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Research article

Combining quince (*Cydonia oblonga*) rootstock with soil-applied calcium chloride solution as a strategy to control brown spot (*Stemphylium vesicarium*) incidence in Abbé Fétel pear fruits

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Abstract: Brown spot (*Stemphylium vesicarium*) is a fungal disease widespread in European pear industry. The high number of fungicide applications required to control the disease can promote strains with fungicide resistance. The aim of the present study was to assess the effectiveness of soil-applied water solution of calcium chloride in combination with rootstock to control the incidence of brown spot in Abbé Fétel pear. Soil salinity, stem water and osmotic potential, along with fruit fraction of calcium and defence-mechanism related-enzymes such as peroxidase, polyphenol oxidase, phenyl-alanine ammonia-lyase, and β-1,3-glucanase were investigate in tree grafted on Fox 11 (*Pyrus communis* L.) and Sydo®, a quince (*Cydonia oblonga*) rootstock. Both grafting combinations were fertigated or not with calcium chloride. Sydo® showed a positive effect in reducing brown spot severity on Abbé Fétel. Compared to Fox 11, fruits from trees grafted on Sydo® showed higher calcium concentrations, fraction of calcium pectate, fruit firmness, polyphenol oxidase, phenyl-alanine ammonia-lyase and β-1,3-glucanase levels. Fruit calcium concentration was positively correlated with disease fruit tolerance. However, soil calcium applications were ineffective in promoting fruit calcium partitioning. Calcium chloride applications decreased leaf osmotic and stem water potential in trees grafted onto Sydo®, but this wasn't found in those grafted on Fox 11. In conclusion, Sydo® rootstock promoted Ca acquisition, osmotic adjustment at leaf and fruit level, fruit synthesis of defence-related enzymes that all together reduced brown spot severity in Abbé Fétel pear.

Keywords: β-1,3-glucanase; Ca-pectate; leaf osmotic potential; PAL; PPO; *Pyrus communis*

Abbreviations: DAFB: days after full bloom; DAFI: days after fungal inoculation; DW: dry weight; EC: electric conductivity; FW: fresh weight; SSC: soluble solids content; TA: titratable acidity; POD: peroxidase; PPO: polyphenol oxidase; PAL: phenyl-alanine ammonia-lyase; SEM: standard error of means

1. Introduction

Brown spot (*Stemphylium vesicarium)* is a fungal disease widespread in the main European pear-growing areas. Firstly recorded in Italy in 1975 [1], it was subsequently reported in Spain and France [2,3], then in Belgium, The Netherland and Portugal [4–7]. Brown spot is included among the most important pear diseases, with high economic impact for pear growers, as fruits with symptoms are not marketable [8].

The causal agent is the Ascomycete *Pleospora allii* (Rabenh.) Ces. and De Not., as teleomorph form, even though the fungus is better known under its anamorphic form of *S. vesicarium* (Wallr.) E. Simmons, which can affect other cultivated species such as garlic and onion [9], tomato [10], asparagus [11] and mango [12].

Symptoms on pear trees usually appear in late spring and consist of necrotic lesions on leaves, fruits and shoots, as a consequence of specific toxins [13] and to the accumulation of phenolic compounds, lignin polymers, etc. [14]. Young leaves are more susceptible than mature ones and fruit susceptibility decreases throughout the season [15]. Symptoms on fruitlets are commonly located on the calyx and/or in the equatorial zone and these may progress to rot, due the secondary colonization by saprophytic fungi such as *Alternaria* spp. Severe infections may induce a rapid defoliation and fruit drop [8].

Many factors affect the disease development, including environmental conditions (e.g., air temperature and humidity, orchard inoculum level), grafting combination, cultural practices (e.g., irrigation, fertilization, floor management), use of net shelter against hailstorm. Among European pear, Abbè Fétel, Bosc, Conference, Comice, General Leclerc, Passe Crassane and Rocha varieties are extremely susceptible, whereas Williams, Blanquilla, Beurre Hardy, Ercolini, Grand Champion and Louis Bonne are moderate or not susceptible [3,15,16]. The different variety susceptibility is mainly due to the synthesis of host-specific toxins (i.e., SV-toxins I and II), responsible of plasma membrane disorders [13]. The high and low susceptibility of the varieties Rocha and Ercolini to *S. vesicarium* infection was related to the late and fast activation of defence mechanisms, respectively [17,18].

Plant water status and mineral composition play an important role in the interaction between pathogen and tree [19,20]. For instance, altering the tissue water status may trigger plant defence strategy against pathogens. Indeed, dehydration of the infection site is the most used mechanism by plants to hinder the bacterial growth. Similarly, reducing intracellular water content, plants limit cell functions, forcing pathogens to adapt to new conditions [19,21,22]. Abd-Elmagid and co-authors [23] concluded that the virulence of *S. sclerotiorum* and *S. minor* in peanut was affected by fungus isolates, osmotic and water potential, highlighting the importance of the plant water balance to improve disease control. Diseases susceptibility increases if plant nutritional status deviates from the optimum [20,24]. Nutrients are involved in most of the active defence mechanisms, and calcium (Ca), magnesium (Mg) and silicon (Si) are important for structural integrity of cell walls, thereby enhancing resistance to fungal (i.e., *Rhizoctonia solani* and *Fusarium solani*) and bacterial (*Erwinia* spp., etc.) enzymatic degradation [20,25].

In Italian pear industry, brown spot is one of the major threat, because of the variety susceptibility and the climatic conditions. The control of the disease requires a high numbers (15–25) of treatments that reduce holdings environmental and economic (the loss of yield can account for as much as 50%) sustainability [8]. In addition, in organic farming, copper-based fungicides are the only allowed, with heavy metal accumulation in the ecosystem. Pear growers generally control brown spot by chemical sprays of fungicides (e.g., dithiocarbamates, captan, iprodione, phosphites, tebuconazole, strobilurins, fludioxonil and SDHIs) generally as a preventive strategy. Curative fungicides, in fact, are ineffective because the inhibition of conidia germination occurs after the release of the fungal toxins [8], but recently some antimicrobial peptides (e.g., BP15, BP22, and BP25) showed a post-infection activity against *S. vesicarium* of pear [26]. Despite the high number of sprays, the effectiveness of the fungicides could be unsatisfactory and the high number of applications can promote the appearance of *S. vesicarium* strains with fungicide resistance, as found for dicarboximide and strobilurins [27,28]. The massive use of chemical forces the European Union to enact regulations towards a meaningful reduction of pesticides use [29], promoting at the same time the development of alternative defence strategies. To decrease the inoculum pressure, alternative and complementary measures, as part of integrated orchard management strategy have been developed and proposed to improve the orchard sanitation, including removal leaf litter [30]. Irrigation and fertilization are agronomic practices that allow growers to manipulate tree water and osmotic potential as well as mineral composition of plant tissues [19,25,31]. Enhancing Ca tissue concentration increases the synthesis of defence-related compounds (e.g., phytoalexin and phenolics substances), as observed in table grape and apple [32,33]. Moreover, it has been demonstrated that Ca can reduce pathogen severity caused by several fungi and bacteria in different species, including *Botrytis cinerea* in sweet cherries [34], brown rot in peach caused by *Monilinia fructicola* [35], fire blight and brown spot in pear [36,37]. Soil-applied $CaCl_2$ solution decreased the leaf susceptibility to brown spot because, along with leaf Ca, it increased also soil salinity and induced a water stress. As a result, brown spot symptoms were positively related to stem water potential and negatively to leaf Ca concentration [36].

Disease resistance in plants is associated with the activation of defense responses including the induction of defense-related enzymes, such as phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), lipoxygenase, superoxide dismutase and β-1,3-glucanase that are activated upon pathogen infection, in many fruits [38] including pear [39]. Pear fruit (*P. pyrifolia* L.) treated with calcium chloride, as an elicitor, showed an increase of these defense-related enzyme activities (i.e., β-1,3-glucanase, PAL, POD and PPO), with a reduction of the disease incidence caused by *Alternaria alternata* [40].

Calcium can be provided through soil application or by an increase of root uptake efficiency. Among the different rootstocks available for pear, seedling-derived (*Pyrus communis*) clones have recently received attention for their grafting compatibility, adaptability to calcareous soils and capability (e.g., Fox 9 and Fox 11) to reduce tree vigour to the same extent of the most widespread quince derived clones (e.g., BA29) in Italy [41].

The aim of this study was to assess the effectiveness of soil-applied $CaCl_2$ in combination with different rootstocks to control the incidence of brown spot in pear.

2. Materials and methods

2.1. Experimental site and plant material

The trial was carried out in a mature commercial pear orchard at the experimental farm of University of Bologna (Cadriano (BO), Italy, 44°33'03''N, 11°24'36''E, 33 m a.s.l.), of the variety Abbé Fétel grafted on two rootstocks: *1)* quince (*Cydonia oblonga* Mill.) Sydo® and *2)* pear (*Pyrus communis* L.) Fox 11. Trees were grown on a silty clay loam soil (Haplic Calcisol soil [42], 20 g 100 g^{-1} sand, 42 g 100 g^{-1} silt and 38 g 100 g^{-1} clay; pH = 7.6) at a density of 1645 and 2193 tree ha^{-1} for Fox 11 and Sydo®, respectively and managed as in a spindle training system. The orchard was drip irrigated and the floor management included herbicide strips along the row (2–3 applications per year of glyphosate) and grassed alleys regularly mown (2–3 times per year). Fertilization (no Ca fertilizers were used) and irrigation, as well as pest and disease control, were carried out according to the Integrated Fruit Production Guidelines of the Emilia-Romagna Region.

2.2. Treatments and measurements

In 2013, the following treatments were soil-applied to four plots (replicates), each one composed by three trees: *1)* Untreated control and *2)* $CaCl_2$, (Ca = 36 g 100 g^{-1}) supplied weekly from April (full bloom) until June. At each application, 72 g of $CaCl_2$ $tree^{-1}$ were dissolved in 8 L of tap water with an electric conductibility (EC) of the solution of 13.3 mS cm^{-1}. The solution was localized in a circular area of a ray of 0.7 m surrounding the tree trunk. Consequently, each tree received 26 g Ca at each application, for a total of 10 applications (260 g Ca $tree^{-1}$). Control trees received tap water only.

Soil cores were collected at 5–30 cm depth and used to measure soil pH and EC after six (June 4th) and ten (July 18th) $CaCl_2$ applications. The same day, stem water potential (Ψ_w) and leaf osmotic potential (Ψ_s) were measured on two fully expanded healthy leaves per tree. Soil pH was determined on oven-dried (105 °C) soils and measured with a pH meter (Basic 20, Crison, Barcelona, Spain) on a 1:2.5 soil:water ratio; EC was determined on 100 g of soil samples shacked 1 hour with 250 mL of deionized water and measured on the supernatant by a conductimeter (Crison 525, Crison Instruments, Barcelona, Spain). Stem Ψ_w was recorded according to Naor et al. [43] on two fully expanded healthy leaves per tree: briefly, leaves were *in vivo* fully covered with aluminium foils and enclosed in plastic bags at least one hour prior measurement. Then, leaves were cut off and the leaf lamina was immediately inserted into a pressure chamber, where the pressure was increased gently until the first drop of water was forced out of the petiole [44]. After stem Ψ_w measurements leaves were rapidly stored at −20 °C and later used to measure leaf Ψ_s: after thawing, the leaf was squeezed and 100 μL of leaf sap was immediately used to determine the leaf Ψ_s by a micro osmometer (Micro osmometer 5B, Roebling, Berlin, Germany).

The susceptibility to brown spot was evaluated on detached fruits, collected 60 days after full bloom (DAFB), with approximately 40 mm of diameter. Fruit surface was immediately disinfected by a 5 mL L^{-1} sodium hypochlorite solution, rinsed several times with deionized water and inoculated by placing on the bottom side four drops (20 μL) of a suspension containing 1×10^5 conidia mL^{-1} of *S. vesicarium.* The spore suspension was prepared from 7-day-old V8 agar plates by adding a few mL of sterile water and gently scratching the colony surface with a spatula [45]. This suspension was then filtered through a 100 μm filter. The fungal strain (ID number 173) was isolated

from leaves coming from a Bologna University experimental orchard located in Altedo (Bologna province) and sensitive to all fungicides authorised in Italy. Four and eleven days after fungal inoculation (DAFI), disease symptoms were assessed by the presence (incidence), number of spots per fruit and surface of spots (severity).

Later, fruits were analysed to evaluate Ca fractions and total Ca, Mg and K concentration. To this end, 10 g fresh weight (FW) of inoculated flesh fruit was used to determine the following fractions of Ca [46]: Ca-ethanol soluble, Ca-water soluble, Ca-pectate, Ca-di- and tri-phosphate, Ca-oxalate and Ca-residual, extracted sequentially on the same sample by 20 mL of ethanol (80%), deionized water, sodium chloride (1 mol), acetic acid (20 mL L^{-1}) and hydrochloric acid (0.6 mol), respectively. Residual Ca was extracted by treating the pellet with 8 mL of nitric acid (650 g L^{-1}) and 2 mL of hydrogen peroxide (300 mL L^{-1}) at 180 °C in an Ethos TC microwave lab station (Milestone, Bergamo, Italy) [47]. Total Ca, Mg and K concentration was determined on fruits lyophilized samples, mineralized as previously described for Ca residual. All Ca fractions, as well as total Ca, Mg and K concentrations were determined by atomic absorption spectrophotometry (SpectrAA-200, Varian Inc., Mulgrave, Australia).

Finally, fruits were also analysed to determine the activity of some defence-related enzymes such as POD, PPO, PAL and β-1,3-glucanase. All extraction procedures were conducted at 4 °C. 10 g of fruit samples were ground with 0.3 g polyvinyl polypyrrolidone with different buffers to assay different enzymes: 30 mL sodium acetate buffer (50 mmol, pH 5.0) for β-1,3-glucanase, 25 mL of 0.05 mol sodium borate buffer (pH 8.8, containing 5 mmol β-mercaptoethanol) for PAL, and 0.2 mol sodium phosphate buffer (pH 6.4) for POD and PPO. Samples were homogenized and centrifuged at 27,000 rpm at 4 °C for 60 min. Then, the supernatant was used as the crude enzyme source to assay enzymatic activities [39], while the protein concentration was determined according to the Bradford method [48], using bovine serum albumin as a standard.

At commercial harvest, tree yield was recorded and a sample of 16 fruits per plot was collected to determine dry matter, flesh firmness by a pressure tester (Effe.Gi, Ravenna, Italy) fitted with an 8 mm diameter plunger on two sides of the fruit previously peeled, soluble solids content (SSC) by a digital refractometer (PR-1, Atago, Tokio, Japan) and titratable acidity (TA, as malic acid) (Compact Tritator I, Crison, Barcelona, Spain).

2.3. Statistical analysis

Data were subjected to analysis of variance, according to a randomised factorial design with two factors. 1) $CaCl_2$ (two levels: 0 and 26 g L^{-1}) and 2) rootstock (two levels: Fox 11 and Sydo®), with four replicates. When a statistically significant ($p \leq 0.05$) effect of factors was observed, mean separation of main effects was performed by Student–Newman–Keuls (SNK) test ($p = 0.05$). When the analysis of variance showed a significant interaction between factors, two standard error of means (2SEM) was used as minimum difference between statistically different values [49]. A correlation analysis was carried out to evaluate the relationship between the disease susceptibility and Ca concentration in leaves and fruits.

3. Results

On June 4 and July 18 (after 6 and 10 applications, respectively), soil-applied $CaCl_2$ decreased soil pH and increased EC (Table 1). Soil EC was statistically higher in quince Sydo®, compared to pear Fox 11 plots, while rootstocks did not affect soil pH (Table 1).

On June 4, stem Ψ_w and leaf Ψ_s were unaffected by $CaCl_2$ applications (Table 2), while trees grafted on quince Sydo® showed a stem Ψ_w and leaf Ψ_s statistically lower than trees grafted on pear Fox 11. A significant interaction between factors on stem Ψ_w and leaf Ψ_s occurred at mid-July. While no effect of Fox 11 rootstock was found, applications of $CaCl_2$ on trees grafted on quince Sydo® significantly decreased Ψ_s compared to untreated control (Table 2).

Table 1. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on soil pH and electric conductivity (EC) after 6 (June 4) and 10 (July 18) calcium chloride applications.

$CaCl_2$ (g L^{-1})	June, 4		July, 18	
	pH	EC (mS cm^{-1})	pH	EC (mS cm^{-1})
0	7.67	102	7.67	152
26	7.09	543	7.06	477
Significance	***	***	***	***
Rootstock				
Sydo®	7.43	347	7.24	360
Fox 11	7.33	299	7.49	269
Significance	*ns*	*	*ns*	*
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*

ns, * and ***: effect not significant or significant at $p \leq 0.05$ and $p \leq 0.001$, respectively. Interaction between factors not significant.

Table 2. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on stem water potential (Ψ_w) and leaf osmotic potential (Ψ_s) after six (June 4) and ten (July 18) calcium chloride applications.

$CaCl_2$ (g L^{-1})	June, 4		July, 18			
	Ψ_w (MPa)	Ψ_s (MPa)	Ψ_w (MPa)		Ψ_s (MPa)	
			Sydo®	Fox11	Sydo®	Fox11
0	−0.66	−1.79	−0.99	−1.29	−1.77	−1.94
26	−0.74	−1.71	−1.26	−1.16	−2.02	−1.87
Significance	*ns*	*ns*	*2SEM = −0.31*		*2SEM = −0.18*	
Rootstock						
Sydo®	−0.79	−1.83				
Fox 11	−0.60	−1.69				
Significance	**	**				
$CaCl_2$ × rootstock	*ns*	*ns*	*		**	

ns, * and **: effect not significant or significant at $p \leq 0.05$ and $p \leq 0.01$, respectively. On July 18, values differing ≥ 2 standard error of means (SEM) are statistically different.

No interaction between $CaCl_2$ and rootstock emerged on leaf and fruit nutrient concentration and Ca fraction in fruits. Leaf and fruit nutrient concentration measured 60 DAFB was not affected by $CaCl_2$ applications (Table 3). In details, Ca in leaf and fruit resulted approximately 7.0 and 1.4 g kg^{-1}, respectively, while K concentration was similar (9.6 g kg^{-1}) in leaves and fruits. Magnesium concentration was 3.4 g kg^{-1} and 0.7 g kg^{-1} in leaves and fruits, respectively (Table 3). Trees grafted on Sydo® showed higher leaf and fruit Ca and Mg concentration, and a lower K concentration than Fox 11. The application of $CaCl_2$ did not alter flesh fruit Ca fractions (Table 4), whereas, with the exception of Ca-phosphate, all Ca fraction concentrations in fruits were increased in trees grafted on Sydo® (Table 4).

Table 3. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on leaf and fruit Ca, K and Mg concentration at the end of June.

$CaCl_2$ (g L^{-1})	Leaf (g kg^{-1} DW)			Fruit (g kg^{-1} DW)		
	Ca	K	Mg	Ca	K	Mg
0	6.85	9.16	3.35	1.27	9.42	0.75
26	7.35	9.87	3.53	1.44	9.84	0.75
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Rootstock						
Sydo®	8.20	7.36	4.25	1.69	8.94	0.83
Fox 11	6.01	11.67	2.64	1.00	10.33	0.67
Significance	***	***	***	***	***	***
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns and ***: effect not significant or significant at $p \leq 0.001$, respectively. Interaction between factors not significant.

Table 4. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on fractions of Ca in flesh fruits experimentally inoculated with *Stemphylium vesicarium.*

$CaCl_2$ (g L^{-1})	Ca fractions (mg kg^{-1} FW)					
	Ethanol soluble	Water soluble	Pectate	Phosphate	Oxalate	Residual
0	43	144	279	109	258	513
26	36	154	261	162	230	641
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Rootstock						
Sydo®	47	170	283	140	267	788
Fox 11	29	123	252	132	214	313
Significance	***	**	**	*ns*	*	***
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns, *, ** and ***: effect not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Interaction between factors not significant.

Soil-applied $CaCl_2$ reduced the occurrence of brown spot symptoms in fruits. Four days after inoculation with *S. vesicarium*, number and area of symptomatic spots per fruit were significantly lower than the untreated control (Table 5). After 11 days, independently of the $CaCl_2$ application, fruits showed a comparable number of spots (3.9 spots $fruit^{-1}$), however, the fruit infected area

resulted statistically higher in untreated control (Table 5). The rootstock genotype affected brown spot symptoms only 11 days after inoculation, when fruits collected from trees grafted on pear Fox 11 showed a larger infected area per fruit compared to those grown on Sydo® (Table 5). Pooling together data of the two rootstocks, a negative linear correlation was found between Ca concentration and symptoms of brown spot (mm^2 $fruit^{-1}$) in both leaves ($p < 0.05$ and r = 0.57, Figure 1) and fruits ($p < 0.05$ and r = 0.53, Figure 2).

Table 5. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on disease incidence, determined as number of spots per fruit and spots area per fruit, four and eleven days after experimental inoculation (DAFI) with *Stemphylium vesicarium*.

	4 DAFI		11 DAFI	
$CaCl_2$ (g L^{-1})	Spots (n $fruit^{-1}$)	Spots area (mm^2 $fruit^{-1}$)	Spots (n $fruit^{-1}$)	Spots area (mm^2 $fruit^{-1}$)
0	3.3	31	3.9	178
26	2.9	25	3.9	155
Significance	*	*	*ns*	*
Rootstock				
Sydo®	3.1	27	4.0	148
Fox 11	3.1	30	3.9	185
Significance	*ns*	*ns*	*ns*	***
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*

ns, * and ***: effect not significant or significant at $p \leq 0.05$ and $p \leq 0.001$, respectively. Interaction between factors not significant.

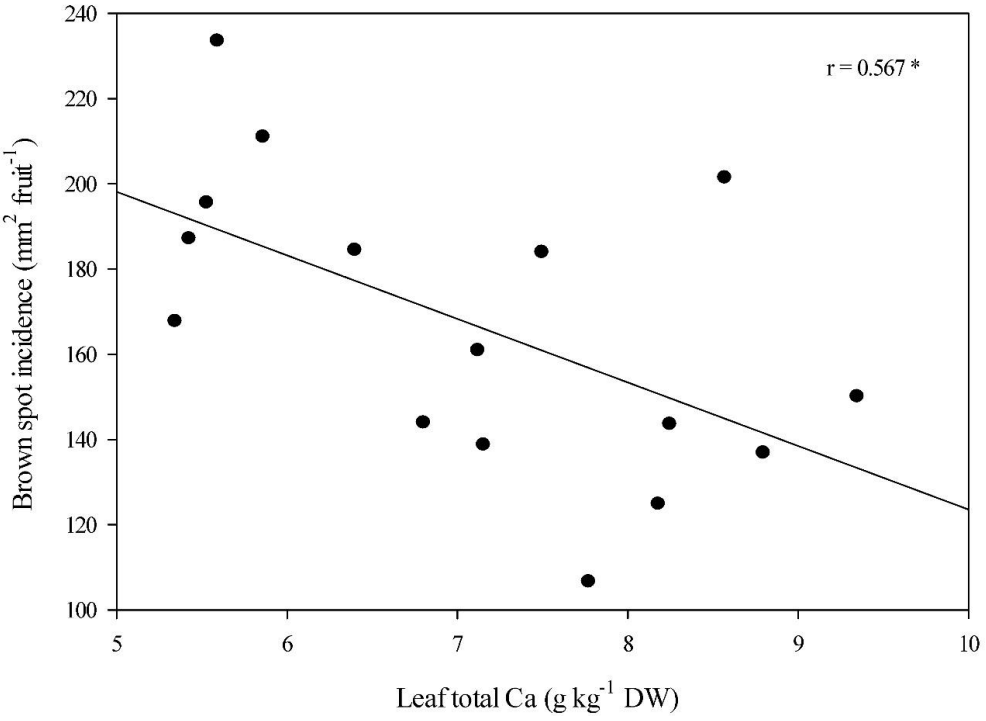

Figure 1. Relationship between leaf-Ca concentration and fruit brown spot incidence (+11 days after inoculation with *S. vesicarium*). *: correlation significant at $p < 0.05$; r = Pearson correlation coefficient.

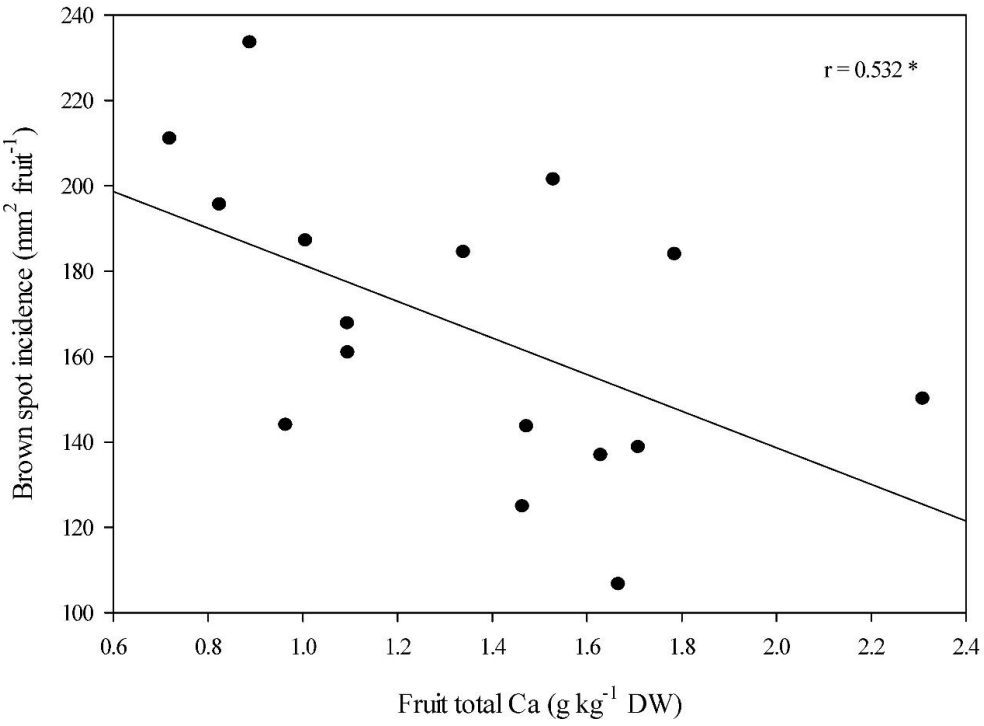

Figure 2. Relationship between fruit-Ca concentration and fruit brown spot incidence (+11 days after inoculation with *S. vesicarium*). *: correlation significant at $p < 0.05$; r = Pearson correlation coefficient.

The application of $CaCl_2$ to soil did not affect the defence-related enzymes POD, PPO, β-1,3-glucanase and PAL in fruits (Table 6). On the other hand, independently of the treatment and with the exception of POD, the enzymatic activity resulted higher in fruits collected from trees grafted on Sydo® particularly significant for β-1,3-glucanase and PAL (Table 6).

Table 6. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on the activity of peroxidase (POD), polyphenol oxidase (PPO), β-1,3-glucanase, and phenyl-alanine ammonia-lyase (PAL) determined on fruits experimentally inoculated with *Stemphylium vesicarium*.

$CaCl_2$ (g L^{-1})	POD (U min.$^{-1}$ µg^{-1} protein)	PPO (U min.$^{-1}$ µg^{-1} protein)	β-1,3-glucanase (µmol glucose h^{-1} µg^{-1} protein)	PAL (µmol cinnamic acid h^{-1} µg^{-1} protein)
0	9.4	0.07	0.91	157
26	6.5	0.05	0.73	125
Significance	*ns*	*ns*	*ns*	*ns*
Rootstock				
Sydo®	8.4	0.10	1.60	271
Fox 11	7.6	0.03	0.25	46
Significance	*ns*	**	***	***
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*

ns, ** and ***: effect not significant or significant at $p \leq 0.01$ and $p \leq 0.001$, respectively. Interaction between factors not significant.

No interaction between $CaCl_2$ and rootstock was observed on yield and fruit quality, while significant effects were ascribed only to the rootstock (Table 7). Tree yield and fruit number were statistically higher in Fox 11 than quince Sydo®, while no differences were recorded on the average fruit weight (Table 7). As regard to fruit quality parameters, the application of $CaCl_2$ increased only fruit juice TA while no effects were recorded on fruit firmness, SSC and dry matter (Table 7). Rootstock did not alter fruit SSC, although fruits from trees grafted onto Fox 11 showed lower values of flesh firmness, TA and dry matter than those measured for Sydo® (Table 7).

Table 7. Effect of soil–applied calcium chloride ($CaCl_2$) and rootstock on yield, fruit number and weight, fruit firmness, soluble solid content (SSC), titratable acidity (TA) and dry matter at commercial harvest.

$CaCl_2$ (g L^{-1})	Yield (kg $tree^{-1}$)	Fruits (n $tree^{-1}$)	Fruit weight (g $fruit^{-1}$)	Firmness (kg)	SSC (°Brix)	TA (g L^{-1})	Dry matter (g kg^{-1})
0	7.75	44	181	5.23	14.8	2.52	212
26	5.87	30	194	5.24	14.5	2.96	209
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	***	*ns*
Rootstock							
Sydo®	4.17	22	188	5.65	14.4	3.05	217
Fox 11	9.44	52	187	4.83	14.9	2.42	205
Significance	**	**	*ns*	***	*ns*	***	**
$CaCl_2$ × rootstock	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns, ** and ***: effect not significant or significant at $p \leq 0.01$ and $p \leq 0.001$, respectively. Interaction between factors not significant.

4. Discussion

Our strategy for control of the brown spot disease included soil application of a concentrated $CaCl_2$ solution, with the aim of increasing the presence of Ca in the disease-susceptible tissues. Our data showed that Ca was effective in promoting pear tolerance to brown spot, confirming earlier reports [37]; however, the increase of Ca in fruit was not achieved by soil Ca application, rather it was induced by rootstock materials. In the present study, the use of quince Sydo® as a rootstock resulted the most effective strategy in promoting Ca accumulation into fruits. The lack of effectiveness of soil-applied Ca solution in increasing fruit Ca concentration was found also in other investigations [36] and it can be in part explained considering the soil type. The investigated soil was sub-alkaline, silt-clay-loam, with a high cation exchange capacity (25 meq 100 g^{-1}) that increased the potential of Ca immobilization and reduced its availability in soil solution. In sub-alkaline soils, phosphorus (P) often reacts with Ca to form a sequence of compounds with low solubility such as tri-calcium phosphate or the insoluble hydroxyl apatites [50]. In this investigation, soil P concentration was 47 mg kg^{-1}, which may justify an insolubilization effect. In addition, Ca^{2+} is a nutrient with a low xylematic mobility, that moves under the transpiration stream [51] that is much higher in leaves than in fruits.

Trees grafted on Sydo® were more affected by the soil modifications related to the application of $CaCl_2$ than those on Fox 11. In fact, unlike Fox 11, Sydo® induced a decrease of stem water and leaf osmotic potential after $CaCl_2$ applications, meaning a physiological adjustment to the higher ion

concentration in the soil, that may have been among the causes that decreased the disease severity. The development of *S. vesicarium* was probably inhibited by the higher solute concentration in cell and the consequent lower water availability that lowered leaf osmotic and stem water potentials. At the same time, all-round the experiment, soil salinity was higher in soil with Sydo® roots compared with that hosting Fox 11, indicating a higher capability of the quince genotype to solubilize ions in soil solution than pear. This response was probably related to the higher root density in the lower volume of soil explored by Sydo® compared to Fox 11. This behaviour resulted in a higher capacity of root system to modify the soil on the tree row, where the $CaCl_2$ solution was applied. All together, these responses increased Ca acquisition and tolerance to brown spot.

On the other hand, Fox 11 root system explored a deeper and wider volume of soil allowing a higher tree vigour than Sydo® [41]. The different tree vigour may be one of the reasons of the different concentration of nutrients in leaf and fruits, which usually decreases with the increase of tree growth. We believe that this was not the case of our study, because the tree density, higher in Sydo® compared to Fox11, compensated for the lower vigour. In addition not all the nutrients showed the same trend, in fact K concentration was higher in tree grafted on Fox 11 than on Sydo®, while the reverse would have been expected, considering the vigour of grafting combinations.

Pear and quince genotypes were reported with a different capability to uptake cations when irrigated with saline water [52]. Therefore, another explanation is that Fox 11 activates defence mechanisms against the excess of soil salinity that prevent an excess of ion absorption (including Ca^{2+}), through a mechanism of ion exclusion, producing a low root uptake and a low Ca concentration in fruits and leaves. This response had a negative impact in term of tolerance to brown spot; in fact, the lower leaf and fruit Ca concentrations increased the susceptibility of tree on Fox 11 to brown spot compared to Sydo®.

With the exception of the fraction of Ca phosphate, the increased fruit Ca concentration induced an increase of all the fractions of Ca investigated, in trees grafted onto quince Sydo®. In particular Ca pectate, the fraction of Ca that strengthens the cell wall and makes it tolerant to both biotic and abiotic stresses [53,20], was found 12% higher in fruits of trees on Sydo® compared with those on Fox 11. This response was accompanied by the higher fruit firmness and higher dry matter concentration of fruit on Sydo® compared to Fox 11. This results were not expected since Fox 11, a *P. communis* rootstock, is more vigorous than Sydo® and consequently should delay fruit ripening, so that at harvest (made on same day for the two rootstocks), fruits were expected more immature than on quince (a dwarfing rootstock).

As already reported in fruit of *P. pyrifolia* infected with *Alternaria alternata* [40], also in our experiment on *P. communis* infected with *S. vesicarium*, an increase of the synthesis of some defence-related enzymes, such as PPO, β-1,3-glucanase and PAL was observed. This result was in relation with the ability of trees to counteract brown spot disease. PPO can oxidize phenolic compounds and produce antimicrobial phenolic substances, such as quinines, which are toxic to pathogens [54]. β-1,3-glucanase has been proven to hydrolyze major components of the cell wall of fungi [55], while PAL is involved in the biosynthesis of phenolics, phytoalexins, and lignins [56]. However, unlike the above-mentioned report [40], in our experiment the enzyme production was not a response to a direct application of an external elicitor (e.g., $CaCl_2$ application) on fruit, but it was the result of a physiological changes induced by a grafting combination between pear variety Abbé Fétel and quince Sydo®. These changes involved ion uptake along with a decrease of water potential in relation with an increase of solute concentration at cellular level. There was a substantial different

methodological approach between the work on *A. alternata* [40] and this experiment. In fact, we provided the condition for tree to take up nutrients and change its physiology (water status), while Tian and co-workers [40] applied directly the potential elicitors to the fruits, looking for a direct effect on the suppression of infection process.

Conclusions

Calcium was found effective in reducing symptoms of pear brown spot; however, in the experimental conditions of our study (i.e., clay soil) soil applications were not effective in promoting Ca fruit accumulation. Contrary to our expectations, the introduction of quince Sydo® rootstock seemed a better strategy than Fox 11 for increasing fruit Ca and reducing brown spot incidence and severity. Among the explanations of this response is the root-soil interaction and the consequent water and osmotic potential adjustment of trees grafted on quince Sydo® roots compared to pear Fox 11, along with the induction of a higher production of defence-related enzymes such as PPO, β-1,3-glucanase and PAL.

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Conflict of interest

All authors declare no conflict of interest in this manuscript.

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