

## Absorption and mobility of radio-labelled calcium in chili pepper plants and sweet cherry trees

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**ABSTRACT:** Calcium (Ca) is often supplied to crop species to prevent the occurrence of Ca-related disorders. Mechanisms of Ca absorption and transport are not fully understood and the effectiveness of root and/or foliar Ca fertilization may be variable. To characterize the rate of Ca absorption and transport, trials were developed with chili pepper and sweet cherry plants, using <sup>45</sup>CaCl<sub>2</sub> as a tracer. The Ca treatments supplied were: (1) No <sup>45</sup>Ca (control); (2) <sup>45</sup>Ca soil application; (3) <sup>45</sup>Ca supply to basal leaves, and (4) <sup>45</sup>Ca application to apical leaves. After two months, plants were harvested for biomass and Ca content determination. The recovery of <sup>45</sup>Ca in different plant parts was measured with a liquid scintillation counter and leaf traits were observed by scanning electronic microscopy. In general, the highest <sup>45</sup>Ca concentrations were recovered in treated organs, while root applications led to highest <sup>45</sup>Ca translocation rates, which varied between chili pepper and cherry plants. For chili pepper, <sup>45</sup>Ca applied to the soil was detected mainly in roots (44 %) followed by leaves (36.6 %) stems (17.4 %) and fruits (2 %). In sweet cherry trees, soil-applied <sup>45</sup>Ca was principally recovered in roots (45.3 %), shoots (28.5 %), leaves (14.3 %) and trunks (11.9 %). The results provide evidence of increased absorption of root-applied Ca, as well as different degrees of Ca mobility between species. Foliar application led to major Ca increases in treated leaves, with Ca transported to other plant organs after apical leaf Ca supply chiefly in cherry trees.

**Keywords:** fertilizers, fertilizer application methods, horticultural crops, plant nutrition, radioisotopes

## Introduction

Calcium is an essential plant nutrient mainly for cell wall formation, cellular signalling responses, and cell membrane stability (Marschner, 2012). However, Ca ion (Ca<sup>2+</sup>), along with organic acids (e.g., in vacuoles), can form strong precipitates, limiting Ca mobility (de Freitas et al., 2015). Calcium is relatively phloem immobile and thus contribution of phloem transport may be limited (Montanaro et al., 2014; Song et al., 2018). Calcium absorbed from the soil solution is transported from the roots via xylem to different tissues and organs (Khalaj et al., 2016; Saure, 2005). However, root Ca<sup>2+</sup> uptake decreases with increasing distance from the root apex (Marschner, 2012) and it is much higher in apical than in basal root zones (Saure, 2005). Transport of Ca to aerial plant organs depends on several factors, such as xylem sap Ca<sup>2+</sup> concentration, balanced mineral nutrition, water uptake and plant water potential, transpiration and growth rate (Hocking et al., 2016; Sourì and Hatamian, 2019). Excessive Ca accumulation may occur in organs with high transpiration rates (i.e., leaves; de Freitas and Mitcham, 2012; de Freitas et al., 2015), while low transpiring organs may suffer localized Ca deficiencies, as reported for several fruit and vegetable crops (e.g., Sampaio et al., 1999; Val et al., 2008; Marschner, 2012).

Nowadays, foliar fertilizers are widely used as complementary treatments to root fertilization (e.g., Sourì and Sooraki, 2019) and for supplying elements with limited plant mobility, such as Ca or micronutrients

(Gomes et al., 2020; Fernández and Brown, 2013; Torres et al., 2017). When Ca is applied to the roots, it is first absorbed and then transported in the xylem before distribution following the transpiration stream (Busse and Palta, 2006; White and Broadley, 2003). In the case of foliar Ca application, the absorption rate is determined by many environmental, physiological, and physicochemical factors, which are currently not fully understood (Fernández and Eichert, 2009; Fernández et al., 2013). The contribution of stomata to the foliar uptake process can be high (Eichert and Burkhardt, 2001; Eichert et al., 2008), but absorption may also occur through the cuticle, cuticular irregularities, trichomes and veins (Bahamonde et al., 2018; Fernández and Bahamonde, 2020). The application of root and foliar Ca fertilizers is currently recommended to improve plant Ca status, both in herbaceous and woody crops; nevertheless, information on how much of the exogenous Ca may be transported outside from the treated organs is still unknown. This study investigated the Ca partitioning and rate of absorption and mobility of <sup>45</sup>Ca supplied to chili pepper (herbaceous) and sweet cherry (woody) plants, after root and foliar application.

## Materials and Methods

### Plant material, experimental design and Ca treatments

Trials were carried out with chili pepper seedlings (*Capsicum annum* L.) and two-year-old non-bearing

sweet cherry (*Prunus avium* L.) trees grown at the facilities of Pontificia Universidad Católica de Chile, Santiago (33°29' S, 70°36' W, 570 m above sea level). Each container was filled with 2:1:1 peat: perlite: vermiculite as substrate. Chili pepper plants were kept in a growth chamber at 24 °C (day/night) air temperature and 16:8 h light: dark photoperiod. Sweet cherry trees were kept outdoors. Chili pepper seedlings were planted in 2.8 L plastic containers in Oct (spring in the southern hemisphere) and cherry trees were transplanted to 40 L plastic containers in Aug (winter end), before bud break. Irrigation frequency and volume were adjusted according to plant water demand. The initial  $^{45}\text{CaCl}_2$  (5 mCi) solution was diluted with  $\text{CaCl}_2$ . Subsequently, the  $^{45}\text{CaCl}_2$  treatment solution had a concentration of  $7.35 \text{ g L}^{-1} \text{ CaCl}_2$  and an activity of  $2,187 \text{ Bq L}^{-1}$ , with no surfactant added. Using a micropipette, the following treatments were applied to the plants on 14<sup>th</sup> Nov, 2018: (1) No  $^{45}\text{Ca}$  application (untreated, control plants), (2) two mL of  $^{45}\text{Ca}$  solution to soil, distributed in four points, (3) foliar application to basal leaves, and (4) foliar application to apical leaves.  $^{45}\text{Ca}$  solutions were supplied once during the growing season. Calcium treatments were applied at the pre-flowering developmental stage of pepper plants and on the second peak of vegetative growth of cherry trees. For foliar treatment to apical or basal leaves,  $25 \mu\text{L}$  of the  $^{45}\text{CaCl}_2$  treatment solution (having  $7.35 \text{ g L}^{-1} \text{ CaCl}_2$  and  $2,187 \text{ Bq L}^{-1}$  activity) was applied to seven chili pepper leaves per plant and 20 sweet cherry leaves per tree, respectively. Trials were carried out following a completely randomized design with three replicates for chili pepper and sweet cherry plants.

#### Plant biomass and Ca concentration partitioning

In Jan 2019, that is, two months after  $^{45}\text{Ca}$  treatment when growth was already arrested, untreated and  $^{45}\text{Ca}$ -treated chili pepper plants and cherry trees were harvested. Plant parts were separated for further analysis, grouping together the leaves, stems, fruits and roots of chili pepper, and the leaves, shoots, trunk and roots of sweet cherry trees. For each species and organ, tissue fresh (FW) and dry (DW) weight (tissues were kept in an oven at 65 °C for 48 h) were consequently determined. Calcium concentration was determined after dry tissue ashing at 500 °C (4 h), ash dissolution in 2 M (Sadzawka et al., 2007) and Ca determination by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Agilent 720 ES axial - Varian, Australia). For Ca content estimations, Ca concentration of each organ was multiplied by its DW, expressing Ca partitioning values as a percentage.

#### Ca transport after $^{45}\text{Ca}$ application

Two months after  $^{45}\text{Ca}$  application,  $^{45}\text{Ca}$  activity (Bq) was measured in different plant fractions and in the soil, in the case of root treatments (0–5 cm and 5–10 cm depth) (Videla et al., 2019). Then, the  $^{45}\text{Ca}$  recovered was

calculated by considering the amount of  $^{45}\text{Ca}$  measured in soil and different tissues, depending on the treatment, plant species, and total  $^{45}\text{Ca}$  amount applied in each treatment.

#### Leaf surface topography and anatomy

The leaf surface features of chili pepper and cherry leaves were analyzed by scanning electronic microscopy (SEM), with focus on veins, stomata, and mesophyll morphology. Images for characterizing stomatal frequency and pore size (length and width) were collected and analyzed using Image-J software.

#### Statistical analysis

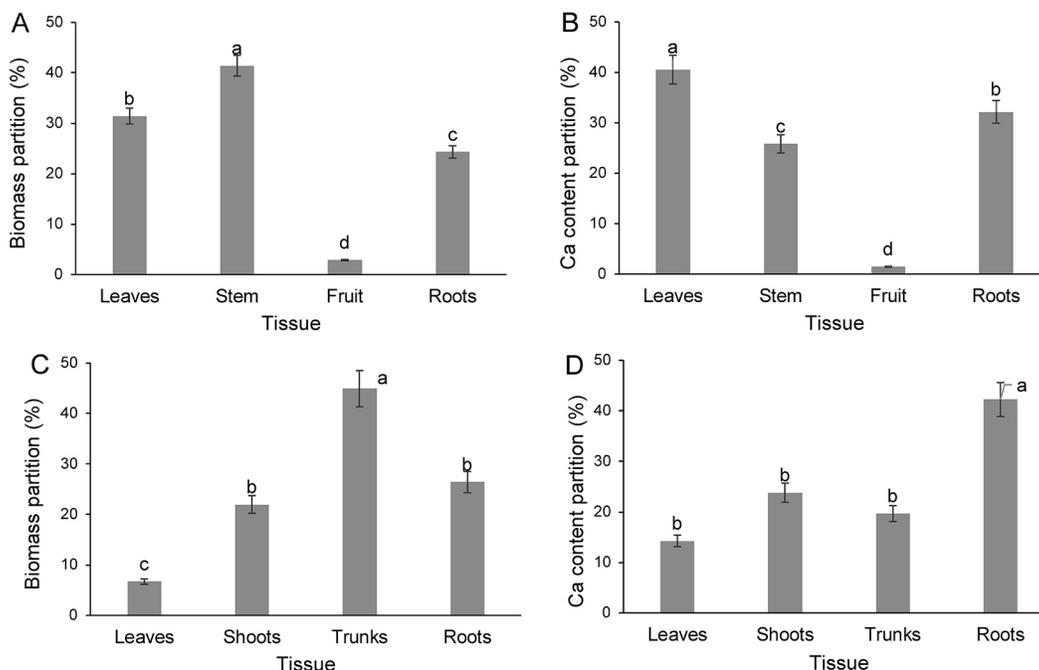
In general, data were statistically analyzed with SPSS 15.0 software. They were subjected to the analysis of variance (ANOVA) and differences between factors ( $^{45}\text{Ca}$  application methods and species) were found with the Tukey HSD Tests, when F-values were significant ( $p < 0.05$ ). For assessing Biomass and Ca content, data were first transformed to arcsin-sqrt before ANOVA. Results concerning  $^{45}\text{Ca}$  detection were transformed to log prior to ANOVA. The number and size of stomata were also compared by ANOVA.

## Results

#### Plants biomass and tissue Ca partitioning

The results for organ biomass and Ca partitioning of chili pepper and sweet cherry plants are shown in Figure 1. For chili pepper, the highest biomass related to stems and leaves (accounting for more than 70 % of plant biomass; Figure 1A). Regarding its distribution in chili pepper (Figure 1B), Ca accumulated mainly in leaves (40 %), followed by roots (approximately 35 %) and stems (around 25 %). Fruits had the least biomass and Ca partitioning rate (Figure 1A and B), representing the lowest values recorded in different plant parts. Hence, similar to organ biomass, Ca partitioning was also higher in aerial parts of the chili pepper plant.

In 2-year-old sweet cherry trees (Figure 1C) the trunk had the highest biomass partitioning (approximately 45 %), followed by shoots (around 23 %) and roots (approximately 30 %), while leaves had the lowest biomass (< 5 %) (Figure 1C). Cherry tree roots had the highest Ca partitioning (more than 40 %), while the remaining aerial parts had lower Ca amounts, which remained within a similar range (Figure 1D). When comparing the results of the two species, no significant differences between aerial (including stems and trunks) and underground organ biomass distribution were observed (from 70 to 74 % for aerial parts and between 26 to 30 % for roots) (Figures 1A and 1C). There were significant differences regarding Ca partitioning in some organs of pepper versus cherry plants, with leaves having the highest Ca amounts in chili pepper (Figure 1 B) and roots showing the greatest Ca accumulation values in cherry trees (Figure 1 D).



**Figure 1** – Chili pepper (A, B) and sweet cherry tree (C, D) biomass partitioning (A, C) and Ca content partitioning (B, D). Data are means  $\pm$  standard deviations (SD,  $n = 3$ ). Different letters indicate different levels of significance according to the Tukey test ( $p < 0.05$ ).

### Radiolabelled Ca trials

For both species, the supply of  $^{45}\text{Ca}$  via foliar treatment was positively correlated with the concentrations recovered in the treated organs, because the applied  $^{45}\text{Ca}$  mostly remained there after leaf application (Figures 2C to 2F). In the case of root Ca supply, significant differences were found in  $^{45}\text{Ca}$  distribution in plant organs. The highest  $^{45}\text{Ca}$  recovery values were recorded for treated roots, which were not significantly different between species.

For chili pepper plants, the highest distribution of  $^{45}\text{Ca}$  was found after soil treatment with Ca, the highest  $^{45}\text{Ca}$  accumulation rate was detected in the roots, followed by leaves and stems (Figure 2A). However, when  $^{45}\text{Ca}$  was applied to apical or basal leaves, around 98 % of the supplied  $^{45}\text{Ca}$  was recovered in the treated tissues (Figures 2B and 2C, respectively), indicating the limited transport capacity of Ca following foliar application. For sweet cherry trees, a similar trend was observed, with the highest rate of  $^{45}\text{Ca}$  distribution recorded when  $^{45}\text{Ca}$  was supplied to the soil (Figure 2D). After root treatment, the highest  $^{45}\text{Ca}$  amounts were measured in roots, followed by shoots, leaves, and trunk (Figure 2D). As for Chili pepper, when  $^{45}\text{Ca}$  was applied to the apical or basal leaves (Figures 2E and 2F), over 90 % of the supplied  $^{45}\text{Ca}$  was found in the treated foliage. However, for this species, the rate of  $^{45}\text{Ca}$  translocation from the treated leaves was significantly higher than for chili pepper plants, leading to detectable  $^{45}\text{Ca}$  increases in the trunk, shoot and basal leaves after  $^{45}\text{Ca}$  supply to apical leaves (Figure 2E). Treatment of basal cherry tree leaves

with  $^{45}\text{Ca}$  also led to the translocation of a small amount of this Ca radiotracer to the trunk (Figure 2F).

Two months after the application of  $^{45}\text{Ca}$  to the soil, chili pepper plants and sweet cherry trees absorbed approximately 8 % and 2 % of the Ca, respectively. When analyzing the treated soil, about 70 % and 30 % of the applied  $^{45}\text{Ca}$  was detected respectively in the top 0–5 cm and 5–10 cm soil layers where chili pepper plants were cultivated. In the case of sweet cherry trees, approximately 65 % and 20 % of the applied  $^{45}\text{Ca}$  was found in the top 0–5 cm and 5–10 cm soil layers, respectively. Hence, between species, significant differences on the recovery of root-applied  $^{45}\text{Ca}$  were found for 5 to 10 cm soil depths, the lower values recorded for cherry trees suggesting a higher root activity of this species in this soil zone compared to chili pepper plants. At 10 cm soil depths and below, no detectable  $^{45}\text{Ca}$  concentrations were measured (data not shown).

### Leaf anatomy

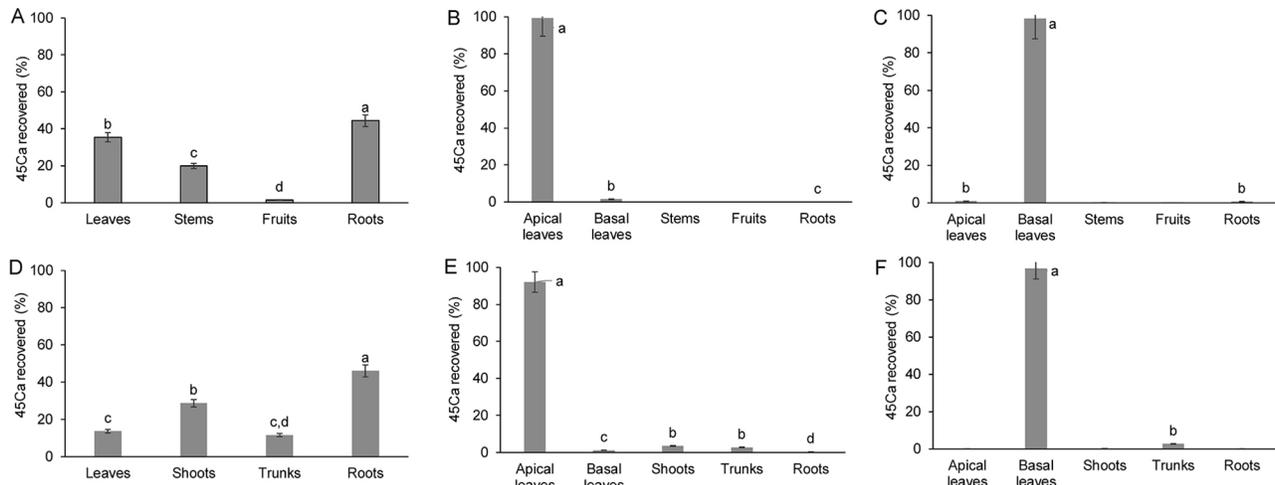
Scanning electron micrographs show that the central vein of chili pepper leaves (Figure 3A) are more prominent and have smaller and more irregular vascular bundles, compared to cherry leaves (Figure 3C). Chili pepper leaves are thinner, have bigger epidermal cells, but a more irregular mesophyll structure (Figure 3B), compared to palisade and spongy parenchyma in cherry leaf (Figure 3D). Stomata were found only in the lower (abaxial) surface of cherry leaf (Figure 3G), while both leaf sides of Chili pepper had stomata, but densities

were higher on the abaxial side (Table 1; Figure 3E). In addition, cherry leaves had smaller stomata (Table 1, Figure 3H) compared to the abaxial side of chili pepper leaf (Figure 3F).

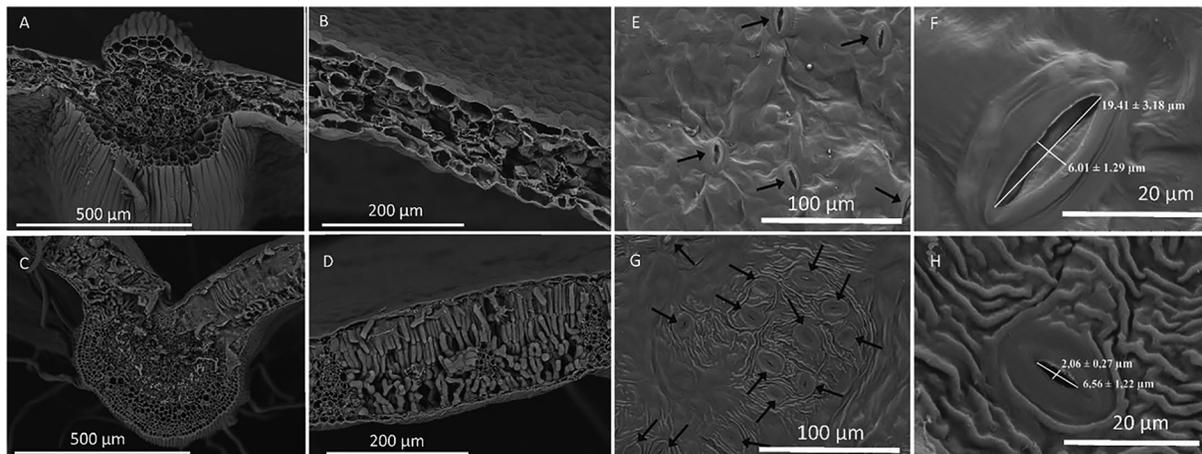
### Discussion

In this study, the rate of Ca accumulation and distribution in chili pepper plants and sweet cherry

trees was evaluated using <sup>45</sup>Ca as a tracer. The total plant biomass of approximately 4-month old chili pepper plants was lower than that of 2-year old cherry trees, mainly due to the absence of lignified, secondary tissues in pepper compared to woody species which develop secondary xylem and phloem (Sun et al., 2003). Shoots and roots of herbaceous plants differ from those of woody plants, for example, concerning their pattern of development, morphology, and functionality (e.g.,



**Figure 2** – Distribution of <sup>45</sup>Ca (%) in different tissues of Chili pepper (A, B, C) and sweet cherry (D, E, F) plants. The recovery of <sup>45</sup>Ca in plant organs is shown following <sup>45</sup>Ca soil application (A, C), <sup>45</sup>Ca supply to apical leaves (B, E) or <sup>45</sup>Ca supply to basal leaves (C, F). Data are means ± SD (n = 3). Different letters indicate different levels of significance according to the Tukey test (p < 0.05).



**Figure 3** – Scanning electron micrographs of Chili pepper (A, B, E, F) and cherry tree (C, D, G, H) leaf parts. (A, C) central veins, (B, D) leaf cross-sections, (E, G) abaxial leaf side (stomata are indicated with arrows), and (F, H) stomata.

**Table 1** – Stomatal density and stomatal pore size of chili pepper and cherry tree leaves. Data are means ± standard error (n = 15). Between species and for abaxial leaf sides, values marked with different letters are significantly different according to the Tukey test (p < 0.05).

Species	Stomatal density (N° mm <sup>-2</sup> )		Pore length (µm)		Pore width (µm)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Pepper	62.7 ± 3.1	125.3 ± 15.7 b	12.4 ± 0.6	19.4 ± 3.1 a	2.4 ± 0.0	6.0 ± 1.2 a
Cherry	-	407.3 ± 59.5 a	-	6.5 ± 1.2 b	-	2.0 ± 0.2 b

absorption and transport of water and nutrients; Shipley and Vu, 2002; Sun et al., 2004). In chili pepper plants, the highest Ca content partitioning was associated with aerial parts (leaves plus stems), while roots had the greatest Ca contents in sweet cherry trees. This may be related to Ca<sup>+2</sup> accumulation and relative immobilization in the trunk and bark (Federer et al., 1989; Turner and Lambert, 2005). In this study, leaf Ca concentration was about 1.5 % for chili pepper plants and 2 % for sweet cherry trees, which is within the Ca sufficiency range (1.2 – 2.4 %) described for these species (Reuter et al., 1997). Chili pepper had approximately 30 % leaf biomass, while sweet cherry trees had a leaf biomass below 10 %, which may be associated with a trade-off between forming woody tissues or leaves in the woody species (Cornelissen et al., 1996). Similar root biomass partitioning values have been reported for cherry (Bonomelli and Artacho, 2013) and sweet orange trees (Mattos Jr. et al., 2003).

When analyzing the rate of <sup>45</sup>Ca transport and accumulation, both species showed certain similarities. In treatments where <sup>45</sup>Ca was applied to apical or basal leaves, <sup>45</sup>Ca was chiefly found in the treated foliage. However, when <sup>45</sup>Ca was applied to the soil, there was a higher rate of <sup>45</sup>Ca distribution within chili pepper plants and cherry trees. The highest rate of delivery of root-applied <sup>45</sup>Ca is associated to xylem transport from roots to shoots, and the subsequent distribution to different aerial organs following the transpiration stream (Montanaro et al., 2014, 2015). Hence, the transport of Ca in the xylem can be considered the natural mechanisms of distribution of the element to aerial organs following root Ca absorption, which can also be limiting for the delivery of sufficient amounts of Ca to fruiting organs (Torres et al., 2017; Val et al., 2008). When <sup>45</sup>Ca was applied to the soil, it remained mostly in the upper 10 cm soil layer. Thus, root-applied Ca can be available to be absorbed in the top soil, where a high root activity may occur (Marschner, 2012; Riekerk, 1971), with some leaching risk. Due to the limited phloem mobility of Ca, foliar Ca supply may be ineffective if absorption rates are low. Following foliar Ca application, most applied Ca may remain in the treated area. Additionally, after foliar/fruit surface Ca absorption of a Ca spray fertilizer, most exogenous Ca, which may have penetrated through the organ surface, may be accumulated in the external tissues, such as the leaf epidermis or fruit peel (Bonomelli and Ruiz, 2010; Kluge et al., 1999; Sampaio et al., 1999; Val et al., 2008). Thereby, direct treatment of low transpiring organs, like fruits with Ca-containing solutions, may help increase Ca contents and avoid the development of Ca-related disorders (Khalaj et al., 2016). However, more research efforts are needed to optimize Ca fertilization, for instance, characterizing optimal fruit developmental stages and application timing of foliar and root Ca fertilizers, or improving Ca foliar fertilizer formulations.

Leaf structure and morphology may affect the absorption and distribution of foliar-applied nutrients (Fernández et al., 2017). The morphology of the central vein was different and in the case of chili pepper, it protruded from the adaxial and abaxial surface (Figure 3A) compared with the concave structure of the adaxial cherry leaf vein (Figure 3C). This may prevent the absorption of a foliar fertilizer solution through the veins of chili pepper leaf, in contrast to the potential accumulation of liquids in the concave veins of the upper cherry leaf side that may facilitate the absorption of foliar-applied Ca, as reported by Bahamonde et al. (2018). Differences in stomatal density and pore size were observed for pepper versus cherry leaves, which may also affect the absorption process of foliar Ca sprays. Pepper leaf is amphistomatous (Weryszko-Chmielewska and Michalajc, 2009) and have larger pore sizes. On the other hand, cherry leaf is hypostomatous (Gonçalves et al., 2008) and have four times smaller stomata compared to pepper leaves. Assuming that the stomatal pathway is relevant for both species, foliar Ca applications should be applied preferably during the day when stomata are open and covering the abaxial side mainly in the case of cherry trees.

When considering root versus foliar Ca fertilization, both methods involve potential advantages and constraints related to limitations associated to Ca absorption and mobility. Furthermore, Ca fertilizer absorption and transport mechanisms in plants may vary to a certain extent between species, as shown in this investigation. Our experiments on <sup>45</sup>Ca mobility showed that the rate of Ca re-translocation after foliar application is low, but higher in cherry trees compared to chili pepper plants. Fruit Ca enrichment at early developmental stages via multiple Ca spray treatments may be a means to increase the concentration of the element and limit the occurrence of Ca-related disorders, as suggested by several authors (e.g., Correia et al., 2020; Torres et al., 2017; Val et al., 2008). However, further research should be carried out to assess, for example, the rate of foliar Ca permeability, potential role of the cuticle, stomata, trichomes, veins, and other potential surface features in the process of foliar absorption and effectiveness of different foliar spray formulations (Fernández and Bahamonde, 2020).

## Conclusion

Soil application of <sup>45</sup>CaCl<sub>2</sub> to chili pepper plants and sweet cherry trees led to the highest rate of Ca transport to aerial plant organs. For both species, <sup>45</sup>CaCl<sub>2</sub> solution application to basal and apical leaves resulted into limited <sup>45</sup>Ca movement from the site of treatment to other tissues, but a higher mobility of foliar-applied <sup>45</sup>Ca was observed for cherry trees. Therefore, it is concluded that the most effective Ca fertilization means to increase fruit Ca concentrations may be direct treatment of developing fruiting organs with multiple Ca spray applications. However, differences in Ca absorption and

translocation rates may be found, for example, between species, varieties, organ ontogeny or environmental conditions at the time of spray application. Furthermore, factors, such as Ca fertilizer formulation, technology of application, and timing should also be considered in future investigations to optimize the response of crop plants to Ca fertilization.

## Authors' Contributions

**Conceptualization:** Bonomelli, C. **Data acquisition:** C.; Alcalde, C.; Videla, X.; Rojas Silva, X. **Data analysis:** Bonomelli, C.; Aguilera, C.; Alcalde, C. **Writing and editing:** Bonomelli, C.; Fernández, V.

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