from untreated trees. In addition to the color, the girdling time also influenced the peel thickness. The peel of the fruits of the early girdled trees (70 and 100 DAFB) was significantly thinner than that of the later girdled trees (130 and 160 DAFB) and of the control. These effects were observed both in the inner layer (albedo) and in the outer layer (flavedo) of the peel. No significant differences were found for the moisture content of the peel, with values ranging between 83.9 and 84.9.

Table 2. Effects of girdling time on the main peel characteristics.

Parameters	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
L*	73.3	72.3	72.9	72.5	72.9	n.s.
a*	6.1 ^{a}	5.7 ^{ab}	5.5 ^{b}	5.3 ^{b}	4.7 ^{b}	**
b*	60.6	59.1	59.1	59.3	59.7	n.s.
Hue angle (h°)	84.3 ^{c}	84.5 ^{bc}	84.7 ^{bc}	84.9 ^{b}	85.9 ^{a}	**
C*	60.9	59.4	59.4	59.6	59.9	n.s.
Citrus color index	1.4 ^{a}	1.3 ^{a}	1.3 ^{a}	1.2 ^{a}	1.1 ^{b}	**
Peel thickness (mm)	6.8 ^{b}	7.0 ^{b}	7.6 ^{a}	7.9 ^{a}	8.1 ^{a}	**
Flavedo thickness (mm)	1.6 ^{d}	1.6 ^{d}	1.7 ^{bc}	1.8 ^{ab}	1.9 ^{a}	**
Albedo thickness (mm)	5.2 ^{b}	5.4 ^{b}	5.9 ^{a}	6.1 ^{a}	6.2 ^{a}	**
Moisture content, peel (%)	83.9	84.9	84.6	84.3	84.1	n.s.

Significant level: n.s. = not significant; ** = $p < 0.001$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

Table 3 reports the influence of girdling on the internal characteristics of the lemon fruits.

Table 3. Effects of girdling time on the main pulp characteristics.

Parameters	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
L*	46.2 ^{a}	46.3 ^{a}	45.0 ^{b}	44.9 ^{b}	44.2 ^{b}	**
a*	0.2 ^{a}	0.2 ^{a}	0.1 ^{a}	−0.2 ^{b}	−0.2 ^{b}	**
b*	12.9	12.6	12.1	11.9	11.7	n.s.
Hue angle (h°)	89.0 ^{b}	89.2 ^{b}	89.4 ^{b}	90.8 ^{a}	91.4 ^{a}	**
C*	19.9	12.6	12.1	11.9	11.8	n.s.
Citrus color index	0.4 ^{a}	0.3 ^{a}	0.2 ^{a}	−0.3 ^{b}	−0.5 ^{b}	**
Normal seeds per fruit (n°)	9.8	9.5	9.9	9.5	9.5	n.s.
Empty seeds per fruit (n°)	2.1	2.2	2.2	1.8	2.3	n.s.
Juice content (%)	40.0 ^{a}	39.2 ^{ab}	36.3 ^{bc}	35.2 ^{c}	34.5 ^{c}	**
Moisture content, pulp (%)	90.0	88.8	90.1	89.5	89.7	n.s.

Significant level: n.s. = not significant; ** = $p < 0.001$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

As regards pulp coloring, significant differences were found for coordinates L* and a*, hue angle (h°) and the citrus color index (CCI). With regard to the coordinate L*, which ranged between a minimum value of 44.2 and a maximum of 46.2, the trees girdled at 70 and 100 DAFB registered significantly higher values than the other treatments compared. The fruits of the trees girdled at 70, 100 and 130 DAFB were characterized by having a pulp with a lighter color than that of the fruits of the trees girdled at 160 DAFB and ungirdled control. In the latter two treatments, the values of the coordinate a* were even negative, an unequivocal sign of the presence of chlorophyll pigments. The greenish color of the pulp of the fruits of these two treatments was also partly confirmed by the values (around 91°) of the hue angle (h°). Conversely, in the other treatments, this value was significantly lower, showing a lighter color, tending to yellowish. The CCI values confirmed those represented by the coordinate a* and the hue angle (h°), clearly highlighting a certain delay in the process of degreening of the fruit pulp of the trees that were annulated later or not girdled. However, no significant difference between treatments was found for the content of the seeds in lemons, regarding both the normal ones and empty ones, the number of which for each fruit was around 10 and 2, respectively. Substantial and important differences were found for the juice content. The results showed that the girdling time strongly influenced this parameter. The fruits of the trees that were early girdled (70 and 100 DAFB) had the highest values, with juice content of around 40%, an increase of 16 and 14%, respectively, compared to the control. No significant difference was found for pulp moisture content, with values ranging between a minimum of 88.8% and a maximum of 90.1%.

In addition to the juice content, the girdling time influenced other internal qualitative characteristics of the fruits (Table 4). The fruits of trees girdled at 70 and 100 DAFB showed a significantly lower total soluble solids content of juice than the other girdled and the control trees, which always abundantly exceeded the threshold of 8 °Brix. No significant differences were found regarding the pH of the juice, with values of 2.4. Significant differences were instead found in relation to the acidity content of the juice. Although the range of variation was quite limited, ranging between a minimum value of 53.0 g L^{-1} and a maximum of 60.7 g L^{-1}, the juice of early girdled trees (70 and 100 DAFB) had a significantly lower acidity content than the other treatments. Conversely, the juice in fruits from trees girdled at 70 and 100 DAFB was characterized by significantly higher ascorbic acid content.

Table 4. Effects of girdling time on the main qualitative characteristics of juice.

Parameters	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
Total soluble solids (°Brix)	7.5 ^{b}	7.7 ^{b}	8.3 ^{a}	8.5 ^{a}	8.6 ^{a}	**
pH	2.4	2.4	2.4	2.4	2.4	n.s.
Titratable acidity (g L^{-1})	53.0 ^{b}	53.7 ^{b}	58.6 ^{a}	59.3 ^{a}	60.7 ^{a}	**
Ascorbic acid (mg 100 mL^{-1})	48.0 ^{a}	47.6 ^{a}	44.4 ^{b}	44.6 ^{b}	43.8 ^{b}	**

Significant level: n.s. = not significant; ** = $p < 0.01$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

Additionally, a chromatographic analysis was applied to define more accurately the organic acids and the individual sugars of juice samples, and the results are shown in Tables 5 and 6, respectively. Regarding the organic acids, five compounds were separated, specifically tartaric, malic, oxalic, ascorbic and citric acid. As already indicated by other researchers, citric acid is the major organic acid found in citrus juices, with values that ranged between 6.88 and 73.93 g L^{-1} [32]. In our samples also (Table 5), the main organic acid was citric acid (4600–4942 mg 100 mL^{-1}), followed by malic acid (144–162 mg 100 mL^{-1}) and ascorbic acid (21–28 mg 100 mL^{-1}), while oxalic and tartaric acids were present in minor amounts.

Table 6 reports the results of the determination of sugars. Between the monosaccharides, the major components analyzed were glucose and fructose, while between the disaccharides, sucrose was the main naturally occurring component in citrus fruits. Among these, sucrose was the main non-reducing sugar. In plants, it works as a transport sugar, while fructose and glucose are used for storage. The sugars were identified in the juice by liquid chromatographic methods.

Table 5. Effects of girdling time on the main organic acid composition of juice (HPLC).

Parameters (mg 100 mL^{-1})	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
Tartaric Acid	12.12 ^{a}	8.52 ^{b}	7.03 ^{b}	8.29 ^{b}	7.91 ^{b}	**
Malic Acid	157.76	152.37	162.45	154.71	144.46	n.s.
Ossalic Acid	11.20 ^{a}	0 ^{d}	3.72 ^{c}	8.86 ^{ab}	6.20 ^{bc}	**
Ascorbic Acid	28.47 ^{a}	26.12 ^{a}	25.50 ^{ab}	21.19 ^{c}	22.62 ^{bc}	**
Citric Acid	4659.94 ^{bc}	4599.75 ^{c}	4942.33 ^{a}	4906.51 ^{a}	4832.92 ^{ab}	**

Significant level: n.s. = not significant; ** = $p < 0.01$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

Table 6. Effects of girdling time on the sugar profile.

Parameters	Girdling Time					Sign.
(g L^{-1})	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
Glucose	7.20 ^{ab}	6.67 ^{b}	8.33 ^{a}	7.52 ^{ab}	8.65 ^{a}	**
Fructose	5.56 ^{b}	7.33 ^{ab}	7.69 ^{a}	6.78 ^{ab}	7.98 ^{a}	**
Sucrose	5.04	4.56	5.17	5.67	4.99	n.s.

Significant level: n.s. = not significant; ** = $p < 0.01$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$).

The concentrations of sugars in the analyzed samples were as follows: glucose ranged from 6.67 to 8.65 g L^{-1}; fructose ranged from 5.56 to 7.98 g L^{-1}, and sucrose ranged from 4.56 to 5.67 g L^{-1}. A clear trend for organic acids was not observed, but for citric acid (the main acid) it was similar to that of titratable acidity. Citric and ascorbic acid concentrations showed a countercurrent trend, both for the chromatographic and titratable measures. Total phenolic content (TPC) and total antioxidant activity (TAA) were determined for all lemon juice samples, and the results are shown in Table 7. Phenolic compounds are one of the most important groups of phytochemical antioxidants present in lemon fruits; in particular, TPC ranged from 1295 to 1492 mg GAE L^{-1} and showed significant differences among the samples ($p > 0.05$). These values were higher than the TPC measured by other authors [33,34].

Table 7. Effects of girdling time on the total antioxidant capacity.

Parameters	Girdling Time					Sign.
	70 DAFB	100 DAFB	130 DAFB	160 DAFB	Control	
TPC	1294.58 ^{bc}	1275.40 ^{c}	1393.78 ^{abc}	1407.01 ^{ab}	1491.67 ^{a}	**
ABTS	7.64 ^{b}	6.70 ^{b}	7.35 ^{b}	10.47 ^{a}	9.501 ^{a}	**
DPPH	7.27	7.01	7.39	7.32	7.02	n.s.

Significant level: n.s. = not significant; ** = $p < 0.01$. The different letters within the rows indicate significant differences according to the Tukey test ($p < 0.05$). TPC: mg GAE L^{-1}; ABTS and DPPH: µmol TE mL^{-1}.

Table 7 also reports the antioxidant activity results for the lemon juice samples. determined with ABTS and DPPH assays. The results are expressed as µmol TE mL^{-1} of juice and represented with lines. The ABTS assay showed significant differences among the samples, with values that ranged between 6.70 and 10.47 µmol TE mL^{-1}, and higher values for the 160 DAFB samples, whereas the DPPH assay showed similar values among the samples with no statistical differences ($p > 0.05$).

The antioxidant values obtained with both assays showed values similar to those reported by Dong et al. [35] in their work on lemon pulp. Antioxidant activity, especially that determined with the ABTS assay, was correlated with total phenolic contents. A positive correlation was shown (r < 0.9).

4. Discussion

The obtained results showed that interruption of the phloem flow carried out on the main branches, at a distance of about 50 cm from the start of the scaffold branches, is able to influence numerous external and internal characteristics of lemon fruits. The effects were more evident when the phloem flow was stopped in the periods immediately following the end of the June drop (70 and 100 DAFB). Undoubtedly, one of the most evident effects caused by the interruption of phloem flow was the increase in fruit size. The results showed that if the girdling was carried out early, a considerable increase in the fruit weight (around 30%) was obtained compared to the control (Table 1). The increase in the size of the fruits of early girdled trees is essentially due to greater cellular enlargement. In fact, since the first girdling was carried out after the June drop, which generally corresponded to the end of fruit growth by cell division [10], it was not plausible to hypothesize interference from the girdling on the cytokinesis process. The greater availability of carbohydrates to the fruits following the girdling of the branches instead positively influenced the period of rapid growth in which the fruits increased in size is due to the cell enlargement and water accumulation [36]. The differences in fruit growth observed relative to girdling time could be related to the dynamics of the amounts of carbohydrates present within the tree epigeal organs that could be used for fruit enlargement. Usually, the period following flowering and fruit set represents a time of scarcity of carbohydrates for citrus trees [37]. In fact, spring flush, floral development, anthesis and fruit set require large amounts of photosynthate that cannot be supplied directly by photosynthesis [38]. The decline in carbohydrate levels throughout the flowering and fruit set period [39–41], which is accentuated by heavy flowering [42], indicates that reserve carbohydrates are effectively used to sustain the early stages of reproductive development [43]. However, the carbohydrate demands to carry out these processes can often exceed the trees' capacity to supply carbohydrates from current photosynthesis and tree reserves [42]. There is ample evidence supporting the existence of a limit on carbohydrate sources for fruit enlargement [37]. In this regard, both girdling and fruit thinning make more photosynthate available per fruit unit. Fruit thinning is a classical means of increasing fruit size at the expense of fruit number [44,45]. The effects of girdling on increasing fruit size are, however, different depending on the time at which this is performed. Girdling carried out during flowering, with the increase in fruit set, generally leads to smaller fruits [46], while summer girdling instead increases fruit size [47,48]. Obviously, the best results are obtained when the girdling is carried out at the beginning of fruit growth by cell enlargement [49].

The height-to-width ratios of the fruits showed that the increase in the axial dimensions of the fruit does not occur in the same way as that of fruit weight. Differences in the direction of enlargement of the juice sacs and the greater degree of cellular expansion may have led the fruits of early girdled trees to have greater growth in width than height (Table 1). Although differences in the development of the two diameters were quite limited, they were sufficient to modify the fruit shape. In fact, while maintaining the classic ellipsoidal shape, the lemons of the early girdled trees were characterized by having a less-elongated fruit shape (Table 1). The greater degree of enlargement of the juice sacs and, consequently, the greater pressure exerted by the pulp against the peel were probably the causes of the smallest peel thickness of fruits of early girdled trees (Table 2). In fact, during the phase of cell enlargement, the peel, after a short initial period in which it increases in thickness mainly due to the albedo cells, gradually becomes thinner following the rapid growth of the pulp segments [45]. This phenomenon occurs both in terms of flavedo and albedo. With advancing fruit growth, following the stretching of this tissue, the shape of the flavedo's oil glands changes [50]. In the albedo, however, the cells expand primarily in the tangential direction via growth of arms. Due to further growth of the arms of these cells, a considerable increase in circumference along with a decrease in rind thickness occurs [51]. The significant differences found regarding peel coloration (Table 2) indicate that in the fruits of the early girdled trees, the peel degreening was more anticipated. This correlated with the greater supply of carbohydrates from which these fruits benefited during the growth phases.

Despite the fact that the peel degreening process in citrus fruits is strongly influenced by environmental factors [52–55], Huff [56,57], based on results from in vitro studies on citrus epicarp, indicated that citrus fruit might partially degreen in response to the accumulation of sugars. Indeed, it has been shown specifically that the chloro- to chromoplast conversion in citrus fruit epicarps is stimulated by sucrose accumulation after an initial decrease in peel nitrogen content [58]. In this context, numerous studies have shown that during the cell enlargement phase of fruit growth, the soluble sugar concentration increased in flavedo [57,59,60] while the chlorophyll content negatively correlated with sugar content in this tissue [57]. Therefore, with the increase in sugars in flavedo, chlorophyll tends to decrease, while carotenoids gradually increase their concentrations as they are synthesized during ripening [61–63]. However, it should be emphasized that the total concentration of carotenoids in lemons is lower than in other citrus fruits [20,21]. Generally, the decrease in peel chlorophyll takes several months, and the onset of carotenoid accumulation almost coincides with the disappearance of chlorophyll [45]. Although external and internal ripening in citrus fruits are two separate and independent processes [10,11], the results relating to pulp coloring show that the degreening pulp process in fruits of trees girdled at 70 and 100 DAFB occurred earlier than that in the fruits of the other treatments (Table 3). The obtained results also showed that the values of the coordinate a* and of the citrus color index (CCI) of the pulp were clearly lower than those of the peel (Tables 2 and 3). Conversely, the highest values of the hue angle (H°) were found in the pulp. Taken together, these results clearly indicate that the pulp degreening process occurs more slowly than that in the peel. Indeed, although the carotenoid profiles of flavedo and pulp are, with few exceptions, very similar [64], and the increase in carotenoid levels and the related changes in gene expression are generally initiated in the pulp before those in the flavedo, the evolution of these processes proceeds more slowly in the pulp [21]. The significantly higher values of the coordinate L* of the fruits of early girdled trees were likely related to the greater turgor of the juice sacs. The absence of significant differences between the different treatments with regard to the number of seeds present in the fruits (Table 3) excludes their possible interference on the results obtained. In fact, theoretically, a different seed content in the fruits could have influenced numerous carpometric and qualitative characteristics of the fruits [65–67], partially masking the results of this study. The interruption of the phloem flow also affected the content of juice, one of the most important qualitative parameters for lemons. The increased availability of carbohydrates for fruit in the initial period of the cell enlargement phase (in trees girdled at 70 and 100 DAFB) allowed the lemons to accumulate more solutes and water in the juice sac cells (Table 3). In fact, the juice sacs, despite being disconnected from the vascular system, which ends in the albedo, are considered the major fruit sink. During fruit growth, following the storage of solutes and water, the vacuoles of the cells of the juice sac become greatly enlarged (over 90% of the total cell volume) and release their content as juice [68].

The obtained results showed a significant decrease in the content of total soluble solids and titratable acidity in the juice from fruits of trees girdled at 70 and 100 DAFB (Table 4). Since the lemon is a species with a very acidic juice, it is inevitable that the content of total soluble solids is closely linked to the acidity of the juice. The lower values for total soluble solids content and titratable acidity of the juice found in the fruits from the trees girdled at 70 and 100 DAFB were likely attributable to an earlier ripening. Generally, as the ripening progresses, there is a decrease in titratable acidity mainly due to the catabolism of citric acid [69]. The hypothesis of early ripening induced by the increased availability of carbohydrates in the fruits of the early girdled trees would also find support in the earlier process of de-greening. Ultimately, the increase in the availability of carbohydrates for fruits would have anticipated the ripening process, confirming what was found in other fruit species [70]. The obtained results also show that the interruption of the phloem flow influenced the ascorbic acid (AA) content of the juice (Table 4). While being inevitably influenced by the probable advancement of the ripening process, the AA content in the juices of fruits from the trees girdled at 70 and 100 DAFB was significantly higher than that from the later girdled trees and the control. This result agrees with what was found by Yang et al. [71] in Satsuma mandarin. In this case, the increase in AA content was also accompanied by changes in the expression of genes involved in the AA L-galactose pathway. Although this mechanism remains unclear, the response of carbohydrate-related gene expression to girdling might be subjected to the regulation of sugar as a signal [72,73]. Other research on spices pointed out that the expression of AA-related genes was stimulated by feeding sucrose or methyl jasmonate [74,75]. Furthermore, a strong correlation between sugar and AA content was found in other studies [76–80]. Based on these results, Yang et al. [71] hypothesized that sugar or phytohormones, or both, synthesized following the girdling, might play a role in regulating the signaling of AA-related gene expression and consequently in the accumulation of AA in the fruit pulp, although no direct proof was provided in their study.

In conclusion, considering that lemon fruit is an important nutraceutical product with important functional properties, the correlation between its chemical components and antioxidant properties is very important. The antioxidant activity of lemon fruits was correlated particularly with the phenolic compounds, as also assumed by Dong et al. [35].

5. Conclusions

The results of the study show that the increased availability of carbohydrates for fruits, following the interruption of phloem flow by branch girdling at the start of the cell enlargement phase of fruit growth, is able to influence both the carpometric and qualitative characteristics of lemons. Although we cannot exclude that a possible change in the hormonal balance caused by girdling may have influenced the results obtained, it is evident, also for lemons, that the productive performance is strongly correlated to the ability of the trees to ensure an adequate supply of carbohydrates to fruits during their growth. The results also show that to improve the qualitative characteristics of lemons and to make the ripening process start earlier, it is essential to ensure an adequate supply of carbohydrates to the lemons in the first part of phase II of fruit growth. The knowledge acquired with this study is very important from a scientific standpoint because it provides new information on factors affecting the growth and ripening of lemons. On the one hand strictly applicative, it provides farmers with new information to improve fruit quality and anticipate harvest time. Regarding the latter, growers that increase the supply of carbohydrates to the fruits during the first half of the summer season, through girdling or thinning (with an increase in the leaves/fruit ratio), could improve the qualitative characteristics of the fruits and initiate ripening earlier. In particular, from an economic point of view, this could be very advantageous for farmers that have the opportunity to initiate earlier harvesting periods for the early varieties. In fact, it would give them the opportunity to place high-quality lemons on the market during a period of the year (between October and November) when the market supply is usually very limited and the prices are consequently very high.

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