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Phosphite applications induce molecular modifications in potato tuber periderm and cortex that enhance resistance to pathogens

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ABSTRACT

Phosphite (Phi) compounds are salts derived from phosphorous acid. These compounds have the ability to protect plants against different pathogens. The aim of the present research was to assess the effect of Phi compounds on components of potato tuber periderm and cortex and to assess their effects on pathogen resistance in the postharvest stage. In a series of field experiments, potassium phosphite (KPhi) was applied to seed potato tubers and foliage. After harvest, several variables were analyzed in tubers obtained from these plants. An increase in pectin content was observed in both periderm and cortex tissue in tubers originating from KPhi-treated plants. After wounding and infection with *Fusarium solani*, a higher amount of pectin accumulation in cortical tissues was observed in tubers following treatment with KPhi. The content and/or activity of polygalacturonase and proteinase inhibitor also increased in tubers from KPhi-treated plants. A new isoform of chitinase was detected in the tuber periderm of treated plants.

These results suggest that KPhi applied to seed tuber and foliage induces defense responses in tuber periderm and cortex and that these reactions are associated with structural and biochemical changes in these tissues.

1. Introduction

Phosphite (Phi) compounds are salts derived from phosphorous acid. These compounds have the ability to protect plants against different pathogens (Bécot et al., 2000; Daniel and Guest, 2006; Jackson et al., 2000; Johnson et al., 2004; Oka et al., 2007; Reuveni et al., 2003). Treatments with Phi could be used within an integrated crop management program as an alternative that would serve to reduce the use of fungicides during the crop cycle. Previously, it has been shown that KPhi applied to seed tubers and foliage produced plants and tubers with increased resistance to *Phytophthora infestans*, *Fusarium solani*, *Rhizoctonia solani* and *Erwinia carotovora* (Lobato et al., 2008, 2011). In addition, the *in vitro* antimicrobial activity of KPhi against the same fungi and *Streptomyces scabies* has been also characterized, showing that KPhi inhibits the growth of these pathogens as a fungistatic compound (Lobato et al., 2010).

Several factors contribute to the resistance of potato tubers to pathogen infections. Among these factors are polygalacturonase-inhibiting proteins (PGIPs), proteinase inhibitors (PIs) and chitinases, previously studied in our laboratory (Feldman et al., 2000; Lobato et al., 2008, 2011; Machinandiarena et al., 2001). PGIPs are proteins associated with the cell wall of all dicotyledoneous plants. They counteract the action of fungal polygalacturonases (PGs), preventing cell wall degradation and therefore limiting the invasion of the pathogen (Di Mateo et al., 2006). In contrast, PIs and chitinases are both pathogenesis-related proteins and are known to be involved in plant defense reactions that inhibit pathogen proteases or degrade fungal wall components, respectively (Van Loon et al., 2006). In this study, we hypothesize that PGIPs, PIs and chitinases participate in defense mechanisms induced by KPhi.

Little is known about the biophysical and biochemical substances that could participate in the periderm response to pathogens. The potato periderm, the outer layer of the tuber, provides protection against pathogen invasion and allows limited gas exchange (Lulai and Freeman, 2001). The protective effect of tuber periderm results from its role as a mechanical barrier. Pectin and suberin are the main constituents of this structural barrier, but proteins could also contribute to this protective effect. The protein

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composition of tuber periderm also changes during maturation and with environmental conditions. A proteomic analysis of skin proteins in mature and immature tissue has shown that the mature skin is enriched in plant-defense-related proteins. This finding indicates that in mature periderm, proteins can also play a role in defense against pathogens (Barel and Ginzberg, 2008).

The quality of the periderm is an important feature for the storability of commercialized potatoes. However, some potato pathogens, such as *F. solani*, penetrate the tuber cortex through wounds, avoiding periderm tissue. Previously, we have shown that the lesion area in tubers coming from KPhi-treated plants after *F. solani* inoculation was reduced by approximately 50%, compared with the lesion size produced in tubers obtained from nontreated plants (Lobato et al., 2011).

The aim of the current work, based on this evidence, was to study the possible effect of KPhi application on tuber periderm and cortex and its relationship with pathogen resistance and storability.

2. Materials and methods

2.1. Field experiments

Three field experiments were performed at Balcarce (37° 45′ S.) during the spring-summer (mid-October to late February) growing seasons of 2007/2008, 2008/2009 and 2009/2010 with cv. Bannock Russet, a long-cycle variety with russet skin, white flesh, high dry-matter content and moderate susceptibility to *F. solani* (Andreu, 2004). The growing area of Bannock Russet has also increased in recent years because the variety is processed to make French fried potatoes. Crops were grown at the McCain Argentina Experimental Field and irrigated by a forward advance system. The experimental design was a four-row plot (0.85 m between rows), 6 m long (20 m^2), replicated 3 times at random. A density of 5.8 plants m^{-2} was used in each season. The other practices used in the study were those common to the area.

The experimental treatments, denoted by letters in square brackets, were applied to seed tubers and/or foliage as follows: [C] seed tubers and crops were sprayed with water; [F-KPhi] seed tubers were treated with 1.5 l ha^{-1} Vitavax 300 (20% Carboxin + 20% Captan, Bayer Cropscience) + 2.5 kg ha^{-1} Acrobat MZ® (9% Dimethomorph + 60% Mancozeb, BASF Company) (conventional fungicide treatment) and crops were sprayed with 3 l ha^{-1} potassium phosphite (KPhi, 1.07 kg ha^{-1} of the active ingredient, Agro-EMCODI, Buenos Aires, Argentina); and [KPhi–KPhi] seed tubers and crops were sprayed with 3 l ha^{-1} KPhi (approximately 5 ml per seed tuber or per plant). Treated seed tubers were planted as described above. Each treatment was then sprayed 6 times with 3 l ha^{-1} KPhi every 2 weeks starting at tuber initiation. Applications were done with a backpack sprayer SHURflo ProPack™ model SRS 600CE. The three central rows of each replication were harvested by hand. The tubers were stored at 8 °C for further analysis.

2.2. Biological material and inoculation process

F. solani f. sp. *eumartii* isolate 3122 (*F. solani*) was obtained from the INTA collection, Balcarce, Argentina. The fungal cultures were grown in solid potato dextrose agar (PDA) for 3 weeks at 25 °C.

The whole tubers were inoculated with the mycelium and spores of *F. solani* by making wounds in the cortex (Olivieri et al., 1998). Briefly, a 0.8 cm disk of PDA colonized by *F. solani* was introduced into the potato cortical tissue using the hollow punch method (Radtke and Escande, 1973). Noninfected, wounded tubers were inoculated with a disk of sterile PDA medium. The inoculated tubers were stored at 25 °C. Ten tubers per treatment were used, and the experiment was performed three times. These tubers were used in the following analysis.

2.3. Extraction of periderm and cortex components

The periderm tissue was peeled from the tuber flesh with a scalpel. During this procedure, contamination with tuber parenchyma cells was avoided. Pieces of periderm were homogenized in a mortar with 1.5 volumes of buffer containing 50 mM sodium acetate at pH 5.2, 0.1% β-mercaptoethanol and 1.5 M NaCl. The homogenate was stirred for 16 h at 4 °C and then centrifuged for 10 min at 10,000 rpm. The supernatant (periderm extract) was collected and stored at −20 °C for PGIP, chitinases and pectin analysis. The cortex tuber tissue surrounding the inoculation site (5–8 mm around the site) at zero (T_0) or 16 days post-inoculation was homogenized in a mortar with 1.5 volumes of buffer containing 50 mM sodium acetate at pH 5.2, 0.1% β-mercaptoethanol and 1.5 M NaCl. The homogenate was centrifuged 10 min at 10,000 rpm. The supernatant (cortex extract) was collected and stored at −20 °C for protein and pectin analysis. A specific pectin extraction was made according to Willats and Knox (1999). Briefly, cortex tissue (1 g fresh weight) was homogenized in 1 ml of a buffer solution containing 50 mM Tris–HCl at pH 7.2, 50 mM *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) and 25 mM dithiothreitol and centrifuged at 7000 rpm for 10 min.

2.4. Pectin detection

For pectin detection, cortex, periderm, or pectin-enriched extracts were diluted in deionized water and loaded onto a nitrocellulose membrane in droplets of 3 μl (equivalent to 1 mg and 2.7 mg of FW for periderm and cortex, respectively). The membranes were blocked with PBS containing 5% milk powder for 1 h prior to incubation with the anti-pectin antibody JIM7, as described by Willats and Knox (1999). The bound antibody was visualized using a second antibody, anti-rat alkaline phosphatase conjugated. Immunodots were quantified with the Scion software (Scion Corporation).

2.5. PGIP activity assay

PGIP activity was determined by measuring the reducing end-groups released by commercial polygalacturonases from polygalacturonic acid (Pectinase and PGA, Sigma, St Louis, MO, USA) according to the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). PG activity was determined in 0.1 ml reaction mixtures containing 0.5% (w/v) polygalacturonic acid as substrate, 50 mM pH 5.2 sodium acetate buffer, and pectinase from *Aspergillus niger* (Sigma, St. Louis, MO, USA). The samples were maintained at 37 °C for 60 min. For the measurement of PGIP activity, the same mixture was used with suitable amounts of protein crude extracts.

One unit (1 U) of PGIP activity was defined to be that amount of crude extract that inhibits PG activity by 50%.

2.6. Partial purification of proteinase inhibitor/s from periderm

The potato periderm of tubers coming from different field treatments was pulverized in a mortar with liquid N_2 and homogenized in 50 mM Tris–HCl at pH 7.5 and containing 0.1% (v/v) β-mercaptoethanol, 0.6 M NaCl and 0.13% (v/v) Tween 20. The homogenate was centrifuged at 10,000 rpm for 30 min and submitted to thermal treatment at 80 °C for 10 min.

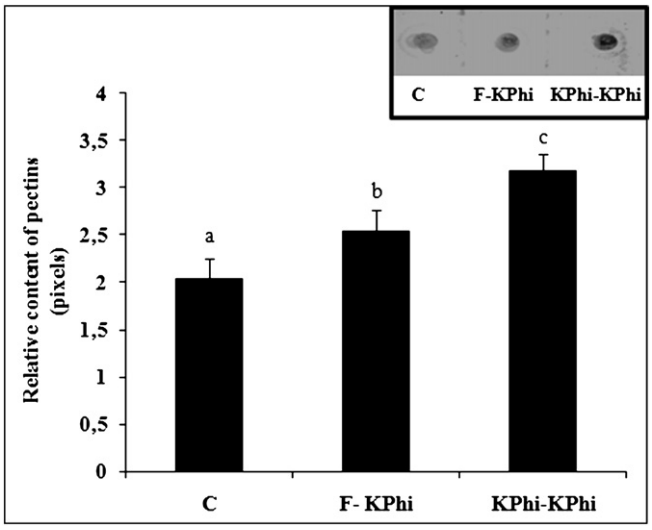

Fig. 1. Pectins content in the periderm of potato tubers from potassium phosphite (KPhi)-treated plants. Immunodot analysis (insert) was performed using a JIM7 monoclonal antibody. Equal quantities of periderm extracts were loaded onto a nitrocellulose membrane. The relative content of pectins was estimated by densitometric scanning of dot-blots. C: nontreated; F-KPhi: fungicide applied to seed tuber and KPhi to foliage; KPhi–KPhi: KPhi applied to seed tuber and foliage. Vertical bars indicate standard deviations ($n = 3$). Columns with the same letters are not significantly different at $P \leq 0.05$.

2.7. Proteinase inhibitory activity

Inhibitory activity was measured by incubating commercial proteinase K (Sigma) with a volume of each sample equivalent to 3.7 mg of fresh weight and 2.5 mg of azocasein as substrate. The reaction mixture also contained 50 mM Tris–HCl at pH 8.0 and 1 mM $CaCl_2$ in a final volume of 0.5 ml. After incubation for 30 min at 37 °C, the reaction was stopped with 1 volume of 10% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant at 335 nm is a measure of the residual proteinase activity. The inhibitory activity of the preparation was calculated as the percentage of proteolytic activity in the absence or presence of the inhibitor (% inhibition).

2.8. Gel electrophoresis and immunoblot analysis

For immunoblotting, protein extracts (volume equivalent to 2 mg of fresh weight) were electrophoresed on SDS-PAGE and transferred onto nitrocellulose using a semi-dry electrophoretic transfer cell (Trans-Blot, Bio-Rad, Hercules, CA, USA). Immunodetection was performed as described by Turner (1986), using either a polyclonal antibody raised against chitinase (Kombrink et al., 1988) or PLPKI, an antibody raised against a proteinase K inhibitor (Feldman et al., 2000). The bound antibody was visualized with a second antibody, goat anti-rabbit conjugated with alkaline phosphatase. Equal amounts of fresh weight were loaded in each lane of the gel.

2.9. Data analysis

The distributions of all variables were approximately normal. Pectin content and PGIP and PI activities were analyzed using one-way ANOVA (Zar, 1999). Multiple comparison (Tukey) tests were performed when significant ($P < 0.05$) differences between means were detected by ANOVA.

3. Results

3.1. Pectin content in periderm tissues

To investigate possible differences in pectin content in periderm tissues from KPhi-treated plants, an immunodot analysis was performed with JIM7 monoclonal antibody (Willats and Knox, 1999). As shown in Fig. 1, pectins were more abundant in tuber periderm extracts from KPhi-treated plants. The highest content was detected in extracts from tubers coming from plants treated with KPhi both in seed tubers and foliage (KPhi–KPhi). A lower pectin content was found in extracts from plants treated with fungicide in seed tubers and with KPhi in foliage (F-KPhi). The lowest pectin value was found in nontreated plants (C).

Different pectin extraction procedures could produce different values of the changes in pectin content. A second specific pectin extraction technique was applied to tuber cortical tissues (Willats and Knox, 1999). No differences in pectin content were detected using the two methods (data not shown).

3.2. Pectin content in cortex tissue infected or not infected with *Fusarium solani*

The pectin content of healthy cortex tissue (Fig. 2a, first column) was similar to that found in the periderm. However, wounding produced a marked increase in pectin content in tubers taken from treated (F-KPhi and KPhi–KPhi) and nontreated plants. In contrast, when the tubers were wounded and infected with *F. solani*, the pectin content decreased relative to that found for the wounding treatment in control tubers, whereas in F-KPhi tubers, this decrease was not as evident. It is noteworthy that the highest level of pectin content was found in the KPhi–KPhi plus *F. solani* treatment (Fig. 2).

3.3. PGIP activity in cortex tissue

In the tubers from all treatments, polygalacturonase-inhibiting protein activity increased in cortex tissue after wounding. This increase was 1.7- (70%) and 1.9- (96%) fold in tissue coming from F-KPhi and KPhi–KPhi treated plants, respectively. In contrast, in tubers from nontreated plants the increase was only 1.2-fold (20%) (Fig. 3).

3.4. Proteinase inhibitor (PI) activity and content in periderm tissue

To assess the ability of KPhi treatment to induce potato PI activity, a partial purification by heat treatment was performed. When partially purified protein extracts from periderm tissue were analyzed, PI activity increased 2.5-fold only when plants were treated with KPhi both in seed tubers and foliage (KPhi–KPhi) (Fig. 4a). Moreover, PI was only detected by the western blot method in plants treated with KPhi–KPhi (not shown).

In cortex tissue, the PI content markedly increased in the KPhi–KPhi treatment in healthy and in *F. solani*-infected tubers (Fig. 4b). However, in healthy and in *F. solani*-infected tubers the PI content of the F-KPhi extracts was lower than that found in the nontreated case.

3.5. Chitinase content in periderm tissue

Fig. 5 shows that an additional chitinase isoform appeared in healthy tuber periderm taken from F-KPhi and KPhi–KPhi treated plants. This isoform was not found in the periderm of nontreated plants. This band corresponds to a protein with the highest electrophoretic mobility of any chitinase detected in the study (Fig. 5, arrow). It is striking that the content of chitinases detected in

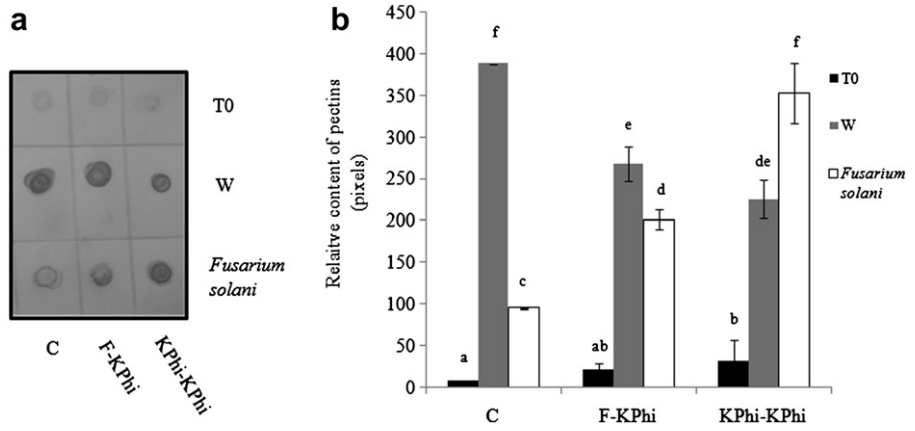

Fig. 2. Pectins content in the cortex of potato tubers from potassium phosphite (KPhi)-treated plants. Immunodot analysis (a) was performed using a JIM7 monoclonal antibody. Equal amounts of cortex extracts were loaded onto a nitrocellulose membrane. The relative content of pectins was estimated by densitometric scanning of dot-blots (b). C: nontreated; F-KPhi: fungicide applied to seed tuber and KPhi applied to foliage; KPhi–KPhi: KPhi applied to seed tuber and foliage; To: cortex tissue extracted from stored healthy tubers; Wounding: cortex tissue extracted from wounded (16 days) tubers; *F. solani*: cortex tissue extracted 16 days after inoculation with the fungus. Vertical bars indicate standard deviations ($n = 3$). Columns with the same letters are not significantly different at $P \leq 0.05$.

healthy tuber periderm coming from nontreated or treated plants has been found to be very high relative to the amounts reported in other potato tissues, like cortex, under nonstress conditions (Lobato et al., 2011).

4. Discussion

Many factors contribute to the resistance of potato tubers to pathogen infections. Among these factors, mechanical and biochemical barriers present in the cell walls of the periderm and parenchymatic external tissues of potatoes play an important role. This study anayzed the effect of seed tuber and foliarly applied KPhi on the periderm and cortex compounds of tubers.

We have previously described the protective effect of KPhi on potato plants against different pathogens (Lobato et al., 2008, 2011). The resistance of plants induced by KPhi was analyzed in leaves, seed tubers or whole tubers. However, this previous analysis did not treat the external protective tissues like periderm and cortex independently. We hypothesized that the resistance induced by KPhi in tubers could be due, at least in part, to modifications in the content and/or activity of the periderm and cortex compounds involved in pathogenic defense reactions. In other systems (e.g., potato-*Phthorimaea operculella*), it has been described that both periderm and cortex tissue can offer resistance to insect attack. However, the authors suggested that the levels of periderm resistance were not correlated with the levels of cortex resistance and that each type of tissue represented an independent resistance source (Horgan et al., 2007).

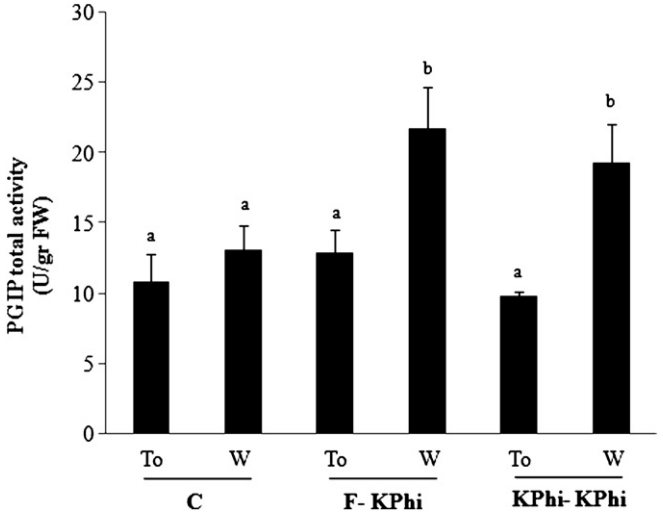

Fig. 3. Polygalacturonase inhibitor activity in the cortex tissue of potato tubers from potassium phosphite (KPhi)-treated plants. Cortical tissue extracts were prepared from healthy tubers (To) or from tubers 16 days after wounding (W). C: nontreated; F-KPhi: fungicide applied to seed tuber and KPhi to foliage; KPhi–KPhi: KPhi applied to seed tuber and foliage. Vertical bars indicate standard deviations ($n = 3$). Columns with the same letters are not significantly different at $P \leq 0.05$.

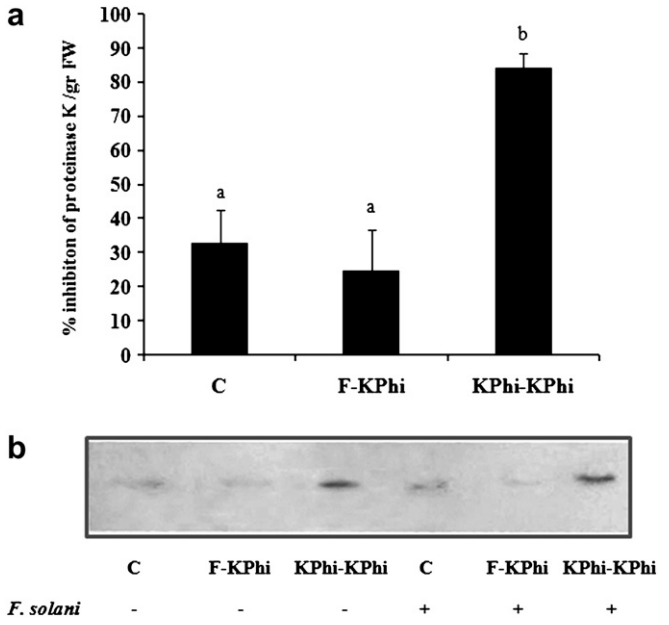

Fig. 4. Proteinase inhibitor activity and content in potato tubers tissues from potassium phosphite (KPhi)-treated plants. Proteinase inhibitor activity (a) was measured in periderm extracts from healthy tubers. The proteinase inhibitor content (b) was analyzed by the western blot method in cortical tissue extracts of healthy (*Fusarium solani* −); or infected (*F. solani* +) tubers. C: nontreated; F-KPhi: fungicide applied to seed tuber and KPhi applied to foliage; KPhi–KPhi: KPhi applied to seed tuber and foliage. Vertical bars indicate standard deviations ($n = 3$). Columns with the same letters are not significantly different at $P \leq 0.05$.

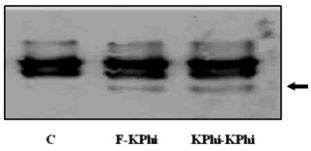

Fig. 5. Chitinase content in the periderm tissue of potato tubers from potassium phosphite (KPhi)-treated plants. Immunoblot analysis was performed using an anti-basic chitinase polyclonal antibody. Equal amounts of periderm extracts were loaded onto electrophoresis gel. C: nontreated; F-KPhi: fungicide applied to seed tuber and KPhi applied to foliage; KPhi–KPhi: KPhi applied to seed tuber and foliage. The arrow indicates the differentially expressed chitinase isoform.

This work investigated compounds associated with the cell wall, like pectins and defense proteins, and studied their content or activities. The pectin content revealed by JIM7 antibody was measured both in periderm and in healthy or infected cortex tissue, and its value was higher in periderm extracted from KPhi-treated plants (Figs. 1 and 2). This result suggests that the cell wall or middle lamella of this tissue might be reinforced by the accumulation of this compound in response to KPhi treatment. The pectin content also changed significantly in extracts from wounded cortex. This increase was high in all treatments. However, in infected tissue the pectin content decreased in nontreated tubers and remained high in tubers coming from KPhi-treated plants, whereas the highest content was found in tuber cortex coming from plants treated with KPhi–KPhi. This effect could be explained either by a reduced level of activity of fungal pectin hydrolases, resulting from tuber protection acquired through KPhi treatment, or by a direct effect of KPhi on *F. solani* (Lobato et al., 2010, 2011). The mode of action of KPhi might involve modifications in the pectin content of the cell wall. These modifications may be associated with its reinforcement and could also help to maintain pectin structure integrity. To our knowledge, no previous reports have related the action of Phi to the structure or pectin content of the cell wall. Further histological studies are needed to support the observations made in this work.

The complex mechanisms of plant–pathogen interactions also include the production by pathogens of enzymes like proteases and polygalacturonases that degrade plant cell walls. As a response to these threats, plants synthesize proteins that are able to inhibit the activity of the enzymes involved in attacks on the cell wall. PGIP are proteins associated with the cell wall. They can act as defense proteins by inhibiting pathogen polygalacturonases and can then elicit defense responses mediated by the oligogalacturonide residues released (Di Mateo et al., 2006). In this work, foliar and seed tuber treatments with KPhi increased PGIP activity in wounded cortex tissue, although we could not detect this activity in periderm (results not shown). This increased activity might help to control invasions of potato cortex tissue by pathogens. Proteinase inhibitors (PIs) are induced by wounding, insect feeding or microbial attacks and play a key role in plant defense mechanisms (Sels et al., 2008). Therefore, PIs can be considered to belong to a general class of proteins responsible, in part, for counteracting pathogen hydrolases (Gvozdeva et al., 2006; Valueva and Mosolov, 2004). In this study, the KPhi–KPhi treatment increased the PI activity and content in both periderm and cortex tissue. It is interesting to remark that these increases in PI content and activity were both quantified with an antibody specific to a proteinase K inhibitor and measured as the inhibition percentage of proteinase K, a protease of microbial origin. The participation of potato PIs in induced defense has been described by Ozeretskovskaya et al. (2009). These authors reported that jasmonic acid (JA) locally and systemically stimulated the synthesis of potato wound periderm and elevated the level of proteinase inhibitors associated with this process. These results allow us to speculate on the possibility that JA may be implicated in the stimulation of PI by KPhi; however, is unlikely to act alone. Further experiments will be necessary to confirm or discard this hypothesis. Recently, Eshraghi et al. (2011) reported that the phytohormones salicylic acid and JA/etilene are involved in defense mechanism induced by Phi, suggesting that this inducer could allow the recruitment of molecules that participate in different pathways of the defense response. Therefore, we can not discard that all these hormones participate in the general mechanism induced by Phi in potato.

As is well documented, necrotrophic fungi secrete several hydrolases to degrade tissue components. In particular, *F. solani* secretes both polygalacturonase and serine protease activities (Olivieri et al., 2004). Our results allow us to speculate that both enzymatic activities could be inhibited by PGIP and PIs, respectively, through a KPhi-induced mechanism. We propose that the induction of these enzymes may be a general response against potato tuber pathogens and not just restricted to *F. solani*. No previous reports have described a possible relationship between these enzymes and KPhi action.

Chitinases were also detected in periderm tissue, and at least one isoform was induced by the KPhi treatments. The fact that the level of one chitinase was increased by the KPhi treatments would suggest that this chitinase may be involved in the battery of responses triggered by KPhi in potato tubers. In addition, in view of the fact that these proteins are commonly expressed under stress conditions, an important aspect of our results is that the periderm showed a high level of chitinases in nontreated plants.

This is the first report to correlate KPhi action with an increase in defense mechanisms at the periderm level. The mechanisms implicated in this increase are the reinforcement of the cell wall and the induction of the activity and/or content of defense-related proteins. Furthermore, these molecules might be implicated in defense mechanisms occurring in inner tissues (e.g., in cortex). It is noteworthy that foliar treatment with KPhi during plant growth produces tubers that exhibit a battery of increased defense mechanisms located in the outer tissues of the tubers, the first area exposed to pathogens.

The fact that KPhi treatment may induce the reinforcement of the cell wall and improve the ability of tubers to withstand pathogen attack during storage could be a very important finding for the purpose of developing methods of preventive treatment.

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