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Phosphite compounds reduce disease severity in potato seed tubers and foliage

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Abstract Phosphites (Phi) are alkali metal salts of phosphorous acid, with the ability to protect plants against different pathogens. In this research, the effect of Phi applied to potato plants on severity of three important potato diseases in Argentina was assessed. Seed tubers and foliage of potato cvs Shepody and Kennebec were treated with Phi to assess effects on resistance against *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani*. Protection resulting from Phi treatment in seed tubers was high against *P. infestans*, intermediate against *F. solani*, and low against *R. solani*. In addition, seed tubers treated with calcium or potassium phosphites (CaPhi and KPhi, respectively) at 1% of commercial product emerged earlier than untreated ones. When Phi were foliarly applied two or four times at different doses, high levels of protection against *P. infestans* were achieved in both cultivars. Higher protection was observed in Kennebec when CaPhi was applied, while in Shepody this was true for KPhi. Expression of β-1,3-glucanases was induced at different times after treatment but no correlation between β-1,3-glucanases expression and foliar protection level was found. On the other hand, Phi positive protection effects did not produce negative effects in plant growth. Leaves from CaPhi-treated plants showed a darker green colour than leaves from control plants; also an increase in Rubisco protein and a delay in crop senescence was observed.

Keywords *Fusarium solani* · Phosphites · *Phytophthora infestans* · Protection · *Rhizoctonia solani* · *Solanum tuberosum*

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Introduction

Potato late blight, caused by the oomycete *Phytophthora infestans* is one of the most important potato (*Solanum tuberosum*) diseases. Other pathogens affecting the crop are *Fusarium solani* f. sp. *eumartii* and *Rhizoctonia solani*, responsible for causing dry rot and stem canker diseases, respectively (Torres 2002). In Argentina, potato cvs Shepody (highly susceptible to late blight, susceptible to dry rot and moderately susceptible to stem canker) and Kennebec (moderately susceptible to late blight, dry rot and stem canker), comprise >20% of the total potato processing production area (Caldiz 2007). This means

that in most growing regions potato agroecosystems are dominated by large patches of genetically homogeneous crop, which could favour rapid disease development. Consequently, one of the tactics utilized for disease management is the intensive use of fungicides which leads to high environmental and production costs. Integrated crop management (ICM) offers an alternative to reduce the use of fungicides. It was reported that certain chemical compounds are capable of naturally increasing plant defence mechanisms (Gozzo 2003, 2004). These compounds could be used as part of ICM.

In previous work, we showed that aminobutyric acid (BABA) and fosetyl–Al, foliarly applied at early stages of crop growth, can increase the resistance of potato foliage and tubers to late blight (Andreu et al. 2006). Phosphites (Phi) are alkali metal salts of phosphorous acid (H_3PO_3) that cannot be directly used by crops as a sole source of nutritional phosphorous (McDonald et al. 2001). However, Phi in general can stimulate plant defence responses and are also active against oomycetes *in vivo* (Guest and Grant 1991). Johnson et al. (2004) assayed phosphorous acid applied to foliage for control of tuber rot diseases of potato cultivars under field conditions. They found that this compound contributed to a reduction in late blight tuber rot and pink rot. Since Phi are environmentally friendly (Guest and Grant 1991) and reduce disease severity (Bécot et al. 2000), they represent a potential alternative for use within an ICM programme. Hence, the aim of this research was to assess the effect of Phi applied both to potato seed tubers for control of diseases produced by *P. infestans*, *F. solani* and *R. solani*, and to the foliage for late blight control. In view of the possible utilization of Phi in ICM programmes, some physiological aspects related to crop yield were also analyzed.

Materials and methods

Pathogen isolates and inoculum production

Phytophthora infestans race R_2 R_3 R_6 R_7 R_9, mating type A2, was isolated and cultivated as described by Andreu et al. (2006). For inoculum production, small pieces of selective medium containing actively growing *P. infestans* hyphae were transferred to tuber slices of cv. Bintje. The slices were incubated in closed plastic boxes in darkness at 18°C and 90% relative humidity (RH). After 7 days the mycelia and sporangia were harvested in sterile water and stimulated to release zoospores by incubation at 4°C for 6 h. After filtration through a 15 μm nylon filter cloth, the zoospore suspension was observed under a light microscope. The concentration of zoospores was adjusted to 4×10^4 zoospores ml^{-1} using a haematocytometer. *Fusarium solani* f. sp. *eumartii* isolate 3122 was provided by the Laboratorio de Fitopatología, INTA Balcarce, Argentina. A stock culture was maintained on 2% glucose-potato agar at 25°C in darkness. Inoculum was produced by washing the cultures in Petri dishes with sterile water and adjusting the concentration to 3×10^3 conidia ml^{-1} using a haematocytometer. Stock culture of a pathogenic isolate of *R. solani* (AG-3), also provided by the Laboratorio de Fitopatología, INTA Balcarce, Argentina, was maintained on 2% glucose-potato agar, at 18°C in darkness. Inoculum was produced as follows. Dried oat grains (Sneh et al. 1986) were soaked overnight in vials with water supplemented with 250 μg of chloramphenicol. Water was decanted and grains were autoclaved at 121°C for 1 h on two consecutive days. Mycelial disks from the margins of actively growing cultures were placed in the vials and incubated at 18°C for 7–10 days. After incubation, the oat grains colonized by the fungi were used as inoculum.

Plant material and growing conditions

Seed tubers of cvs Shepody (highly susceptible to late blight, susceptible to dry rot and moderately susceptible to stem canker) and Kennebec (moderately susceptible to late blight, dry rot and stem canker) were used due to their different susceptibility indices (Andreu and Caldiz 2004; Caldiz 2007). For seed tuber assays, seed pieces (45 g) were cut from tubers harvested 4 months previously, and planted at 6 cm depth in steam-pasteurized substrate immediately after treatments (Phi application and pathogen inoculations). Pots were placed in growth chambers at different temperatures depending on the pathogen being tested. Fifteen seed pieces were used per treatment and each experiment was repeated three times.

For foliage assays, non-treated seed tuber pieces (45 g) were planted at 6 cm depth in steam-

pasteurized substrate. Pots were maintained in a greenhouse under a day–night regime temperature 24–15°C and natural daylight supplemented by high-pressure sodium lamps (400 W) to provide 16 h daylight. Emerged plants were maintained under these conditions and irrigated with a sprinkler system. These growing conditions were applied to all foliage experiments (Phi treatments 1 and 2, see below), which were performed at least three times each. Fifty plants were used in each experiment and they were randomly distributed within the greenhouse.

Application to seed tubers

Phi were provided by Agro-EMCODI SA (Buenos Aires, Argentina) Ca and K phosphite salts (CaPhi and KPhi, respectively) were applied to seed tuber pieces at 1% (*v*/*v*) of the commercial product (equivalent to 3 l ha^{-1}) by spraying immediately after cutting at planting time, using an atomizer (ESAC SA) operating at 200 kPa. Approximately 50 ml of the dilution were used per 50 seed pieces. Water was applied to seed tuber pieces as a control treatment.

Phytopathological assays in seed tubers

Control of late blight was evaluated by quantifying the number of emerged plants without disease symptoms (Powelson et al. 2002), 5 weeks after planting. For this purpose, Phi-treated and control seed tuber pieces were sprayed with a zoospore suspension of *P. infestans* (4×10^4 zoospores ml^{-1}) immediately before planting. Individual seed pieces were planted and pots were placed in a growth chamber (18±2°C and 16 h daylight).

Dry rot severity was evaluated in seed tubers treated with Phi or in non-treated controls, inoculated with a solution of *F. solani* f. sp. *eumartii* conidia. Individual seed pieces were planted and placed in a growth chamber (25±2°C, 16 h daylight). Two weeks after planting, disease severity was evaluated using an arbitrary scale of 0=no symptoms, 1=<2.5% of cut area with symptoms, 2=2.5–10% cut area with symptoms, 3=>10–25% cut area with symptoms, 4=>25–50% cut area with symptoms and 5=>50% cut area with symptoms of susceptibility.

Disease severity caused by *R. solani* was evaluated in emerged plants from control and Phi-treated seed tubers. Individual seed pieces were planted at 6 cm depth in steam-pasteurized substrate and ten oat kernels colonized by *R. solani* were placed at 1–2 cm depth. After planting, pots were placed in a growth chamber (25±2°C, 16 h daylight). Five weeks after planting the symptoms in each emerged plant were evaluated using an arbitrary scale described by Escande & Echandi (1991) with some modifications, where 0=no lesions, 1=lesions <5 mm long, 2=lesions >5 mm long, 3=lesions girdling the stem and 4=death of the pre-emerging sprout.

Evaluation of emergence

Seed tuber pieces (45 g) of cvs Shepody and Kennebec were sprayed with Phi or water as described above and planted in steam-pasteurized substrate at 6 cm depth. The effect of Phi on emergence was evaluated by determining the percentage of emerged plants when the control treatment achieved approximately 80% emergence.

Foliage treatment

CaPhi and KPhi were applied to the foliage at 33 and 66 days after emergence (dae) (Treatment 1) or 36, 51, 66 and 80 dae (Treatment 2). CaPhi or KPhi were applied at 30 ml per plant by using an atomizer (ESAC SA) operating at 200 kPa. Doses utilized were 1%, 1.5% and 2% of the commercial product (equivalent to 3, 4.5 or 6 l ha^{-1}, respectively) in Treatment 1; and 1 and 1.5% in Treatment 2. In both treatments, control plants were sprayed with water each time Phi were applied.

Evaluation of foliage protection and persistence

Percentage of foliage protection against *P. infestans* was evaluated by the detached leaf method (Goth and Keane 1997). In Treatment 1 foliage protection was assessed at 5, 14, 21 and 33 days after application (daa), while in Treatment 2 foliage protection was assessed at 7 and 14 daa. In each test two leaflets per plant were detached from ten plants per treatment. In the laboratory, these leaflets were artificially inoculated with two 50 µl droplets of a zoospore suspension (4×10^4 zoospores ml^{-1}) of *P. infestans* applied to the centre of each abaxial side. The inoculated leaflets were placed in Petri dishes with wet filter paper and incubated at 4°C for 24 h. After

this period, incubation continued at 18°C for 6 days. Disease severity index in treated and control leaflets was recorded 7 days after inoculation by estimating the leaf area showing late blight lesions on a scale from 1 to 10, where 1=no lesions, 2=a few circles, 3 =up to 5%, 4=>5–10%, 5=>10–25%, 6=>25–50%, 7=>50–75%, 8=>75–85%, 9=>85–95% and 10= >95–100% of the total area with late blight symptoms. Foliage protection percentage (FPP) was calculated as follow: FPP (%)=100 $(1-x/y)$, where x and y are disease severity indices for treated and control plants, respectively (Andreu et al. 2006).

Preparation of soluble leaf extracts

Either 5 or 21 days after treatment with Phi (1%), leaflets were inoculated with *P. infestans* and 72 h later processed for biochemical analysis. Potato leaflets (1 g) were homogenized in 100 mM sodium acetate buffer pH 5.2 containing 0.5 g l^{-1} aqueous sodium metabisulphite using a mortar. Homogenates were filtered through cheesecloth and centrifuged at 12,000×g for 20 min. The resulting supernatant represented the soluble extract used for further processing and was stored at −20°C.

Gel electrophoresis and immunoblot analysis

For immunoblot analysis, the soluble extract was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 120 g l^{-1} aqueous acrylamide (Laemmli 1970) and subsequently transferred electrophoretically (15 V, 20 min, 25 mM Tris–HCl, pH 8, 0.192 M glycine, 20% methanol) onto nitrocellulose membranes in a semi-dry electrophoretic transfer cell (Trans-Blot, Bio Rad, Hercules, CA, USA). The immunodetection was performed as described by Turner (1986) using a polyclonal antibody raised against a β-1,3-glucanase (36 kDa) purified from intercellular fluid of potato leaves infected with *P. infestans* (Kombrink et al. 1988). The nitrocellulose sheet was soaked for 2 h with a solution containing 100 mM Tris–HCl, pH 8.0 and 10 g l^{-1} bovine serum albumin (BSA). The membrane was washed four times with 100 mM Tris–HCl, pH 8.0 containing 0.15 M NaCl and 0.05% (v/v) Tween 20 (TBST) and then incubated overnight with rabbit anti-β-1,3-glucanase (1:3,000 v/v) in 100 mM Tris–HCl, pH 8.0, and 10 g l^{-1} BSA. After four washes with TBST solution, the blot was allowed to react for 2 h with goat anti-rabbit antibody (1:10,000 v/v) labelled with alkaline phosphatase (Sigma). Bound antibody was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue-tetrazolium (BCIP/NBT) according to procedures recommended by the manufacturer (Sigma).

The intensity of the immuno-positive bands on the western blots was measured using densitometric analysis (TN-Image, Image Analysis Software, Compuserve, IBMAPP, Rockville, MD USA). Equal amounts of fresh weight (7.5 mg) were loaded in each lane of the gel and molecular weight was estimated using standard molecular markers from Sigma. Protein concentration was measured by the Bradford method (Bradford 1976), using BSA as standard. Rubisco (ribulose bisphosphate carboxylase/oxygenase) was visualized in leaf soluble extracts by SDS-PAGE stained with Coomassie-Blue.

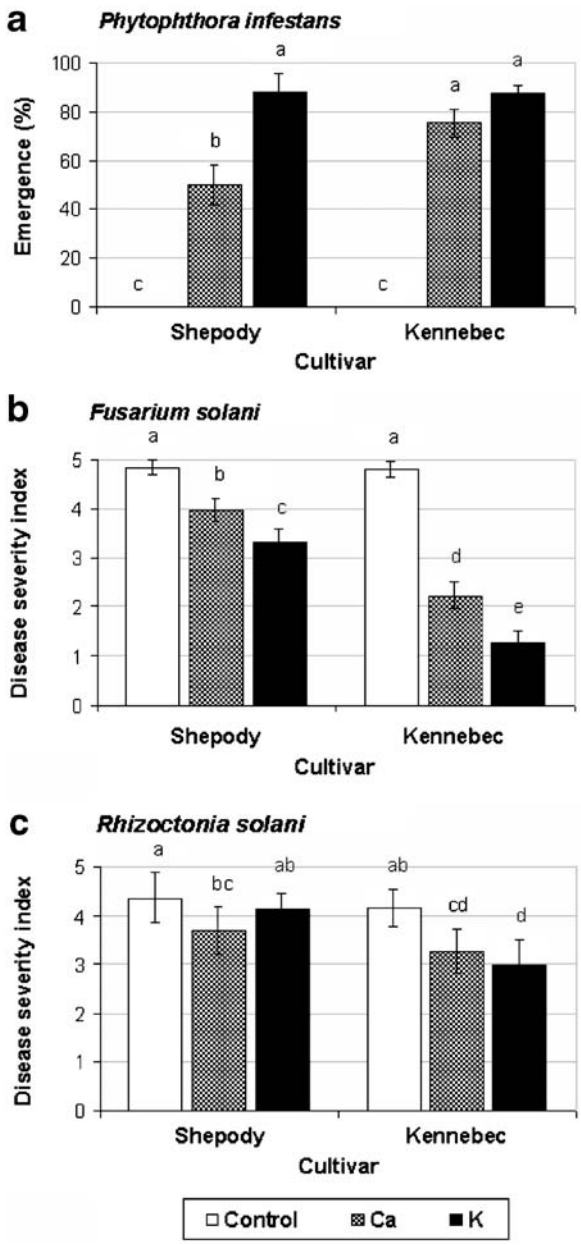
Data analysis

The null hypothesis of no differences between treatments in the emergence, severity and protection data was evaluated independently for each variable by one-way ANOVAs (Zar 1999). When a significant interaction was found, main effects of factors were not considered due to the lack of independence between them. *A posteriori* multiple comparison tests (Tukey test) were performed when significant differences between factors were detected (Underwood 1997).

Results

Effects of seed tuber treatments

Control of late blight by Phi treatment to seed tubers was evaluated through the percentage of emerged plants from treated-inoculated and control-inoculated seed tubers. KPhi reduced late blight incidence in seed tubers of Shepody and Kennebec inoculated with *P. infestans*. KPhi-treated inoculated seed reached almost 90% emergence while no emergence was observed in control-inoculated seed tubers (Fig. 1a). CaPhi-treated seed tubers resulted in a 50 and 75% of



◀ **Fig. 1** Effect of phosphite treatment to seed tubers on control of three potato pathogens. Seed tuber pieces were sprayed with water (control), 1% CaPhi (Ca) or 1% KPhi (K). **a** Infection with *P. infestans* was done by spraying seed pieces with a zoospore suspension. **b** Infection with *F. solani* f. sp. *eumartii* was achieved by spraying seed pieces with a conidial suspension. **c** Soil was infected with oat kernels colonized by *R. solani*. Protection against *P. infestans* was evaluated by comparing the number of emerged plants from treated-inoculated and control-inoculated seed pieces. Severity of disease caused by *F. solani* and *R. solani* was evaluated using a semi-quantitative scale (see "Materials and methods" for details). *Bars* represent standard deviations. *Bars with the same letter* are not significantly different at $P<0.05$ (Tukey multiple comparison)

emergence for Shepody and Kennebec, respectively. All plants from treated and inoculated seed tubers emerged without late blight symptoms.

Phi-treated seed tubers showed a reduced dry rot severity due to *F. solani*. In both cultivars efficacy of KPhi was higher than CaPhi. In Kennebec average disease severity indices decreased by 55 and 75% approximately by CaPhi and KPhi treatments, respectively, while in Shepody average disease severity indices decreased by 20 and 30% approximately by CaPhi and KPhi, respectively (Fig. 1b). Phi treatment of seed tubers had little effect in reducing disease severity caused by *R. solani*. Only CaPhi reduced the disease severity index by 15% in Shepody while in Kennebec a decrease of approximately 20 and 25% was observed by CaPhi and KPhi treatments, respectively (Fig. 1c).

In addition, Phi application to seed tubers immediately after cutting resulted in earlier emergence of plants. By the time that control plants reached approximately 80% emergence, Phi-treated seed tubers reached at least 93 and 99% emergence for the CaPhi and KPhi treatments, respectively, in both cultivars (Fig. 2). Seven days later, all seed tubers (treated and non-treated) reached 100% emergence (data not shown).

Effect of phosphites on foliage protection

CaPhi and KPhi applied twice (Treatment 1) protected both the moderately susceptible cv. Kennebec and the highly susceptible cv. Shepody from late blight infections. Highest protection was observed at the highest doses (2 and 1.5%), decreasing 14 daa (Fig. 3 a). There was a cultivar by compound interaction; higher protection was obtained in Kennebec with CaPhi than with KPhi, while in Shepody best results were obtained with KPhi applications.

When four applications were performed (Treatment 2), a similar behaviour was observed (Fig. 3b). In both cultivars protection decreased 14 daa and also with plant age, as was observed in Treatment 1.

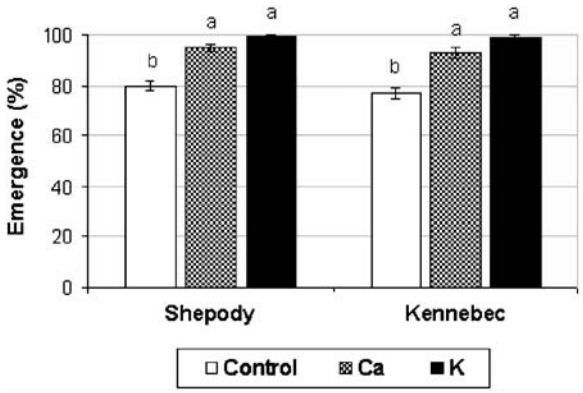


Fig. 2 Effect of phosphite application to seed tubers on emergence. Seed tuber pieces of cvs Shepody and Kennebec were treated with CaPhi and KPhi (1%) by spraying immediately after cutting at planting time. The emergence percentage was determined when the control treatment (water-treated only) reached approximately 80% emergence. *Bars* represent standard deviations. *Bars with the same letter* are not significantly different at $P<0.05$ (Tukey multiple comparison)

Effect of phosphites on the expression of β-1,3-glucanases

In Shepody, expression of β-1,3-glucanases increased in infected leaflets of both CaPhi and KPhi-treated plants 5 daa, when compared with expression in control infected leaflets (Fig. 4). However, in infected leaflets of Kennebec, glucanases only increased by KPhi application. Twenty-one daa, the expression pattern of β-1,3-glucanases differed from that at 5 daa. Only CaPhi induced the isoforms of glucanases in Shepody, but both CaPhi and KPhi had this effect in Kennebec.

Effects on Rubisco expression and length of crop cycle

Although no apparent differences in plant growth were observed between Phi-treated and control plants at early stages, leaves from plants treated with CaPhi were darker green than those coming from KPhi-treated or control plants (data not shown). This observation coincides with an increase in an approximately 55 kDa protein band in soluble leaf extracts from CaPhi-treated plants compared to KPhi and controls. This was the most abundant protein in these extracts and, according to its molecular weight, it corresponded to Rubisco. In KPhi-treated plants this increase was less evident, and was the only increase in protein observed by SDS-PAGE of soluble leaf extracts from plants treated with Phi (Fig. 5). Phi effect on length of crop cycle was also analyzed. For both cultivars, leaf senescence was effectively retard-

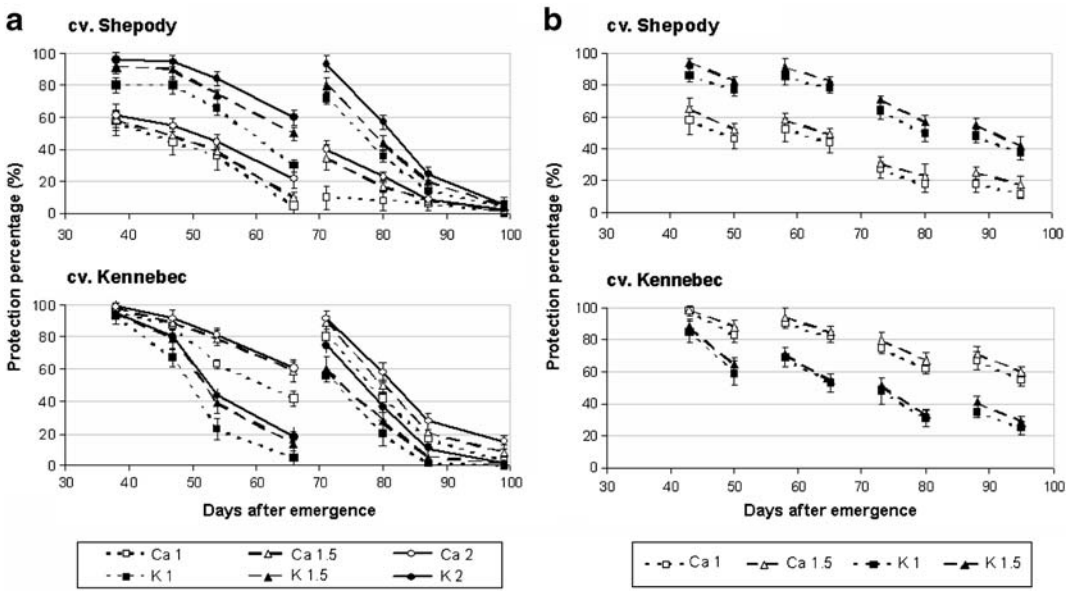


Fig. 3 Effect of foliar applications of phosphite compounds on protection against late blight over time. **a** Two applications of CaPhi (Ca) or KPhi (K) at 1, 1.5 or 2%, were made at 33 and 66 dae. Five, 14, 21 and 33 daa leaves were cut and inoculated with *P. infestans*. **b** Four applications of CaPhi or KPhi at 1 or 1.5%, were made at 36, 51, 66 and 80 dae. Seven and 14 daa leaves were cut and inoculated with *P. infestans*. For both assays, protection was analyzed 7 days after inoculation by estimating the area showing late blight lesions. *Bars* represent standard deviations

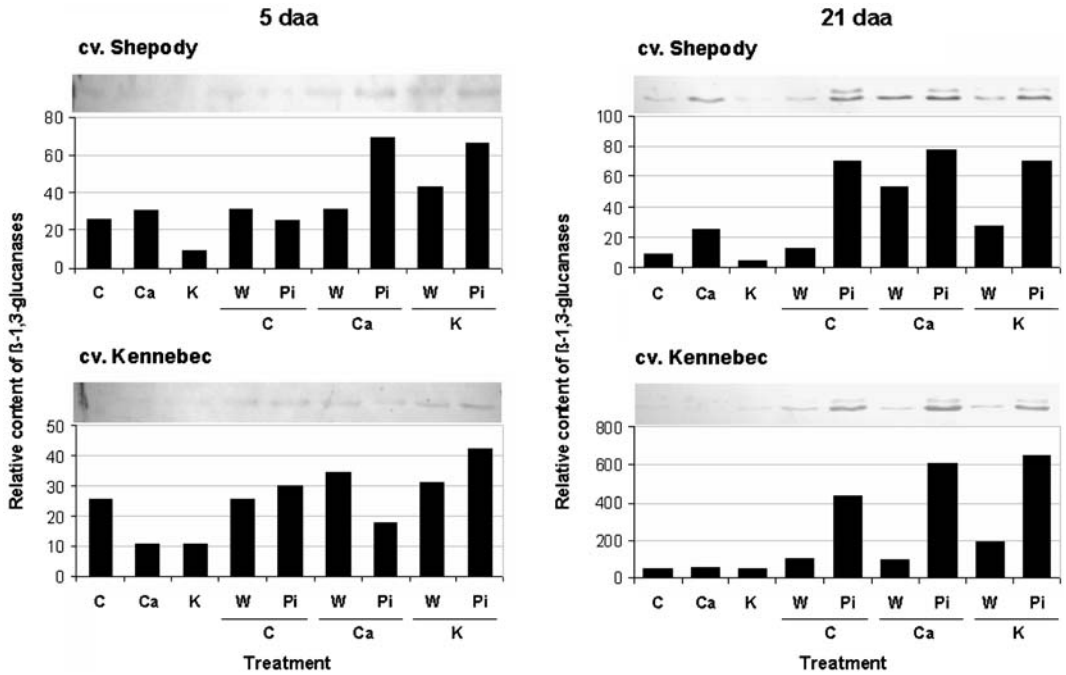


Fig. 4 Expression of β-1,3-glucanases in leaf extracts from phosphite-treated plants. Immunoblot analysis of β-1,3-glucanases from leaf extracts prepared 72 h after inoculation with *P. infestans* corresponding to 5 or 21 daa of CaPhi or KPhi (1%). The relative content of β-1,3-glucanases was estimated by densitometric scanning of western blot. *C*, control; *Ca*, CaPhi; *K*, KPhi; *W*, wounding; and *Pi*, *P. infestans*

ed by Phi treatments. In Kennebec at 80 dae, plants treated with both phosphites were taller than controls and had darker green leaves than those treated with water (Fig. 6). Phi-treated foliage stayed alive even at 100 dae, when control foliage was already dead. For Shepody, most foliage of control plants was dead at 80 dae; however Phi-treated foliage remained green until 100 dae (Fig. 6).

Discussion

CaPhi and KPhi are commercialized as 'nutritional compounds with antifungal action' (Agro-EMCODI), although little is known about their role in disease control in potato seed tubers and foliage. It has been reported that phosphorous acid is able to control crop diseases caused by oomycetes (Cooke and Little 1996, 2001), both through a direct effect inhibiting oxidative phosphorylation in oomycete metabolism (McGrath 2004), and by an indirect effect stimulating the plant's natural defence responses (Smillie et al. 1989). Incorporation of Phi in ICM programmes might reduce pesticide use and also production costs, while maintaining disease levels under the threshold of economic damage.

The aim of this study was to assess the effect of Phi compounds on disease control as well as on some physiological variables. In the present work, CaPhi and KPhi were studied as preventive compounds against three potato pathogens: *P. infestans*, *F. solani*, and *R. solani* (Fig. 1). Phi significantly reduced susceptibility of seed tubers to infections. This reduction was greater in Kennebec than in Shepody

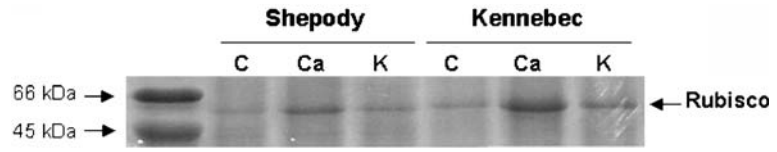


Fig. 5 Electrophoretic analysis of soluble proteins from leaf extracts of phosphite-treated plants. Soluble protein extracts were made 21 days after two phosphite applications at 1%. Protein profile was analyzed by SDS-PAGE (12%) and subsequent staining with Coomassie Blue

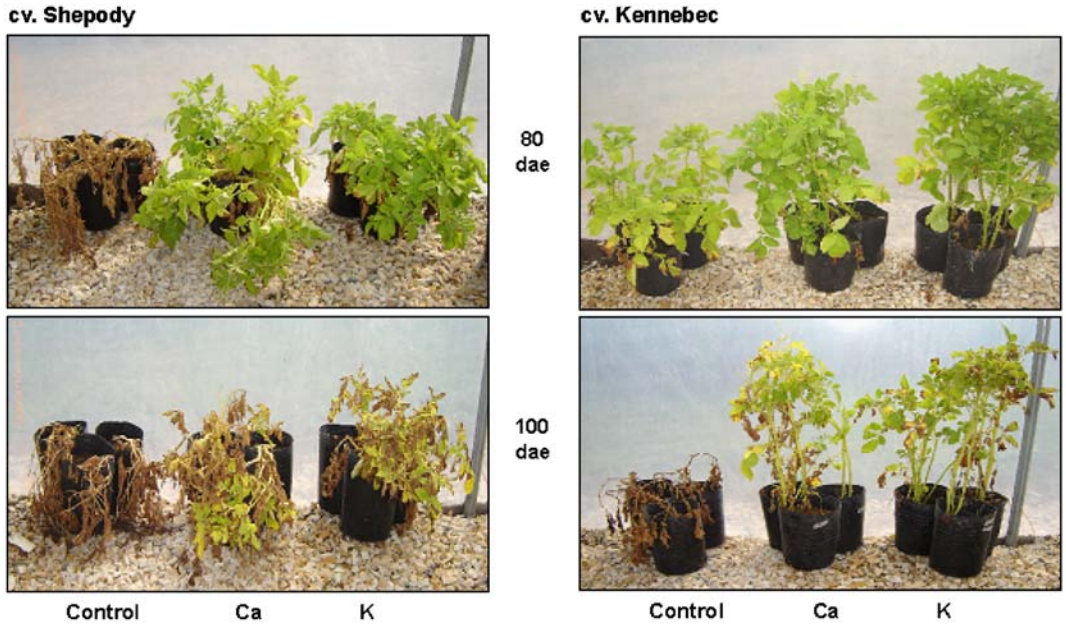


Fig. 6 Appearance of phosphite-treated plants. Plants of Shepody and Kennebec cultivars were treated with four applications of CaPhi or KPhi at 1% or water (control). Photographs were taken 80 and 100 dae

for the three pathogens studied where KPhi resulted in more efficient triggering of this response.

Phi applied to seed tubers appeared to have some additional effects on plant physiology. Plants emerged earlier when grown from treated seed tubers than from un-treated tubers (Fig. 2). Earlier emergence is an important advantage regarding both escape from possible soil diseases and radiation interception.

Foliarly applied Phi resulted in different protection levels against late blight depending on dose and plant age at application time. CaPhi showed a higher effect than KPhi in Kennebec, whereas KPhi was more effective in Shepody (Fig. 3), suggesting that the protective effect of Phi against pathogens can be affected by the cationic form of Phi used and by the host genotype. In addition, the effects of lower doses applied more frequently (1 and 1.5%, in four applications separated by 15 days each) were also assayed. In this case, protection also decreased with plant age; however, the final protective effect resulting from four cumulative applications was higher than that obtained after only two applications with the highest doses in the same period (Fig. 3b). Consistent with our results, Johnson et al. (2004) found that the effect of Phi varied depending on cultivar and pathogen, and also showed that the application schedule influences disease incidence and severity in the field. Disease suppression was greater when application began at initial tuber bulking rather than four weeks later. They also concluded that although phosphorous acid reduced tuber rot in the field under conditions favouring disease development, cultural practices, such as proper irrigation, and use of other fungicides, are critical for managing potato tuber rots.

Since the chemical nature of Phi and plant genotype influence protection obtained both in seed tubers and in foliage, it is suggested that a differential mobility of the compounds or a selective induction of natural defence responses in each cultivar might occur. Considering the last hypothesis, and since other chemical inducers like BABA and BTH (acibenzolar-*S*-methyl) showed an increase in β-1,3-glucanase expression (Andreu et al. 2006; Bokshi et al. 2003), the effect of Phi in the accumulation of these enzymes after wounding or infection was analyzed. Results indicated that the isoforms of β-1,3-glucanases analyzed increased their expression more in CaPhi-treated plants after inoculation with *P. infestans* than in infected tissue from non-treated plants (Fig. 4). KPhi only increased glucanase accumulation in Kennebec 5 daa. This increase does not correlate strictly with the level of protection observed. Other pathogenesis-related proteins (PRs) have not been reported to be induced by Phi although Bécot et al. (2000) showed a weak induction of β-1,3-glucanase activity and PR2 expression following

Phytogard® (K_2HPO_3) application in cauliflower. However, they concluded that these inductions were not enough to provide complete systemic protection. The response of these PRs suggests that the β-1,3-glucanases might participate in defence reactions induced by Phi in potato inoculated with *P. infestans*. The effect of Phi on other inducible defence mechanisms should be analyzed.

Induction of defence responses by Phi or other inducers might involve a metabolic cost resulting from the production of compounds or defence structures. We have analyzed the effect of Phi applications on plant growth through the analysis of two physiological parameters, Rubisco content and length of crop cycle (Figs 5 and 6). Data suggest that improvement in defence by Phi was not detrimental to plant growth. Increase in Rubisco by CaPhi and length of crop cycle support this hypothesis. Thus, we suggest that these inducers might improve crop performance because their photosynthetically active period is extended. Other researchers also report an increase in vigour in Phytogard®-treated plants (Bécot et al. 2000). Further studies will contribute to assessing the relative importance of different factors involved in the protection given by phosphites: a direct toxic effect on fungi, induced defence responses and improvement in the general nutritional state of the plant.

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