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Apple scab control and activation of plant defence responses using potassium phosphite and chitosan

Ricardo B. Felipini · José I. Boneti · Yoshinori Katsurayama · Argus C. Rocha Neto · Beatriz Veleirinho · Marcelo Maraschin · Robson M. Di Piero

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Abstract In this study, the effects of two elicitors (potassium phosphite and chitosan) on apple scab (*Venturia inaequalis*) and their ability to modulate plant defences were assessed. Potassium phosphite and chitosan were sprayed on apple seedlings 7 days before fungus inoculation, and disease intensity was evaluated 14 days after inoculation. Samples of leaves treated with phosphite or chitosan that proved to be susceptible and moderately susceptible to disease were then collected for analysis of their metabolic profile by attenuated total reflectance–Fourier transform infrared spectroscopy. The activity of the plant defence enzymes and the phenolic compound content were also determined by spectrophotometry and high performance liquid chromatography, respectively. The effect of product application on the germination of *V. inaequalis* was also evaluated. Moderately susceptible leaves presented higher peroxidase activity, regardless of the application of a product. Although it reduced spore germination by 45 %, chitosan did not affect the intensity of the disease. On the other hand, potassium phosphite (2 μL mL^{-1}) reduced significantly the severity of scab by up to 62 % and it promoted the accumulation of salicylic acid, protocatechuic acid, and epicatechin in susceptible leaves, especially after the challenge with *V. inaequalis*. The salt did not exhibit antimicrobial activity. The resistance induced by potassium phosphite could thus play a significant role in scab control.

Keywords Induced resistance · Pathogenesis-related proteins · Phenolic compounds · HPLC · ATR-FTIR

R. B. Felipini (✉) · R. M. Di Piero
Laboratório de Fitopatologia, Universidade Federal de Santa Catarina, Caixa Postal 476, Florianópolis, SC CEP 88034-000, Brazil
e-mail: ricardo_felipini@yahoo.com.br

J. I. Boneti · Y. Katsurayama
Estação Experimental de São Joaquim, Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, Caixa Postal 81, São Joaquim, SC CEP 88600-000, Brazil

A. C. R. Neto · B. Veleirinho · M. Maraschin
Laboratório de Morfogênese e Bioquímica Vegetal, Universidade Federal de Santa Catarina, Caixa Postal 476, Florianópolis, SC CEP 88034-000, Brazil

Introduction

Apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is one of the most important fungal diseases on apple (*Malus* x *domestica* Bork) (MacHardy 1996). The fungus overwinters in the ascocarps of pseudothecia. In spring, fungal spores can cause severe primary infections on susceptible plant parts under favourable weather conditions. Secondary infections are forced by conidia of *Spilocea pomi* causing secondary scab epidemics (MacHardy 1996). Young leaves are more susceptible to the disease than older ones and this age-related resistance is often called 'ontogenic resistance' (Schwabe 1979; MacHardy 1996; Li and Xu 2002). Attempts to control the disease have been based on the eradication of primary inocula by sanitization practices, and on the application of protective and post-infection

synthetic fungicides (Sutton et al. 2000; Holb 2006, 2009). However, some fungicide groups such as benzimidazole and demethylation inhibitor (DMI) have lost their effectiveness because populations of pathogens resistant to the products have evolved and as a consequence these facts highlighted a need for alternative and more sustainable scab control methods (Ma and Michailides 2005; Holb 2009).

In the strategy known as induced resistance, biotic and abiotic elicitors are used to trigger a cascade of reactions that result in the formation of structural defence mechanisms such as cell wall thickening (Daniel and Guest 2005), or biochemical responses such as the synthesis of pathogenesis-related proteins (PR-proteins). These proteins can act directly against pathogens, or catalyse reactions that culminate in the formation of compounds toxic to the pathogen, usually phenolic compounds (Nicholson and Wood 2001). Some approaches with less environmental impact, such as the use of polysaccharides and phosphite salts, have been able to control plant diseases, both through their direct effect on plant pathogens and also through the activation of defence mechanisms.

Chitosan is a polysaccharide of low toxicity, derived from the deacetylation of chitin extracted mainly from the shells of crustaceans. Besides having an antimicrobial effect (El Ghaouth et al. 1992, 1994; Devlieghere et al. 2004; Qiu et al. 2014), this polysaccharide induces resistance in plants (Khan et al. 2003; Lin et al. 2005; Faoro et al. 2008; Chen et al. 2014). Another compound of interest, potassium phosphite, is marketed as a foliar fertilizer and applied to provide plants with phosphorus and potassium. It is well known, however, that its use may also contribute to disease control, mainly those caused by oomycetes (Förster et al. 1998; Silva et al. 2011; Machinandiarena et al. 2012), although diseases caused by true fungi can be controlled by phosphites as well (Reuveni et al. 2003; Yogev et al. 2006; Amiri and Bompeix 2011). The protective action of phosphite salts is attributed mostly to its antimicrobial activity (Niere et al. 1994; Schwinn and Staub 1995; Matheron and Porchas 2000), but its ability to induce resistance has also been mentioned (Daniel and Guest 2005; Percival et al. 2009; Machinandiarena et al. 2012).

In light of this background, our study was designed to assess the effects of potassium phosphite and chitosan as a preventive spray for the control of apple scab. Subsequently, we evaluated whether the observed reductions in disease severity could be attributed to the antimicrobial action of the products or to their ability to activate defence mechanisms in apple plants.

Material and methods

Plant, pathogen, and products

Seedling apple trees (cultivar Gala), grafted on the Marubakaido rootstock, with a single stem, were grown in pots containing 2 L of soil. The soil was prepared by mixing bovine manure compost, vermiculite, and sand at proportions of 1:2:2 (*v*/*v*/*v*). The seedlings were kept in a cold chamber (1 °C) and later were transferred to a greenhouse. No additional treatment was performed to control diseases or insects. The experiments were conducted in a greenhouse at the EPAGRI experimental station in São Joaquim, Santa Catarina, in southern Brazil. The treatments were sprayed over eight-leaf plants on a single stem.

Venturia inaequalis spores were obtained from symptomatic leaves of apple trees collected at the same experimental station. The collection of spores occurred under vigorous agitation of the symptomatic leaves in sterile distilled water, followed by filtering of the suspension with a cheesecloth. The concentration was adjusted to 3×10^5 spores mL^{-1} as determined by a hemacytometer. For inoculation, the plants were sprayed with spore suspension, transferred to a moist chamber for 48 h at 18 °C (±1 °C), and subsequently returned to the greenhouse.

Potassium phosphite solutions were prepared using a commercially available product formulated with 40 % P_2O_2 and 20 % K_2O (Fitofós K Plus, Wiser, Brazil), and the concentrations were adjusted by dilutions in distilled water. The chitosan solution (85 % deacetylated) was prepared by dissolving the polysaccharide in 0.05 N HCl under constant stirring, followed by pH adjustment to 5.6 with 2 M NaOH. No adjuvant was used in the preparation of the products.

Effects of the alternative products on scab severity and leaf sampling

In the first experiment, potassium phosphite (2 μL mL^{-1}), chitosan (5 mg mL^{-1}), or distilled water (control) was applied at 7 days before inoculation (DBI). This experiment was conducted with four replicates per treatment and each experimental plot consisted of a

single potted plant. Disease severity was assessed 14 days after inoculation and five apical leaves of each plant were observed for disease symptoms using a scale with grade 0 standing for a leaf with no symptoms and grades 1, 2, 3, 4, and 5 standing for leaves with severity less than or equal to 1 %, 5 %, 10 %, 25 %, and 50 %, respectively. Grade 6 was assigned to leaves with disease severity above 50 %. Subsequently, the grades were transformed into a disease index through the formula (Townsend and Heuberger 1943):

$$DS(\%) = \frac{\sum (nv)}{NV} \times 100$$

where: DS = disease severity (%); n = degree of infection on the scale of 1–6; v = number of leaves with a particular grade in the parcel; N = value of the highest grade of the parcel; and V = total leaves assessed in the parcel.

In the second experiment, apple seedlings were sprayed with the same treatments, and the application of the products and the pathogen inoculation were performed as described previously. Six plants of each treatment were used only for the assessment of disease severity, and the other plants provided samples for biochemical analysis. In this case, each plot consisted of three plants, and three young leaves (susceptible) and three more developed leaves (moderately susceptible) were collected from each plant. The leaves were collected immediately before inoculation and 2 days after pathogen challenge, and they were placed into aluminium foil and stored at −80 °C for further analysis. Six replicates were performed per treatment each time.

Metabolic profile by Fourier transform infrared (FTIR) vibrational spectroscopy

For this analysis, 250 mg of apple leaf tissue was macerated in the presence of liquid nitrogen in 3 mL of 80 % methanol (*v*/*v*) acidified with 1.25 % HCl (*v*/*v*). The extraction occurred during 1 h in the dark at room temperature. After this period, the extract was filtered on 14 μm filter paper.

In order to determine the metabolic profiles of the studied samples, aliquots (500 μL) of the organosolvent leaf extract were submitted to attenuated total reflectance–Fourier transform infrared (ATR-FTIR) vibrational spectroscopy. The spectra were acquired in a Bruker IFS 55 spectrometer with a glycerin-sulfate detector (DTGS) and attenuated total reflectance accessories (ATR, Golden Gate – 45° incidence-angle, Specac, New Jersey, USA). Prior to analysis, a background spectrum was collected from the clean crystal. 128 co-added scans/sample before Fourier transform were collected in a spectral window of 4000–500 waves cm^{-1} and a resolution of 2 waves cm^{-1}. For each sample, five spectra were recorded in absorbance mode, followed by spectral data processing using the OPUS software system version 5.0 (Bruker BioSpin, Billerica, USA); i.e., the delimitation of the spectral window of interest (3000–600 waves cm^{-1}), the correction of the baseline, the normalization of the signal/noise ratio, smoothing, and spectrum deconvolution (k factor = 1.5) (van Soest et al., 1995).

Peroxidase and β-1,3-glucanase activity levels

In order to determine enzyme activity levels, 200 mg of the sampled leaves was macerated with liquid nitrogen and homogenized in 1.5 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethanesulfonyl fluoride, 0.5 % polyvinylpyrrolidone (*w*/*v*), and 1 mM ethylenediamine tetraacetic acid. Homogenates were centrifuged at 20,000×*g* (30 min, 4 °C) and the supernatant, constituting the protein extract of the sample, was collected.

Peroxidase activity (EC 1.11.1.7) was measured in a reaction medium containing 45 μL of protein extract, 2.55 mL of 50 mM phosphate buffer (pH 6.0) containing 0.25 % guaiacol (*v*/*v*), and 0.1 M hydrogen peroxide, at 40 °C. The reaction was carried out for 4 min and the values of absorbance (470 nm) were recorded in a Femto 700 Plus spectrophotometer (São Paulo, Brazil) at 30 s intervals, starting from the first minute of reaction. After determining the sample protein content according to the method of Bradford (1976), the peroxidase activity was expressed as optical density units at 470 nm per minute per mg of protein (OD_{470nm} min^{-1} mg $protein^{-1}$) (Hammerschmidt et al. 1982).

To determine the β-1,3-glucanase (EC 3.2.1.39) activity, 40 μL protein extract were added to 460 μL of 100 mM acetate buffer (pH 5.0) containing 1 mg mL^{-1} laminarin. The reaction was conducted at 44 °C during 1 h. For each sample a blank cuvette containing all reagents in the amounts previously mentioned, except laminarin, was used. After this period, the reducing sugars were quantified (Lever 1972), and 500 μL (40 μL of incubated sample diluted in 460 μL of

100 mM acetate buffer) was kept at 100 °C for 5 min in the presence of 1.5 mL of 1 % hydrazide *p*-hydroxybenzoic acid (*w*/*v*) in 0.5 M NaOH. The absorbance of the reaction product was then measured at 595 nm. Reducing sugar content was determined through a glucose standard curve (0–50 μg mL^{-1}, y = 0.0359 x; r^2 = 0.991) and the results were expressed as μkatal glucose formed from the hydrolysis of laminarin per mg protein (μkatal mg $protein^{-1}$).

Reverse phase–high performance liquid chromatography (RP-HPLC) of phenolic compounds

The identification of phenolic compounds present in the extracts was performed as described by Schmidt et al. (2012), with some modifications. For that, 200 mg of the sampled leaves were macerated with 3 mL of 99 % methanol (*v*/*v*) and centrifuged (6000 rpm, 4 °C) for recovery of the supernatant. Ten μL of the methanolic extract was injected into a liquid chromatograph Shimadzu LC-10 AD (Shimadzu Co., Kyoto, Japan) equipped with a C_{18} column (Shim-Pack; 250 mm × 4.6 mm internal Ø, 5 μm particle) and a UV spectrophotometer detector operating at 280 nm. The elution used a water:acetic-acid:η-butanol (350:1:10, *v*/*v*/*v*) mobile-phase solution at 0.8 mL min^{-1}. The identification of the phenolic compounds was carried out by comparison of the retention times and co-chromatography of standard compounds (Sigma-Aldrich, St. Louis, USA) under the same experimental conditions. The quantification of the phenolic compounds was performed through an external standard curve of gallic acid (0.5–300 μg mL^{-1}, y = 35,158 x; r^2 = 0.99) considering the peak area of interest for the calculation of the concentration. The concentration values, expressed as mg of gallic acid equivalents (GAE) per g of dried biomass, represent the average of three consecutive injections per sample.

Germination of *Venturia inaequalis* spores

Venturia inaequalis spores were obtained as described above for the process of inoculation, and the suspension concentration was adjusted to 5 × 10^5 spores mL^{-1}. In single-cavity slides, 20 μL of chitosan or potassium phosphite solution was added to 20 μL of spore suspension. The dilutions were prepared to obtain chitosan solutions at 0, 125, 250, 500, or 1000 μg mL^{-1}, and potassium phosphite solutions at 0, 0.625, 1.25, 2.5, or 5 μL mL^{-1}. The slides were placed inside a Petri dish under high humidity and incubated at 25 °C under a photoperiod of 12 h. After 48 h, the germination rate was quantified by looking at 100 spores using an optical microscope. The spores were considered to be germinated when showing a germ tube larger than their greatest length (El Ghaouth et al. 1994). The experiment was performed with five replicates per treatment.

Experimental design and statistical analysis

All experiments followed a completely randomized experimental design. The homogeneity of variances was verified using the Bartlett test ($p < 0.05$), and when the results were not satisfactory, the transformation of data to √x or log x was applied. Further, the dataset was subjected to analysis of variance (ANOVA, $p < 0.05$), and for the experiments with qualitative parameters the Student-Newman-Keuls (SNK) test of mean separation was applied using the Statistica package (version 6.0, StatSoft Tulsa, USA). Additionally, linear regression analysis was performed when the doses of products were evaluated.

For purpose of chemometric analysis, the FTIR dataset for the whole spectrum (3000–600 cm^1) and for the fingerprint regions of proteins at 1650–1550 cm^{-1}, carbohydrates (1200–950 cm^{-1}), and lipids (2900–2850 cm^{-1} and 1740 cm^{-1}) were transferred (JCAMP.DX format) into the data analysis software for the principal component analysis (PCA) (Unscramble v. 9.1, CAMO Software Inc., Woodbridge, NJ, USA). Prior to PCA analysis each spectrum within the regions of interest was standard normal deviates corrected (Copikova et al. 2006; Kuhnen et al. 2010).

Results

Effects of phosphite and chitosan on scab severity

Potassium phosphite and chitosan were applied to apple plants 7 days before inoculation. Phosphite significantly reduced the scab severity by 62 % and 56 % in the two experiments compared to control, while chitosan had no effect on the disease (Table 1).

Table 1 Effects of different products on the severity of apple scab on leaves. Seedlings were treated at 7 days before inoculation with *Venturia inaequalis*. Disease index was calculated 14 days post-inoculation

Treatments	Disease severity (%)		
	Experiment 1	Experiment 2	Mean
WTR	[a] 53 a	39 a	46
CHT	36 a	34 a	35
PHO	16 b	18 b	17

WTR = Distilled water; CHT = 5 mg ml^{-1} Chitosan; PHO = 2 μl ml^{-1} Potassium phosphite

[a] Means with the same letter in the column do not differ by the SNK test ($p < 0.05$)

Metabolic profile by Fourier transform infrared vibrational spectroscopy (FTIR)

The metabolic profiles of the leaf samples were investigated through FTIR spectroscopy. Representative FTIR spectra are shown in Fig. 1a, suggesting discrepancies between the chemical composition of susceptible (I) and moderately susceptible (II) apple leaf samples at day three of the experimental period in particular, but observable also at the other sampling times (data not shown). Discrepant bands related to carbohydrate at 1204 cm^{-1} and to phenolic compounds at 880 cm^{-1} and 831 cm^{-1} were detected in susceptible leaf samples (Schulz and Baranska 2007). FTIR spectra of both susceptible and moderately susceptible leaf samples showed typical bands in the 1650–1550 cm^{-1} region related to amines I and II (Lambert et al. 2001), revealing the occurrence of proteins. Signals of aliphatic hydrocarbons at 2900–2850 cm^{-1} derived from the axial and angular deformation of the methyl and/or methylene group(s) of the alkyl portion of fatty acids or lipids were also found (Lambert et al. 2001; Schulz and Baranska 2007).

Further analysis of the FTIR data set by visual inspection took into account the spectral profiles of the assayed treatments (Fig. 1b). Some discrepancies could be detected, mainly for the chitosan/susceptible treatment with bands at 1513 cm^{-1}, and also in the fingerprint region of the phenolic compounds where prominent IR bands due to C–H wagging vibration between 800 cm^{-1} and 920 cm^{-1} were found, e.g., at 917 cm^{-1}, 880 cm^{-1}, and 820 cm^{-1}. For the phosphite/moderately susceptible treatment, bands due to the C–O stretching mode (# in Fig. 1b) of carboxylic acids were detected at 1260–1180 cm^{-1} (Schulz and Baranska 2007).

Peroxidase and β-1,3-glucanase activity

In addition to the FTIR bands that detected discrepancies between susceptible and moderately susceptible leaves, and in order to verify whether the control of the disease by phosphite was related to the synthesis of pathogenesis-related proteins, peroxidase and β-1,3-glucanase activity were quantified in apple leaf samples. When moderately susceptible leaves were compared to susceptible ones, it was noted that the former showed higher peroxidase activity at all sampling times (Fig. 2a). However, plants treated with phosphite and

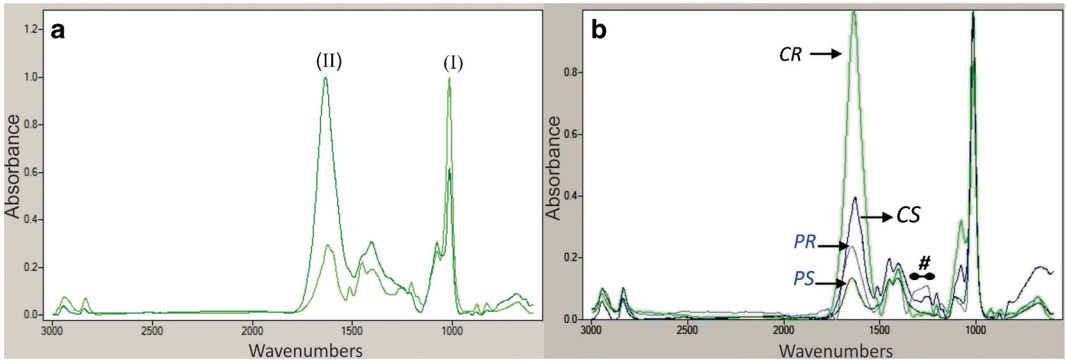

Fig. 1 Representative attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectral profiles of susceptible (I) and moderately susceptible (II) apple leaf samples, i.e., controls, of the Gala cultivar 3 days after the treatments (**a**); and ATR-FTIR spectra of the susceptible and moderately susceptible apple leaf samples according to the treatments, i.e., potassium phosphite and chitosan, 3 days after the treatments (**b**). CR = chitosan, moderately susceptible; CS = chitosan, susceptible, PR = potassium phosphite, moderately susceptible, and PS = potassium phosphite, susceptible. The symbol # denotes the bands due to the out-of-phase C–C–O stretching mode of phenolic compounds

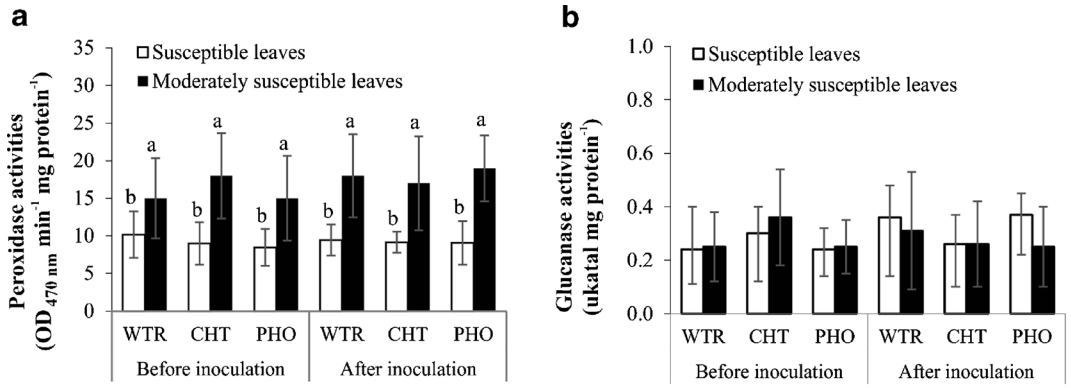

Fig. 2 Peroxidase (**a**) and glucanase (**b**) activity in susceptible and moderately susceptible apple leaves sprayed with distilled water (WTR), 5 mg mL^{-1} chitosan (CHT), or 2 μL mL^{-1} potassium phosphite (PHO). Seedlings were treated 7 days before inoculation with *Venturia inaequalis*. Leaf samples were collected immediately before inoculation and 48 h after inoculation. Significant differences between susceptible and moderately susceptible leaves were detected for peroxidase (Fischer's test, $p < 0.05$). The results represent the mean of two independent experiments

chitosan did not exhibit changes in peroxidase activity in relation to the control, and no difference in the glucanase activity of plants that received the treatments was detected, nor between susceptible and moderately susceptible leaves (Fig. 2b).

Reverse phase–high performance liquid chromatography (RP-HPLC) of phenolic compounds

The chromatographic profiles of the samples revealed that potassium phosphite led to a significant increase in three phenolic compounds, identified as salicylic acid, protocatechuic acid, and epicatechin, in susceptible leaves after inoculation with *V. inaequalis* (Fig. 3).

The susceptible leaves exhibited an increment in salicylic acid 48 h after the challenge with the pathogen. In that time, the concentration of salicylic acid in the leaves treated with potassium phosphite was 36 % higher than the control (distilled water) (Fig. 3a).

In addition to the salicylic acid increment, an augment in the protocatechuic acid content in phosphite-treated leaves was observed even before the inoculation. However, it was only after 48 h of inoculation with *V. inaequalis* that the maximum concentration of protocatechuic acid (3900 % higher than control) was achieved. Scab-susceptible leaves that were treated with potassium phosphite also exhibited a significant increase in this phenolic compound compared to the control (Fig. 3b).

Finally, it was observed that in susceptible leaves the epicatechin content was higher than in more resistant leaves, both before and after inoculation. Nevertheless, plants treated with potassium phosphite also exhibited substantial levels of this compound (Fig. 3c).

Germination of venturia inaequalis spores

Although chitosan had not controlled the disease, this polysaccharide linearly reduced the germination of the fungus at the doses examined. A reduction in germination of 45 % was detected when the polysaccharide was added to the spore suspension at 1000 μg mL^{-1}. There was a chitosan dose effect and the linear equation had the best fit to the data ($R^2 = 0.94$) (Fig. 4a), while potassium phosphite did not affect the germination of fungal spores (Fig. 4b).

Discussion

Younger apple leaves are more susceptible to scab than older ones, through a process known as ontogenic resistance. In a study of ontogenic resistance to scab in leaves of a susceptible cultivar of apple, Li and Xu (2002) showed that the mycelial growth of *V. inaequalis* in moderately susceptible leaves was slower and the leaves visually asymptomatic, whereas in susceptible leaves of the shoot apex the fungus growth was more intense.

In the present study, we found that the leaves more resistant to scab showed increased peroxidase activity. These enzymes are involved in the elimination of some reactive oxygen species (ROS) which, when excessive, can react either with proteins, reducing the activity of

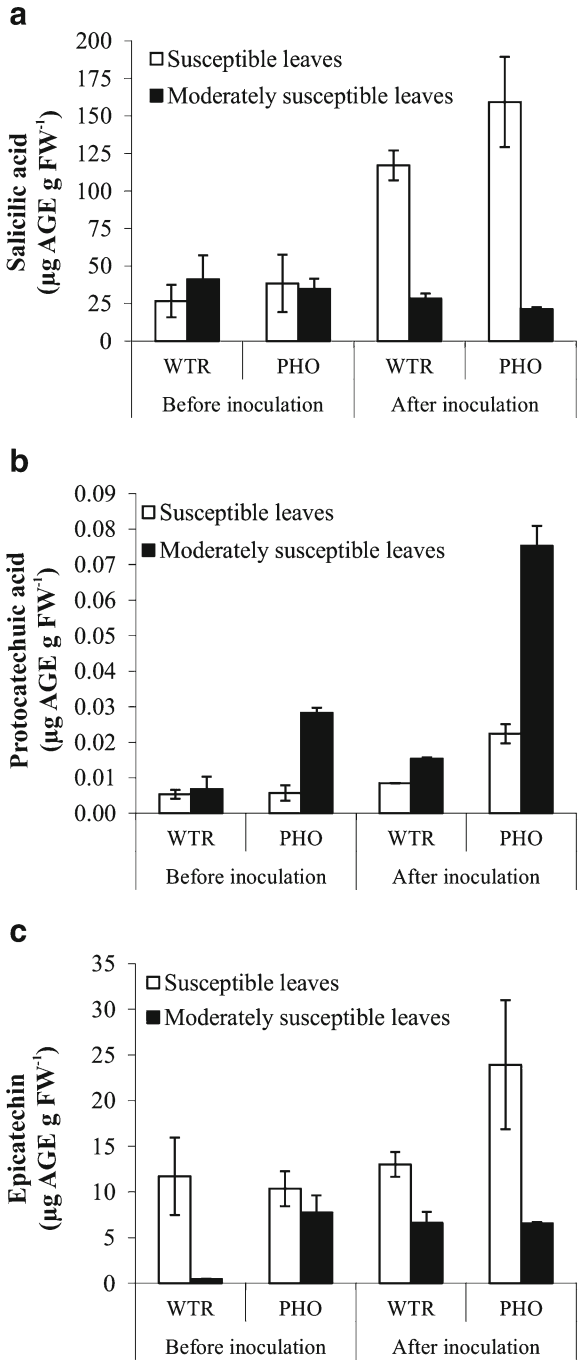

Fig. 3 Concentrations of salicylic acid (**a**), protocatechuic acid (**b**), and epicatechin (**c**) in susceptible and moderately susceptible leaves sprayed with distilled water (WTR) or 2 µL mL^{-1} potassium phosphite (PHO). The inoculation with *Venturia inaequalis* occurred at 7 days after the treatments. Leaf samples were collected immediately before inoculation and 48 h after inoculation. The results represent the means of three replicates

enzymes; with lipids, increasing the permeability of membranes; or with DNA, causing mutations (Moller 2001). The peroxidases are also related to the synthesis and the deposition of lignin in the cell walls, giving greater resistance against the penetration of pathogens (Gaspar et al. 1982). Thus, it is suggested that increased peroxidase activity can act indirectly in plant defence, catalysing reactions that result in the production of substances that give greater mechanical resistance to the tissue, and that thereby hinder the infection of older leaves by the causal agent of apple scab.

The great difficulty in controlling scab is the need for constant spraying, considering that apple plants constantly emit new branches and leaves during the vegetative growth phase. Although there are some fungicides registered for scab control, the evolution of pathogen populations resistant to those pesticides has diminished the effectiveness of this kind of control (Ma and Michailides 2005). In the present study, we found that potassium phosphite reduced scab severity by 60 % on average, and it could be applied as an alternative to fungicides, contributing to apple scab management through a preventive pulverization. Percival et al. (2009) also had observed a reduction of the severity of apple scab (*V. inaequalis*) and pear scab (*V. pirina*) by 35 % and 28 %, respectively, after application of a commercial product based on phosphite at a concentration of 10 µL mL^{-1} in the field.

Disease control through preventive application of phosphite has been reported in the literature, especially for diseases caused by oomycetes (Matheron and Porchas 2000). Jackson et al. (2000) suggested that the control of *Phytophthora cinnamomi* in *Eucalyptus marginata* is related to antimicrobial activity when phosphite is used in high concentrations. It is known that against oomycetes, phosphites can cause changes in the metabolism of phosphorus and amino acids (Niere et al. 1994; Schwinn and Staub 1995), reducing mycelial growth, the formation of sporangia, and the germination of zoospores. However, at low concentrations, reduction of disease seems more related to the increase in the activity of plant defence enzymes promoted by the salt.

Although potassium phosphite also affects the germination of true fungi such as *Alternaria alternata*, the doses reported to cause this effect were up to 200 times greater (Reuveni et al. 2003; Yogev et al. 2006) than those used in our study (2 µL mL^{-1}), which found no effect on the germination of spores of *V. inaequalis*.

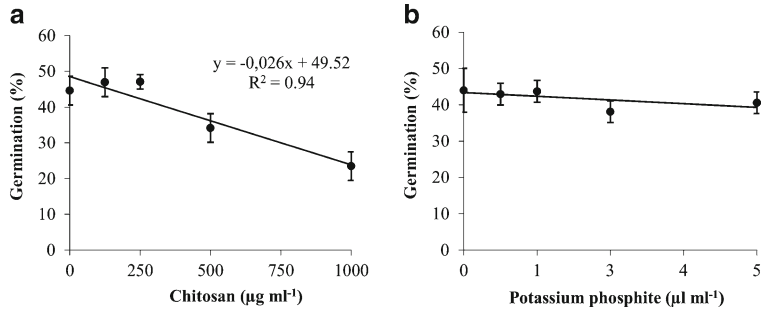

Fig. 4 *Venturia inaequalis* spore germination at different concentrations of chitosan (**a**) or potassium phosphite (**b**) after 48 h of incubation under a photoperiod of 12 h at 25 °C ± 1. A dose effect was observed only for chitosan (Fischer's test, $p < 0.01$)

These results reinforce the need to investigate the effect of potassium phosphite on the host metabolism.

Few studies have been done to explain the phosphite action mode involved in controlling apple scab, especially with regard to the activation of plant defence mechanisms (Percival et al. 2009). In the present work, potassium phosphite application did not induce an increment in the activity of either enzyme type (peroxidase and glucanase), showing that the disease-control action of the product follows a pathway different from ontogenic resistance, where the role of peroxidases had been demonstrated.

Phosphite increased the content of salicylic acid, mostly in susceptible leaves. Several papers report that salicylic acid could act as a signalling molecule in systematic acquired resistance, or even in the hypersensitivity responses against avirulent pathogens (Durner et al. 1997; Kunkel and Brooks 2002; Loake and Grant 2007), since it is capable of boosting the expression of defence responses through different pathways (Siegrist et al. 1994; Kauss and Jeblick 1995; Shirasu et al. 1997). In this way, the increase in salicylic acid levels in susceptible leaves after inoculation with *V. inaequalis* could be related to an attempt by the plant to systematically defend itself, triggering a molecular signalling cascade that leads to the production of enzymes and chemical compounds related to defence (Nicholson and Hammerschmidt 1992; Shirasu et al. 1997; van Loon 1997; Durrant and Dong 2004).

The levels of other phenolic compounds were also higher in apple leaves after the application of potassium phosphite. Several phenolic compounds are related to disease resistance, including protocatechuic acid, catechol, epicatechin, and various flavonoids (Vidhysekaran 1988). Protocatechuic acid is derived from salicylic acid, formed through salicylate oxidase action. It is a precursor in catechol synthesis, being one of the first phytoalexins isolated, and it exhibits antimicrobial activity in *Allium cepa* against *Colletotrichum circinans* (Link and Walker 1933). In our studies, we found that seedlings sprayed with potassium phosphite exhibited a considerable increase of protocatechuic acid in both susceptible and moderately susceptible leaves, mainly after the challenge with the pathogen. In this way, it is suggested that a part of the additional salicylic acid formed after the treatment with potassium phosphite may be converted into protocatechuic acid, leading to an increment in resistance against the disease. Such a finding was observed by Zhang et al. (2012), who described an accumulation of polyphenols, including salicylic acid and catechol, in peach (*Pyrus* × *bretschneideri* cv. Yali and *Pyrus* × *ussuriensis* cv. Jinbaili) when exposed to *V. nashicola*.

Another phenolic compound related to resistance against several apple diseases is epicatechin, which acts as an antioxidant against lipid peroxidation. Slatnar et al. (2010) suggest that flavonoids like epicatechin might accumulate around infection sites, forming a chemical barrier against colonization by *V. inaequalis*. Yin et al. (2013) verified that apple trees resistant to *Diplocarpon mali* exhibited higher concentrations of epicatechin after pathogen inoculation, probably contributing to resistance against the disease. In our studies we observed that susceptible leaves pulverized with potassium phosphite exhibited higher levels of epicatechin after fungal inoculation. Therefore, the induction of epicatechin biosynthesis is a major biochemical indicator of the way potassium phosphite could be acting for the control of apple scab.

A further indication that defence mechanism activation has played a more relevant role than direct antimicrobial activity can be seen in the results obtained with chitosan. Unlike potassium phosphite, chitosan showed some antifungal effect on the germination of *V. inaequalis* spores, but it did not control the disease. The antifungal and fungistatic

effects of chitosan have been reported on pathogenic fungi such as *Pythium aphanidermatum*, *Rhizopus stolonifer*, and *Colletotrichum acutatum* (El Ghaouth et al. 1992, 1994; Felipini and Di Piero 2009), but the sensitivity of those fungus species to the polysaccharide varies. Felipini and Di Piero (2009) showed a fungistatic effect of chitosan at 100 μg mL^{-1} on *C. acutatum*, a value 10 times lower than the effective dose found in this study. The relatively low sensitivity of *V. inaequalis* to chitosan and its inability to activate defensive mechanisms in susceptible leaves explains the absence of significant control of scab, even at a concentration five times higher than the one used on in vitro assays.

Thus, potassium phosphite has been shown to be effective in controlling scab. Apple plants treated with this salt exhibited changes in the concentrations of molecules related to defence mechanisms, such as salicylic acid, protocatechuic acid, and epicatechin. In this way, the induced resistance could play a significant role in scab control exerted by the potassium phosphite.

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