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Potassium phosphite primes defense responses in potato against *Phytophthora infestans*

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ABSTRACT

Although phosphite is widely used to protect plants from pathogenic oomycetes on a wide range of horticultural crops, the molecular mechanisms behind phosphite induced resistance are poorly understood. The aim of this work was to assess the effects of potassium phosphite (KPhi) on potato plant defense responses to infection with *Phytophtora infestans* (*Pi*). Pathogen development was severely restricted and there was also an important decrease in lesion size in infected KPhi-treated leaves. We demonstrated that KPhi primed hydrogen peroxide and superoxide anion production in potato leaves at 12 h post-inoculation with *Pi*. Moreover, the KPhi-treated leaves showed an increased and earlier callose deposition as compared with water-treated plants, beginning 48 h after inoculation. In contrast, callose deposition was not detected in water-treated leaves until 72 h after inoculation. In addition, we carried out RNA gel blot analysis of genes implicated in the responses mediated by salicylic (SA) and jasmonic acid (JA). To this end, we examined the temporal expression pattern of *StNPR1* and *StWRKY1*, two transcription factors related to SA pathway, and *StPR1* and *StIPII*, marker genes related to SA and JA pathways, respectively. The expression of *StNPR1* and *StWRKY1* was enhanced in response to KPhi treatment. In contrast, *StIPII* was down regulated in both KPhi- and water-treated leaves, until 48 h after infection with *Pi*, suggesting that the regulation of this gene could be independent of the KPhi treatment. Our results indicate that KPhi primes the plant for an earlier and more intense response to infection and that SA would mediate this response.

Introduction

Induced resistance (IR) is defined as the mechanism that, upon abiotic or biotic stimuli, plants can increase their level of resistance against a future stress. Based on differences in signaling pathways and spectra of effectiveness, IR is classified in various types. Among them, the classic form is systemic acquired resistance (SAR), and occurs on distal parts of the plant after a localized infection by a necrotizing pathogen and is controlled by a signal pathway that involved salicylic acid (SA) accumulation and the defense regulatory protein NPR1 (Spoel et al., 2009; Vlot et al., 2009). Downstream and upstream of NPR1, several WRKY transcription factors play important roles in the regulation of SA-dependent defense responses (Chen and Chen, 2000; Yu et al., 2001; Wang et al., 2006; Van der Ent et al., 2009). Another type, induced systemic resistance (ISR) is induced by non pathogenic bacteria and in *Arabidopsis* it shown to be independent of SA but requires jasmonic acid (JA) and ethylene (ET).

The IR does not necessarily require direct activation of defense responses, but can also result from a sensitization of the tissue to express basal defense mechanisms more rapidly and strongly upon pathogen or insect attack. This capacity for augmented defense expression is called "priming", so this primed state appears to be an immune system that offers protection to a wide spectrum of stresses caused by biotic or abiotic agents (Beckers and Conrath, 2007; Goellner and Conrath, 2008). Primed responses include an oxidative burst that consists in a rapid accumulation of reactive oxygen species (ROS), the deposition of cell wall reinforcement components such as callose and lignin, and the induction of pathogenesis-related proteins (PRs) (Ahn et al., 2007; Taheri and Tarighi, 2010). Moreover, it has also been suggested that this responses would be a consequence of the accumulation of signaling components that enhance the transcription of defense genes after stress recognition (Conrath et al., 2006).

Abbreviations: DAB, diaminobenzidine; hpi, hours post-inoculation; IR, induced resistance; JA, jasmonic acid; KPhi, potassium phosphite; NBT, nitrobluetetrazolium; *Pi*, *Phytophthora infestans*; PRs, pathogenesis-related proteins; ROS, reactive oxygen species; SA, salicylic acid.

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Potato late blight, caused by the oomycete pathogen *Phytophthora infestans* (*Pi*), is the most important disease of this crop, considered as a serious threat to tuber production. Therefore, in Argentina and other regions of the world, potato production is not possible without the use of fungicides. Their use not only increase production costs but also generates environmental and health damage (Cooke et al., 2011). In this scenario, it is clear the need of an environmental and economic more sustainable late blight control method. An innovative strategy, within integrated crop management (ICM), is the use of biocompatible chemical compounds that enhance disease resistance in plants through the IR (Daayf et al., 2000; Shibuya and Minami, 2001; Altamiranda et al., 2008). Among them, phosphites (inorganic salts of phosphorous acid) have received particular attention, and they have been described as capable of controlling crop diseases caused by oomycetes and bacteria through both, a direct effect on the pathogen and an indirect effect by stimulating host defense responses (Deliopoulos et al., 2010). Direct effects include the inhibition of mycelial growth and the reduction or alteration of the pathogen metabolism (Grant et al., 1990; Guest and Grant, 1991; Wilkinson et al., 2001; King et al., 2010). The indirect effect involves the stimulation of plant defense mechanisms such as the enhanced production of phytoalexins and ROS, the induction of PRs and the reinforcement of the cell wall (Guest and Grant, 1991; Lobato et al., 2008, 2011; Pilbeam et al., 2011; Eshraghi et al., 2011).

In our lab, the effect of phosphite applications directly to seed tubers and foliage of potato plants has been studied (Lobato et al., 2008). Phosphites reduced seed tuber disease symptoms caused by the oomycete *Pi*, but also by *Fusarium solani* and *Rhizoctonia solani* infections. Protection in foliage against *Pi* was also obtained by foliar applications of calcium phosphite and potassium phosphite (KPhi). In addition, foliar applications of KPhi to field grown crops resulted in post-harvest tubers with a reduced susceptibility to *Pi*, *F. solani* and *Erwinia carotovora* infections, suggesting that this compound induced a systemic defense response (Lobato et al., 2011). These phosphites were able to induce PRs, phytoalexins and other defense related enzymes in the foliage and tubers which could explain, at least in part, the protection observed (Lobato et al., 2008, 2011). Interestingly, KPhi was able to prime the accumulation of molecules involved in defense reaction only in *Pi*-infected tubers from plants with foliar KPhi treatments (Lobato et al., 2011). Despite the common use of phosphites, their complex mechanism underlying their ability to protect plants against infections by different pathogens remains unclear.

Taken together, our previous results suggest that phosphites might act inducing plant defense responses through a priming mechanism, increasing the level of potato resistance against a future pathogen challenge. In order to elucidate the molecular mechanisms underlying these responses, the aim of the present work was to analyze if KPhi primes defense responses mediated by SA, analyzing the expression of marker genes and key regulators of this pathway, and the induction of cellular defense responses like ROS accumulation and callose deposition.

Materials and methods

Biological material

For foliage assays, *Solanum tuberosum* seed tubers (cv. Kennebec) were planted in pots containing a pasteurized mixture of soil: vermiculite (3:1, v/v). Pots were maintained under greenhouse conditions (18 °C day-night temperature, 16 h of light per day). These growing conditions were applied to all foliage experiments which were performed at least three times each.

Phytophthora infestans (*Pi*) (Mont.) De Bary (race R_2 R_3 R_6 R_7 R_9, mating type A2) (Andreu et al., 2010) was grown on potato tuber slices of cv. Spunta at 18 °C for seven days. Mycelia 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 sporangial suspension was observed under light microscope for quantification before being used as inoculum.

Chemical treatment

KPhi (Afital Potassium Phosphite, Agro-EMCODI SA) was applied to the foliage at 10 mL per plant (3 L/ha) by using an atomizer (ESAC SA) operating at 200 kPa, 21 days after emergence. The dose utilized was 1% (v/v) of the commercial product. Control plants were sprayed with water.

Pathogen challenge

Three days after KPhi or water treatment, two leaflets per plant were detached from ten plants per treatment. These leaflets were immediately placed on a wet filter paper in Petri dishes and artificially inoculated with a zoospore suspension (2×10^4 zoospores/mL) of *Pi* either by spray (RNA extraction/foliage protection assay) or drops (15 μL each, histochemical assays). The inoculated leaflets were then incubated at 18 °C. At different times post-inoculation, leaves were used for RNA extraction or histochemical assays.

Seven days post-inoculation, typical leaf disease symptoms were observed (Hooker, 1980).

RNA isolation

At four time points (0, 24, 48 and 72 h) post-inoculation with *Pi*, total RNA from each treatment was isolated using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. RNA concentration was evaluated by measuring the absorbance at 260 nm and its integrity was visualized by 1% agarose gel electrophoresis.

Relative quantification of Pi in potato leaves

Approximately 2 μg of total RNA (DNA-free), were used for first-strand cDNA synthesis using the M-MLV Reverse transcriptase enzyme (Promega) according to the manufacturer's instructions.

The ITS region of *Pi* (Gen Bank accession number JF834703) was used to generate the primer pair *ITS1*-R/*ITS2*-F (listed in Table 1). Amplifications were performed with an automated thermal cycler (Thermo) in a 25 μL reaction volume containing 125 μM of the four dNTPs (Promega), 0.5 μM of each primer, 1 U of GoTaq DNA polymerase (Promega), template cDNA and water. The reaction mixture was subjected to 30 cycles at the following incubations: 30 s denaturation at 95 °C (120 s for the first cycle), 30 s annealing at 55 °C and 60 s extension at 72 °C (10 min for the final cycle). *Stactin* was used as the internal control standard for reverse transcription PCR in the same samples. Primers for this gene are listed in Table 1. PCR products were analyzed by gel electrophoresis in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8) in the presence of ethidium bromide. DNA bands were visualized using a UV transilluminator.

Histochemical detection of reactive oxygen species (ROS) and callose

ROS detection

The *in situ* accumulation of ROS, hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) was determined by histochemical