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Calcium biofortification of Rocha pears, tissues accumulation and physicochemical implications in fresh and heat-treated fruits

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ABSTRACT

Low dietary intake of Ca in humans has been epidemiologically linked to various diseases, which can have serious health consequences over time. Accordingly, the development of an agronomic itinerary for Ca biofortification of Rocha pears and the assessment of physicochemical deviations prompted this study. Two orchards with contrasting soil and water characteristics were selected, characterized through orthophotomaping and, during fruits development, leaves were sprayed twice with $Ca(NO_3)_2$ (0.1, 0.3 and 0.6 kg ha^{-1}) or $CaCl_2$ (0.4, 0.8 and 1.6 kg ha^{-1}), followed by pulverization only with $CaCl_2$ (first once with 4 kg ha^{-1} and then four times with 8 kg ha^{-1}). During fruits development net photosynthesis, stomatal conductance, transpiration rates, instantaneous and water use efficiency, only showed minor deviations, which indicated that the threshold of toxicity was not surpassed. Calcium contents varied during fruits development and at harvesting the average biofortification index varied between 47 %–63 % and 24 %–59 % in each of the orchards. Besides, the equatorial region of the fruits showed for all treatments (substantially in Ca treated samples) higher Ca contents in the epidermal and in the central regions. Fresh and heat-treated fruits (in a thermomix at 90 °C, during 10 min) biofortified with Ca only revealed minor differences and the sensory acceptability did not vary markedly. It is concluded that, although prevailing a heterogeneous distribution of Ca in fruit tissues, high indexes of biofortification in Rocha pears can be prompt in the orchards, without substantial physicochemical changes. Accordingly, agronomic biofortification with Ca can be used as a strategy for benefiting consumer's health.

1. Introduction

Calcium is the most abundant mineral in the human body, predominating in bones and teeth and being at a physiological level critical, namely at a vascular level (IOM - Institute of Medicine, 2011). Still, Ca malnutrition can promote osteoporosis, rickets, hypertension and infantile bone deformity (IOM - Institute of Medicine, 2011; EFSA, 2015). To surpass Ca malnutrition (*i.e.*, the daily average level of this nutrient

Abbreviations: E, Transpiration rate; g_s, Stomatal conductance; iWUE, Instantaneous water use efficiency; P_n, Net photosynthesis.

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intake might vary between 800−1300 mg, depending of the age, or whether a woman is pregnant or lactating - NIH, 2017), agronomic biofortification itineraries can be adopted as a strategy to enhance minerals content in vegetable food products. Yet, Ca accumulation in the edible parts of vegetables largely varies (Dayod et al., 2010), depending of assimilable forms (thus, solubilization rates) by plant roots (which, further depends of soil composition) and of leaves spray concentrations and application times during plants life cycle.

Calcium is the third-most important available nutrient in soils (Kabata-Pendias, 2011; D'Imperio et al., 2016). Uptake of soluble ions (Ca^{2+}) prevails in the growing meristematic regions of root tips (Bonomelli and Ruiz, 2010) and in plant organs acts as a signal transduction agent (Wei et al., 2017) promoting cell walls integrity (Hocking et al., 2016). Additionally, is a cofactor for some enzymes of ATP and phospholipids catabolism and acts as second messenger in metabolic regulation (Taiz and Zeiger, 2002). If assimilable forms of Ca in soils are limited, deficiency symptoms in plants might develop, occurring necrosis of young meristematic regions in young leaves or root tips. Besides, Ca stunting also promotes deformation in young leaves and in fruit tissues development (Taiz and Zeiger, 2002). These effects are closely associated to low mobilization rates of Ca from older tissues, distribution through the phloem sap and its uptake *via* xylem, in a unidirectional transpiration stream (White and Broadley, 2003; Dayod et al., 2010).

If there is limited absorption of inorganic elements at the root level, or if there is an agronomic aims for increasing the amount of a specific nutrient in the edible part of vegetables, foliar spraying may became an alternative. This agronomic biofortification process is being used for Ca, but this mineral has low mobility in plants (Dayod et al., 2010). In pears, leaf sprays with $CaCl_2$, after full bloom, at 2−5 kg $CaCl_2$ ha^{-1}, and before harvest, at a rate of 20 or 25 kg ha^{-1}, increased Ca concentration (respectively, 796 and 803 mg kg^{-1}_{DW}, after one and two years, relatively to the control with 587 and 601 mg kg^{-1}_{DW}) (Wójcik et al., 2014). For understanding the effect of $CaCl_2$ in hard end pear disorder, Wang et al. (2018) also sprayed 2 % of $CaCl_2$ on fruits at 30, 45 and 75 days after anthesis, finding that Ca content in the fruit, inhibited the hard end fruit through a mechanism associated to lignin synthesis blockage. In other vegetables like basil, mizuna, tatsoi and endive, it was also found that after application of two solutions of Ca (100 and 200 mg L^{-1}), the accumulation of this nutrient also increased in plants, without affecting growth (D'Imperio et al., 2016). Wei et al. (2017) also found that Ca spray inhibited pear ripening in a refrigerated air storage and increased the content of volatile aromatic substances, through decomposition of bound aroma substances to free aromatic forms, while promoted the synthesis of volatile aromatic substances in the fruits. Chen et al. (2010), after harvesting, also divided strawberries in three groups, submitting them to 10 L of $CaCl_2$ (0 %, 1 % and 4 %) solution for 15 min. After dipped, strawberries were stored at 4 °C for 15 days, being found that $CaCl_2$ treatments delayed some changes of physicochemical properties and degradation of pectins. Bonomelli and Ruiz (2010) further revealed that $CaCl_2$ application in grapes turned the fruits larger, more turgent, with low dry matter and larger cells.

Pyrus communis L. variety Rocha originated in Sintra, Portugal, in the middle of the 19th century. Nowadays is mostly produced in the West region of Portugal, in about 12,000 ha (INE – Instituto Nacional de Estatística, 2016), with an average production of *ca.* 135,000 ton (WAPA – The world apple and pear association, 2016) and being largely export to the United Kingdom, France, Brazil and Russia (Silva et al., 2010). Considering the growing importance of *Pyrus communis* L. variety Rocha worldwide, and that Ca biofortification was never done in the fruit, this study aimed to develop an agronomic itinerary for this mineral enrichment and the characterization of its tissue accumulation and physicochemical implications in the fresh and heat-treated fruits.

2. Materials and methods

2.1. Experimental fields

Two orchards of *Pyrus communis* L. variety Rocha were selected in the West Region of Portugal, being located at: 39° 23′ 28.997″N; 9° 4′ 52.483″W (orchard 1); 39° 29′ 52.641″N; 9° 1′ 19.604″W (orchard 2). The experimental period, from 12 May to 4 September, was characterized by maximum and minimum average temperatures of 23 °C and 15 °C (with maximum and minimum values of 41 °C and 6 °C, respectively), with an average rainfall of 0.41 mm, a daily maximum of 18.03 mm and an accumulation of 60.4 mm.

In each orchard, to avoid toxicity and considering that Ca has low mobility in plant organs (Dayod et al., 2010) the agronomic Ca biofortification comprised three distinct pulverization phases. Between fruit set and 30 mm diameter, two leaves spraying, with fifteen days range, were made with $Ca(NO_3)_2$ (0.1, 0.3 and 0.6 kg ha^{-1}, for each treatment four trees). In parallel, two other initial leaves pulverization with $CaCl_2$ (0.4, 0.8 and 1.6 kg ha^{-1}, for each treatment four trees) were also carried out. Thereafter, with fifteen days interval, for all treatments, all the 24 trees were sprayed with $CaCl_2$ (firstly at a concentration of 4 kg ha^{-1}, followed by four with 8 kg ha^{-1}). Control trees (8 trees) were not sprayed at any time with $Ca(NO_3)_2$ or $CaCl_2$. Orchards 1 and 2 were harvest on 4/09/2018 and 10/9/2018, respectively.

2.2. Orthophotomaping

On 4 May 2018, for each plot (*i.e.,* prior to leaves spraying) of both orchards, data collected by a drone was used to produce orthophotomaps. High-definition and multi-sector RGB (*i.e.*, with three Electromagnetic Spectra Bands – Red, Green and Blue) and Parrot Sequoia (*i.e.*, with five Electromagnetic Spectra Bands – NIR, REG, Green, Red and RGB) cameras were used. Calibration of the multispectral Parrot Sequoia camera further consider the environmental brightness conditions. Images were processed in a Workstation (AORUS, GIGA-BYTE Technology Co., Ltd - 2019), to produce the final mapping. To assess the general morphology and surface water drainage areas of the experimental fields, Agisoft PhotoScan Professional (Version 1.2.6, Software of 2016 and the ESRI of 2011 and ArcGIS Desktop - Release 10 from Redlands, CA: Environmental Systems Research Institute) was used. The evaluation of drainage areas of surface waters was carried out according to Direcção Geral de Agricultura Desenvolvimento Rural (1972). The highest class corresponded to the land that, due to its morphology, enhances the surface runoff of the water and does not promote infiltration. Conversely, the lower class corresponded to flattened surfaces, as potential infiltration areas, since they promote the accumulation of surface water.

2.3. Soil and irrigation water analysis

For quantification of organic matter in the soils, 16 samples (about 100 g, using a rectangular grid of 5.70 × 4 m for orchard 1 and 4.50 × 3.30 m for orchard 2, collected from surface to a 30 cm deep) from the experimental fields were sieved (2.0 mm mesh). Samples weight was record, and after drying, at 105 °C, for 24 h (followed by 1 h desiccation), dry mass and percentage of moisture were determined. Samples were then heated, to 550 °C, for 4 h (*i.e.*, until constant weight), followed by desiccation until room temperature and weighted to determine the percentage of organic matter. Following Pessoa et al. (2016), pH and electrical conductivity of soil samples were determined in the decanted supernatant of a mixture at a ratio of 1: 2.5 (g_{soil} $mL^{-1}_{water\ milli\text{-}q}$), for 1 h with stirring (at 25 °C, for 30 min) in a thermal bath. Additionally, mineral elements were analyzed using an XRF analyzer (model XL3t 950 He GOLDD+) under helium atmosphere, according to Pelica et al. (2018).

Water quality of the orchards considered physical (pH, temperature

and electrical conductivity) and chemical (bicarbonate, sulfate, chloride, sodium, calcium, magnesium, potassium, nitrate and phosphate) parameters. Electrical conductivity (EC) and pH were determined using a Consort multiparameter analyzer (C 6030) and SP21 (pH) and SK20 T (CE) electrodes. Calcium, Na, K and Mg ions were quantified using a Metrohm (Model 761 Compact IC) chromatograph, equipped with column and pre-column (Metrosep cation 1–2, 6.1010.000), using an eluent mixture (4 mM tartaric acid / 1 mM dipicolinic acid) at a flow rate of 1.00 mL / minute and a sample injection of 10.0 μL. Alkalinity / bicarbonate was determined by titration, in 100 mL of water samples, using 0.1 N hydrochloric acid as titrant, in the presence of 0.1 % methyl orange (Rodier et al., 2009). Chloride, sulphate, nitrate and phosphate ions were quantified by photometry (Spectroquant NOVA 60, Merck), using specific kits (1.14897, 1.14779, 1.14773 and 1.14842). Water classification in the soils of both orchards, considering dominant ions, followed Piper (1944). Sodium adsorption index was determined and related to the electrical conductivity, in classes C and S. The Langelier saturation index was also estimated from the pHe (equilibrium pH), at a reference temperature a 20 °C, to determine the fouling or aggressiveness of the water relatively to calcium carbonate.

2.4. Leaf gas exchange measurements

Leaf gas exchange parameters were determined in orchard 1, using 4–6 randomized leaves per treatment, in 19 June, 20 July and 11 September, following Rodrigues et al. (2016). Leaf rates of net photosynthesis (P_n), stomatal conductance to water vapor (g_s) and transpiration (E) were obtained under photosynthetic steady-state conditions after *ca.* 2 h of illumination (in the middle morning). A portable open-system infrared gas analyzer (Li-Cor 6400, LiCor, Lincoln, NE, USA) was used under environmental conditions, with external CO_2 (*ca.* 400 ppm) and PPFD ranging between 1200−1400 μmol m^{-2} s^{-1}. Leaf instantaneous water-use efficiency (iWUE) was calculate as the P_n-to-E ratio, representing the units of assimilated CO_2 per unit of water lost through transpiration.

2.5. Analysis of calcium contents and tissues location in the fruits

After the 2nd, 3rd and 5th leaf spraying, according to Pelica et al. (2018), Ca contents were determined in randomized fruits using a XRF analyzer (model XL3t 950 He GOLDD+) under He atmosphere. Whole fruits were cut, dried (at 60 °C, until constant weight), grounded and processed into pellets.

The location of Ca in the tissues of the fruits collected at harvest was determined using the μ-EDXRF system (M4 Tornado™, Bruker, Germany), according to Cardoso et al. (2018). The X-ray generator was operate at 50 kV and 100 μA without the use of filters, to enhance the ionization of low-Z elements. To a better quantification of Ca, a set of filters between the X-ray tube and the sample, composed of three foils of Al/Ti/Cu (with a thickness of 100/50/25 μm, respectively) was used. All the measurements with filters were performed with 600 μA current. Detection of fluorescence radiation was performed by an energy-dispersive silicon drift detector, XFlash™, with 30 mm^2 sensitive area and energy resolution of 142 eV for Mn Kα. To better measure the distribution mapping of Ca, fruits were cutted, at the equatorial region, into slices with a stainless steel surgical blade. Measurements were than carried out under 20 mbar vacuum conditions. These point spectra were acquire during 200 s.

Following Lidon et al. (2018), with minor modifications, in the control and treated fruits, collected at harvest and sprayed with the highest concentrations of $Ca(NO_3)_2$ (0.6 kg ha^{-1}) and $CaCl_2$ (1.6 kg ha^{-1}), the rates of Ca / Mg were assessed and evaluated, through scanning electron microscopy. A JEOL JSMT330A model coupled with Energy Dispersive X-Ray Spectroscopy (EDS) and a Tracor Northern Series II microanalyzer was use. Fruits were cutted transversely in the equatorial region, being previously dehydrated and dried in CO_2, using a Balzers Union CPD 020 system. Thereafter were adhered to 13 mm aluminum stubs with conductive carbon adhesive pads and sputter-coated with a 60/40 Au/Pd alloy, to an approximate thickness of 10–15 nm, using a Polaron equipment coating unit. Sputter-coated were examined, imaged and analyzed at 20 Kv, using an static electron beam that interact with the sample producing a variety of emissions, including characteristic X-rays of different elements. The abundance of specific elements was determined, in 200 s and after 5000 readings, with the EDS system software.

2.6. Fruits morphometric, colorimetric and quality analysis

Height, diameter, density and dry weight were measured considering four randomized fruits per treatment. Colorimetric parameters, using fixed wavelength, followed Ramalho et al. (2018). Brightness (L) and chromaticity parameters (a* and b* coordinates) were obtained with a Minolta CR 300 colorimeter (Minolta Corp., Ramsey, NJ, USA) coupled to a sample vessel (CR-A504). Using the illuminant D_{65}, the system of the *Commission Internationale d'Éclaire* (CIE) was applied. The parameter L represents the brightness of the sample, indicating the variation of the tonality between dark and light, with a range between 0 (black) and 100 (white). Parameters a* and b*, indicate color variations between red (+60) and green (-60), and between yellow (+60) to blue (-60), respectively. The approximation of these coordinates to the null value translates neutral colors like white, gray and black. Measurements were carried out in triplicates in the pulp of the pears at harvest. Total soluble solids were measure in fruit juice, using a digital refractometer Atago (Atago, Tokyo, Japan), being the obtained values expressed as °Brix. Fruit firmness was evaluate with a Bellevue type penetrometer with a diameter of 0.5 cm (values expressed in kg). Following Soares et al. (2001), acidity was quantified in the juice of 5 randomized fruits per sample (4 samples per treatment), by acid-base titration and using 0.1 N sodium hydroxide and a pH meter Jenway, 350 model, England (values expressed in grams of malic acid per liter).

2.7. Heat-treated Rocha pear pulp: physical and chemical characteristics and sensory analysis

For monitoring the physicochemical characteristics and sensory analysis of the biofortified heat-treated Rocha pears, fruits were collected from orchard 1. Pulps of control and biofortified Rocha pears were separated, washed, peeled and weighed, being therefore added 1.5 % of lemon juice. The pulps were thermally treated in a thermomix (at 90 °C, during 10 min) and then, in triplicates, containers (glass bottles and plastic tubes with sanitized lid) were filled with the pulps in a water bath (under the same temperature and time conditions). To evaluate the rheological behavior, the consistency index (k, Pa.sn) was determined from the apparent viscosity values of the pulps (3 determinations per sample). The apparent viscosity of the pulps (expressed as Pa.s) was performed using a Brookfield viscometer model RVT (spindle 5), at rotational speeds of 2, 4, 10 and 20 rpm, under controlled temperature conditions (20 ± 1 °C) and ambient pressure. The obtained results were transformed into rheological measurements: strain velocity (s^{-1}) and apparent viscosity (Pa.s), which were use for elaboration of creep curves. Finally, from the power law, the consistency index (k) and flow index (n) was obtained. The color parameters were determined using the Minolta CR 300 colorimeter (Osaka, Japan) in the CIELab system (illuminant C). The calibration was performed with the white reference standard (L = 97.10; a* = 0.19; b* = 1.95). The mean values were obtained from 5 measurements per sample. By direct reading, parameters L, a* and b* were obtained. The pH was determined using potentiometer (Crison Micro pH 2001, Crison Instruments, Spain), by immersing the probe directly into the pulp. The mean values resulted from the average of three determinations per sample.

Soluble solids content was determined using a refractometer (DR-A1, ATAGO Co. Ltd., Japan), from juice obtained directly from the pulp

filtration. The results were expressed as °Brix, representing the percentage of soluble solids per 100 g of product, resulting from 3 determinations per sample.

A semi-trained panel of tasters (n = 19) carried out the tests for sensory evaluation, in a Sensory Analysis Laboratory, being the tasters individually located in booths according to ISO 8589 (1988). Based on a preset 5-point hedonic scale, varying gradually from "I greatly disliked" to "I liked it a lot", tasters were asked to classify the samples by noting the following sensory attributes appearance, color, consistency, aroma / taste and overall appreciation.

2.8. Statistical analysis

Data were statistically analyzed using a One-Way or Two-Way ANOVA ($P \leq 0.05$), to assess differences between treatments and experimental periods and, based on the results, a Tukey's for mean comparison was performed, considering a 95 % confidence level. For the Two-Way ANOVA, different letters in the columns and lines indicate significant differences between testing parameters for the same treatment, or between different treatments for the same parameter, respectively.

3. Results

3.1. Orchards characteristics for agronomic biofortification

Considering the contribution of soils to the enrichment of Ca in the edible part of Rocha pears, in this study two orchards with different characteristics were chosen for implementation of the biofortification itinerary. Orchard 1 presented planar morphology, varying its heights between 15.5 and 34 m, for the whole land (Fig. 1A-D), while orchard 2, although also of mild morphology, showed a slight slope to NE, with a variation of 12-meter dimensions, for the whole plot (Fig. 1E-H). Considering that the morphology of the orchards strongly influences the drainage of the surface waters, the slopes for each plot of land were calculated. Therefore, slope maps were classified in slope classes to differentiate the drainage, or surface drainage zones, from the planar zones that accumulates surface water and, consequently, promote its infiltration into the ground. Accordingly, orchard 1 presented (Table 1) a larger area with a propensity for surface water accumulation (about 80 %), relative to orchard 2 (which only had about 1/3 of the area with potential for accumulation of surface water). Therefore, unlike the orchard 2, relatively to superficial drainage, orchard 1 had a less need of artificial soil irrigation. Besides, from a geological point of view, both orchards are situated in the Upper Jurassic (Kimmeridgian, J^{3-4}) complex of sandstones and shales (*i.e.,* covering the sheets of Caldas da Rainha - 26D and Alcobaça - 26B of the 1:50000 scale geological mapping of Portugal). Orchard 2 is allocated on a modern alluvium overlying the aforementioned Jurassic complex, while orchard 1 is situated on sandstones and shales. Since soils preparation and fertilization in both orchards remained similar, differences in their constituents (lower average levels of nutrients in orchard 2) are not only related to this geological aspect (Table 1), but also with differentiate surface drainage (Fig. 1; Table 1). Similarly, relatively to orchard 2, the higher content of organic matter (*ca.* 18 %) in orchard 1 is linked to its upper tendency for accumulation. Moreover, in both orchards, the soils are slightly basic, being in the range of pH 6.5–7.5, which is ideal for agriculture / forestry, but the electrochemical conductivity of the soil was about 60 % higher in orchard 2, indicating a higher salt content (Table 1) and, consequently, a greater energy expenditure for water absorption (*i.e.*, osmotic effect) by roots. Soil analysis further showed significantly different levels of Ca, K, P, Fe, S, and Zn (Table 1), being higher in orchard 1 (2.96, 1.30, 1.58, 1.66, 1.18, 1.84 fold, respectively), but the threshold of toxicity was not reached (Table 1). Nevertheless, high levels of Pb and As contaminantion were found in both orchards. Water for irrigation in both orchards (Table 1) was of underground origin (facies hydrochemic bicarbonated sodic in orchard 2 and bicarbonated sodium calcium in orchard 1), with high salinity (concentration of salts evaluated, in terms of electrical conductivity, between 750 and 2250 µS / cm, at 20 °C). Waters belong to class C3S1, with an sodium adsorption index 3.6 and

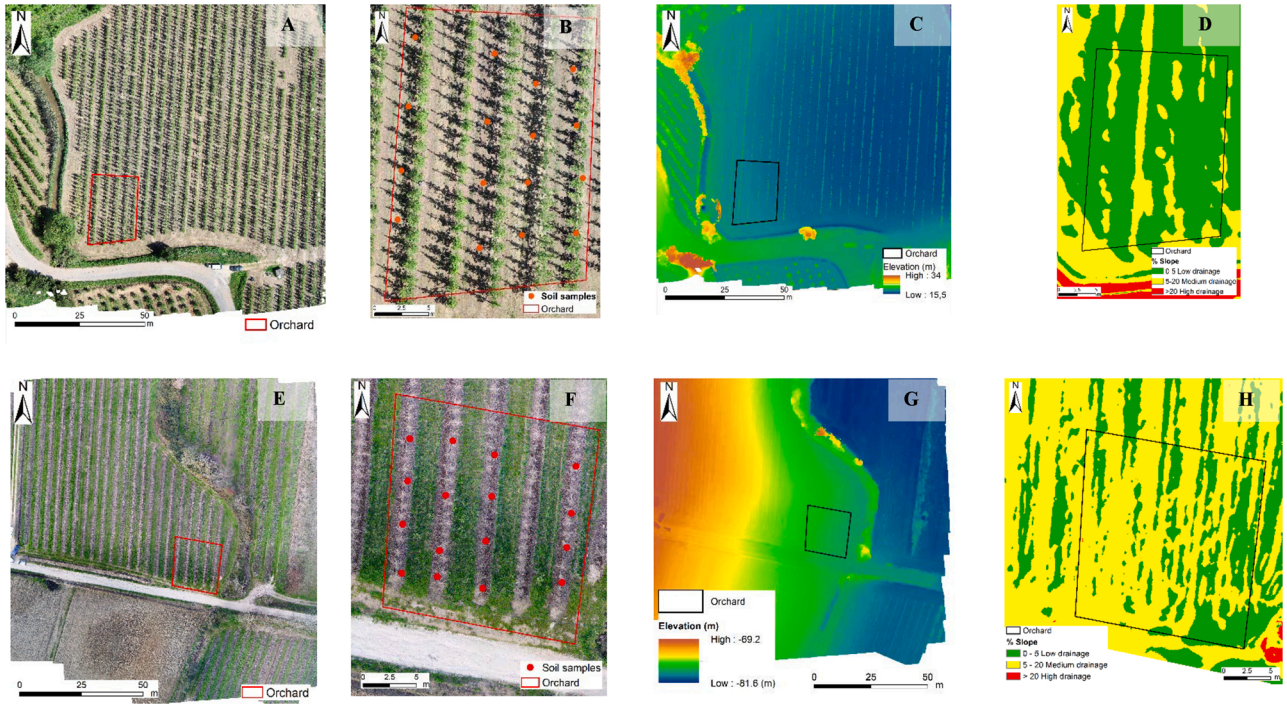


Fig. 1. Orthophotomaps of orchards 1 and 2 of *Pyrus communis* L., variety Rocha pear. Indication (in red) of limits of both orchards (A and E); Sampling points (in orange) for soil samples analysis for minerals (B and F); Digital elevation model of orchards (C and G); Digital map of slopes of orchards (D and H). Information collected before fruits harvesting and biofortification treatments (4 May for orchards 1 and 2, respectively). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

Table 1
Analysis of soils (0-30 cm deep) and irrigation water (n = 16) of orchards 1 and 2 of *Pyrus communis* L., variety Rocha pear. Letters a, b indicate significant differences, of each parameter, between both orchards (statistical analysis using the single factor ANOVA test, P ≤0.05).

Orchards	Ability to accumulate or drain surface water			
	Slope class (%)	Surface drainage	Area ()	Area (%)
1	1 - [0-5%]	Low	375.7	79.4
	2 -]5-20%]	Moderate	97.6	20.6
	3 - > 20%	High	-	-
2	1 - [0-5%]	Low	120.7	32.5
	2 -]5-20%]	Moderate	250.9	67.5
	3 - > 20%	High	0.04	0.01

	Soil analysis (0-30 cm deep)													
	pH	Electrical Conductivity	Organic Matter	Ca	K	Mg	P	Fe	S	Zn	Mn	Pb	As	Cu
		µS cm $^{-1}$			%						ppm			
1	7.41	205a	4.28a	1.33a	2.75a	0.42	0.19a	3.07a	71.4a	50.3a	481	17.2a	13.5a	41.9
2	7.30	332a	3.63b	0.45b	2.11b	0.42	0.12b	1.85b	60.7b	27.4b	234	15.0a	13.0a	66.5

	Water analysis										
	pH	Electrical Conductivity	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	Cl^{-}	HCO_3^{-}	SO_4^{2-}	NO_3^{-}	PO_4^{3-}
		µS cm $^{-1}$				mg L^{-1} (meq L^{-1})					
1	7.3	1467	94.9 (4.7)	2.8 (0.07)	37.8 (3.1)	99.5 (4.3)	113 (3.2)	312.3 (5.1)	48 (1.0)	5.5 (0.08)	0.4 (0.01)
2	7.5	1233	40.5 (2.0)	2.0 (0.05)	22.6 (1.8)	116.6 (5.0)	142 (4.0)	353.1 (5.7)	73 (1.5)	1.8 (0.02)	< 1.5 (<0.04)

Table 2
Average ± S.E of leaf gas exchange parameters, net photosynthesis (P_n), stomatal conductance to water vapor (g_s), and transpiration (E) rates, as well as variation in the instantaneous water use efficiency (iWUE) in leaves of *Pyrus communis* L., variety Rocha pear, submitted to Ca biofortification, from orchard 1, at 19 June 2018, 20 July 2018 and 11 September 2018 (after the 3rd, 4th and at harvest, respectively). For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between treatments for the same analytical date (a, b, c), or between analytical dates for each treatment (r, s, t) (statistical analysis using the single factor ANOVA test, P ≤0.05).

Leaves Spray	19 June 2018				20 July 2018				11 September 2018			
					P_n (µmo $CO_2 m^{-2} s^{-1}$)							
Ctr	14.86	±	0.44	b,s	17.74	±	0.63	a,r	16.03	±	0.59	a,r
0.4CCa	13.55	±	0.48	b,s	16.56	±	0.77	a,r	15.03	±	0.35	a,b,r
0.8CCa	13.95	±	0.44	b,s	16.88	±	0.24	a,r	12.58	±	0.20	b,r
1.6CCa	13.32	±	0.61	b,t	17.26	±	0.11	a,r	14.85	±	0.45	b,r,s
NCa0.1	16.91	±	0.34	a,r	16.54	±	0.32	a,b,r	14.63	±	0.24	b,r,s
NCa0.3	16.32	±	0.52	a,b,r,s	17.62	±	0.38	a,r	15.00	±	0.25	b,r
NCa0.6	15.00	±	0.38	b,s	17.22	±	0.25	a,r	12.73	±	0.42	c,s
					g_s (mmo H_2O $m^{-2} s^{-1}$)							
Ctr	171.25	±	14.70	c,s	218.44	±	9.19	b,r	330.33	±	16.72	a,r
0.4CCa	128.19	±	10.14	c,t	209.00	±	8.59	b,r	324.42	±	6.12	a,r
0.8CCa	132.61	±	7.94	c,t	239.39	±	3.99	b,r	222.50	±	4.86	a,s
1.6CCa	141.93	±	9.59	c,s,t	213.75	±	2.76	b,r	293.50	±	19.95	a,r
NCa0.1	227.57	±	9.86	b,r	204.14	±	5.84	b,r	294.17	±	13.46	a,r
NCa0.3	198.23	±	12.48	b,r,s	205.00	±	4.10	b,r	288.22	±	10.36	a,r
NCa0.6	177.05	±	8.37	b,s	232.05	±	4.87	a,r	234.41	±	13.42	a,s
					E (mmo H_2O $m^{-2} s^{-1}$)							
Ctr	3.44	±	0.07	b,r	2.30	±	0.06	c,r	5.87	±	0.17	a,t
0.4CCa	3.77	±	0.11	b,r	2.42	±	0.06	c,r	7.98	±	0.13	a,r
0.8CCa	3.85	±	0.08	b,r	2.75	±	0.03	c,r	7.15	±	0.05	a,s
1.6CCa	4.14	±	0.11	b,r	2.67	±	0.01	c,r	7.99	±	0.25	a,r
NCa0.1	4.23	±	0.08	b,r	2.27	±	0.04	c,r	6.61	±	0.13	a,s
NCa0.3	4.03	±	0.13	b,r	2.39	±	0.04	c,r	6.70	±	0.12	a,s
NCa0.6	4.05	±	0.09	b,r	2.61	±	0.03	c,r	6.69	±	0.14	a,s
					iWUE (mmol CO_2 mol^{-1} H_2O)							
Ctr	4.32	±	0.09	b,r	7.69	±	0.11	a,r	2.74	±	0.08	c,r
0.4CCa	3.58	±	0.05	b,s	6.79	±	0.19	a,s	1.89	±	0.04	c,s
0.8CCa	3.62	±	0.08	b,s	6.16	±	0.12	a,s,t	1.76	±	0.03	c,s
1.6CCa	3.19	±	0.09	b,t	6.47	±	0.01	a,s	1.86	±	0.03	c,s
NCa0.1	4.00	±	0.07	b,s	7.29	±	0.11	a,r	2.21	±	0.02	c,s
NCa0.3	4.05	±	0.05	b,r,s	7.38	±	0.12	a,r	2.24	±	0.03	c,s
NCa0.6	3.71	±	0.06	b,s	6.62	±	0.12	a,s	1.90	±	0.04	c,s

2.1 (orchards 2 and 1, respectively). Additionally, these waters were subsaturated in calcium carbonate, having pHe of 7.9 and 7.6 and a Langelier saturation index of -0.38 and -0.30, in orchards 2 and 1, respectively.

3.2. Physiological monitoring during biofortification

Assuming orchard 1 as a test system, pulverization with increasing amounts of $CaCl_2$ showed a relatively small but persistent drop of P_n (ranging between 5–18 %), but spraying with $Ca(NO_3)_2$ promoted a marginal, null increase or a decrease of P_n (Table 2). During the experimental periods, in each treatment, leaves sprayed with Ca significantly increased (Table 2) g_s and E (excepting, for all treatments at 20 July) from the 1st to the last determination. With few exceptions, the application of $CaCl_2$ and $Ca(NO_3)_2$ did not alter these variation patterns. However, in the 1st and 3rd applications of $CaCl_2$, the increase of g_s became attenuated (relatively to the control, significantly in several cases) and iWUE decreased (between 12 % and 27 % up to the 5th application and mainly between 31 % and 36 % after the 7th application). Moreover, within each experimental period, relatively to the control, $CaCl_2$ in general promoted a decrease of g_s, but for $Ca(NO_3)_2$ this effect only did not occur at 19 June. Also, within each experimental period, relatively to the control, E in general increased.

3.3. Kinetics of Ca accumulation in fruits

The kinetics of Ca accumulation in the whole fruits was assessed in orchard 1 (Table 3), being found, after the 2nd spray (8 June 2018), that Ca contents tended to be lower with application of $Ca(NO_3)_2$, when compared to the application of $CaCl_2$. Comparing all treatments, it was also found that only 0.8CCa showed the highest Ca content. After the 3rd spray, now only with $CaCl_2$ (4 kg ha^{-1}), lower Ca values were measured in the control (with the exception of the treatment with NCa0.6), being found significant differences regarding the treatments NCa0.4 and 1.6CCa. At this stage, macroscopic symptoms of phytotoxicity could not be detected, which determined the increase of $CaCl_2$ leaves sprays to 8

Table 3
Average of Ca contents ± SE (n = 3) in all, or considering 5 sections (ranging from the epidermis to the centre, 1, 2, 3, 4, 5), of the equatorial region of the fruits of *Pyrus communis* L., variety Rocha pear, from orchards 1 and 2. Letters a, b, c, d, e, f indicate significant differences of Ca contents between treatments (statistical analysis using the single factor ANOVA test, $P \leq 0.05$). Ctr = control; NCa0.1, NCa0.3, NCa0.6 correspond, respectively, to the initial application of 0.1 %, 0.3 % and 0.6 % Ca $(NO_3)_2$; 0.4CCa, 0.8CCa, and 1.6CCa, correspond respectively to the initial application of $CaCl_2$ 0.4 %, 0.8 % and 1.6 %.

Treatments	Ca contents ($\%_{DW}$ ± S.E.)							
	Orchard 1							
	Average value in the whole fruit							
	2nd leaf application 8/6/2018		3rd leaf application 15/6/2018		5th leaf application 20/7/2018		At harvesting 4/9/2018	
Ctr	0.17a,b	±0.01	0.18c	±0.01	0.23d	±0.01	0.06d	±0.00
NCa0.1	0.16b	±0.01	0.20b,c	±0.00	0.33a,b,c	±0.01	0.09b	±0.00
NCa0.3	0.16b	±0.00	0.24a	±0.00	0.27c,d	±0.01	0.10a	±0.00
NCa0.6	0.18a,b	±0.01	0.15d	±0.00	0.38a,b	±0.03	0.09b	±0.00
0.4CCa	0.17a,b	±0.01	0.19c	±0.01	0.41a	±0.03	0.09b	±0.00
0.8CCa	0.20a	±0.00	0.18c	±0.01	0.32b,c,d	±0.01	0.08b,c	±0.00
1.6CCa	0.17a,b	±0.01	0.22a,b	±0.00	0.31b,c,d	±0.01	0.08c	±0.00

Location of the 5 regions	Mean values in the equatorial region of the fruit					
	1	2	3	4	5	Total
Ctr	0.10±0.00	0.06±0.00	0.06±0.00	0.07±0.00	0.11±0.01	0.10±0.00
NCa0.1	0.42±0.02	0.16±0.01	0.14±0.01	0.27±0.01	0.24±0.01	0.29±0.01
NCa0.3	0.11±0.01	0.09±0.01	0.09±0.00	0.12±0.01	0.13±0.01	0.16±0.01
NCa0.6	0.40±0.02	0.12±0.01	0.15±0.01	0.18±0.01	0.31±0.02	0.28±0.01
0.4CCa	0.27±0.01	0.16±0.01	0.52±0.03	0.65±0.03	0.90±0.05	0.64±0.03
0.8CCa	0.28±0.01	0.20±0.01	0.13±0.01	0.14±0.01	0.18±0.01	0.19±0.01
1.6CCa	0.49±0.02	0.09±0.00	0.08±0.00	0.08±0.00	0.14±0.01	0.14±0.01

Treatments	Orchard 2							
	Average value in the whole fruit							
	2nd leaf application 8/6/2018		3rd leaf application 15/6/2018		5th leaf application 20/7/2018		At harvesting 10/9/2018	
Ctr	0.20a	±0.01	0.35a	±0.02	0.33a	±0.03	0.14c	±0.01
NCa0.1	0.17b	±0.00	0.24b	±0.01	0.31a,b	±0.01	0.12d	±0.00
NCa0.3	0.16b	±0.00	0.24b	±0.00	0.34a,b	±0.03	0.18b	±0.01
NCa0.6	0.15b	±0.00	0.22b,c	±0.01	0.22b	±0.01	0.09e	±0.00
0.4CCa	0.15b	±0.01	0.20b,c	±0.01	0.25b	±0.02	0.06f	±0.00
0.8CCa	0.15b	±0.00	0.24b	±0.01	0.22b	±0.01	0.22a	±0.01
1.6CCa	0.14b	±0.00	0.19c	±0.01	0.23b	±0.01	0.17b	±0.01

Location of the 5 regions	Mean values in the equatorial region of the fruit					
	1	2	3	4	5	Total
Ctr	0.12±0.01	0.05±0.00	0.10±0.01	0.10±0.01	0.14±0.01	0.14±0.01
NCa0.1	0.73±0.04	0.06±0.00	0.09±0.00	0.14±0.01	0.28±0.01	0.22±0.01
NCa0.3	0.44±0.02	0.10±0.00	0.10±0.01	0.10±0.01	0.34±0.02	0.26±0.01
NCa0.6	0.32±0.02	0.07±0.00	0.08±0.00	0.10±0.00	0.59±0.03	0.31±0.02
0.4CCa	0.77±0.04	0.09±0.00	0.11±0.01	0.13±0.01	0.41±0.02	0.27±0.01
0.8CCa	0.26±0.01	0.09±0.00	0.11±0.01	0.19±0.01	0.80±0.04	0.35±0.02
1.6CCa	0.65±0.03	0.10±0.00	0.22±0.01	0.31±0.02	0.44±0.02	0.30±0.01

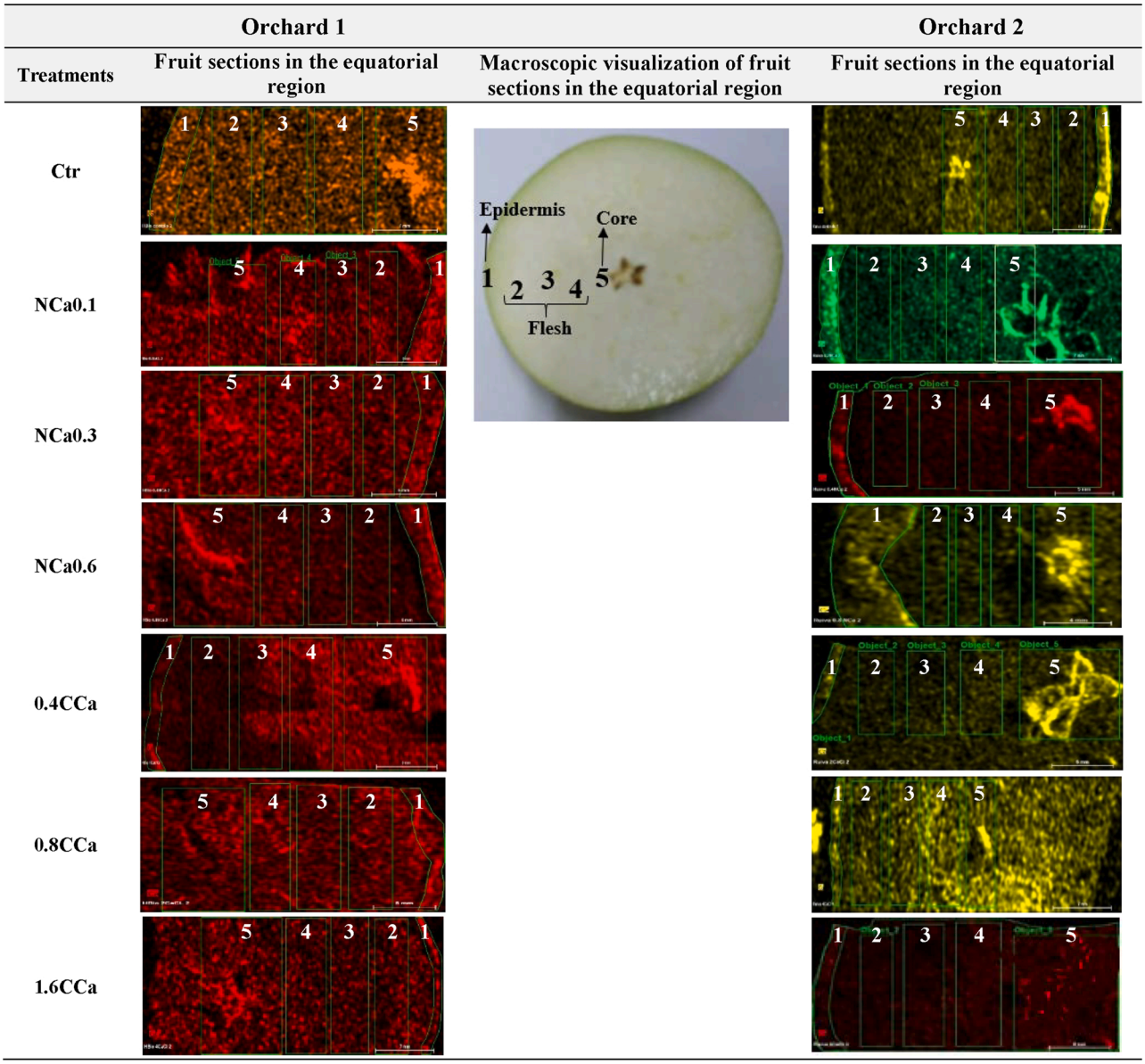


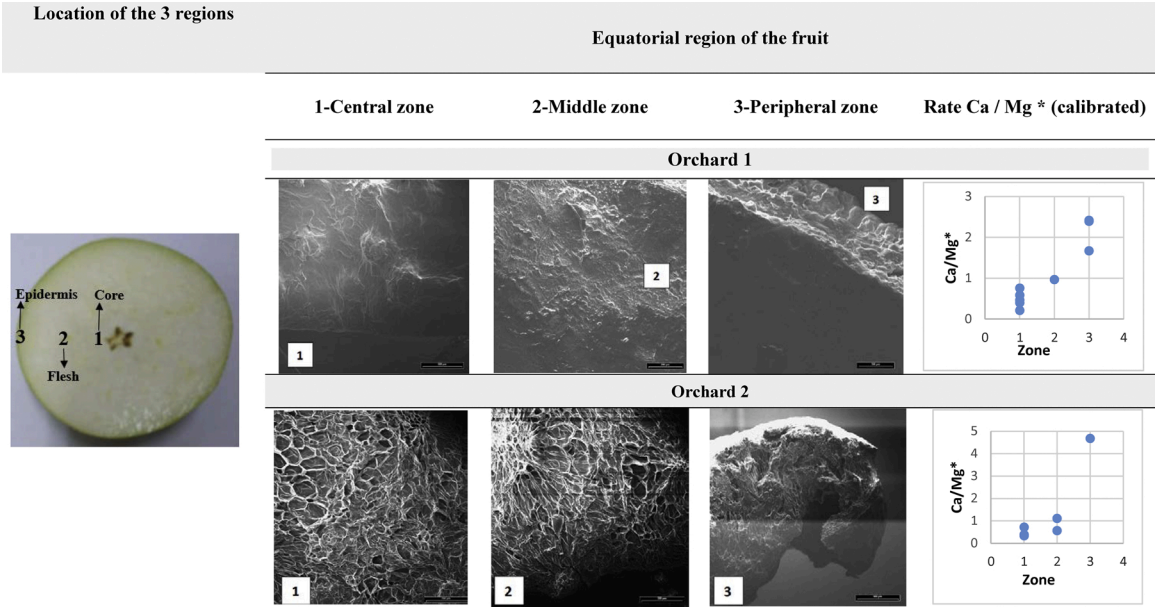
Fig. 2. Fruit sections represented with green lines (from the epidermis to the centre, 1, 2, 3, 4, 5) of each treatment, from the equatorial region of the fruits of *Pyrus communis* L., variety Rocha pear, of orchards 1 and 2. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

kg ha^{-1}. Monitoring Ca accumulation persisted after the 5th spray, and the respective contents (except for treatment 1.6CCa) were found to be substantially lower in the control (relatively to all treatments). At harvest, Ca contents in the whole fruits was still significantly lower in the control, being found in all treatments an average biofortification index for Ca ranging from 47 %–63 %. Moreover, a heterogeneous distribution of Ca within fruits was found (Fig. 2; Table 3), with its equatorial region showing for all treatments (substantially after the control) higher contents in the epidermal and in the central regions. In orchard 2 (Fig. 2; Table 3), after the 2nd spray with $CaCl_2$ and $Ca(NO_3)_2$, significantly higher Ca contents were found in the control. After the 3rd spray, now also only with $CaCl_2$ (4 kg ha^{-1}), significantly higher levels of Ca were measured in the control (relative to the remaining treatments). After the 5th spray, at this stage also after leaves spray with $CaCl_2$ (8 kg ha^{-1}), it was found that Ca values were significantly lower in all treatments previously submitted to $CaCl_2$ (0.4CCa, 0.8CCa and 1.6CCa) and NCa0.6. Moreover, at harvest, accumulation of mineral elements, showed against the control, significantly higher Ca contents in treatments NCa0.3, 0.8CCa and 1.6CCa. The average biofortification index in Ca ranged between 24 %–59 % but, like in orchard 1, total Ca accumulation in the equatorial region relative to the control, was substantially higher in all treatments (Fig. 2; Table 3). Besides, Ca contents in all treatments (substantially after the control) were higher in the epidermal region and in the central region of the fruit. Considering that Mg contents remained similar among treatments (data not shown), the ratio Ca/Mg in the equatorial region of Rocha pears was further assessed (Table 4), being again confirmed that at harvest, in both orchards, Ca contents also prevailed in the vicinity of the epidermis.

3.4. Physicochemical characteristics of fresh fruits

In orchard 1, no significant variations in height and diameter were observed (except on 15 June, treatments 0.4CCa, 0.8CCa and 1.6CCa) during the phenological development of fruits (Table S_1). At harvest, fruits height varied between 72.2–84.4 mm, with the diameter oscillating between 61.4–70.0 mm. The colorimetric analysis of the fruit pulp

Table 4
X-rays dispersive spectroscopy (SEM-EDS) of fruit samples of *Pyrus communis* L., variety Rocha pear, from orchards 1 and 2, with indication of the 3 equatorial regions (1-central zone, 2-middle zone and 3- peripheral zone next to the epidermis), where the readings were taken, to calculate the ratio between the Ca / Mg* ratio (calibrated) of treatment 1.6CCa (in the phase immediately after harvest – 4 September 2018). Reference samples were analyzed and, for biofortification in Ca, the Mg was used as calibration element, to avoid interference of concentration variations of the most abundant elements, such as N.



during its development, relatively to the control, showed lower values for parameter L in treatments NCa0.3 and 0.8CCa, after the 3rd and 5th leaves sprays (Table S_1). Parameter a* showed, following the 2nd leaves spray, the highest values in the control, with the lowest positive values being found in NCa0.3 following the 3rd leaves spray, and the highest positive value occurring in 0.8CCa after the 5th application (Table S_1). Parameter b*, after the 2nd pulverization showed, relatively to the control and all the treatments with $Ca(NO_3)_2$, significantly lower values in all treatments with $CaCl_2$. After the 3rd and 5th leaves spray the lowest values were detected with 1.6CCa and 0.8CCa, but at harvesting no significant differences were found (Table S_1).

In orchard 2, no significant variations in height and diameter were observed (except on 15 June and 10 July, in treatments NCa0,3 and NCa0.6, respectively) during the phenological development of the fruits (Table S_2). At harvest the height of the fruits ranged from 79.6 to 93.6 mm, with the diameter oscillating between 61.3–71.0 mm. The colorimetric analysis of fruits pulp, in the course of its phenological development (Table S_2), relative to parameters L and b*, following the 2nd leaves application, showed the highest value in 0.8CCa but, thereafter, significant variations could be found among treatments, until harvesting. Parameter a* after the 2nd pulverization also showed (Table S_2) the highest value in 0.8CCa. After the 3rd leaves spray maximum values for parameter a* were found in the control, but thereafter significant variations could not be found too (although at harvesting negative values were detected).

At harvest, density and dry weight of fruits collected in orchard 1 did not show significant variations between treatments (Table 5), with density oscillating around 1029 - 1109 kg m^{-3} and dry weight ranging

Table 5
Average ± SE (n = 4) of density, dry weight, firmness, °Brix and malic acid of fruits from *Pyrus communis* L, variety Rocha pear, from orchards 1 and 2, at harvesting (4/09/2018 and 10/09/2018). Letters a, b, c, d indicate significant differences, of each parameter, between the treatments (statistical analysis using the single factor ANOVA test, $P \leq 0.05$). Ctr = control; NCa0.1, NCa0.3, NCa0.6 correspond, respectively, to the initial application of 0.1 %, 0.3 % and 0.6 % $Ca(NO_3)_2$; 0.4CCa, 0.8CCa, and 1.6CCa, correspond respectively to the initial application of $CaCl_2$ 0.4 %, 0.8 % and 1.6 %.

Treatments	Density (kg m^{-3})		Dry weight (%)		Firmness (N)		°Brix		Malic acid (g/L)	
	Fruits	S.E.	Fruits	S.E.	Fruits	S.E.	Fruits	S.E.	Fruits	S.E.
					Orchard 1					
Ctr	1029a	±15	13.39a	±0.22	68.99b,c	±0.78	11.0a	±0.2	1.05b	±0.13
NCa0.1	1064a	±20	13.99a	±0.35	65.76c,d	±0.69	11.4a	±0.1	1.54a	±0.07
NCa0.3	1037a	±13	14.05a	±0.47	67.33b,c,d	±0.69	11.5a	±0.1	1.47a	±0.03
NCa0.6	1050a	±5	13.18a	±0.36	67,62d	±0.78	11.1a	±0.1	1.48a	±0.03
0.4CCa	1056a	±2	14.49a	±0.44	74.28a	±0.69	11.0a	±0.2	1.41a	±0.03
0.8CCa	1072a	±26	14.59a	±0.21	71.70b	±1.67	11.2a	±0.2	1.38a	±0.04
1.6CCa	1109a	±6	13.77a	±0.26	66.64b,c,d	±1.27	11.1a	±0.1	1.37a	±0.03
					Orchard 2					
Ctr	1056a	±7	14.35b,c	±0.24	60.17a	±0.69	10.9b	±0.1	1.20b,c,d	±0.03
NCa0.1	1034a	±22	12.85d	±0.13	56.35b,c	±0.78	11.7a	±0.1	1.39a,b	±0.02
NCa0.3	1049a	±16	15.00a	±0.04	58.70a,b	±0.49	11.9a	±0.2	1.44a	±0.10
NCa0.6	1045a	±12	14.04c	±0.09	58.70a,b	±0.59	11.2b	±0.1	1.37a,b,c	±0.02
0.4CCa	1019a	±20	15.06a	±0.15	56.15b,c	±0.78	10.5c	±0.1	1.04d	±0.03
0.8CCa	1051a	±20	13.94c	±0.15	58.70a,b	±0.59	10.7b,c	±0.2	1.14c,d	±0.05
1.6CCa	1030a	±26	14.76a,b	±0.14	55.47c	±0.69	10.5c	±0.1	1.20b,c,d	±0.05

between 13.18 % and 14.59 %. Fruits treated with $CaCl_2$ showed an increased firmness in 0.4CCa, but a reduction therefore occurred until 1.6CCa (Table 5). No alterations were observed for total soluble solids content in any treatments, but all the modalities had higher values of acidity relatively to the control fruits (Table 5). In orchard 2, at harvesting, the density did not show significant variations among treatments, ranging from 1019 - 1056 kg m^{-3} and dry weight between 12.9–15 % (Table 5). However, dry weight, relative to the control, had significantly higher values in NCa0.3 and 0.4CCa. Treatment NCa0.6 showed, relative to the control, a significant decrease in firmness and treatments with $CaCl_2$, showed a progressive firmness reduction (Table 5). Moreover, the contents of total soluble solids did not vary significantly among $Ca(NO_3)_2$ treatments, but fruits treated with $CaCl_2$, showed lower values than the control (Table 5). Regarding acidity, relatively to the control fruits treated with $Ca(NO_3)_2$ showed higher values, whereas application of $CaCl_2$ did not revealed significant deviations (Table 5).

3.5. Physicochemical characteristics and sensory analysis of heat-treated fruits

Assuming, as a test system, heat-treated Rocha pears pulp, collected at harvest in orchard 1, significant variations could not be found (Table 6) for pH, colorimetric parameters (excepting parameter b*) and total soluble solids, between the control and the highest $CaCl_2$ treatment (1.6CCa). Nevertheless, a higher viscosity was detected in control fruits. Sensory analysis of the Rocha pears pulp (Control and 1.6CCa) collected in orchard 1 showed a slight tendency towards greater acceptability in parameters "Appearance" and "Color" (only external attributes). However, in general, samples in the sensory plane were similar, and biofortification did not influence their acceptability.

4. Discussion

Although soils are natural bodies, having both mineral and organic components, as well as physical, chemical and biological processes, agronomic biofortification also is not a simple reflection of the combined properties of all soil components. Bioavailability of nutrients throughout the entire pathway from soils to plants further depends on the relief and type of land use, with the slope and slope length being the most important factors that control the intensity and frequency of surface runoff and therefore sediment / fertilizers loss. Considering this background, to verify the effectiveness of natural Ca enrichment in Rocha pears, the biofortification itinerary was applied in two orchards having contrasting characteristics, namely soils with different sloping surfaces (Fig. 1), electric conductivity and contents of organic matter and minerals, as well as irrigation waters that presented a sharply different composition (Table 1). It is well known that soil erosion tends to occur faster on sloping surfaces, but rates may also be sensitive to other factors, namely to the amounts of water supplied (*eg*, irrigation watering). Accordingly, relatively to orchard 1, in the soil of orchard 2, with a higher sloping surface, prevailed a higher rate of alluviums and a lower organic matter accumulation (which additionally decreased water retention) (Teixeira et al., 2011). Besides, although soils of both orchards revealed a slightly basic pH, orchard 2 also had a higher electric conductivity and a lower amount of Ca. Considering that in slightly alkaline soils Ca^{2+} is the dominant cation, and that organic matter has higher levels of colloidal materials, ions (K, P, Fe, S, Zn, Mn and Pb) also predominated in orchard 1. Additionally prevailed a lower energy expenditure for roots uptake in the soil of orchard 1, as seen by its lower electrical conductivity, even though the pH be suitable for agriculture in both orchards. Concerning to the irrigation water (Table 1), as indicated by the Langelier saturation index, orchard 2 had a slightly higher tendency to dissolve calcium carbonate, which favors root uptake and accumulation of Ca in plants. Nevertheless, water of both orchards did not represented a danger for soils alkalization, due to the low Na concentration relatively to Ca and Mg.

Calcium is involved in many pathways of plant cells, including plant growth, development, resistance to environmental stress (Liang et al., 2009; Knight et al., 2010) and photosynthesis (Quan et al., 2019). As Ca signals constitute a massive and complex signaling network in plant cells, the responses of multiple pathways might be affected by only one Ca pathway with different response levels. Following, this assumption, trees from orchard 1 were used as a test system for monitoring the physiological state of the trees during the Ca biofortification process. It was found that the small drop of P_n found during fruits development, when increasing amounts of $CaCl_2$ were applied, counteracted with a marginal, null increase or a decrease after application of $Ca(NO_3)_2$, with g_s and E keeping a relatively similar increasing tendency (Table 2). Accordingly, P_n inhibition suggested a cumulative effect of Ca, especially at the highest dose (0.8 kg $CaCl_2$ ha^{-1}) after the 7th application, where the largest decrease was recorded (21 %) compared to that day's control. These data suggested that leaves spray with increasing Ca concentrations triggered downstream events at the metabolic pathways in the chloroplasts, which eventually could implicate Ca^{2+}-binding proteins, such as s-adenosylmethionine transporter-like (Stael et al., 2011) and chloroplast inner envelope protein (Aronsson and Jarvis, 2009). Moreover, the pattern of g_s and E in conjunction with the variations of P_n, triggered a decreasing iWUE. It is well known that stomatal movement is regulated by the water content (*eg*, soluble sugars) of guard cells and the presence of amylase in these cells can regulate water content, through soluble sugars produced by degradation of starch to regulate stomatal movement (Lawson, 2009; Leshem and Levine, 2013). Therefore the patterns of g_s and E, developed during the itinerary of Ca biofortification (Table 2), further suggested that increasing leaves Ca spraying promoted their metabolic unbalance, potentially implicating reactive oxygen species. Indeed, as a key signal of stomatal regulation (Song et al., 2014), reactive oxygen species are mainly produced in chloroplasts (Asada, 2006), with the production of H_2O_2activating $[Ca^{2+}]_{cyt}$ channels (Allen et al., 2000; Sparla et al., 2006), and the Ca^{2+} sensors involved in further generation of H_2O_2, which induces $[Ca^{2+}]_{cyt}$ oscillation, causing stomatal closure. Nevertheless, the extent of this physiological disorder was restricted, since a high metabolic activity and sink strength to utilize photosynthates are decisive to sustain high C acquisition in response to increasing $[CO_2]$ (Rodrigues et al., 2016). This was the case in pear trees, as plants displayed a high allocation of resources to continuous vegetative growth of leaves and branches, with fruits production occurring without relevant deviations in all the biofortified treatments (Table 5).

Calcium is involved in nearly all aspects of plants development (Hocking et al., 2016). During the early stages of young fruit development, Ca absorption is mainly involved in cell division and metabolism, specifically during initial expansion, but during the later stages development is mainly involved in cell-to-cell junction (Hocking et al., 2016).

Table 6
Physical and chemical characteristics of heat-treated pulp (at 90 °C during 10 min) of fruits (n = 3) from *Pyrus communis* L, variety Rocha pear (average values ± SE for Ctr and treatment 1.6CCa) produced in orchard 1. Ctr = Control; 1.6CCa, correspond to the initial application of 1.6 % $CaCl_2$. Letters a, b indicate significant differences of each parameter, between the treatments (statistical analysis using the single factor ANOVA test, P ≤0.05).

Heat-treated Parameters		Ctr		1.6CCa	
		Average	S.E	Average	S.E.
pH		4.58a	±0.08	4.66a	±0.00
Brix (%)		11.53a	±0.31	11.10a	±0.14
Colorimeter parameters	L	35.61a	±0.36	34.67a	±0.01
	a*	2.69a	±0.48	1.25a	±0.30
	b*	3.65a	±0.02	3.44b	±0.03
Viscosity	K (Pa.sn)	5459a	±296	3118a	±1215
	n	0.23a	±0.04	0.20a	±0.00

Yet, as Ca deficiency drop off, younger fruit vulnerable to this mineral deficiency and can lead to membrane breakdown implicating fruit disorders (Freitas et al., 2012). Thus, in both orchards, after fruit set, the agronomic biofortification started with two leaf spraying with increasing levels of $CaCl_2$ or $Ca(NO_3)_2$. Besides, both chemical forms were sprayed to determine if each one could trigger toxicity or develop abnormalities (Hocking et al., 2016) in the higher concentrations, but no symptoms of toxicity were detected. The experimental design at this stage considered that Ca application can be more effective during the early fruits development stage. For instance, Ca^{2+} deficiency during tomato development causes blossom-end rot (Freitas et al., 2012). Additionally, Zhou (1999) also found that $CaCl_2$ treatment increased the electron density of the cell wall, cell gap, plastid, and enhanced the stability of the cell wall structure. Following these initial treatments, the successive leaf application of increasing $CaCl_2$ concentrations in both orchards considered the enrichment of cell-to-cell intersections in Ca (Hocking et al., 2016), therefore further increasing the biofortification index. Indeed, Ciccarese et al. (2013) found that pre-veraison Ca^{2+} applications greatly enhanced Ca^{2+} concentrations in the skin tissues of grapes, and Ca^{2+} applications significantly improved flesh firmness and reduced the occurrence of *Botrytis cinerea* rots when in storage. Zhang and Wang (2019) also found that pectic acid in cell walls could be combined with Ca to form calcium pectate, constituting the cellular skeletal structure, preventing the disintegration of the gel layer in the cells. Nevertheless, after application of this biofortification itinerary, different accumulation indexes were obtained (Table 3). These data strongly correlated with the heterogeneity of both orchards. Orchard 1, relatively to orchard 2, tended to accumulate more irrigation water and had higher level of organic matter, lower electric conductivity and higher levels of Ca in the soil (Fig. 1; Table 1), which determined an additional Ca supply from the root system and transport to fruit development. Nevertheless, through this pathway, differences between Ca biofortification indexes found in both orchards were limited confirming that Ca accumulations in the fruits were also dependent on xylem delivery, due to the low phloem mobility of Ca^{2+} (Dražeta et al., 2004).

Calcium leaves spray and root uptake from soils further determined a heterogeneous distribution within fruits tissues (Fig. 2; Table 3), or considering the ratio Ca/Mg (Table 4). Calcium prevailed in the epidermal region mostly due to leaves spray but at a lesser extent further accumulated in the central region because of xylem delivery after roots uptake and transport. This unbalanced and higher accumulations of Ca did not affect substantially the physicochemical characteristics of the fruits in both orchards (Tables S_1, S_2, 5) and eventually could reduce fruits respiration rates and ethylene production during storage (Ferguson, 1984). In general, at harvest, and among treatments, the height, diameter and color parameters of the fruits remained similar in both orchards (Tables S_1, S_2). Moreover, during fruits development, some significant differences among these parameters could be attributed to some physiological heterogeneity associated to leaves spray and xylem distribution. In fact, until harvesting, exogenous Ca applications could interact with protein and chlorophyll content, cell membrane fluidity, and respiration rates, which are important parameters of senescence or reduce the accessibility of cell wall-degrading enzymes to their substrates by binding to the cell wall components (Shewfelt and Prussia, 2009). Calcium could further crosslink pectic acid residues, stabilizing at a different extent the cell membrane (Hui et al., 2009; Poovaiah et al., 1988).

The biofortified fruits of both orchards did not vary significantly relatively to the control, but again some heterogeneity occurred with the dry weight in the fruits of orchard 2 (Table 5). Interesting was to notice that, relatively to orchard 2, firmness remained higher in the fruits of orchard 1, which correlated with the higher biofortification index for Ca (Table 3). Indeed, Ca effectively maintain fruit firmness and delay fruit softening and ripening (Zhang and Wang, 2019). Moreover, the levels of malic acid and total soluble solids (Table 5), although showing some variation among treatments, remained quite similar between both orchards, which indicated the absence of inhibitory interactions with Ca. Heat-treated pear pulp, further revealed the absence of depreciative the physical and chemical characteristics prompt by Ca biofortification (Table 6), with the sensory analysis confirming their acceptability (Fig. 3).

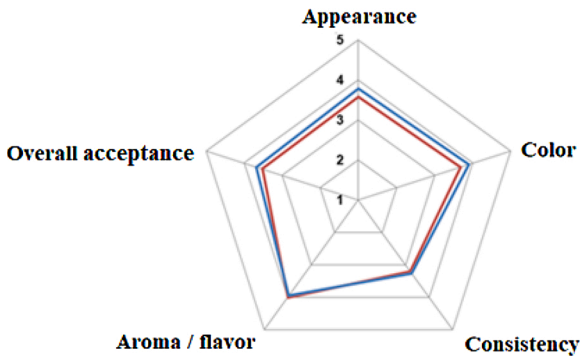


Fig. 3. Average values (n = 19) of the sensory analysis of heat-treated (90 °C / 10 min) Rocha pear pulp collected in orchard 1. Ctr (▬) and biofortified with 1.6CCa (▬). Ctr = control; 1.6CCa, correspond to the initial application of 1.6 % $CaCl_2$.

5. Conclusion

Orchards of *Pyrus communis* L. variety Rocha, having different soil and water irrigation types, did not reach the threshold of toxicity after leaves spray in the early stages of fruits development with 0.6 kg Ca $(NO_3)_2$ ha^{-1} or 1.6 kg $CaCl_2$ ha^{-1}. Thereafter, promoting Ca biofortification with leaves pulverization up to 8 kg $CaCl_2$ ha^{-1} revealed only minor inhibitions at the photosynthetic machinery, but visual symptoms of toxicity could not be found. Nevertheless, although soil and irrigation water heterogeneity could triggered slightly different biofortification indexes, Ca gathering prevailed in the epidermal region of the fruits, mostly due to leaves spray, whereas at a lesser extent accumulated in the central region due of xylem delivery from roots uptake and transport. Calcium biofortification did not substantially affected the physicochemical characteristics of the fresh or heat-treated fruits or its sensory acceptability after heat treatment, which becomes an additional warranty for consumer's expectations.

Declaration of Competing Interest

The authors declare that there are not any kind of conflict of interest at an institution or personal levels.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2020.109834.

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