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Antimicrobial activity of phosphites against different potato pathogens

Antimikrobielle Aktivität von Phosphiten gegenüber verschiedenen Kartoffelpathogenen

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Abstract

Phosphites have low-toxicity on the environment and show high efficacy in controlling oomycete diseases in plants, both by a direct and an indirect mechanism. We have shown that they are also effective in reducing disease symptoms produced by *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani* when applied to potato seed tubers. To gain better insight into the direct mode of action of phosphites on different potato pathogens, and to ascertain chemical determinants in their direct antimicrobial activity, four potato pathogens were assayed with respect to sensitivity toward calcium, potassium and copper phosphites (CaPhi, KPhi and CuPhi, respectively). The influence of acidification and ionic strength changes after Phi addition on the antimicrobial activity, and the fungicidal or fungistatic activity, were evaluated. Results showed that phosphites were able to inhibit growth of all pathogens. *Phytophthora infestans* was the most inhibited pathogen by all phosphites, followed by *Streptomyces scabies*, while *Rhizoctonia solani* and *Fusarium solani* were less inhibited. CuPhi had the highest antimicrobial activity against the four pathogens analysed, and CaPhi and KPhi showed similar antimicrobial activities. Inhibitions by CuPhi and CaPhi could be partially explained by acidification of the media. However, results obtained with KPhi demonstrated that the phosphite anion has antimicrobial activity itself. The increase in ionic strength after Phi addition was not important in the antimicrobial activity of Phi. The activity of phosphites on germination of *F. solani* spores showed to be fungistatic rather than fungicidal.

Key words: disease control, *Fusarium solani*, growth inhibition, *Phytophthora infestans*, *Rhizoctonia solani*, *Streptomyces scabies*

Zusammenfassung

Phosphite besitzen eine geringe Umwelttoxizität sowie eine gute direkte und indirekte Wirkung gegenüber Oomyceten-Pathogenen von Pflanzen. Wir zeigen hier, dass sie ebenfalls die durch *Phytophthora infestans*, *Fusarium solani* und *Rhizoctonia solani* verursachten Symptome an Kartoffeln nach einer Knollenbehandlung vermindern. Vier Kartoffelpathogene wurden mit dem Ziel untersucht, die direkte Wirkungsweise von Calcium-, Kalium- und Kupfer-Phosphiten (CaPhi, KPhi und CuPhi) auf die Erreger sowie die chemischen Determinanten ihrer direkten antimikrobiellen Aktivität zu ermitteln. Weiterhin wurde die Wirkung von Azidifizierung und Ionenstärke nach Zugabe von Phosphiten auf antimikrobielle, fungizide und fungistatische Aktivität untersucht. Die Phosphite beeinträchtigten das Wachstum aller untersuchten Erreger. *Phytophthora infestans* wurde durch alle verwendeten Phosphite am stärksten inhibiert, gefolgt von *Streptomyces scabies*, während die Wirkung auf *Rhizoctonia solani* und *Fusarium solani* geringer war. CuPhi besaß die höchste antimikrobielle Aktivität gegenüber den vier untersuchten Pathogene, gefolgt von den ähnlich wirksamen CaPhi und KPhi. Die Wirkung von CuPhi und CaPhi kann zum Teil durch die Ansäuerung der verwendeten Medien erklärt werden. Die mit KPhi erhaltenen Ergebnisse zeigen dagegen, dass das Phosphit-Anion selbst antimikrobiell wirksam ist. Die Zunahme der Ionenstärke nach Phosphit-Applikation war nicht für die antimikrobielle Wirkung verantwortlich. Die Beeinträchtigung der Sporenkeimung von *F. solani* zeigte, dass die Wirkung der Phosphite eher fungistatisch als fungizid ist.

Stichwörter: *Fusarium solani*, Krankheitsbekämpfung, *Phytophthora infestans*, *Rhizoctonia solani*, *Streptomyces scabies*, Wachstumsbeeinträchtigung

1 Introduction

In the world, conventional potato production is not possible without fungicides. However, these increase production costs, and those commonly used are considered as environmental and human health hazards. An alternative to fungicide use are the phosphites (Phi), compounds derived from phosphorous acid (H_3PO_3) with low toxicity for the environment.

Phosphorous acid is classified by the US Environmental Protection Agency (US-EPA) as a biopesticide (http://www.epa.gov/pesticides/biopesticides/). Pesticide properties of this compound were discovered by scientists at Rhône-Poulenc Agrochimie Laboratories in France during the 1970 s. Soon after, fosetyl-Al (aluminium tris O-ethyl phosphonate) was formulated under the trade name Aliette and released for commercial use (Guest and Grant 1991). Upon the expiration of the patent for the active ingredient in the fosetyl-Al, several formulations of phosphite biopesticides have become available in Argentina, United States, Brazil, Ecuador and Peru. Differences in the efficacy of phosphorous acid-related products against oomycetes have been reported for different crops (Ouimette and Coffey 1989; Panicker and Gangadharan 1999; Cooke and Little 2002; Brown et al. 2004; Vawdrey and Westerhuis 2007).

Phosphites, as biopesticides, have both direct and indirect mode of action (Guest and Bompeix 1990) and in crops, they can not be directly used as a sole source of nutritional phosphorus (McDonald et al. 2001). Direct effects on the oomycetes include: inhibition of mycelial growth, changes in the composition of the mycelium surface, increased activity of the pentose phosphate pathway, changes in the metabolism of pyrophosphate and inhibition of enzymes allosterically regulated by phosphate (Guest and Bompeix 1990; Guest and Grant 1991; Niere et al. 1994; Stehmann and Grant 2000). Sporulation and germination of *Phytophthora* species have also been shown to be suppressed by these biopesticides (Cohen and Coffey 1986; Garbelotto et al. 2009). Indirect effects of phosphites are thought to be the activation of plant defence responses (Guest and Bompeix 1990; Andreu et al. 2006; Lobato et al. 2008a).

Previously, we have tested calcium and potassium phosphites (CaPhi and KPhi, respectively) commercialized as "nutritional

compounds with antifungal action" (Agro-EMCODI), against several diseases in the potato crop. These Phi applied to potato seed tubers immediately after cutting, increased resistance against *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani*. Protection was high against *P. infestans*, intermediate against *F. solani* and low against *R. solani* for all cultivars tested (Lobato et al. 2008b). On the other hand, foliar applications of CaPhi or KPhi provided protection against *P. infestans* in all cultivars analysed, although protection degree was cultivar-specific (Andreu et al. 2006; Lobato et al. 2008b). Tubers from plants treated in foliage showed a smaller colony diameter of *P. infestans* and a reduction of the lesion area caused by *F. solani* and *Erwinia carotovora* when artificially infected; KPhi had a stronger effect than CaPhi (Lobato et al. 2008a).

Although we have described some responses *in planta* after Phi application, a direct mode of action of these Phi can not be discarded within the general defence response, due to the different resistance levels obtained for the different pathogens tested. To gain better insight into the direct mode of action of phosphites on different potato pathogens, and to ascertain chemical determinants in their direct antimicrobial activity, four potato pathogens were assayed with respect to *i*) sensitivity toward CaPhi, KPhi and CuPhi, *ii*) contribution of acidification and ionic strength changes after Phi addition on the inhibitory activity of these compounds, and *iii*) fungicidal or fungistatic activity.

2 Materials and methods

2.1 Potato pathogens and culture conditions

Phytophthora infestans race $R_5R_8R_9$, mating type A2, was isolated from potato plants showing single lesions in a field in Buenos Aires Province. Pieces of infected tissue surrounding the sporulating region of the lesion were inoculated on potato tuber slices of cv. Spunta and incubated in darkness at 18°C for 7 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), supplemented with ampicillin (200 mg l^{-1}), Benlate (50% WP, 100 mg l^{-1}), PCNB (75% WP, 67 mg l^{-1}), olymixin B (50 mg l^{-1}) and rifampicin (20 mg l^{-1}), and then incubated at 18°C for 10 days. Mycelium from 10-day-old culture was used for growth inhibition assays.

A pathogenic isolate of *Rhizoctonia solani* (AG-3) and *Fusarium solani* f. sp. *eumartii* isolate 3122 were provided by the Laboratorio de Fitopatología, INTA Balcarce, Argentina and maintained on potato dextrose agar (PDA) at 18°C and 25°C, respectively, in darkness.

Streptomyces scabies was provided by Diagnósticos Vegetales SRL, Mar del Plata, Argentina. The strain was grown on an isolation medium containing 0.4 g casein, 1.0 g starch, 0.5 g potassium nitrate, 0.2 g potassium monohydrogen phosphate, 0.1 g magnesium phosphate, 0.1 g calcium carbonate and 15 g agar per litre.

2.2 Chemicals

Calcium, potassium and copper phosphites (CaPhi: 25% P_2O_5, 8% Ca; KPhi: 30% P_2O_5, 20% K_2O and CuPhi: 25% P_2O_5, 5% Cu, respectively), were formulated by PFG International SA (Lerida, España) and provided by Agro-EMCODI SA (Buenos Aires, Argentina). Final concentrations of phosphites were expressed as dilution percentage (% v/v) of the commercial products, or as mg ml^{-1} of the phosphite anion, as shown in Table 1.

2.3 Effect of phosphites on growth of potato pathogens

For all growth inhibition assays CaPhi, KPhi or CuPhi were added to growth media at final concentrations of 1, 0.67, 0.1,

Table 1: Final concentrations of calcium, potassium and copper phosphites and the resultant pH and ionic strength of the media in the growth inhibitory assays

Phi concentration* (% v/v)	Phi anion concentration* (mg ml^{-1})	pH			Calculated ionic strength (mM)
		Rye A agar	PDA	LB	
CaPhi					
1	2.25	2.9 ± 0.1	3.8 ± 0.4	4.6 ± 0.3	80.95
0.67	1.5	3.4 ± 0.1	4.6 ± 0.3	5.1 ± 0.3	53.95
0.1	0.23	5.1 ± 0.3	5.5 ± 0.1	6.4 ± 0.2	8.1
0.02	0.03	5.5 ± 0.3	5.8 ± 0.3	6.6 ± 0.3	1.62
0.01	0.02	6.0 ± 0.4	6.4 ± 0.4	6.7 ± 0.2	0.81
KPhi					
1	3.56	5.5 ± 0.3	5.4 ± 0.1	5.6 ± 0.1	72.35
0.67	2.37	5.5 ± 0.3	5.5 ± 0.1	5.5 ± 0.1	48.2
0.1	0.36	5.6 ± 0.2	5.8 ± 0.3	6.4 ± 0.1	7.24
0.02	0.07	6.0 ± 0.4	5.9 ± 0.4	6.5 ± 0.1	1.45
0.01	0.04	6.0 ± 0.3	6.4 ± 0.4	6.6 ± 0.0	0.72
CuPhi					
1	3.82	2.0 ± 0.2	1.5 ± 0.6	2.2 ± 0.3	65.75
0.67	2.55	2.5 ± 0.3	2.0 ± 0.6	2.8 ± 0.1	43.85
0.1	0.38	3.0 ± 0.2	4.5 ± 0.4	5.1 ± 0.1	6.58
0.02	0.08	5.2 ± 0.3	5.5 ± 0.1	5.9 ± 0.3	1.32
0.01	0.04	5.5 ± 0.1	5.8 ± 0.1	6.4 ± 0.1	0.66

* Concentrations of phosphite were expressed as dilution percentage (% v/v) of the commercial products, or as mg ml^{-1} of the phosphite anion.

0.02 or 0.01% (v/v) of the commercial products. Phi were sterilized by passing them through a 0.2 μm Millipore filter and then added to the autoclaved media.

For *P. infestans* growth inhibition assays, 9 cm plastic Petri dishes containing 25 ml of rye A agar were supplemented or not (controls) with different amounts of either CaPhi, KPhi or CuPhi solutions. A 7 mm cork borer was used to cut discs from the edge of an actively growing culture. One disc was placed, mycelium side down, at the edge of a plastic Petri dish and then sealed with Parafilm. Plates were incubated at 18°C for 10 days and then the radial growth of mycelia in Phi supplemented and control media was measured.

Rhizoctonia solani and *Fusarium solani* growth inhibition assays were performed as above, but PDA was used as growth medium for both fungi. After a 5-day incubation period at 18°C in darkness for *R. solani* or a 15-day incubation period at 25°C in darkness for *F. solani*, the radial growth of mycelia in Phi supplemented and control media was measured.

The effect of phosphites on the growth of *Streptomyces scabies* was evaluated in test tubes containing 5 ml of Luria-Bertani liquid medium (LB) supplemented with Phi or not (controls) with different amounts of Phi as described above. *S. scabies* was first transferred from the isolation medium to LB liquid medium and 10 μl of a culture growing at an exponential rate (OD_{600} = 1.6), were added to the test tubes containing the supplemented LB. Tubes were incubated in a shaking chamber (200 rpm) at 30°C for 8 h. After this period, optical density of Phi supplemented and control cultures (OD_{600}) was measured.

2.4 Determination of sensitivity to phosphites of different potato pathogens

EC_{50} values (concentration of compound inhibiting growth by 50%) for each potato pathogen was calculated by plotting inhibition percentage against Phi concentration (logarithmic scale).

2.5 Effect of pH and ionic strength on potato pathogen growth

The influence of pH and ionic strength due to Phi addition on growth inhibition was evaluated. The pH of rye A agar, PDA or LB media supplemented with CaPhi, KPhi or CuPhi was measured with a pHmeter before the addition of agar for solid media; while ionic strength changes due to Phi addition to the different media was calculated from the composition of each phosphite used (Table 1). Controls of pH and ionic strength were performed as follows. Prior to cooling and solidification of rye A agar or PDA mediums, or prior to inoculation of LB liquid medium, pH and ionic strength were adjusted with HCl and NaCl, respectively, before solidification, to reach values comparable to those obtained by the addition of the different amounts of CaPhi, KPhi or CuPhi. Each experiment contained two replicates at each Phi concentration and at least three independent experiments were performed for each microorganism.

2.6 Effect of phosphites on spore germination

Fusarium solani conidial production and germination assays were performed as described by Mendieta et al. (2006). Tests samples contained 50 mM sodium acetate buffer pH 5.2, 4% sucrose, 1×10^5 spores ml^{-1} and water (controls) or different amounts of CaPhi or KPhi (1, 0.67, 0.1, 0.02 or 0.01% v/v), in a final volume of 100 μl. After incubation at 25°C for 14 h, the slides were evaluated for inhibition of germination under a light microscope in a haematocytometer. A conidium was considered germinated if the germ tube was longer than one-half of conidium length. Three independent experiments were performed.

2.7 Evaluation of fungicidal or fungistatic activity of phosphites

Reversibility of inhibition of *F. solani* spore germination was evaluated after a 14 h-incubation with Phi period at 25°C. For this, 30 μl of a dilution 1:6 of the germination test samples were spread on Phi unsupplemented PDA plates, and then incubated at 25°C in darkness. Two and 3 days later, colonies were counted. Experiments were performed twice.

2.8 Statistical analysis

The null hypothesis of no differences between treatments in growth and germination inhibition percentage data was evaluated independently for each variable by two-way ANOVAs (Zar 1999). *A posteriori* multiple comparison tests (LSD test) were performed when significant ($P < 0.05$) differences between means were detected.

3 Results

3.1 Effect of phosphites on growth of potato pathogens

3.1.1 CaPhi effect on pathogen growth. The antimicrobial activity of CaPhi against potato pathogens is shown in Figure 1. CaPhi at highest concentrations, 1 and 0.67%, almost completely inhibited *P. infestans* and *S. scabies* growth, and even 0.1% CaPhi inhibited *P. infestans* growth by 96%. At lower concentrations, no inhibition of *S. scabies* was found, although *P. infestans* was still inhibited by approximately 30 and 20% by 0.02 and 0.01% CaPhi, respectively (Fig. 1a,d). The antimicrobial activity of 1, 0.67 and 0.1% CaPhi against *P. infestans* could be attributed to pH decrease, since pH corresponding to rye A agar medium supplemented with CaPhi at those concentrations, completely inhibited the oomycete growth (Fig. 1a). On the other hand, the effect of ionic strength increment due to addition of 1 and 0.67% CaPhi only inhibited *P. infestans* growth by 45 to 38%, respectively. *S. scabies* was less sensitive than *P. infestans* to pH decrease and ionic strength change due to CaPhi addition, ranging from 60 and 90% growth inhibition by pH decrease corresponding to 0.67 and 1% CaPhi, respectively, to less than 20% by ionic strength changes at the same conditions (Fig. 1d).

CaPhi had a minor but significant antimicrobial activity against fungi than against *P. infestans* or *S. scabies*, inhibiting *R. solani* growth by 76 and 60% and *F. solani* growth by 60 and 40%, at 1 and 0.67%, respectively (Fig. 1b,c). At these concentrations inhibition of both pathogens could not be attributed to ionic strength changes whereas pH decease could partially (20 to 30%) contribute to inhibition produced by CaPhi at 1%. No fungal growth inhibition was found at lower concentrations of this phosphite.

3.1.2 KPhi effect on pathogen growth. Figure 2 shows the antimicrobial activity of KPhi against potato pathogens. This phosphite had a similar antimicrobial activity against the pathogens studied than CaPhi, although growth inhibition percentages were usually lower. *P. infestans* was completely inhibited by 1 and 0.67% KPhi and strongly inhibited (85% approximately) at 0.1%, whereas at lower concentrations, inhibition showed no differences from those obtained with CaPhi (Fig. 2a). However, unlike inhibition by CaPhi, the contribution of pH decrease to inhibition by KPhi was approximately 5%, although ionic strength partially contributed to the antimicrobial activity by this compound, since the inhibition values for this factor corresponding to KPhi at 1 and 0.67% were approximately 50 and 35%, respectively (Fig. 2a).

R. solani and *F. solani* were less inhibited by KPhi than the oomycete (Fig. 2b,c). At 1 and 0.67%, *R. solani* growth inhibition reached approximately 50%, whereas, *F. solani* was inhib-

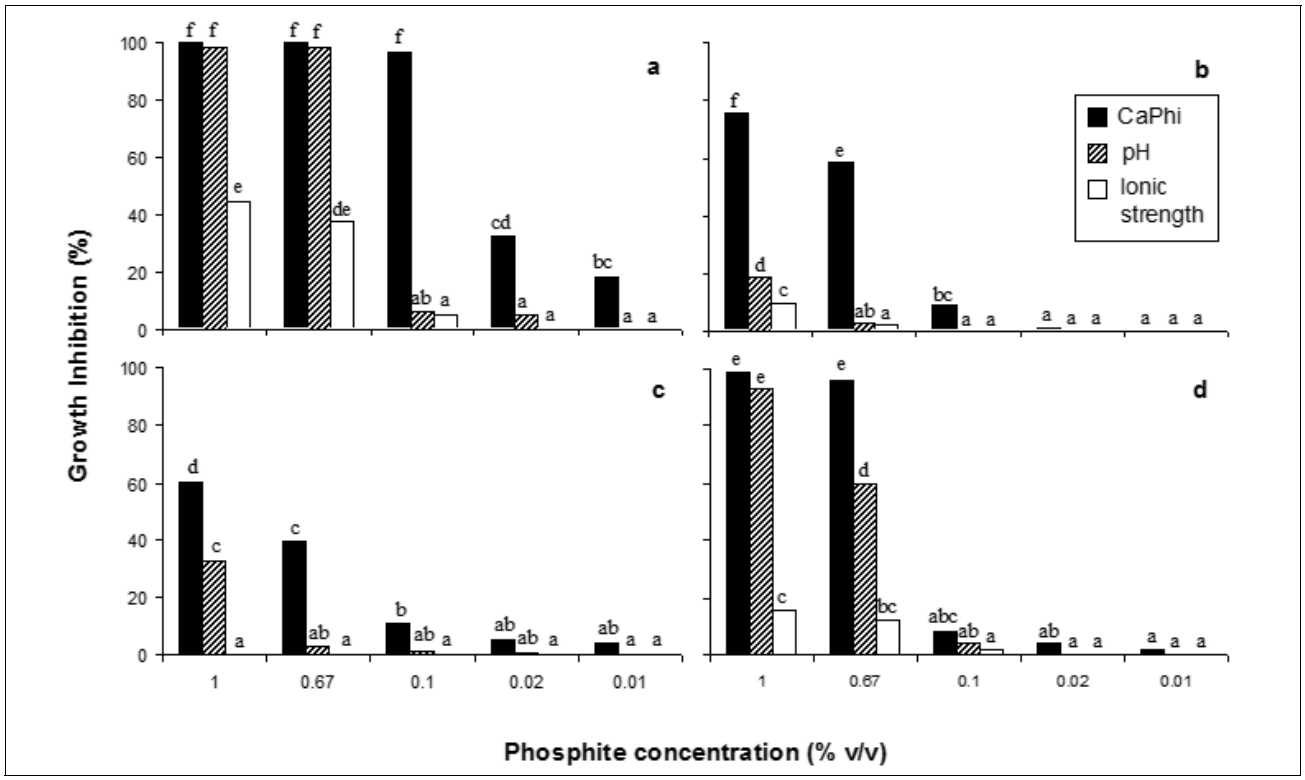

Fig. 1: Growth inhibition of four potato pathogens by different concentrations of calcium phosphite (CaPhi). (a) *Phytophthora infestans*, (b) *Rhizoctonia solani*, (c) *Fusarium solani* and (d) *Streptomyces scabies* growth inhibitions. Black bars represent total growth inhibition by CaPhi. Striped and white bars represent growth inhibition by pH and ionic strength equivalent to CaPhi concentrations added to media, respectively. The letters in each bar represent the statistical analysis of growth inhibition percentages by CaPhi, pH and ionic strength for each pathogen analysed. Bars with the same letter are not significantly different at a *P* value of 0.05.

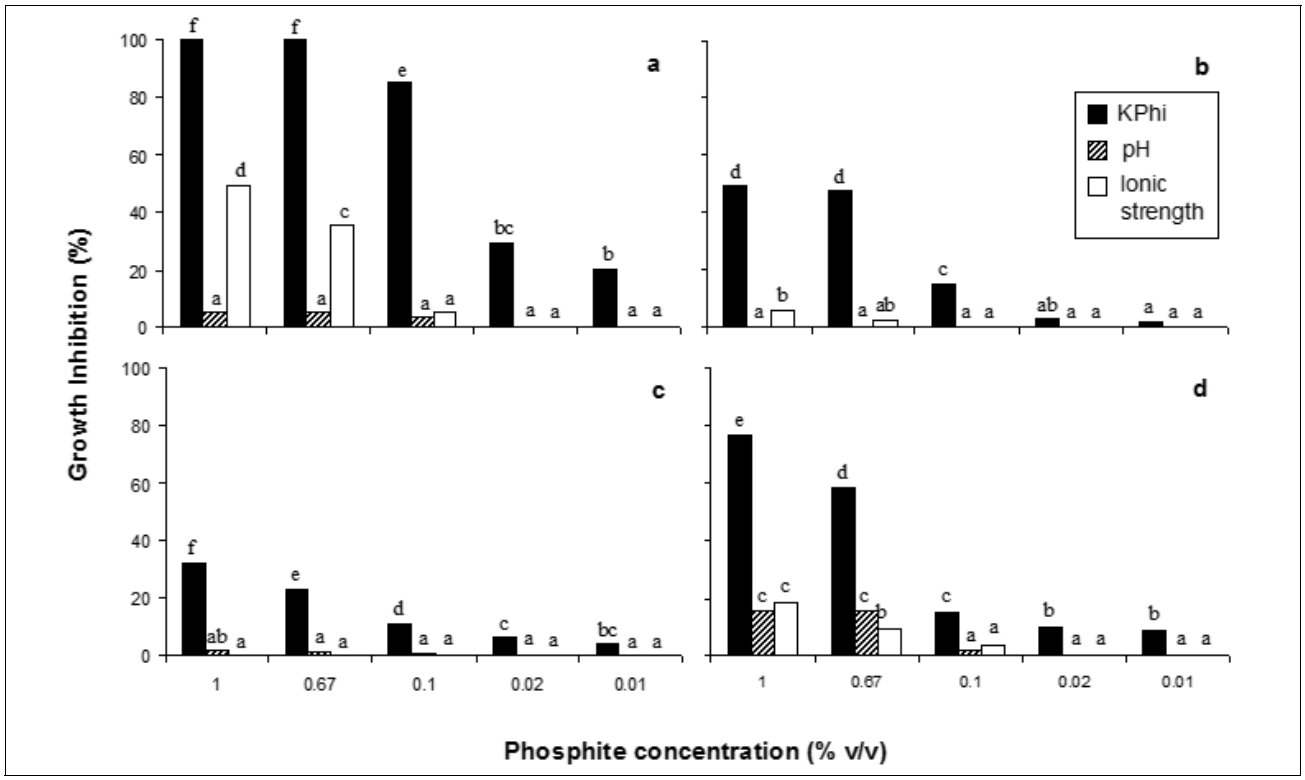

Fig. 2: Growth inhibition of four potato pathogens by different concentrations of potassium phosphite (KPhi). (a) *Phytophthora infestans*, (b) *Rhizoctonia solani*, (c) *Fusarium solani* and (d) *Streptomyces scabies* growth inhibitions. Black bars represent total growth inhibition by KPhi. Striped and white bars represent growth inhibition by pH and ionic strength equivalent to KPhi concentrations added to media, respectively. The letters in each bar represent the statistical analysis of growth inhibition percentages by KPhi, pH and ionic strength for each pathogen analysed. Bars with the same letter are not significantly different at a *P* value of 0.05.

ited by 32 and 23%, respectively. No inhibition was obtained by pH and ionic strength comparable to those obtained by KPhi addition at 0.67% or lower concentrations, whereas a little inhibition (approximately 6%) was found at the ionic strength corresponding to PDA medium supplemented with KPhi at 1%.

The bacterium *S. scabies* was inhibited by 1 and 0.67% KPhi by almost 80 and 60%, respectively. However, at lower concentrations, inhibition of bacterial growth was less than 15%. Only a small contribution could be attributed to pH decrease or ionic strength changes, since inhibition by these changes corresponding to 1 or 0.67% KPhi were less than 18% (Fig. 2d).

3.1.3 CuPhi effect on pathogen growth. The antimicrobial activity of CuPhi against potato pathogens is shown in Figure 3. CuPhi at 1 and 0.67% completely inhibited the growth of the four pathogens studied, and this effect could be due to acidification of the media. *P. infestans* and *S. scabies* were completely inhibited even at 0.1% CuPhi (Fig. 3a,d). Whereas no growth of oomycete was observed at pH corresponding to 0.1% concentration of CuPhi, *S. scabies* grew 40% respect to control at that pH. The antimicrobial activity of CuPhi against *S. scabies* abruptly dropped to less than 10% at lower concentrations of this phosphite, but completely inhibited *P. infestans* even at 0.01% CuPhi. *R. solani* and *F. solani* were partially inhibited at 0.1% (40 and 26%, respectively) (Fig. 3b,c). Ionic strength changes due to CuPhi addition had little inhibitory effect on the growth of these pathogens, being the most important the inhibition of *P. infestans* growth by ionic strength changes corresponding to CuPhi at 1 and 0.67%, with values of 40 and 30%, respectively (Fig. 3b,c).

3.2 Sensitivity of different potato pathogens to phosphites

To compare the sensitivity of different potato pathogens to Phi, EC_{50} values were calculated for CaPhi, KPhi and CuPhi. Results showed that the lowest EC_{50} values were obtained with the oomycete *P. infestans*, followed by the bacterium *S. scabies*, while the fungus *F. solani* was the most tolerant. For all the pathogens tested, CuPhi exhibited the smallest EC_{50}, meaning that this phosphite is the strongest inhibitory compound. The response of *P. infestans* growth to CaPhi and KPhi was very similar, with EC_{50} values of 0.04% for both compounds, whereas EC_{50} values for *F. solani* were very different (0.83 and > 1% for CaPhi and KPhi, respectively). However, in all cases, the EC_{50} was smaller for CaPhi than for KPhi (Table 2).

3.3 Fungicidal or fungistatic activity of phosphites

Reversibility of inhibition of *F. solani* spores germination was evaluated. After incubation, no germinated spores were found in the samples supplemented with CaPhi or KPhi at all, except the lowest concentration tested (Fig. 4). In addition, at 0.01% of CaPhi and KPhi the percentage of germinated spores was lower, (30–66% for CaPhi or KPhi treatments, > 95% for control treatment) and the germ tubes were shorter than in control samples. To determine the viability of Phi incubated spores, samples of all treatments after the incubation period for germination assay were transferred to Phi unsuplemented media. No differences in the number of colonies grown were found between control and Phi incubated spores (not shown), indicating that Phi have fungistatic and not fungicidal activity against *F. solani*.

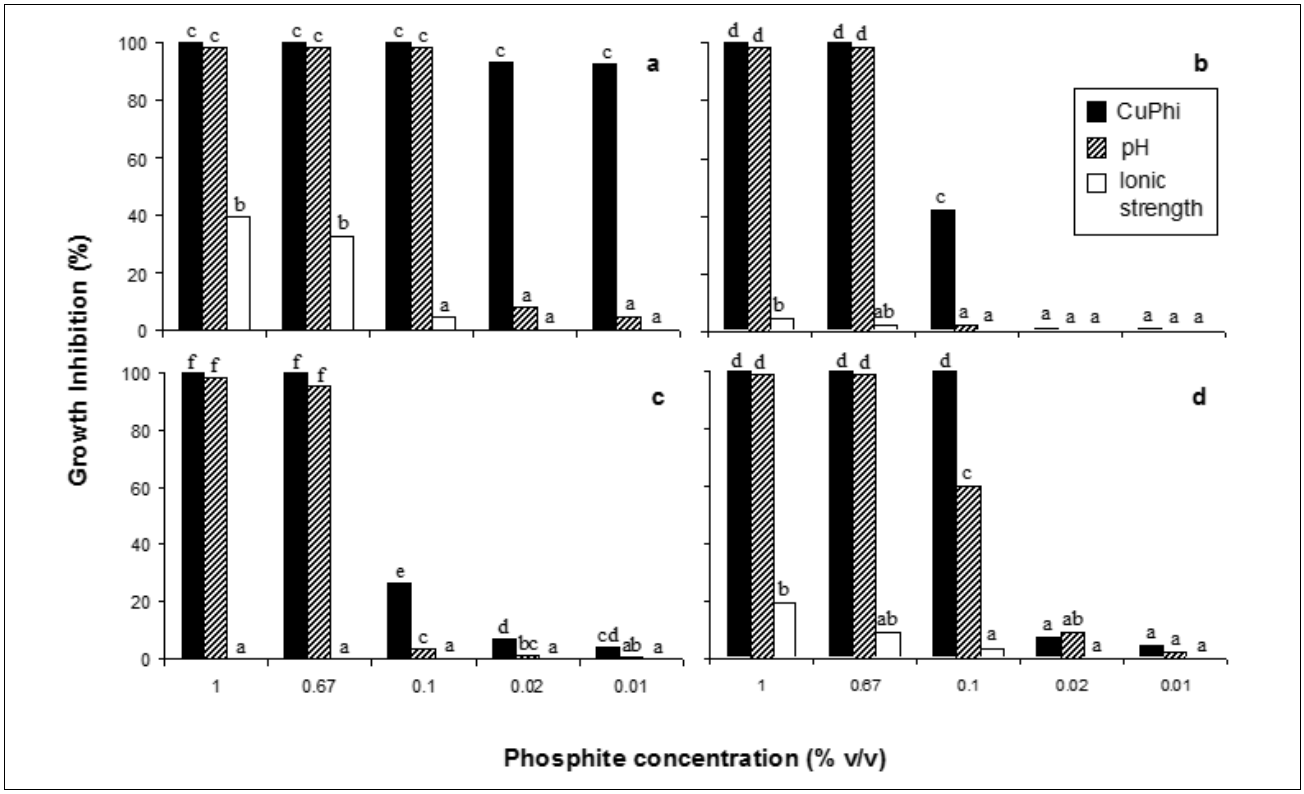

Fig. 3: Growth inhibition of four potato pathogens by different concentrations of copper phosphite (CuPhi). (a) *Phytophthora infestans*, (b) *Rhizoctonia solani*, (c) *Fusarium solani* and (d) *Streptomyces scabies* growth inhibitions. Black bars represent total growth inhibition by CuPhi. Striped and white bars represent growth inhibition by pH and ionic strength equivalent to CuPhi concentrations added to media, respectively. The letters in each bar represent the statistical analysis of growth inhibition percentages by CuPhi, pH and ionic strength for each pathogen analysed. Bars with the same letter are not significantly different at a *P* value of 0.05.

Table 2: EC_{50}s of calcium, potassium and copper phosphites for four potato pathogens

Pathogen	CaPhi		KPhi		CuPhi	
	EC_{50} (%)	EC_{50} (mg ml^{-1})	EC_{50} (%)	EC_{50} (mg ml^{-1})	EC_{50} (%)	EC_{50} (mg ml^{-1})
Phytophthora infestans	0.04	0.09	0.04	0.15	< 0.01	< 0.04
Rhizoctonia solani	0.83	1.87	> 1.00	> 3.56	0.27	1.04
Fusarium solani	0.57	1.28	> 1.00	> 3.56	0.18	0.68
Streptomyces scabies	0.37	0.83	0.56	1.99	0.06	0.22

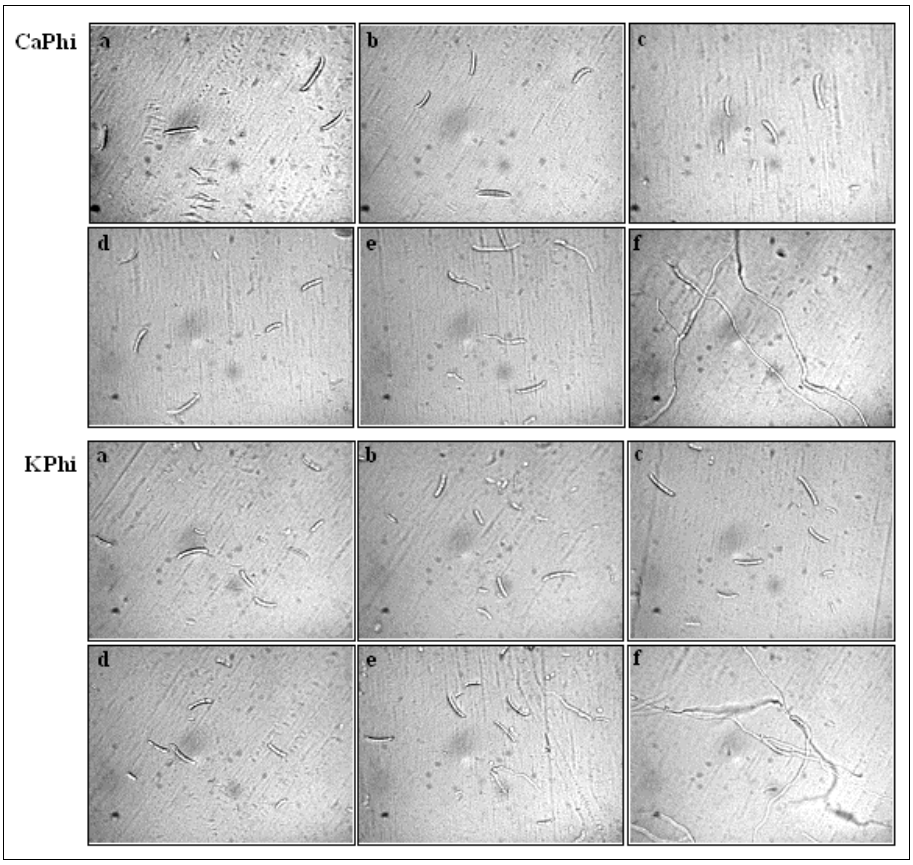

Fig. 4: Inhibition of germination of *Fusarium solani* spores by calcium and potassium phosphites (CaPhi and KPhi). Inhibition by CaPhi or KPhi at the concentrations of: (a) 1, (b) 0.67, (c) 0.1, (d) 0.02, (e) 0.01 and (f) 0% v/v (control).

4 Discussion

Phosphites, in general, are environmentally harmless compounds (Guest and Grant 1991) that can stimulate plant defence responses and also reduce disease severity in different plants (Panicker and Gangadharan 1999; Bécot et al. 2000; Reuveni et al. 2003; Johnson et al. 2004; Lobato et al. 2008a), representing a potential alternative to be used in an integrated crop management programme.

CaPhi, KPhi and CuPhi used in this work have shown high efficacy at 3 l ha^{-1} (1% commercial dose) in controlling pathogens on potato seed tubers and foliage. Moreover, the defence responses were cultivar, pathogen and compound dependent (Lobato et al. 2008a). The different levels of induced resistance obtained against the pathogens studied could be explained, in part, by considering a direct action of Phi on each pathogen.

The aim of this study was to know about the direct mode of action of Phi on different potato pathogens and, in particular, to know which is the contribution of acidification and ionic strength changes after CaPhi, KPhi and CuPhi application, on their antimicrobial activity. The fungicidal or fungistatic activity of these compounds was also evaluated.

Four potato pathogens from a broad range of taxonomic groups were tested in terms of their susceptibility to CaPhi, KPhi and CuPhi at different commercial doses. Results showed that CuPhi had the highest antimicrobial activity against the four potato pathogens analysed (*Phytophthora infestans*, *Rhizoctonia solani*, *Fusarium solani* and *Streptomyces scabies*). This might be due to the toxicity of copper ion itself, to the fact that CuPhi has the highest concentration of the phosphite anion of the three Phi tested, as well as to acidification of media after CuPhi addition at the different doses. When comparing between CaPhi and KPhi at the same doses, similar antimicrobial activities against the different pathogens were found. However, KPhi, in general, inhibited these pathogens less than CaPhi, despite the fact that the amount of the phosphite anion in KPhi is greater than in CaPhi, although, KPhi did not produce the important acidification effect of the media than did CaPhi. In a previous work we analysed the protection against *P. infestans*, *F. solani* and *R. solani* by applications of CaPhi or KPhi to seed tubers before planting. Highest protection levels were obtained against *P. infestans*, while the protection levels against the fungi *F. solani* and *R. solani* were intermediate and very low, respectively. However, in all cases, protection was equal or higher when KPhi was applied rather

than CaPhi (Lobato et al. 2008a), contrary to *in vitro* results. This apparent contradiction can be explained by considering that the effect of acidification of the media in *in vitro* assays was stronger when CaPhi was added, as mentioned above. No adjustments to a given pH value after the addition of Phi to the different media were made, as our objective was to compare the three phosphite compounds like are already marketed and used in field. However, the contribution of pH and ionic strength changes were evaluated. The effect of acidification on growth inhibitions by KPhi was low; contrary, this effect highly contributed to the growth inhibitions obtained by CaPhi and CuPhi at the highest concentrations. This effect was high against *P. infestans*, followed by that against *S. scabies*. Nevertheless, additional inhibition only by Phi was observed. On the other hand, negative effects on pathogen growth by increasing ionic strength after the addition of all Phi were not very significant except for *P. infestans*, but still in this case, additional contribution of Phi to the growth inhibition was obtained. All these results suggest that antimicrobial activity of Phi can not be attributed to a single reason, but is the result of the combination of three effects: the nature of the cation, the concentration of phosphite anion and the acidification of the media. For example, when acidification is not important, the concentration of phosphite anion plays the main role in growth inhibition of the pathogens.

To examine the different sensitivity of the pathogens studied, EC_{50} values were calculated for each pathogen isolate and phosphite analysed. For all Phi tested, the lowest EC_{50} values were obtained with the oomycete *P. infestans*. The contrary was found for the fungi *R. solani* and *F. solani*, while the bacterium *S. scabies* showed intermediate sensitivity to Phi. EC_{50}s for each phosphite tested differed in more than one order of magnitude between the isolates of the fungi and oomycete analysed. Fenn and Coffey (1984) reported similar relative sensitivity results and compared the inhibition percentage of radial growth of various *Phytophthora* species with those of several fungi, by addition of H_3PO_3 at different concentrations (69–552 $\mu g\ ml^{-1}$). They calculated EC_{50} values for three isolates of *P. infestans*, with EC_{50} values ranging from 173 to 221 $\mu g\ ml^{-1}$ of H_3PO_3, consistent with the EC_{50}s calculated in the present work and also with those reported by Bashan et al. (1990). When comparing oomycetes with fungi, Fenn and Coffey (1984) found that, except at the lowest concentration of that compound, growth of *Phytophthora* species was inhibited by 80% or more, while *R. solani* was inhibited by 8 to 38% at all the concentrations assayed. Similar results were obtained by Mills et al. (2004) when they compared *P. infestans*, *P. erythroseptyca* and *F. solani* var. *coeruleum*. Despite the similarities of our results with those mentioned above regarding relative sensitivity to Phi between oomycetes and fungi, to make extrapolations of the sensitivity of these pathogens to the entire kingdoms could be a mistake, because significant differences in the sensitivity to phosphorous acid related compounds were found within various classes of Oomycetes, and moreover, within a species (Bashan et al. 1990; Wilkinson et al. 2001). On the other hand, Avis et al. (2007) reported that sensitivity to low doses of aluminum chloride and sodium metabisulfite, two chemicals capable of controlling post-harvest pathogens on potato tubers, showed similar behaviour in terms of relative sensitivity between oomycetes and fungi as in the present study with phosphites. In that report, they showed that EC_{50}s of aluminum chloride and sodium metabisulfite for *P. infestans*, *R. solani* and *F. sambucinum* also differed in at least one order of magnitude, being the oomycete the most sensitive of these pathogens analysed.

In the future, it would be important to elucidate the reason that makes pathogens from very different taxonomic groups so different in sensitivity to phosphite compounds, and in particular, to know which is the main target of Phi in the pathogens. As a starting point, lipid composition and the effect of Phi on lipid peroxidation in the different pathogens could be analysed. This is based on the work reported by Avis et al. (2007), who analysed the lipid composition and lipid peroxidation as playing a role in the different sensitivities of the pathogens to the chemicals tested.

There has been controversy regarding the fungicidal properties of phosphorous acid related compounds. The present work supports the hypothesis that these compounds do not behave as common fungicides unless the cation combined with the phosphite has toxicity itself. Moreover, our results show that CaPhi and KPhi completely inhibit *F. solani* spore germination but this effect was reversible after the 14-h incubation period. Consistent to our results, Guest and Grant (1991) reported that Phi cause disturbances at several metabolic sites in the mycelial phase, for example, inhibiting sporulation at low concentrations without affecting mycelial growth, and that, even at high concentrations, Phi are fungistatic rather than fungitoxic.

Based on the fact that the Phi concentration required for inhibition of *F. solani* spore germination was very low, and together with the *in vitro* results, we can not discard that Phi have a direct effect in protection of post-harvest tubers, in addition to the previously reported effect of resistance induction. Moreover, regarding the broad spectrum of the pathogens directly inhibited by Phi and the known effects of Phi in inducing plant defence responses, results support the use of Phi in an integrated potato crop management program with the purpose to minimize the use of fungicides.

The knowledge of the direct and indirect mode of action of Phi compounds will contribute to improve the use and management of these products.

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