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Effect of foliar applications of phosphite on post-harvest potato tubers

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Abstract The utilization of phosphites (Phi) could be considered as another strategy to be included in integrated disease management programmes to reduce the intensive use of fungicides and production costs. The aim of the present work was to analyze whether the beneficial effects of phosphite treatment previously observed in potato plants grown under greenhouse conditions, were reflected after harvest of field grown potatoes, both in disease protection and in yield. In addition, biochemical compounds possibly involved in induced defence responses by Phi, like phytoalexins, pathogenesis related proteins and oxidative stress enzymes were measured. Foliar applications of KPhi to field grown crops resulted in post-harvest tubers with a reduced susceptibility to *Phytophthora infestans*, *Fusarium solani* and *Erwinia carotovora* infections, suggesting that this compound induced a systemic defence response. An increase in phytoalexin content in *P. infestans* inoculated tubers obtained from Phi-treated plants suggests their participation in the defence response. Chitinase content increased 72 h after wounding or inoculation with *P. infestans* in tubers from KPhi-treated plants compared to wounded or infected tubers from non-treated plants. Contrary to this, the isoforms of β-1,3-glucanases analyzed did not increase in the tubers of Phi-treated plants. The increment in peroxidase and polyphenol oxidase activities indicated that these enzymes could be part of the Phi defence mechanism. No negative effects were observed in potato yield at harvest, measured as total tuber weight and dry matter, after foliar KPhi treatment. This suggests that the energetic cost involved in the defence response activation would not be detrimental to plant growth.

Keywords Potato · Phosphite · Plant defence · *Phytophthora infestans* · Phytoalexins · Induced resistance

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Introduction

Numerous phosphite products are currently utilized in field crops; some of them have been registered as fungicides and others are sold as fertilizers. Several of

these commercial products are reviewed by Leymonie (2007). Potassium phosphite (KPhi) utilized in the present work is commercialized as a "nutritional compound with antifungal action" (Agro-EMCODI). Several plant species have been treated with phosphites to protect them against pathogens (Cooke and Little 2002; Guest and Grant 1991; Johnson et al. 2004; Lobato et al. 2008; Ouimette and Coffey 1989; Panicker and Gangadharan 1999; Reuveni et al. 2003); however, their mode of action is not completely understood yet. As alkali metal salts of phosphorous acid (H_3PO_3), phosphites can not be directly used by crops as a sole source of nutritional phosphorus (McDonald et al. 2001). It has been reported that phosphorous acid is able to control crop diseases caused by oomycetes (Cooke and Little 1996, 2002) through both a direct effect by inhibiting oxidative phosphorylation and other processes in oomycete metabolism (McGrath 2004), and an indirect effect by stimulating the plant's natural defence responses (Daniel and Guest 2006; Smillie et al. 1989).

The utilization of Phi could be considered as another strategy to be included in an integrated disease management programme to reduce the intensive use of fungicides as well as production costs. We have obtained evidence that foliar treatment with Phi increased the length of crop cycle and that could improve tuber yield (Lobato et al. 2008). Recently, Thao and Yamakawa discussed in a review article the role of Phi as a fungicide, fertilizer or bio-stimulator and showed that Phi can act with at least one of these properties in different study systems (Thao and Yamakawa 2009).

In a previous study (Lobato et al. 2008), the effect of the application of potassium phosphite (KPhi) and calcium phosphite (CaPhi) directly to seed tubers and foliage of potato plants was compared. Both phosphites reduced seed tuber disease symptoms caused by *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani* infections; although KPhi resulted in a more efficient triggering of this effective control. Protection in foliage against *P. infestans* was also obtained by foliar applications of CaPhi and KPhi; however, in cv. Shepody, the latter was shown to be more effective. In addition, different protection levels against late blight were induced by Phi, depending on the dose and the plant age at the time of application.

In order to continue our previous studies, the present study aimed to evaluate the effect of foliar applications of KPhi on post-harvest tubers. We analyzed protection against different potato pathogens, in stored tubers obtained from plants whose foliage was treated with KPhi.

Since phytoalexin accumulation is part of the general defence response of potato tubers against *P. infestans* (Andreu et al. 2001), and their accumulation was also reported in tubers after treatment with the plant activator β-aminobutyric acid (BABA) (Olivieri et al. 2009), phytoalexin content and the expression of different enzymes that could participate in the defence mechanism were measured. Taking into account that treatment with Phi produced changes in yield related parameters, like darker green colour in leaves and a delay in crop senescence, we also analyzed the effects of foliar treatment on crop yield.

Materials and methods

Isolation of pathogens and inoculum production

An isolate of *P. infestans* race R_2 R_3 R_6 R_7 R_9, mating type A2, was isolated from infected leaflets of a field grown potato crop, showing single lesions. Pieces of this infected tissue surrounding the lesion were placed on potato tuber slices and incubated at 17–19°C for 5 days until new sporulation appeared. An inoculation needle was used to transfer mycelium from the tuber slice to rye A agar (Caten and Jinks 1968). For inoculum production, small pieces of rye A agar medium containing actively growing *P. infestans* hyphae were transferred on tuber slices of cv. Bintje. The slices were incubated in closed plastic boxes containing wet filter paper, in the dark, at 18°C and 90% relative humidity (RH). After 7 days, mycelium was harvested in sterile water and stimulated to release zoospores by incubation at 4°C for 6 h. After filtration through a nylon filter cloth, the suspension of sporangia was observed under a light microscope for quantification before being used as inoculum. The concentration of sporangia was adjusted to 4×10^4 sporangia ml^{-1} using a haematocytometer.

The pathogenic fungus *Fusarium solani* f. sp. *eumartii* isolate 3122 (*F. solani*) was obtained from the INTA collection, Balcarce, Argentina. Fungal

cultures were grown in solid potato dextrose agar (PDA) for 3 weeks at 25°C.

Erwinia carotovora ssp. *carotovora* strain SCC3193 (*E. carotovora*) was kindly provided by Dr. Sabina Vidal (Biología Molecular Vegetal, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay). The bacterial culture was maintained on LB (Luria-Bertani) agar medium, and propagated in LB liquid medium at 30°C prior to use in the experiments.

Plant material, treatments and growing conditions

Three field experiments were carried out in Balcarce area (Southern latitude 37° 45′) during the summer growing seasons (mid-October to late February) of 2006/2007, 2007/2008 and 2008/2009. Trials were performed with cv. Shepody (highly susceptible to infection by *P. infestans* and susceptible to infection by *F. solani*), grown at the McCain Argentina Experimental Field, and irrigated with a forward advance irrigation system. Plots of four rows (0.85 m between rows) and 6 m long (20 m^2) with three replications in a randomized block design were used. In all seasons plant density was equivalent to 5.8 plants m^{-2}, similar to commercial crops grown in the area.

KPhi (Agro-EMCODI SA, Buenos Aires, Argentina) was applied to the foliage every 15 days, alternating with fungicide each 15 days from initiation of tuberization, using a rechargeable electric backpack sprayer SHURflo ProPack™ Model SRS 600CE. It was applied at 1% of the commercial product (equivalent to 3 l ha^{-1}, 5 ml per plant). Control plants were sprayed with water.

The yield was measured after harvesting at random all the tubers present in 3 m of row from three places in each plot, and from each of the three replicate plots (27 m in total).

The percentage dry matter of tubers was determined by measuring the specific gravity (SG), weighing in air and water a sample of 3.5 kg of healthy washed tubers and calculating SG as follows: $SG = \text{Weight of tubers in air}/[(\text{weight in air}) - (\text{weight in water})]$. A conversion table was used based on the high correlation between specific gravity and dry matter (http://new.dpi.vie.gov.au/notes/horticulture/vegetables/ag0323-potatoes-factors-affecting-dry-matter2).

Phytopathological assays

After harvest, tubers coming from the KPhi-treated and control plants of cv. Shepody were stored at 10°C and 50% RH for 2 months to obtain better artificial infection and adequate levels of biochemical markers. At this time, tubers were washed with distilled water, disinfected by immersion in 2.5 g l^{-1} sodium hypochlorite for 5 min and washed again with distilled water, and then used for phytopathological assays.

Protection against *P. infestans* and *F. solani* was evaluated as described by Olivieri et al. (2009). Briefly, for evaluation of *P. infestans* 20 tubers per treatment were used in five independent replicates. Two slices per tuber were cut and inoculated with 50 µl of a sporangial suspension (4×10^4 sporangia ml^{-1}), or with sterile water, and incubated at 18°C in the dark. After 7 days the mycelium colony diameter was measured. For *F. solani*, ten tubers per treatment were used and the experiment was performed three times. A disk of fungus grown on PDA medium was introduced in potato cortical tissue by wounding, according to the hollow punch method (Olivieri et al. 1998). Control tubers (wounded) were inoculated with a disk of sterile PDA medium. Inoculated tubers were incubated at 25°C in the dark over 16 days.

For evaluation of protection against *E. carotovora*, sterile potato disks (two disks per tuber, 4–6 cm diameter, 10 mm thick) were inoculated with 15 µl of a 1:3 dilution of a bacterial culture growing at an exponential rate (OD_{600}=1.6), or with sterile water, and incubated at 30°C in the dark. The area in the upper surface showing infection symptoms were compared 4 days after inoculation. In this study, 5 tubers per treatment were used in three independent replicates.

Extraction and determination of phytoalexins

Slices of sterilized tubers (4–6 cm diam, 10 mm thick) taken from the control or the KPhi-treated plants, were inoculated with 50 µl of sporangial suspension of *P. infestans* (4×10^4 sporangia ml^{-1}) and incubated at 18°C in the dark. Seven days after inoculation, phytoalexins were extracted and measured according to Andreu et al. (2006).

Preparation of soluble tuber extracts

Disks (1 g) of tubers obtained from control and KPhi-treated plants, infected or not infected with *P.*

infestans, were homogenized in a mortar and pestle 0, 12, 24 and 72 h after inoculation or wounding. The extraction solution was 100 mM sodium phosphate buffer pH 7.5 containing 2 mM sodium metabisulfite, 1% polyvinylpyrrolidone (PVP) and 0.5 M sodium chloride. Homogenates were filtered through cheesecloth and centrifuged at 10,000 × *g* for 15 min. The supernatant, representing the soluble crude extract, was stored at −20°C for further processing.

Peroxidase and polyphenol oxidase activities

Peroxidase and polyphenol oxidase activities were determined in the crude extracts according to the procedure described by Chen et al. (2000), with some modifications. Briefly, the reaction mixture for peroxidase activity measurements consisted of 100 µl of crude extract, 400 µl of sodium phosphate buffer 100 mM pH 6 containing 8.6 mM guaiacol. The reaction was started by the addition of H_2O_2 (3.6 mM final concentration) to the mixture, and the linear increase in absorbance at 470 nm due to the formation of tetraguaiacol was measured over 1 min. The polyphenol oxidase activity was measured in a 1 ml reaction mixture consisting of 20 µl of crude extract, sodium phosphate buffer 35 mM pH 6 and 100 µl 0.2 M catechol. The reaction was started by the addition of catechol to the mixture, and the linear increase in absorbance at 420 nm was measured over 1 min.

Gel electrophoresis and immunoblot analysis

For protein separation and for immunoblot analysis, the soluble crude extract corresponding to 0 and 72 h of wounding or infection, was submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% aqueous acrylamide, and subsequently electro-blotted onto nitrocellulose membrane in a Semi-dry electrophoretic transfer cell (Trans-Blot, Bio Rad, Hercules, CA, USA). Immunodetection was performed as described by Turner (1986) using polyclonal antibodies raised against chitinase or β-1,3-glucanase (Kombrink et al. 1988). Bound antibody was visualized with a second antibody, goat anti-rabbit conjugated with alkaline phosphatase. The intensity of the immuno-positive bands on the western blots was evaluated by densitometry analysis (TN-Image, Image Analysis Software, Compuserve, IBMAPP, Rockville, MD USA). Equal amount of fresh weight (3 mg for glucanases and 2 mg for chitinases) were loaded in each lane of the gel and molecular masses were estimated by comparison with standard molecular markers (10 to 170 kDa) (Sigma).

Data analysis

All variables approximated to normality. Surface area showing lesion symptoms, phytoalexin content or tuber yield and dry matter data were analyzed by the *t*-test. Enzyme content and activity data were analyzed by one-way ANOVA (Zar 1999), and *a posteriori* multiple comparison tests (Tukey test) were performed when significant ($P<0.05$) differences between means were detected.

Results

Effect of KPhi on tuber protection

Foliar applications of KPhi resulted in post-harvest tubers with a reduced susceptibility to several pathogen infections. A reduction in the lesion size produced by *P. infestans*, *F. solani*, and *E. carotovora* was observed (Fig. 1). For these three pathogens (oomycete, fungus and bacterium) the lesion area in tubers coming from KPhi-treated plants after inoculations was reduced by approximately 50% with respect to lesion size produced in tubers obtained from non-treated plants.

Phytoalexin, chitinase and glucanase responses after KPhi treatment

The level of phytoalexin expression was determined in tuber slices obtained from KPhi-treated or control plants, and infected with *P. infestans* (Fig. 2). Phytoalexins accumulated after *P. infestans* infection and they were not detected in healthy tubers. KPhi treatment was able to produce a three-fold increase in phytoalexin content in infected tubers compared to the infected tubers coming from non-treated plants. An increase in chitinase expression after wounding and *P. infestans* infection was observed in control tubers (Fig. 3a, lanes 3 and 4). In wounded or infected tubers obtained from KPhi-treated plants the level of

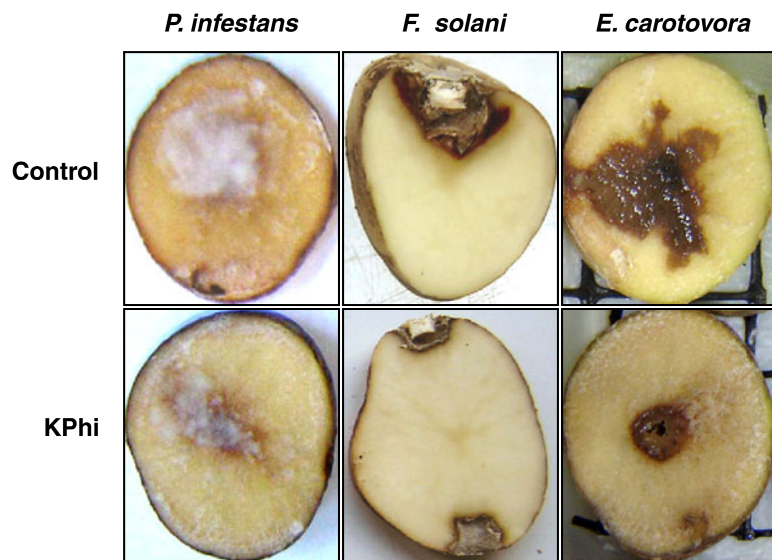

Fig. 1 Effect of foliar applications of KPhi on the lesion area caused by the potato pathogens *Phytophthora infestans*, *Fusarium solani* f. sp. *eumartii* and *Erwinia carotovora* spp. *carotovora* in post-harvest tubers. Symptoms were observed for each pathogen as indicated in materials and methods

Treatment	Lesion area (cm^2)*		
	P. infestans	*F. solani*	*E. carotovora*
Control	10.43 ± 0.43 b	4.69 ± 0.89 b	7.08 ± 2.75 b
KPhi	5.92 ± 1.5 a	2.40 ± 1.08 a	3.07 ± 2.38 a

*For each fungus, values followed by the same letter are not significantly different according to the *t*-test, at a *P* value of 0.05.

chitinase expression increased by two- and three-fold, respectively, compared to their corresponding controls. In contrast to chitinases, the expression of glucanases did not change significantly in tubers coming from treated plants (Fig. 3b). As previously reported, glucanases increased in potato tubers after *P. infestans* infection, but no increase was observed in infected tubers from KPhi treated plants, compared to non-treated ones, with or without wounding.

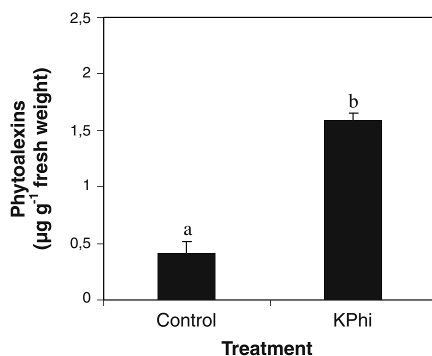

Fig. 2 Phytoalexins accumulation after *P. infestans* infection in tuber slices obtained from KPhi-treated or control plants. Phytoalexins were measured seven days after inoculation with *P. infestans*

Peroxidase and polyphenol oxidase responses after KPhi treatment

Peroxidase (POD) and polyphenol oxidase (PPO) activities were quantified at different times after wounding or *P. infestans* inoculation. For POD activity the major values were obtained 72 h after inoculation. At this time, the activity increased even more (almost 40%) in infected tubers obtained from KPhi-treated plants (Fig. 4a). Results of PPO activity show a similar behaviour but the peak of activity was detected earlier, 12 h after inoculation or wounding. At this time, tubers obtained from KPhi-treated plants showed an increase in PPO activity of approximately six-fold with respect to infected tubers obtained from non-treated plants (Fig. 4b).

Effect of foliar KPhi treatment on tuber yield

The results indicate that in the periods 2007/2008 and 2008/2009, total tuber yield at harvest, expressed as tons per hectare, did not differ significantly between KPhi treatment and the non-treated control. In 2006/2007 tuber yield of KPhi-treated plants increased 40%

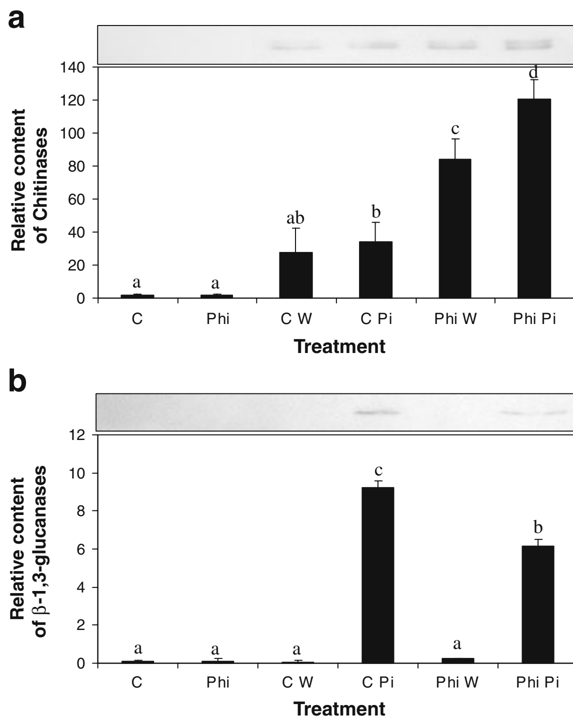

Fig. 3 Expression of chitinases (**a**) and β-1,3-glucanases (**b**) in tubers obtained from KPhi-treated plants. Immunoblot analysis of chitinases and β-1,3-glucanases from tuber extracts corresponding to 0 and 72 h after wounding or inoculation with *P. infestans.* The relative content of chitinases and β-1, 3-glucanases was estimated by densitometric scanning of western blot. C, control tubers; Phi, tubers obtained from KPhi-treated plants; C W, control tubers + wounding; C Pi, control tubers inoculated with *P. infestans*; Phi W, tubers obtained from KPhi-treated plants + wounding; Phi Pi, tubers obtained from KPhi-treated plants inoculated with *P. infestans*

compared to control, although this, again, was not statistically significant. However, yields of both treatments in that period were lower than in the other periods (Table 1).

There was a significant increase in dry matter production in tubers obtained from KPhi-treated plants in the 2008/2009 period. In the two other periods no significant differences were observed in this variable between treatments (Table 1).

Discussion

In our previous research we have observed that Phi application to seed tubers or foliage during potato crop grown in greenhouse, resulted in beneficial phytopathological and physiological effects (Lobato et al. 2008). To continue this study, and with the objective to determine whether Phi foliar treatment is beneficial for post-harvest tubers, analysis was made in tubers obtained from field grown crop.

In the present work, foliar applications of KPhi resulted in post-harvest tubers with significantly reduced disease symptoms after inoculation with: *P. infestans*, *F. solani* and *E. carotovora*. The most important aspects of this result are the durability and the systemic character of the defence response produced by Phi to a broad range of pathogens, at least under the field conditions of this study and for this potato cultivar. Some comparable results were obtained by Mayton et al. (2008). They analyzed different phosphite compounds (phosphonates) on the protection against *P. infestans* in foliage and in post-harvest tubers. In general, the phosphonates tested in their experiments suppressed foliar late blight and were as effective as a conventional fungicide in controlling tuber blight.

Biochemical responses possibly involved in the Phi action mechanism were studied. Phytoalexin accumulation is a typical potato defence response, and in this study it was observed that it was also increased by KPhi treatment. This means that KPhi could be activating a general plant defence response in which phytoalexins participate. The involvement of phytoalexins in the response of phosphonate-treated plants to infection by *Phytophthora* species had been previously reported, and several studies were reviewed by Saindrenan and Guest (1994). Other components of the plant defence response are chitinases and glucanases, which are pathogenesis related proteins (PRPs), and their accumulation in potato tubers after wounding or infection has been reported (Machinandiarena et al. 2001; Tonón et al. 2002). In the current study, an increase in chitinase expression was observed by KPhi treatment but not for glucanases. Moreover, the accumulation of these glucanases in infected tubers from KPhi-treated plant seems to be less than in non-treated but infected ones. Again, there is no correlation between defence response and PRPs accumulation, as we have previously observed (Lobato et al. 2008); however, chitinase participation in the response induced by KPhi can not be discounted.

In order to know if stress oxidative enzymes can participate in the defence mechanism induced by KPhi, POD and PPO activities were measured in post-

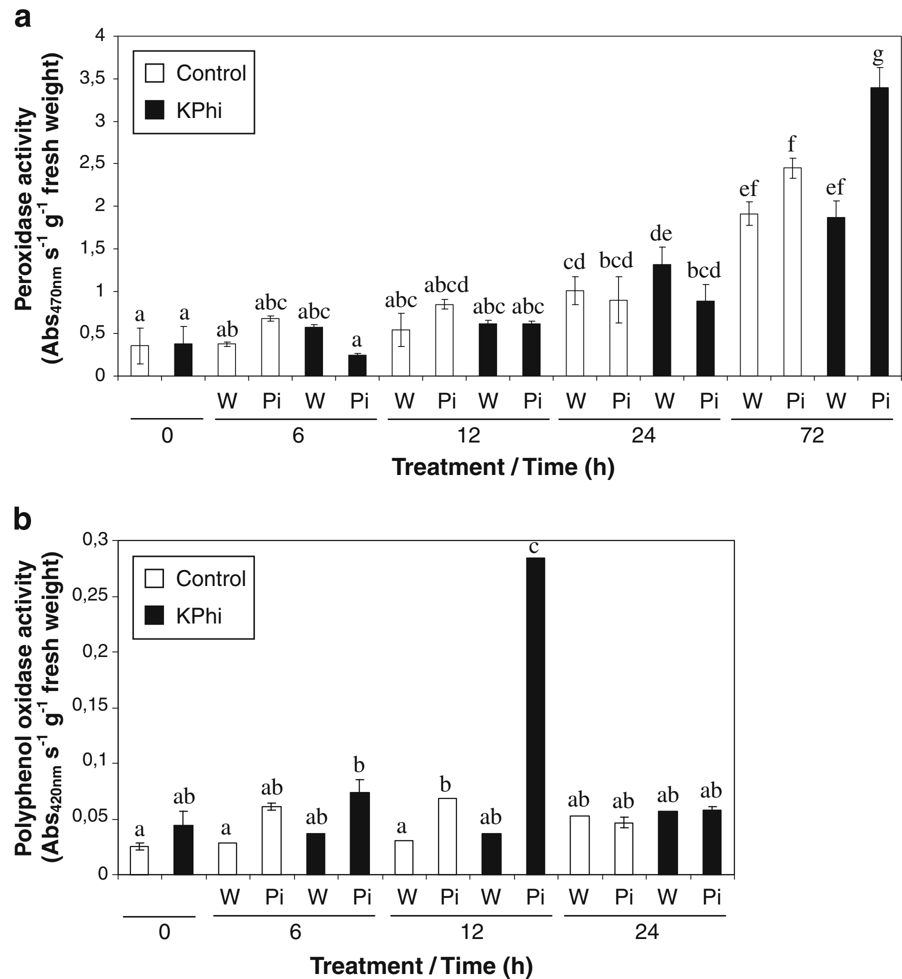

Fig. 4 Peroxidase (**a**) and Polyphenol oxidase (**b**) activities in tubers coming from KPhi-treated plants. Enzymatic activities were quantified in crude extracts of tuber from control and KPhi-treated plants at different times after wounding or *P. infestans* inoculation

harvest tubers. The peroxidase activity is modified after wounding or infection. In tubers obtained from KPhi-treated plants the activity increased even more compared to non-treated ones after *P. infestans* inoculation. PPO activity had an earlier increase in infected tubers obtained from treated plants. There is no evidence in the literature showing that these oxidative stress enzymes are involved in the mechanism induced by KPhi. Further investigations will be made to understand the relationship between Phi activation of stress oxidative pathways and the defence responses observed.

Table 1 Post-harvest tuber yield of KPhi treated and untreated control plants

Crop period	Treatment	Yield (tn ha^{-1})[a]	Dry matter (%)[a]
2006–2007	Control	16.56 a	18.7 a
	KPhi	23.7 a	18.6 a
2007–2008	Control	41.1 a	18.23 a
	KPhi	37.28 a	17.9 a
2008–2009	Control	29.68 a	17.63 a
	KPhi	31.33 a	18.49 b

[a] Values in each crop period followed by the same letter do not differ significantly at $P<0.05$.

It has been reported that the cation potassium (K) could play a role in alleviating adverse effects of different abiotic stress factors on crop production (Cakmak 2005). Taking into account these results, we can not discount that the mechanism involved in abiotic stress tolerance induced by K, could be a part of the mechanism involved in biotic stress resistance induced by KPhi in our study system.

Finally, we analyzed whether the beneficial effects previously observed in plant growth were reflected in

tuber yield at harvest. The effect of Phi on growth and yield of different crops has been discussed in the literature. For example, Thao and Yamakawa (2009) reviewed the effects of Phi as fungicide, fertilizer or biostimulant, and proposed that Phi effect is strongly dependent on the phosphate status of plants. In particular, for potato cultivars, Johnson et al. (2004) reported that tuber yield at harvest did not differ significantly among the phosphorous acid treatments and the untreated controls. They also mentioned the potential influences of potato cultivar, rate and timing of Phi application, and geographic location on tuber yield. Under the field conditions of our study, no deleterious effects of Phi were observed and we did not observe any significant modifications in total tuber weight between the 2007/2008 and 2008/2009 growing seasons. In contrast, in 2006/2007, when soil conditions were unfavourable, the total yield was low for both treated and non-treated plants. However, total weight of tubers obtained from KPhi-treated plants was 40% higher compared to non-treated ones. Together, these results indicated that foliar treatments with KPhi usually do not decrease tuber yield. The consequences on total tuber weight could hide effects on dry matter concentration, a quality indicator of economic importance for industry. KPhi application could have negative effects in dry matter concentration in tubers due to a high energetic cost involved in the defence response activation. The results showed that KPhi application did not have a negative effect on this variable. In addition, our findings suggest that KPhi could have a beneficial effect on crop yield mainly under some unfavourable climatic or soil conditions, but this must be confirmed in future experiments.

The results of our study, strongly suggest that the use of KPhi on field grown crops together with an integrated disease control strategy can be used to effectively reduce the use of toxic pesticides whilst maintaining yields.

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