



# **Tetraploid Carrizo citrange rootstock (*Citrus sinensis* Osb.\**Poncirus trifoliata* L. Raf.) enhances natural chilling stress tolerance of common clementine (*Citrus clementina* Hort. ex Tan)**

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## Original article

# Tetraploid Carrizo citrange rootstock (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) enhances natural chilling stress tolerance of common clementine (*Citrus clementina* Hort. ex Tan)

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## ABSTRACT

Low temperatures can disturb the development, growth and geographic distribution of plants, particularly cold-sensitive plants in the Mediterranean area, where temperatures can reach seasonally low levels. In citrus crops, scion/rootstock combinations are used to improve fruit production and quality, and increase tolerance to biotic and abiotic stresses. In the last decade, several studies have shown that tetraploid citrus seedlings or rootstocks are more tolerant to abiotic stress than their respective diploid. The objective of this study was to test whether the use of tetraploid rootstocks can improve the chilling tolerance of the scion. We compared physiological and biochemical responses to low seasonal temperatures of common Clementine (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) grafted on diploid and tetraploid Carrizo citrange rootstocks, named C/2xCC and C/4xCC, respectively. During the coldest months, C/4xCC showed a smaller decrease in net photosynthesis (P<sub>n</sub>), stomatal conductance (G<sub>s</sub>), chlorophyll fluorescence (F<sub>v</sub>/F<sub>m</sub>), and starch levels, and lower levels of malondialdehyde and electrolyte leakage than C/2xCC. Specific activities of catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) were higher in C/4xCC during the cold period, whereas chlorophyll, proline, ascorbate and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels and superoxide dismutase (SOD) activity did not vary significantly between C/4xCC and C/2xCC throughout the study period. Taken together, these results demonstrate that tetraploid Carrizo citrange rootstock improves the chilling tolerance of common clementine (scion) thanks to a part of the antioxidant system.

## 1. Introduction

Polyploidy is a common biological phenomenon and a major force of plant evolution (Chen, 2007; Soltis and Soltis, 2009). In nature, 50–70% of angiosperms have undergone at least one episode of polyploidization (Masterson, 1994). In citrus, tetraploid can be divided into two categories – doubled diploid or allotetraploid (Stebbins, 1947) – resulting from either somatic chromosome doubling or sexual

reproduction via 2n gametes, respectively. Doubled diploid citrus genotypes arise from somatic chromosome doubling and from intraspecific hybridization or self-fertilization through 2n gametes, with the subgenomes in doubled diploid thus being considered identical (Aleza et al., 2011). Allotetraploids citrus inherited subgenomes from two different parents after interspecific hybridization. Tetraploidization may lead to specific genome expression changes that can in turn induce phenotypic changes that trigger an increase in productivity and

**Abbreviations:** ABA, abscisic acid; Asa, reduced ascorbate; APX, ascorbate peroxidase; CAT, catalase; CBFs, C-repeat-binding factor; CO<sub>2</sub>, carbon dioxide concentration; DHA, oxidized ascorbate; DHAR, dehydroascorbate reductase; F<sub>0</sub>, minimal value of chlorophyll a fluorescence; F<sub>m</sub>, maximum fluorescence; F<sub>v</sub>, variable fluorescence of photosystem II; F<sub>v</sub>/F<sub>m</sub>, the maximal quantum yield of photosystem II; FE%, electrolyte leakage percentage; G<sub>s</sub>, stomatal conductance; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; QA, quinone acceptor; MDA, malondialdehyde; O<sub>2</sub><sup>•−</sup>, superoxide radical; OH<sup>•</sup>, hydroxyl radical; P<sub>net</sub>, net photosynthesis rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; tAsa, total ascorbate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; C/2xCC, clementine grafted onto diploid carrizo citrange rootstock; C/4xCC, clementine grafted onto tetraploid Carrizo citrange rootstock

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efficiency, especially by enhancing stress tolerance (Tan et al., 2015). Polyploid species are more common in extreme environments, with low temperatures, high radiation, low fertility soils and dry conditions (Brochmann et al., 2004; Ehrenforfer, 1980; Levin, 1983). Based on several reports, the use of tetraploid genotypes is an alternative way to improve stress tolerance in many crops (Saleh et al., 2008; Meng et al., 2012; Tan et al., 2015; Ruiz et al., 2016a,b,c). In a previous study, we showed that tetraploid Rangpur lime (*Citrus limonia* Osbeck) rootstock improved the water deficit tolerance of Valencia scion compared to the use of diploid Rangpur lime rootstock grafted with Valencia scion (Allario et al., 2013).

Rootstock/scion combinations are frequently employed in the citrus industry to improve fruit production and quality, and the tolerance to biotic and abiotic stresses. The rootstocks are selected for root traits linked to resistance to pests and pathogens from the soil but also to various abiotic stresses such as salinity, drought, floods and cold. Roots subjected to cold have decreased water absorption due to increased root hydraulic resistance (Kramer, 1940) and decreased membrane permeability (McElhaney et al., 1973). This results in decreased nutrient and water absorption causing a disruption of plant growth and development (Ahn et al., 1999). Usually, citrus rootstocks are diploid with a basic chromosome number  $x = 9$  (Krug, 1943). Citrus rootstocks are propagated by polyembryonic seeds since citrus species are partially apomictic. Spontaneous doubled diploid ( $4\times$ ) seedlings may arise from chromosome set doubling of nucellar cells (maternal tissue) (Cameron and Frost, 1968).

Carrizo citrange (*Citrus sinensis* Osb.  $\times$  *Poncirus trifoliata* L. Raf.) and its parent *Poncirus trifoliata* are two rootstocks classically used in Corsica. When grown as seedlings, these genotypes have deciduous leaves and undergo winter dormancy. In *Poncirus trifoliata*, this marked difference was probably acquired during the evolutionary process in northeastern Asia when the trees were exposed to cold temperatures (Ziegler and Wolfe, 2017). However, when grafted, these cultivars are no longer deciduous and their cold tolerance is much more limited, although the use of *Poncirus trifoliata* and Carrizo citrange rootstock is still one of the most effective ways to boost cold tolerance. However, little is known regarding the mechanisms that allow the rootstock to improve the scion's cold tolerance.

When plants are exposed to cold temperatures, profound changes in gene expression are observed. The C-repeat-binding factor (CBFs) pathway is known to play a very important role in the response to cold stress (Chan et al., 2016). Huang et al. (2015) recently showed that, under cold conditions, ICE1 (inducer of CBF expression 1) of *Poncirus trifoliata* modulates polyamine levels through interactions with arginine decarboxylase. This leads to lower hydrogen peroxide ( $H_2O_2$ ) and superoxide radical ( $O_2^{\cdot-}$ ) contents, and higher activity of antioxidant enzymes, such as superoxide dismutase and catalase. Thus, it is likely that Carrizo citrange inherited this capacity to better tolerate cold from its *Poncirus trifoliata* parent. Carrizo citrange rootstock is also widely used since it is tolerant to *Citrus Tristeza Virus* and flooding. In addition, it promotes good fruit productivity and quality (Pérez-Clemente et al., 2012).

In the Mediterranean region, the climate is dry and hot during the summer and relatively cold during the winter. Regions such as Corsica, which is the most northern citrus growing area, can sometimes have relatively low temperatures. For example, during the coldest months (January and February) of winter 2010–2011, the average daily minimum air temperatures were 4.1 and 4.6 °C (Santini et al., 2013) in Corsica. Below 13 °C, the development and growth of citrus is disturbed (Ribeiro and Machado, 2007).

Low temperatures can interfere with photosynthesis and redox homeostasis in cells (Asada, 1999). This disruption of the photosynthetic machinery induces an over-production of reactive oxygen species (ROS) leading to an oxidative stress, one of the most harmful consequences of cold stress (Asada, 2006). The main ROS are  $O_2^{\cdot-}$ ,  $H_2O_2$  or the hydroxyl radical ( $OH\cdot$ ). They are produced on a regular

basis in biological pathways as by-products or signal transducers; however, an excess of ROS can cause oxidative damage to organic molecules like deoxyribonucleic acid (DNA) mutation, protein denaturation and membrane lipid peroxidation (Mittler, 2002; Apel and Hirt, 2004). The latter can lead to changes in the permeability and fluidity of the membrane phospholipid bilayer and can significantly alter cellular integrity. Plants possess antioxidant enzymes (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and dehydroascorbate reductase (DHAR)) and non-enzymatic antioxidants (ascorbate and proline) for scavenging ROS. This antioxidant system has already been shown to have a crucial role in the cold tolerance of plants (Sabehat et al., 1998).

In this study, we tested whether the use of tetraploid rootstocks can increase the chilling stress tolerance of the citrus graft. The effects of seasonal temperature fluctuations were investigated on common clementine (*Citrus clementina* Hort. ex Tan) grafted on diploid and tetraploid Carrizo citrange rootstocks.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

The experiment was performed on 40-year-old Clementine trees (*Citrus clementina* Hort. ex Tan; SRA 63; ICVN 0100059) grafted onto Carrizo citrange rootstock (*Citrus sinensis* (L.) Osb.  $\times$  *Poncirus trifoliata* (L.) Raf.; ICVN 0110476). Bark samples were collected (1 cm<sup>2</sup> area cut from below the bud union) from each single rootstock to determine its ploidy using flow cytometry (Froelicher et al., 2007). In the orchard, 11 tetraploid Carrizo citrange were identified. Four clementine trees grafted onto diploid Carrizo citrange (*Citrus sinensis* (L.) Osb.  $\times$  *Poncirus trifoliata* (L.) Raf.) (ICVN 0110476) rootstock (C/2xCC) and four clementine trees grafted onto the respective tetraploid form of the same rootstock (C/4xCC) were selected for investigation. All trees had the same South orientation and were a similar height above ground (about 1.5 m). Trees were grown under organic farming conditions in an orchard located in Moriani, Corsica, France (42° 23' 08" N and 09° 31' 47" E). Measurements and sampling were carried out from October 2015 to May 2016. The same study was also carried out in 2014–2015 and the results obtained were identical (data not shown). The coldest months were January, February and March. We focused on the coldest sunny days of those three months, where the minimum temperature was  $-1$ ,  $0.8$  and  $0.9$  °C, respectively (Table 1). Climatic data were collected throughout the sampling period (Table 1).

Resistance to chilling stress was evaluated through measurement of the main photosynthetic traits (net photosynthesis, stomatal conductance, chlorophyll content and chlorophyll *a* fluorescence), electrolyte leakage, starch levels, oxidative status (malondialdehyde, hydrogen peroxide); the contribution of the antioxidant system was assessed by enzymatic (scavenging and recycling enzymes) and non-enzymatic assays (ascorbate, proline). For each physiological measurement, 3 mature leaves per tree and per genotype were used (12 replicates). Leaves were selected on 1-year branches subjected to the same light exposure (East, Southeast). Each parameter was measured between 9 am and 11 am. For biochemical analyses, 4 samples of 20 fully expanded leaves were sampled for each genotype (4 replicates) once a month from October 2015 to May 2016. This sampling was carried out as described in previous studies (Allario et al., 2013; Hussain et al., 2012; Santini et al., 2012) in which four replicates were used for biochemical parameters. Harvested leaves were immediately immersed in liquid nitrogen and then stored at  $-80$  °C. Before performing biochemical studies, each leaf sample was ground to a fine powder in liquid nitrogen.

### 2.2. Measurements of gas exchange and chlorophyll content

Leaf net photosynthetic rate ( $P_{net}$ ) and stomatal conductance ( $G_s$ )

**Table 1**  
Meteorological data from October 2015 to May 2016 at the experimental plot.

Sampling period	Mean temperature (°C)		Minimum temperature (°C)		Maximum temperature (°C)		Average minimum temperature (°C)	Average maximum temperature (°C)
	<sup>1</sup> SM	<sup>2</sup> SD	<sup>1</sup> SM	<sup>2</sup> SD	<sup>1</sup> SM	<sup>2</sup> SD	<sup>1</sup> SM	<sup>1</sup> SM
October	16.3	14.2	5.6	8.2	24.9	18.9	11.6	21.3
November	15.4	13.5	0.9	8.8	22.1	20.9	8.1	18.6
December	13.8	7.8	2.4	6.1	17.7	17.1	5.1	16.6
January	9.6	7.8	-1	3.9	20.9	9.1	4.5	15.1
February	10.6	12.2	0.8	4.2	20.4	16.2	5.4	15.7
March	11.3	12	0.9	9.4	18.3	14.8	6.3	15.9
April	14.1	13	2.9	4.8	22.1	18.7	8.5	19.3
May	16.9	16.3	5.8	14	24.7	20.9	10.9	21.9

<sup>1</sup> SM: Sampling months.

<sup>2</sup> SD: Sampling days.

were measured using a LI6400 portable photosynthesis system (Li-COR, Lincoln, NE, USA) with the LI6400-40 Leaf Chamber. For measuring  $P_{net}$  and  $G_s$ , airflow rate was set at  $500 \mu\text{mol s}^{-1}$ , carbon dioxide concentration ( $\text{CO}_2$ ) at  $380 \mu\text{mol mol}^{-1}$  and the temperature at  $25^\circ\text{C}$ . Photosynthetic photon flux density (PPFD) was provided by a red-blue light source (6400-02B no. SI-710, Li-Cor, Lincoln) in a gas exchange chamber and was fixed at  $1400 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

The chlorophyll content was measured non-invasively with a Dualex Scientific + <sup>TM</sup> meter (FORCE-A, Paris). Values were obtained in Dualex units, convertibles to  $\text{g cm}^{-2}$ .

### 2.3. Measurements of chlorophyll a fluorescence

Chlorophyll a fluorescence was measured using a Handy PEA fluorometer (Hansatech, Instruments Ltd) with a resolution of 10 s, in a non-destructive and reproducible manner. Before the measurements, each leaf was dark-adapted with a “leafclip” during 30 min. Following the dark adaptation, a bright flash of saturation intensity ( $10,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was applied on the leaf surface using three light-emitting diodes (650 nm). Two parameters were measured: the minimal value of chlorophyll a fluorescence ( $F_0$ ), when all photosystem II (PSII) centers were open (all the primary quinone acceptors “QA” were oxidized) and the maximum fluorescence ( $F_m$ ) when all PSII centers were closed (all QA were reduced) after the emission of a saturating flash of light. The variable fluorescence ( $F_v$ ) was obtained by subtracting  $F_0$  from  $F_m$ . The maximum quantum efficiency of PSII primary photochemistry was calculated as the ratio  $F_v/F_m$  [=  $(F_m - F_0)/F_m$ ] (Maxwell and Johnson, 2000).

### 2.4. Determination of electrolyte leakage

Efflux of electrolytes was determined according to the protocol of Thiaw (2003). For each tree, three discs of three different fully expanded leaves were cut with a cork borer, rinsed with distilled water and then placed in test tubes containing 10 mL of distilled water. Test tubes were submerged in a water bath for 2 h at  $45^\circ\text{C}$ . Solution conductivity ( $C_1$ ) was measured with a conductivity meter (Mettler Toledo Seven2Go) after cooling to room temperature. Conductivity was measured a second time ( $C_2$ ) after placing the samples in the water bath for 1 h at  $100^\circ\text{C}$  and then cooled at room temperature. Electrolyte leakage percentage (FE%) was calculated with the formula:  $\text{FE} (\%) = (C_1/C_2) \times 100$  (Tripathy et al., 2000).

### 2.5. Determination of oxidative stress response

Ascorbate and antioxidant enzyme assays were performed as described by Santini et al. (2013).

$\text{H}_2\text{O}_2$  levels were measured with the method described by Zhou et al. (2006), as modified by Santini et al. (2013).  $\text{H}_2\text{O}_2$  was extracted

by homogenizing 80 mg of leaf powder with 2.0 mL of 5% trichloroacetic acid (TCA) (w:v) and 60 mg of activated charcoal. The homogenate was centrifuged at  $5000g$  for 20 min at  $4^\circ\text{C}$ . The supernatant was collected. Absorbance was determined at a wavelength of 505 nm. The  $\text{H}_2\text{O}_2$  concentration was calculated using a standard curve of  $\text{H}_2\text{O}_2$ .

MDA levels were determined using a thiobarbituric acid (TBA) reaction described by Hodges et al. (1999) and modified by Santini et al. (2013). MDA was extracted by homogenizing 80 mg of leaf powder in 2.0 mL of 80% ethanol (v:v). Homogenates were centrifuged at  $3000g$  for 10 min at  $4^\circ\text{C}$ . Absorbance was determined at wavelengths of 440, 534 and 500 nm against a blank.

A V-630 spectrophotometer was used for all measurements (Jasco Inc., Tokyo, Japan).

### 2.6. Determination of starch content

Starch content was determined enzymatically using the Megazyme total starch HK assay kit (Megazyme International Ireland Ltd., Co., Wicklow, Ireland) and by following the manufacturer's recommended procedure. Extraction was carried out at pH 5.0 from 100 mg of leaf powder with 200  $\mu\text{L}$  of aqueous ethanol (80% v:v) and 3 mL of thermostable alpha-amylase diluted with 100 mM sodium acetate (pH 7.5). Samples were incubated in boiling water bath for 6 min and then in a bath at  $50^\circ\text{C}$  for 30 min with 100  $\mu\text{L}$  of amyloglucosidase. The full contents were transferred in 100 mL volumetric flasks and the volume was adjusted with distilled water. Aliquots were centrifuged at  $1077g$  for 10 min. The reaction mixture contained 50  $\mu\text{L}$  of crude extract, distilled water, 10  $\mu\text{L}$  of buffer (pH 7.6) with sodium azide (0.02% w:v) and 10  $\mu\text{L}$  of NADP<sup>+</sup>/ATP. Starch content was measured with a spectrophotometer at 340 nm with hexokinase/glucose-6-phosphate dehydrogenase reagent. Total starch was expressed in mg/mg of fresh weight.

### 2.7. Determination of proline content

Proline contents were measured using the ninhydrin reaction described by Bates et al. (1973), with slight modifications. Proline was extracted from 20 mg of leaf powder, suspended in 1 mL of 100 mM potassium phosphate buffer (pH 7.5). Homogenates were then centrifuged at  $14,000g$  for 15 min at  $4^\circ\text{C}$ . The reaction mixture contained 400  $\mu\text{L}$  of supernatant and 600  $\mu\text{L}$  of 1% ninhydrin (w:v). After 20 min of incubation at  $95^\circ\text{C}$ , 2 mL of toluene was added to extracted red products and samples were kept at room temperature and protected from light for 3 h. The absorbance of the toluene layer was determined at 520 nm. Proline content was calculated using a standard curve of proline. A V-630 spectrophotometer was used for all measurements (Jasco Inc., Tokyo, Japan).

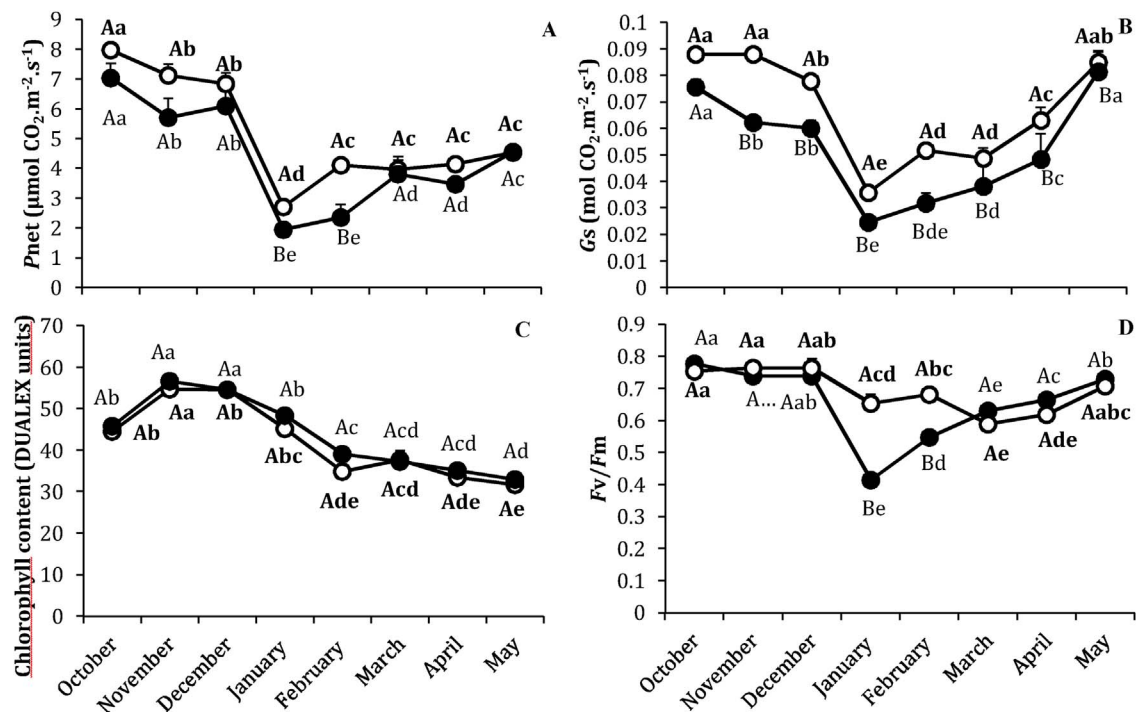


Fig. 1. Change in net photosynthesis (Pnet) (A), stomatal conductance (Gs) (B), chlorophyll fluorescence (Fv/Fm) (C) and chlorophyll content (D) in C/2xCC (●) and C/4xCC (○) throughout the experiment. All data are presented as mean ( $\pm$  S.E.) of twelve independent measurements ( $n = 12$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.

## 2.8. Statistical analyses

The experimental design was a split-plot, with C/4xCC and C/2xCC as the main plots and sampling period (months) as the subplot. All statistical measurements were assessed with R statistical software (<http://www.R-project.org>). Influence of genotype and sampling period were analysed using two-way ANOVA followed by LSD test at  $P < 0.05$ .

## 3. Results

$P_{net}$  and  $G_s$  decreased from October to January and then increased until May in both C/4xCC and C/2xCC (Fig. 1A and B). However, a higher photosynthetic capacity was observed in C/4xCC than C/2xCC in January and February for  $P_{net}$ , and from November to May for  $G_s$ . While no significant difference in chlorophyll content was observed between C/4xCC and C/2xCC throughout the experiment (Fig. 1C), C/2xCC showed a marked decrease in  $F_v/F_m$  compared to C/4xCC in January and February (Fig. 1D). Also, electrolyte leakage increased significantly from the warm to the cold period in C/2xCC whereas it did not change significantly in C/4xCC (Table 2).

Equivalent starch content was measured in C/2xCC and C/4xCC at

each sample point of the experiment, except in March, where C/4xCC showed a greater accumulation of starch (Fig. 2A). Proline content increased at the beginning of the experiment, and then began to decrease in January in both clementine/rootstocks combinations (Fig. 2B). No difference was observed except in November.

Investigations of leaf  $H_2O_2$  contents throughout the experiment revealed no large changes induced by low temperatures (Fig. 3A). Also, no significant difference between C/4xCC and C/2xCC was observed except in October.

However, lower wintertime temperatures induced a marked decrease of MDA content from November to March in C/4xCC while it increased from December to February in C/2xCC (Fig. 3B). From March on, MDA contents remained stable until May in C/4xCC and increased in C/2xCC.

Antioxidant activities (CAT, APX, DHAR and SOD) were monitored throughout the experiment (Fig. 4). During the cold period, C/4xCC had higher CAT, APX and DHAR activities than C/2xCC (Fig. 4A, B and C). C/4xCC had a greater CAT activity than C/2xCC from January to May. A significant increase in APX activity was observed in C/4xCC during some of the coldest months (February and March) (Table 1). Similarly, C/4xCC had a significantly higher DHAR activity than C/2xCC in January and February. Finally, SOD activity decreased in both scion/rootstock combinations with the winter period. No significant difference in SOD activity was observed between C/4xCC and C/2xCC (Fig. 4D).

Reduced ascorbate (Asa), total ascorbate (tAsa) and oxidized ascorbate (DHA) contents increased from October to April in both C/4xCC and C/2xCC (Fig. 5A B and C) while Asa/DHA ratio decreased during the same period (Fig. 5D). Conversely, in May, Asa, tAsa and DHA contents dropped while Asa/DHA ratio rose. No significant difference was observed between C/4xCC and C/2xCC throughout the time period.

Table 2  
Change in electrolyte leakage between the warm and the cold period.

	Electrolyte leakage (%)	
	Warm period	Cold period
C/2xCC	20.95 $\pm$ 3.38 <sup>Ab</sup>	31.18 $\pm$ 2.77 <sup>Aa</sup>
C/4xCC	21.35 $\pm$ 2.57 <sup>Aa</sup>	23.64 $\pm$ 1.19 <sup>Ba</sup>

All data are presented as mean ( $\pm$  S.E.) of four independent measurements ( $n = 4$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ).

Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.



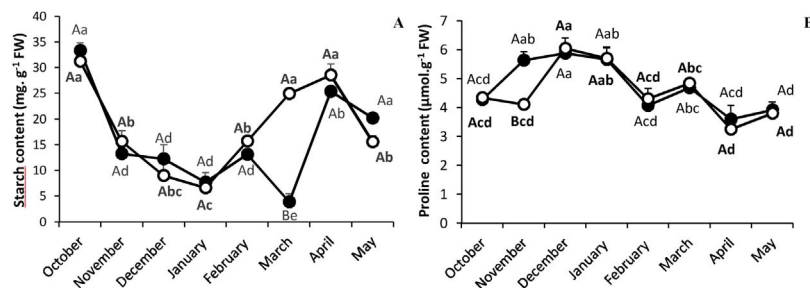


Fig. 2. Change in starch (A) and proline (B) concentration in C/2xCC (●) and C/4xCC (○) throughout the experiment. All data are presented as mean ( $\pm$  S.E.) of four independent measurements ( $n = 4$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.

#### 4. Discussion

According to their origin, plants have an optimum temperature for their growth and development. *Poncirus trifoliata* and Carrizo citrange seedlings are deciduous during winter, which favors cold adaptation. Most citrus trees have an optimum temperature between 22 °C and 30 °C (Sun and Ma, 1998). During this experiment, the coldest months were January, February and March. The data were collected during the coldest sunny days of those three months where the minimum temperature was  $-1$ ,  $0.8$  and  $0.9$  °C, respectively. These low temperatures affected the physiology of the trees.

##### 4.1. Comparison of physiological and oxidative status of common clementine grafted onto diploid and tetraploid Carrizo citrange rootstocks during the cold period

When exposed to low temperatures, plant cells are confronted with physiological and biochemical disturbances (Ruelland and Zachowski, 2010). As expected, the decline in  $P_{net}$ ,  $G_s$  and  $F_v/F_m$  was observed during the coldest period for both C/2xCC and C/4xCC (Fig. 1A, B and D), as a consequence of the very low temperatures measured for this period (Table 1). In response to low temperatures, plants can maintain their water potential by closing their stomata to prevent water loss by transpiration (Wilkinson et al., 2001). During the time course of the experiment,  $P_{net}$  and  $G_s$  appeared to be highly correlated. Thus, the  $P_{net}$  decrease in C/4xCC and C/2xCC is due to the stomatal closure induced by low temperatures. However, a faster and significant decline for these parameters was observed in C/2xCC than in C/4xCC, suggesting that tetraploid rootstock maintains higher photosynthetic activities of the scion during the cold period. The lower photoinhibition observed in C/4xCC appears to result in more efficient light use for photosynthesis and therefore  $P_{net}$ . This is in agreement with previously reported findings (Brugnoli and Björkman, 1992; Loreto et al., 2009). Indeed, higher maximum quantum yield was also observed in tetraploid citrus genotypes compared to diploid under salt stress or water deficit

conditions (Mouhaya et al., 2010; Allario et al., 2013).

We assume that the increased chilling tolerance observed in C/4xCC is due to an overexpression of genes induced by the tetraploid rootstock. For example, RDM4 that regulates CBF gene expression could have a direct or indirect function during the accumulation of ROS under stress conditions, as indicated by changes in the expression of genes related to ROS (manganese superoxide dismutase I, catalase, microsomal ascorbate peroxidase 5 and cytochrome b561 genes) and the activity of antioxidant enzymes (CAT and SOD), as well as the accumulation of  $H_2O_2$  (Chan et al., 2016). Moreover, Dong et al. (2013) also found increased enzymatic activity (SOD and CAT) in transgenic tobacco overexpressing ICE 1 from *Vitis amurens* upstream transcription factor (VaICE1) which regulates the transcription of CBFs genes.

Moreover, a study conducted on Valencia scions grafted on diploid and tetraploid Rangpur lime rootstocks showed that tetraploidy changes gene expression in Rangpur lime roots, thereby controlling the adjustment to water stress (Allario et al., 2013). After the coldest months, the recovery of photosynthetic parameters in C/4xCC and C/2xCC indicates that changes induced by seasonal temperature fluctuations are reversible. The tetraploid rootstock boosts its cold resistance only during the coldest months. Thus, the use of tetraploid rootstocks could be a solution for crops in colder regions.

Chlorophyll is a key biomolecule in the photosynthesis process (Xu et al., 2000) and it is very sensitive to abiotic stress. When investigating C/4xCC and C/2xCC, the decrease in chlorophyll content due to cold was not significantly different suggesting that tetraploidization of the Carrizo citrange rootstock does not confer protection against chlorophyll damage. However, many studies have shown a lower decrease in chlorophyll content suggesting a more stable photosynthetic system in tetraploid plants under stress conditions. For example, a smaller decrease in chlorophyll content was observed in tetraploid rice under water stress conditions (Yang et al., 2014) and turnip greens subjected to high salt stress (Meng et al., 2012) than in their respective diploid. These results can be explained by the large differentiation observed

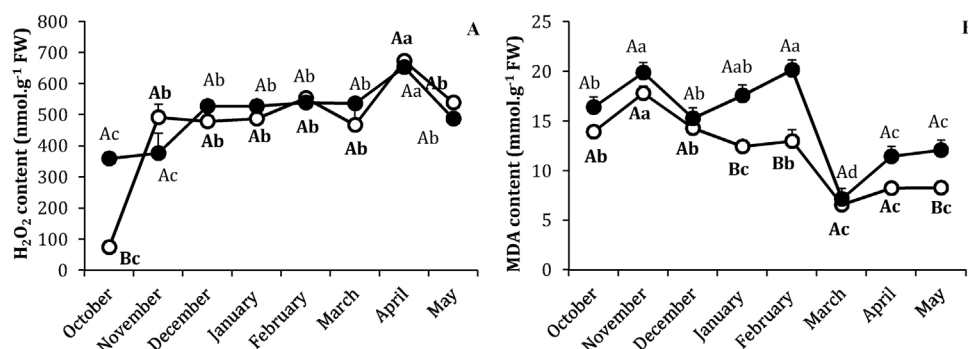


Fig. 3. Change in  $H_2O_2$  (A) and MDA (B) contents in C/2xCC (●) and C/4xCC (○) throughout the experiment. All data are presented as mean ( $\pm$  S.E.) of four independent measurements ( $n = 4$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.

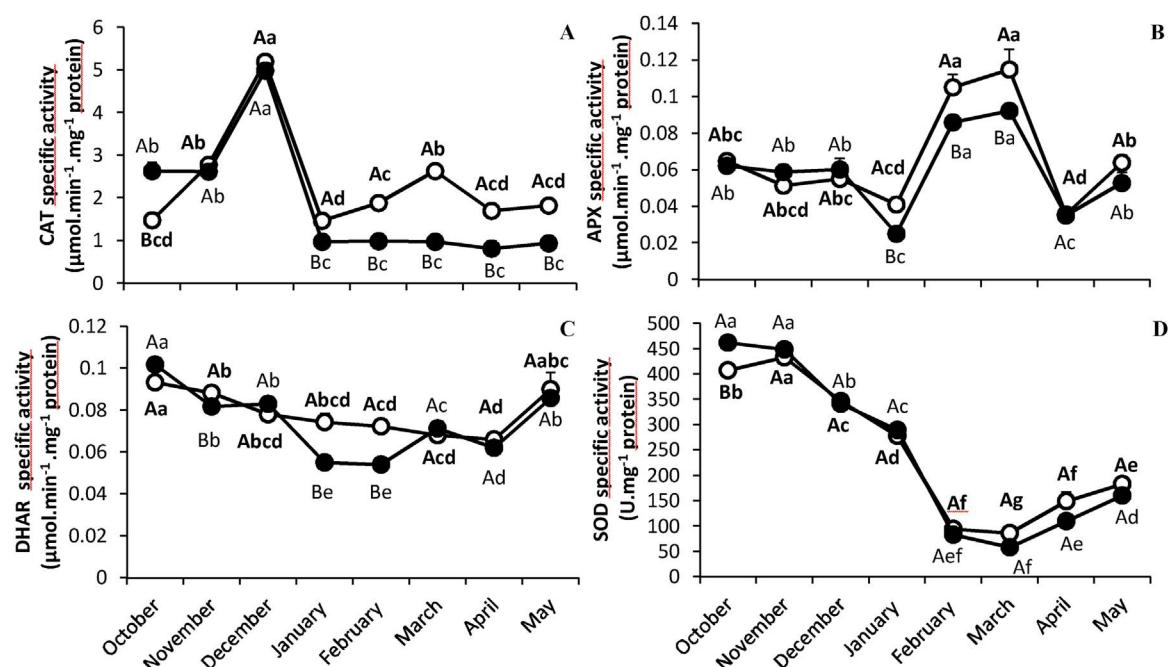


Fig. 4. Change in antioxidant enzyme specific activities: CAT (A), APX (B) et DHAR (C) SOD (D), in C/2xCC (●) and C/4xCC (○) throughout the experiment. All data are presented as mean ( $\pm$  S.E.) of four independent measurements ( $n = 4$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.

between diploid and tetraploid (Mouhaya et al., 2010; Allario et al., 2011) leading to better adaptation.

From October to February, leaf starch contents were similar in C/2xCC and C/4xCC. Cold-sensitive plants that change their rates of starch metabolism would indicate a more rapid degradation of starch compared to the rate of photosynthesis (Hodges et al., 1997). A faster accumulation of starch starting in February was found in C/4xCC, while

it increased only after March in C/2xCC (Fig. 2A); this suggests an earlier response to increased temperatures in C/4xCC. In that study, tetraploid rootstocks react to photosynthetic sink limitations by increasing starch synthesis. The increase of photosynthesis in February in C/4xCC and its stabilization in the next months could explain starch accumulation during this period. Indeed, during the warm period, leaf starch and sugar contents were found to be much higher in Clementine

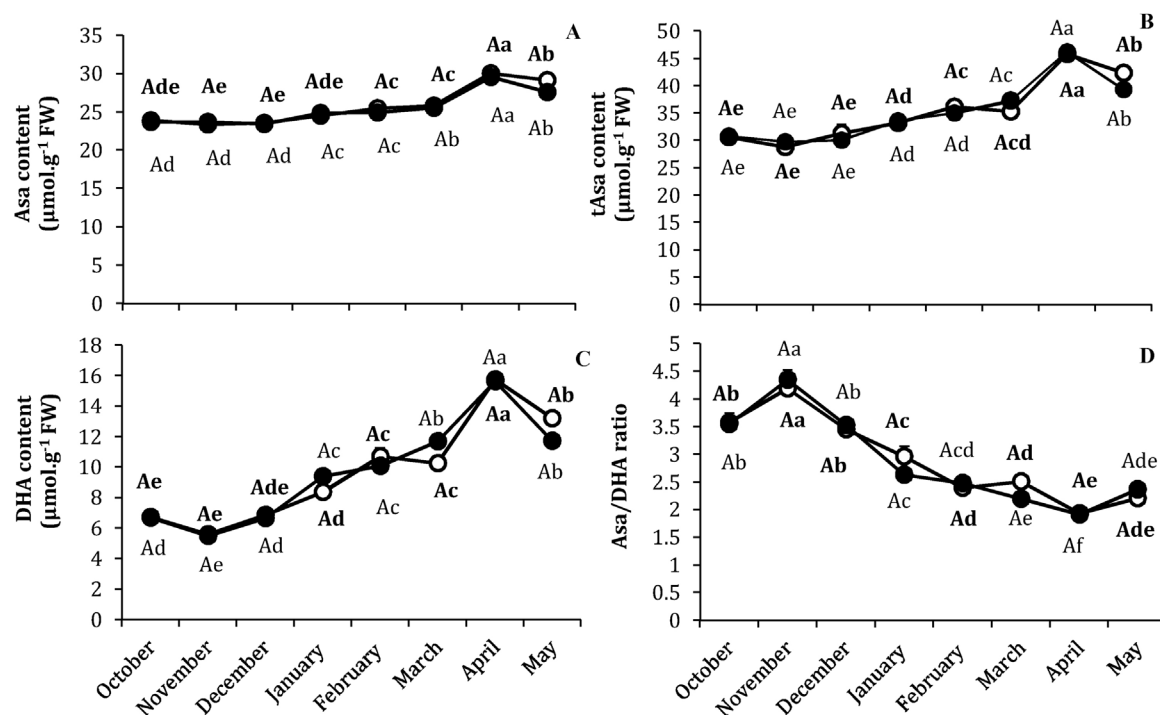


Fig. 5. Change in (A) ascorbate (Asa), (B) total ascorbate (tAsa), (C) oxidized ascorbate (DHA) concentration and (D) redox status (Asa/DHA ratio) in C/2xCC (●) and C/4xCC (○) throughout the experiment. All data are presented as mean ( $\pm$  S.E.) of four independent measurements ( $n = 4$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between C/2xCC and C/4xCC at a point of the time course and different lower case letters indicate significant differences throughout the time course for C/2xCC or for C/4xCC.

grafted onto tetraploid *Poncirus* rootstocks than onto diploid *Poncirus* (Hussain et al., 2012).

The increase in  $H_2O_2$  and MDA due to environmental stress is commonly used as oxidation indicators. In C/4xCC, the lowest accumulation of MDA and maintenance of  $H_2O_2$  levels from January to March would suggest that use of a tetraploid rootstock increases the performance of the antioxidant system and therefore prevents the accumulation of these toxic compounds in the scion leaves during cold periods (Fig. 2A and B). These results are consistent with greater sensitivity of the photosynthetic system observed in C/2xCC. In the literature, similar results were observed in tetraploid plants. For example, a greater accumulation of MDA was found in “Dez orange” (Shafieizargar et al., 2013) and honeysuckle (Li et al., 2009) diploid compared to their tetraploid counterparts subjected to salt stress. This finding indicates that tetraploidization can improve resistance to abiotic stress and thus to oxidative stress.

The accumulation of MDA during the cold period in C/2xCC is supported by the increase of electrolyte leakage, whereas electrolyte leakage did not increase in C/4xCC (Table 2). The increased electrolyte leakage and MDA content during cold period in C/2xCC could indicate cell membrane damage due to lipid peroxidation and thus larger changes in biophysical plant properties at low temperatures. These findings are also consistent with the lower  $F_v/F_m$  values observed in C/2xCC. Studies on cucumber and watermelon have shown that grafting onto cold tolerant rootstocks might reduce the degree of lipid peroxidation and electrolyte leakage induced by cold stress (Gao et al., 2008).

#### 4.2. Could antioxidant systems explain the better chilling resistance of leaves from clementines grafted on tetraploid rootstocks compared to diploid ones?

Plant tolerance to oxidative damage may be associated with their ability to eliminate ROS. The antioxidant mechanisms include detoxification enzymes such as SOD, CAT, APX (Huang and Guo, 2005; Almeselmani et al., 2006). In C/4xCC, an increase in CAT and APX activities (Fig. 4A and B) was found from January to March, while their activities remained stable or increased to a lesser extent, respectively, in C/2xCC. This could explain the lower cell damage in C/4xCC. On the whole, tetraploidization of the Carrizo citrange rootstock seems to confer protection against oxidative damage to the scion. The enzymatic response in the scion leaves is dependent upon signalling regulated by rootstocks as suggested by Hippler et al. (2016). This indicates that the communication mechanisms between the scion and rootstock are complex and little is known about them in the literature.

Some studies have highlighted a drop in antioxidant enzyme activities in cold sensitive plants while cold tolerant plants over-expressed genes encoding detoxification enzymes such as SOD, CAT or APX (Mutlu et al., 2013; Xu et al., 2013). A rise in detoxification enzyme activity was often observed in non-grafted tetraploid plants under numerous abiotic stress conditions. For example, in tetraploid *Acacia* subjected to salt stress (Meng et al., 2016) and in tetraploid wild yam exposed to high temperatures (Zhang et al., 2010), higher enzymatic activity (CAT and APX) and lower concentrations of oxidation indicators (MDA and  $H_2O_2$ ) were found compared to their diploid counterparts. An increase in SOD activity was also observed in tetraploid varieties of chrysanthemums (Liu et al., 2011) and in *Acacia* (Meng et al., 2012) subjected to water and salt stress, respectively, but not in their respective diploid. In addition, a study of two citrus tetraploid rootstocks (*Willow leaf mandarin* and *Cleopatra mandarin*) found higher constitutive levels of antioxidant enzymes than their respective diploids during salt stress (Podda et al., 2013).

Another possible reason for increased activity of the antioxidant enzyme system of a scion grafted on a rootstock could be the increased intake of abscisic acid (ABA) and cytokine root to shoot (Schwarz et al., 2010). Indeed, few studies have shown that detoxification enzymes can be up-regulated by phytohormones such as ABA and cytokinins (Jiang

and Zhang, 2002; Wang et al., 2003) mainly produced in roots and that can regulate the stress response in the aerial part of the plant. Under control conditions, ABA content in the root of Valencia grafted onto tetraploid rootstock was higher than in roots of Valencia grafted onto diploid rootstock (Allario et al., 2013). This was associated with greater expression of stress responsive genes in tetraploid than in diploid roots, including SOD, DHAR, and alternative oxidase.

Whether in C/4xCC or C/2xCC, the Asa/DHA ratio (Fig. 5D) is greater than 1 throughout the sampling period indicating that more reduced forms (Asa) than oxidized forms (DHA) of ascorbate are produced. This could enable them to fight effectively against oxidative stress over time. The change in Asa/DHA ratio is not always linked to DHAR activity (Fig. 4C). However the Asa/DHA ratio remains above 1, indicating sufficient DHAR activity and/or *de novo* ascorbate synthesis. Some studies have shown the fundamental role of DHAR in the stress response (Chen et al., 2003; Lee et al., 2007; Mai et al., 2010; Mostofa et al., 2015).

The osmoregulation of proline reduces the water potential of the plant and prevents cold-induced dehydration (Liu et al., 2013). In our study, proline content was not significantly changed no matter the type of rootstock used from the beginning to the end of the sampling period (Fig. 2B). A few studies have shown a greater accumulation of proline in tetraploid plants compared to their diploid counterparts (Li et al., 2009; Shafieizargar et al., 2013).

## 5. Conclusion

A typical response to low temperatures was observed in common clementine grafted onto diploid or tetraploid Carrizo citrange rootstocks. Smaller decrease in photosynthetic capacity, lower levels of MDA, less electrolyte leakage and higher specific activities of CAT, APX and DHAR were observed in trees grafted onto tetraploid Carrizo citrange rootstocks during cold months (mainly January and February). These results show that tetraploid Citrange Carrizo rootstock improves the cold tolerance of the scions. To confirm the relevance of Citrange Carrizo tetraploid rootstock, it would be interesting to study how they impact production and fruit quality of the scion.

## Disclosures

The authors have no conflicts of interest to declare.

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