



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 *Phytophthora 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.

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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 R₂ R₃ R₆ R₇ R₉, 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 (H₂O₂) and superoxide anion (O₂⁻) was determined by histochemical

Table 1

Primers used for the generation of probes for RNA gel blot analysis and for RT-PCR analysis.

Stgene	GenBank accession number	Reverse primer	Forward primer
<i>StNPR1</i>	AY615281	5'-CGCATCTCTCTCCAAACAT-3'	5'-GAGCTTCTCACXTCAATGCGTT-3'
<i>StPR1</i>	AY050221	5'-CATGAACATATGGTACGTGGAA-3'	5'-CCTAAAGCAAAATGGGGTTG-3'
<i>StWRKY1</i>	AY615273	5'-ATGGGATGTGAATGCATGC-3'	5'-CGGGTCTTGGGACTAATG-3'
<i>StPII</i>	BQ113673	5'-TAGCCGTGGTAAAGGTCCAC-3'	5'-TCAATCAAAGCAAGAGTTGAGA-3'
<i>Stactin</i>	DQ252512	5'-CGACCACCTTAATCTTCATGC-3'	5'-TACTCGTTCACCACCTCAGC-3'
<i>P. infestans</i> ITS region	JF834703	5'-TACTCGTTCACCACCTCAGC-3'	5'-GTTTGACTTCGGTCTGAGC-3'

analysis at 0, 12 and 24 hours post-inoculation (hpi). Hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were detected macroscopically and microscopically by diaminobenzidine (DAB, Sigma) and nitrobluetetrazolium (NBT) staining methods, respectively (Thordal-Christensen et al., 1997; Hükelhoven and Kogel, 1998). Leaf disks containing the inoculation site were stirred in 1 mg/mL DAB (pH 3.8) or a 0.2% NBT solution (sodium phosphate buffer 10 mM, pH 7.8 containing 0.2% NBT and 5 mM sodium azide) for 3 h at room temperature. Boiling ethanol was used to stop the reactions and bleach the disks. DAB polymerizes instantly, and develops a localized brown color as soon as it comes into contact with H_2O_2 in the presence of peroxidase, whilst the occurrence of dark blue insoluble precipitates after NBT staining corresponds to O_2^- accumulation.

A minimum of 6 disks were examined for each collection time and treatment. The experiment was repeated three times. Quantification of both, H_2O_2 and O_2^- was performed with ImageJ software.

Callose detection

For callose visualization within the cells, leaves were collected at 12, 24, 48 and 72 hpi with *Pi*. Leaf disks containing the inoculation site were discolored in boiling ethanol and stained with aniline blue 0.05% (M6900, Sigma–Aldrich) as described by Cohen et al. (1990). Leaf disks stained with aniline blue were examined for callose deposition using a Nikon Eclipse E200 epifluorescence microscope (Ex 365/10, Dichroic 400 LP, Emis 460/50). Callose-like deposits appeared as fluorescent yellow-white-stained cells surrounding the site of infection. A minimum of 6 disks were examined for each collection time and treatment. The experiment was repeated three times. Quantification of callose was performed with ImageJ software.

RNA gel blot hybridization

Total RNA (10 µg) was denatured using gloxal and dimethyl-sulfoxide (Sambrook et al., 1989). RNAs were size fractionated on 1.5% agarose gels, blotted to Hybond N membranes, and hybridized with the following potato cDNA probes *StNPR1* (AY615281), *StPR1* (AY050221), *StWRKY1* (AY615273) and *StPII* (BQ113673), as described by Sambrook et al. (1989). cDNA probes were labeled with ^{32}P -dCTP using the Megaprime DNA labeling system according to the manufacturer's description (Amersham Biosciences). Equal loading was assured by ethidium bromide staining of rRNA. After a high stringency wash, the membranes were exposed to a phosphorimager plate (FUJI Photo Film) for 2–3 days. Hybridization patterns were visualized by phosphorimaging (Storm; Amersham). RNA quantification was performed by plot analysis using Image-QuantTL (version v2005). The expression levels were quantified as a ratio against *Stactin* levels and expressed as a fold increase taking *Stactin* levels as 1.

The sequences of the primers used for the synthesis of the different probes are listed in Table 1.

Statistical analysis

ROS accumulation and callose deposition data was analyzed by the *t*-test and one-way Kruskal–Wallis test, respectively. Gene expression data was analyzed by one-way ANOVA (Zar, 1999). *A posteriori* multiple comparison tests (Tukey test) were performed when significant ($P < 0.05$) differences between means were detected. Data transformation ($\log_{10} Y$) was used when needed to meet homoscedasticity.

Results

P. infestans (*Pi*) growth in potato leaves

Pathogen biomass was analyzed through semi-quantitative RT-PCR, in a time course post-inoculation. Leaves from water-treated plants showed an increase in *Pi* biomass at 72 hours post-inoculation (hpi), reaching its highest value at 96 hpi (Fig. 1A). However, *Pi* biomass was slightly detectable only after 72 hpi in leaves from KPhi-treated plants.

Pi-Infected leaves from water-treated plants showed the typical necrotic lesion caused by this oomycete at 7 days post-inoculation (Fig. 1B). On the other hand, KPhi-treated leaves were protected against the pathogen since almost no disease symptoms were developed (Fig. 1C).

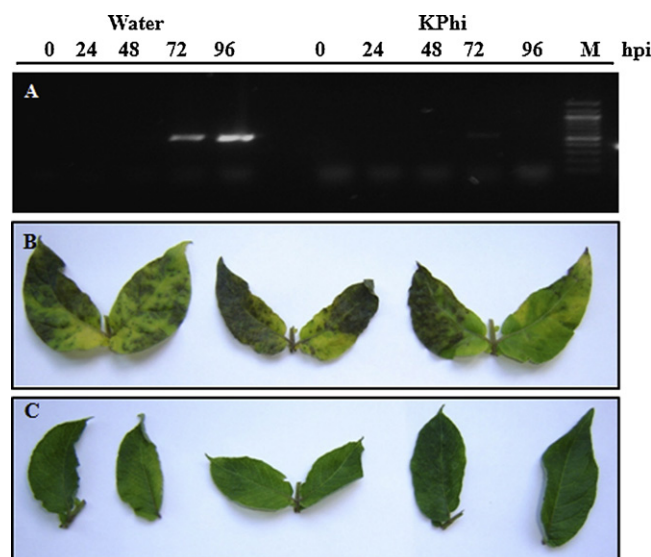


Fig. 1. Effect of KPhi on induced resistance against *Phytophthora infestans*. (A) *P. infestans* biomass detection by RT-PCR on potato leaves. Amplification products of primer set *ITS1* and *ITS2* and template DNA leaves infected with *P. infestans* from water- or KPhi-treated plants. Symptoms of *P. infestans* infection on leaves from (B) water-treated and (C) KPhi-treated plants, 7 days post-inoculation. hpi: hours post-inoculation. M: molecular markers.

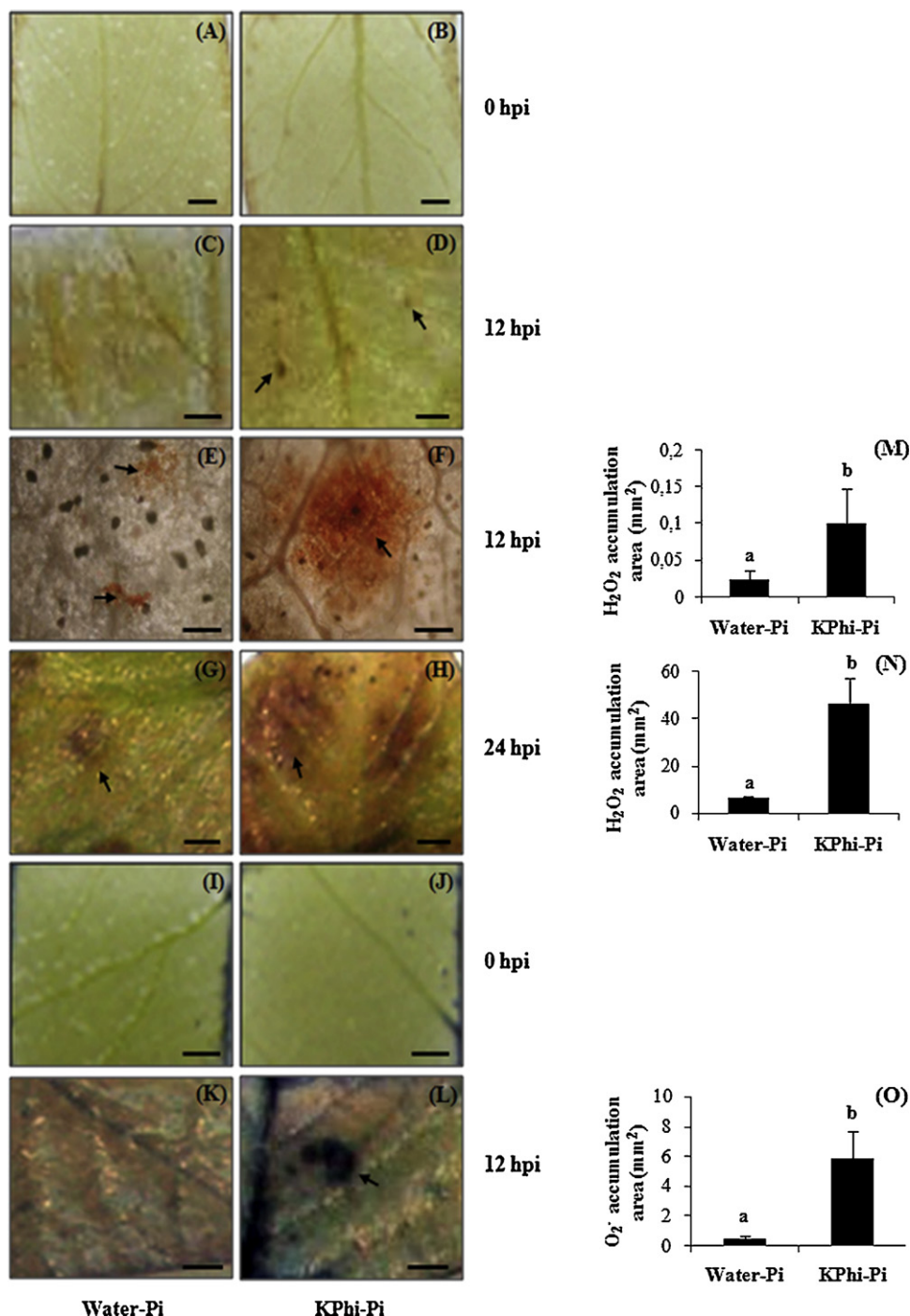


Fig. 2. Effect of KPhi on the H_2O_2 and O_2^- accumulation in potato leaves following infection with *P. infestans*. (A–H) Hydrogen peroxide (H_2O_2) detection by the 3,3-diaminobenzidine (DAB) staining method. H_2O_2 was observed (A–D, G, and H) macroscopically and (E and F) microscopically in infected leaves from water-treated and KPhi-treated plants. Deep-brown color shows H_2O_2 production (arrows indicate). (I–L) Analysis of superoxide anion (O_2^-) accumulation by the nitrobluetetrazolium (NBT) staining method. O_2^- was detected macroscopically in infected leaves from (I and K) water-treated and (J and L) KPhi-treated plants. Blue formazan precipitate indicates O_2^- production (arrows indicate). Scale bars represent 2 mm (A–D and G–L plates) and 0.1 mm (E and F plates). Quantification of both, H_2O_2 and O_2^- are represented in bar plots as it follows: M, N and O for E, F, G, H and K–L plates, respectively. Each bar represents the mean \pm SD. Bars with the same letter do not differ significantly at $P < 0.05$.

Production of ROS during potato–Pi interaction

Oxidative burst has often been implicated in early defense responses; therefore we analyzed the accumulation of ROS, hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), in leaves from

KPhi- and water-treated potato plants. KPhi treatment by itself did not induce H_2O_2 or O_2^- accumulation (Fig. 2A, B, I and J). At 12 hpi with Pi, H_2O_2 and O_2^- production was higher in leaves from KPhi-treated plants as compared with leaves from water-treated plants (Fig. 2C–F, K–M and O). This response was also observed at 24 hpi

for H_2O_2 (Fig. 2G, H and N). However, no differences were detected in O_2^- accumulation between leaves from KPhi- and water-treated plants at this time (not shown).

Callose deposition during potato-Pi interaction

Late blight development on potato leaves led to callose deposition in the walls of epidermal cells surrounding the lesions. Leaves from KPhi-treated plants showed an earlier callose deposition than leaves from water-treated plants, post-inoculation. At 48 hpi, fluorescence was only observed in leaves from KPhi-treated plants (Fig. 3A, B and E). Moreover, callose deposition was much stronger 72 hpi in leaves from KPhi-treated than from water-treated plants (Fig. 3C, D and E).

Analysis of the expression of *StNPR1* and *StWRKY1* during potato-Pi interaction

In order to establish whether the SA-mediated signal transduction pathway participates in the mechanism involved in phosphite-induced priming, we performed transcriptional analysis of genes implicated in the response mediated by SA. For this purpose, we examined the temporal expression pattern of *StNPR1* and *StWRKY1* transcription factors in potato leaves from water- or KPhi-treated plants at different time post-inoculation with *Pi* (Fig. 4A and B). RNA gel blot analysis of *StNPR1* and *StWRKY1* showed a differential induction of both genes in leaves from KPhi-treated plants as compared with leaves from water-treated plants. KPhi-treatment potentiated *StWRKY* expression at 24 and 48 hpi, reaching its highest level at 48 hpi (2.6-fold induction). Similarly, *StNPR1* expression was induced by KPhi treatment only at 48 hpi (1.7-fold induction) being also its highest expression level. In addition, the highest expression of these genes in leaves from water-treated plants was observed at 72 hpi.

Induction of the *StPR1* and *StIPII* genes during potato-Pi interaction

Expression profiling of marker genes for both, the SA (*PR1*) and JA (*IPII*) signal transduction pathways, were used to further determine their role in the observed KPhi-induced priming. Analysis of the expression of *StPR1* showed an earlier induction in leaves from KPhi-treated plants as compared with leaves from water-treated plants, starting at 24 hpi. This increase reached its highest level after 48 hpi, showing a 5.4-fold induction as compared with leaves from water-treated plants (Fig. 5A). On the other hand, the expression of *StIPII* showed a high constitutive expression for both, KPhi-treated and water-treated plants, showing a dramatic down regulation until 48 hpi. However, at 72 hpi in KPhi-treated plants, this value was not significantly different than the basal expression of this gene in both treatments (Fig. 5B).

Discussion

The results emerged from this study confirm our hypothesis that KPhi induces resistance in potato against *Pi* through a priming mechanism. In addition, they provide more evidences about the role of these compounds as plant defense activators and explain, at least in part, that the mechanism by which phosphites are able to trigger priming in the potato-Pi pathosystem involves SA signaling pathway.

KPhi application restricted lesion development and pathogen biomass in potato leaves. These results are consistent with our previous findings where we have demonstrated that KPhi was able to protect potato leaves against *Pi* infection (Lobato et al.,

2008). To further study the early events that occur when *Pi* colonize potato leaves, we analyzed the production of ROS (Jabs et al., 1997; Lamb and Dixon, 1997). Our results show that KPhi treatment by itself did not induce H_2O_2 or O_2^- accumulation. However, a rapid accumulation of H_2O_2 and O_2^- was observed in leaves from KPhi-treated plants after pathogen inoculation, suggesting a priming effect mediated by KPhi on pathogen-induced oxidative burst. Similar results were reported by Daniel and Guest (2006) and Eshraghi et al. (2011) in KPhi-treated *Arabidopsis thaliana* plants infected with *P. palmivora* and *P. cinnamomi*, respectively. The ability to respond more rapidly and efficiently may be determinant for avoiding the success of the infection. In addition, an increase in the production of ROS would contribute not only to the resistance induced by KPhi, but also would increase lignin formation, a compound that is implicated in reinforcement of the cell wall (Boerjan et al., 2003). Among the molecules involved in defense responses, callose deposition within the cells has been well documented in plant-*Phytophthora* interactions and has been correlated to resistance in several plant-pathogen systems because of its well established role in cell wall reinforcement (Zimmerli et al., 2000; Hamiduzzaman et al., 2005). Our results showed that KPhi treatment not only increased considerably callose deposition following leaf inoculation with the pathogen, but it was also earlier produced than in water-treated plants. However, Daniel and Guest (2006) found an increase in callose accumulation in the *A. thaliana*-*P. palmivora* pathosystem regardless of phosphite treatment, suggesting that the mechanism of action of this compound is highly influenced by the nature of host and pathogen.

Many chemical compounds have been described as effectors of IR. Most of these agents elicit the SAR pathway, activating similar PR genes and failing to induce these responses in SAR mutants (Dong et al., 1999). However, β -aminobutyric acid (BABA), a well studied chemical inducer, not only impaired its effect through the SA signaling pathway but, depending on the pathosystem, it triggers priming through an independent *via*. Despite the differences between the different forms of IR, both BABA-mediated mechanisms are characterized by primed resistance (Van der Ent et al., 2009).

SA has been identified as a crucial signaling molecule required for the expression of plant defense responses. Potato is described to have high basal levels of this molecule. Moreover, potato varieties with race-nonspecific resistance contain higher levels of SA and PRs gene expression than susceptible ones (Coquoz et al., 1995), suggesting an important role of SA-dependent responses for resistance in potato (Halim et al., 2007; Vleeshouwers et al., 2000). Thus, in order to analyze the mechanisms underlying KPhi priming in the potato-Pi pathosystem, we evaluated the expression pattern of two transcription factors that are reported to be involved in SA-mediated processes, *StNPR1* and *StWRKY* (Chen and Chen, 2000; Subramaniam et al., 2001; Koornneef and Pieterse, 2008).

KPhi treatment induced the expression of both *StNPR1* and *StWRKY1* genes following inoculation, showing an elevated and earlier transcription in leaves from KPhi-treated compared to water-treated plants. Moreover, we analyzed the effect of KPhi on the expression of *StPR1* as a marker gene of SA pathway. *StPR1* showed a higher and earlier induction in leaves from KPhi-treated as compared with water-treated plants, following infection. Since SA activates defense responses through its downstream component NPR1 and some transcriptional factors belonging to the WRKY family are regulated by NPR1 (Bari and Jones, 2009; Van der Ent et al., 2009), these transcription factors might play a significant role in priming SA-inducible defense mechanisms in potato. Additionally, PR1 has shown a positive correlation with WRKY transcription factor up-regulation at transcript levels (Wang et al., 2005; Tian et al., 2006). These results support the hypothesis that KPhi-induced priming could be mediated by SA.

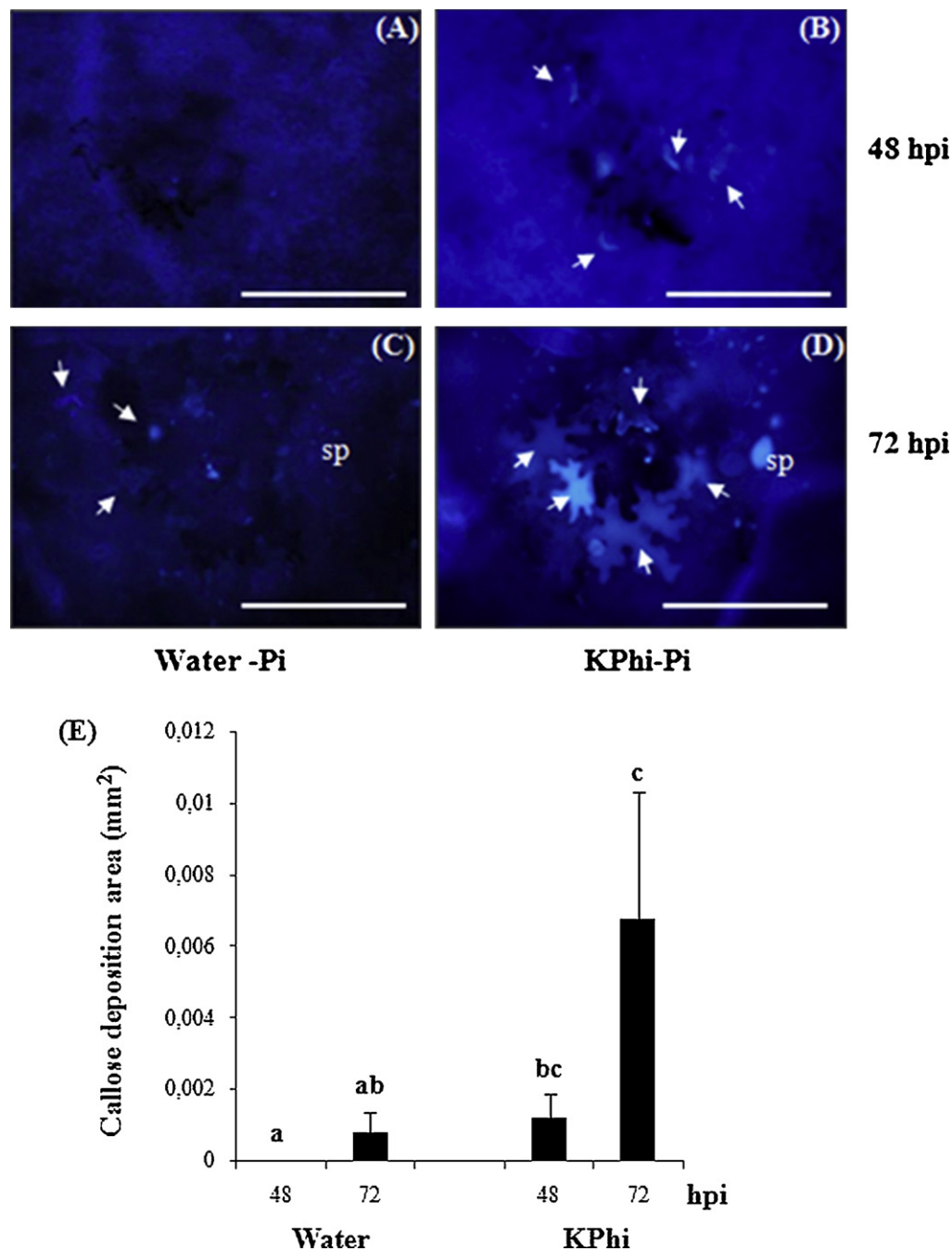


Fig. 3. Effect of KPhi on the callose deposition in potato leaves following infection with *P. infestans*. Microscopic observations of leaves from (A and C) water-treated and (B and D) KPhi-treated plants at 48 and 72 hpi with *P. infestans*. Leaves were stained with aniline blue for callose detection (fluorescent areas indicated by arrows). sp: sporangia. Scale bars represent 0.1 mm. Quantification of callose deposition is represented in the bar plot (E). Each bar represents the mean \pm SD. Bars with the same letter do not differ significantly at $P < 0.05$.

NPR1 not only plays a key role in SA dependent IR, but has also been implicated in the regulation of JA/ET-dependent pathways (Leon-Reyes et al., 2009). Moreover, *Arabidopsis npr1* mutant plants are compromised in the SA-mediated suppression of JA responsive gene expression, indicating that NPR1 plays an important role in SA–JA interaction (Spoel et al., 2003). Accordingly, we analyzed the involvement of JA pathway in KPhi-induced priming as there is ample evidence that priming is mediated not only by SA but also by JA and ET (Conrath et al., 2006; Taheri and Tarighi, 2010; Kravchuk et al., 2011; Tonelli et al., 2011). The expression of *StIPII*, a key marker gene of this pathway evidenced a high constitutive expression for both KPhi- and water-treated plants,

showing a dramatic down regulation until 48 hpi. However, at 72 hpi in KPhi-treated plants, this value was not significantly different than the basal expression of this gene in both treatments. It has been demonstrated that NPR1 is required for SA-mediated suppression of JA-dependent defense responses. Thus, the increase in the expression of *StNPR1* in leaves from water- and KPhi-treated plants after infection, could explain the downregulation of *StIPII*, regardless of the KPhi treatment. Similar results were obtained by Restrepo et al. (2005) who, based on indirect evidence (reduced expression of marker genes, *IPII* among them), showed that JA defense pathway was down-regulated in potato-*Pi* compatible interaction.

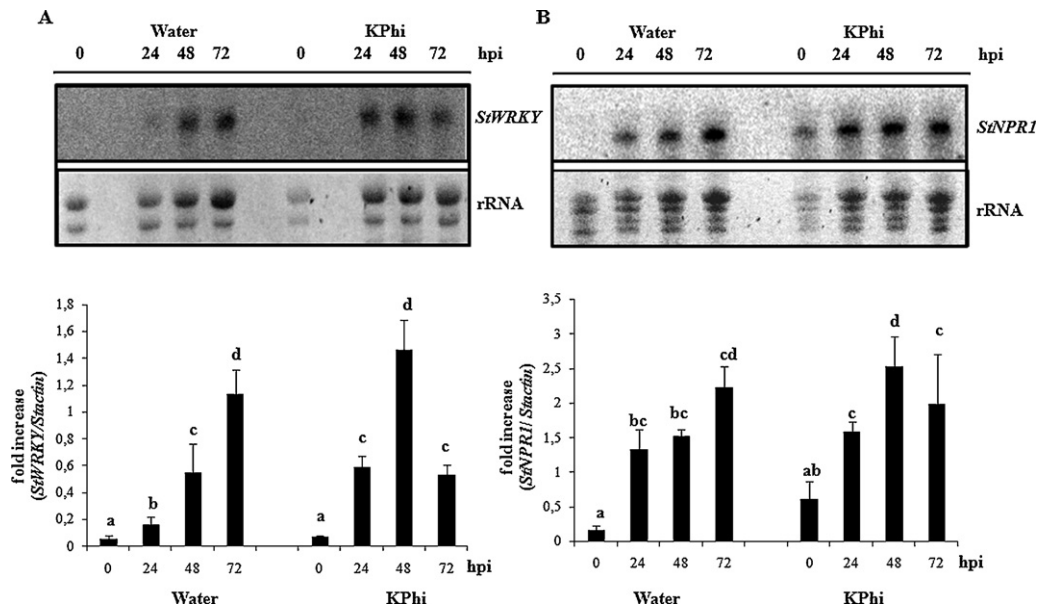


Fig. 4. Effect of KPhi treatments on the gene expression upon *P. infestans* infection. Expression of *StNPR1* and *StWRKY1* genes was analyzed in potato leaves from water- and KPhi-treated plants at 0, 24, 48 and 72 hpi with *P. infestans*. RNA gel blot analysis was performed. Following electrophoresis, RNA was transferred to nylon membrane and hybridized with dCTP-[α - 32 P]-labeled probes synthesized with the fragments of (A) *StNPR1* and (B) *StWRKY1*. Equal loading (10 μ g of total RNA) was assured by ethidium bromide staining of rRNA. Radioactivity was visualized and quantified by phosphorimaging. The radioactivity of the *StNPR1* and *StWRKY1* was normalized against the radioactivity of *Stactin*, and expressed as a fold increase taking *Stactin* level as 1. The quantification of three (*StNPR1*) or five (*StWRKY1*) independent experiments is plotted. Each bar represents the mean \pm SD. Bars with the same letter do not differ significantly at $P < 0.05$.

Taken together, our results support the idea that KPhi treatment may enhance resistance by the increased expression of defense molecules that would otherwise be induced in the plant, but at a later stage and to a lesser degree. We propose that the early events triggered by KPhi treatment are related to the reinforcement of cell wall. In a coordinated manner, transcription factors *StNPR1* and *StWRKY* may act as amplifiers of defense signaling cascades. In this context, probably even a slight induction during priming could be

enough to prime the plant to an immune state to counteract future stresses. Further studies with mutant plants where SA signaling pathway is impaired should be performed in order to confirm this hypothesis.

In this scenario, the potential use of KPhi as a “priming inducer” seems to be a promising feature, potentiating cellular defense responses just when it is needed (i.e. upon pathogen attack), avoiding a metabolic cost that could affect crop yield.

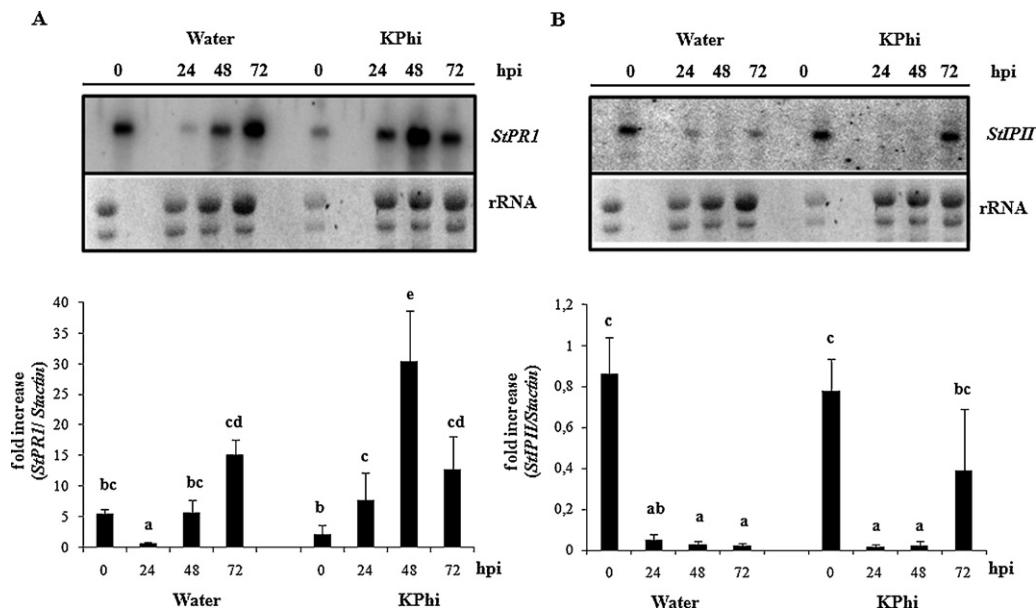


Fig. 5. Effect of KPhi treatments on the gene expression upon *P. infestans* infection. Expression of *StPR1* and *StPII* genes were analyzed in potato leaves from water- and KPhi-treated plants at 0, 24, 48 and 72 hpi with *P. infestans*. RNA gel blot analysis was performed. Following electrophoresis, RNA was transferred to nylon membrane and hybridized with dCTP-[α - 32 P]-labeled probes synthesized with the fragments of (A) *StPR1* and (B) *StPII*. Equal loading (10 μ g of total RNA) was assured by ethidium bromide staining of rRNA. Radioactivity was visualized and quantified by phosphorimaging. The radioactivity of *StPR1* and *StPII* was normalized against the radioactivity of *Stactin*, and expressed as a fold increase taking *Stactin* level as 1. The quantification of three independent experiments is plotted. Each bar represents the mean \pm SD. Bars with the same letter do not differ significantly at $P < 0.05$.

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References

- Ahn I-P, Kim S, Lee Y-H, Suh S-C. Vitamin B₁-induced priming is dependent on hydrogen peroxide and the NPR1 gene in *Arabidopsis*. *Plant Physiol* 2007;143: 838–48.
- Altamiranda EAC, Andreu AB, Daleo GR, Olivieri FP. Effect of β -aminobutyric acid (BABA) on protection against *Phytophthora infestans* throughout the potato crop cycle. *Australas Plant Pathol* 2008;37:421–7.
- Andreu AB, Caldiz DO, Forbes GA. Phenotypic description of resistance to *Phytophthora infestans* in processing potatoes in Argentina. *Am J Potato Res* 2010;87:177–87.
- Bari R, Jones JD. Role of plant hormones in plant defence responses. *Plant Mol Biol* 2009;69:473–88.
- Beckers GJM, Conrath U. Priming for stress resistance: from the lab to the field. *Curr Opin Plant Biol* 2007;10:425–31.
- Boerjan W, Ralph J, Baucher M. Lignin biosynthesis. *Annu Rev Plant Biol* 2003;54:519–46.
- Chen C, Chen Z. Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. *Plant Mol Biol* 2000;42:387–96.
- Cohen Y, Eyal H, Hanania J. Ultrastructure, autofluorescence, callose deposition and lignification in susceptible and resistant muskmelon leaves infected with the powdery mildew fungus *Sphaerotheca fuliginea*. *Physiol Mol Plant Pathol* 1990;36:191–204.
- Conrath U, Beckers GJ, Flors V, García-Agustín P, Jakab G, Mauch F, et al. Priming: getting ready for battle. *Mol Plant Microbe Interact* 2006;19:1062–71.
- Cooke LR, Schepers HTAM, Hermansen A, Bain RA, Bradshaw NJ, Ritchie F, et al. Epidemiology and integrated control of potato late blight in Europe. *Potato Res* 2011;54:183–222.
- Coquoz J-L, Buchala A, Meuwly P, Métraux J-P. Arachidonic acid induces local but not systemic synthesis of salicylic acid and confers systemic resistance in potato plants to *Phytophthora infestans* and *Alternaria solani*. *Phytopathology* 1995;85:1219–24.
- Daayf F, Ongena M, Boulanger RN, El Hadrami I, Bélanger RR. Induction of phenolic compounds in two cultivars of cucumber by treatment of healthy and powdery mildew-infected plants with extracts of *Reynoutria sachalinensis*. *J Chem Ecol* 2000;26:1579–93.
- Daniel R, Guest D. Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. *Physiol Mol Plant Pathol* 2006;67:194–201.
- Deliopoulos T, Kettlewell PS, Hare MC. Fungal disease suppression by inorganic salts: a review. *Crop Prot* 2010;29:1059–75.
- Dong H, Delaney TP, Bauer DW, Beer SV. Harping induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J* 1999;20:207–15.
- Eshraghi L, Anderson J, Aryamanesh N, Shearer B, McComb J, Hardy GESJ, O'Brien PA. Phosphite primed defence responses and enhanced expression of defence genes in *Arabidopsis thaliana* infected with *Phytophthora cinnamomi*. *Plant Pathol* 2011;60:1086–95.
- Goellner K, Conrath U. Priming: it's all the world to induced disease resistance. *Eur J Plant Pathol* 2008;121:233–42.
- Grant BR, Dunstan RH, Griffith JM, Niere JO, Smillie RH. The mechanism of phosphonic (phosphorous) acid action in *Phytophthora*. *Australas Plant Pathol* 1990;19:115–21.
- Guest D, Grant B. The complex action of phosphonates as antifungal agents. *Biol Rev* 1991;66:159–87.
- Halim V, Eschen-Lippold L, Altmann S, Birschwilks M, Scheel D, Rosahl S. Salicylic acid is important for basal defense of *Solanum tuberosum* against *Phytophthora infestans*. *Mol Plant Microbe Interact* 2007;20:1346–52.
- Hamiduzzaman MM, Jakab G, Barnavon L, Neuhaus J-M, Mauch-Mani B. β -Aminobutyric acid-induced resistance against Downy Mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. *Mol Plant Microbe Interact* 2005;18:819–29.
- Hooker WJ. Compendio de Enfermedades de la Papa. St. Paul, MN, USA: The American Phytopathological Society; 1980. p. 56–60.
- Hückelhoven R, Kogel KH. Tissue-specific superoxide generation at interaction sites in resistant and susceptible near-isogenic barley lines attacked by the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*). *Mol Plant Microbe Interact* 1998;11:292–300.
- Jabs T, Tschöpe M, Colling C, Hahlbrock K, Scheel D. Elicitor-stimulated ion fluxes and O₂⁻ from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci USA* 1997;94:4800–5.
- King M, Reeve W, Van der Hoek MB, Williams N, McComb J, O'Brien PA, Hardy GE. Defining the phosphite-regulated transcriptome of the plant pathogen *Phytophthora cinnamomi*. *Mol Genet Genomics* 2010;284:425–35.
- Koornneef A, Pieterse CMJ. Cross talk in defense signaling. *Plant Physiol* 2008;146:839–44.
- Kravchuk Z, Vicedo B, Flors V, Camañes G, González-Bosch C, García-Agustín P. Priming for JA-dependent defenses using hexanoic acid is an effective mechanism to protect *Arabidopsis* against *B. cinerea*. *J Plant Physiol* 2011;168:359–66.
- Lamb C, Dixon RA. The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol* 1997;48:251–75.
- Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, et al. Ethylene modulates the role of nonexpressor of pathogenesis-related genes1 in cross talk between salicylate and jasmonate signaling. *Plant Physiol* 2009;149:1797–809.
- Lobato MC, Olivieri FP, González Altamiranda EA, Wolski EA, Daleo GR, Caldiz DO, Andreu AB. Phosphite compounds reduce disease severity in potato seed tubers and foliage. *Eur J Plant Pathol* 2008;122:349–58.
- Lobato MC, Machinandiarena MF, Tambascio C, Dosio GAA, Caldiz DO, Daleo GR, et al. Effect of foliar applications of phosphite on post-harvest potato tubers. *Eur J Plant Pathol* 2011;130:155–63.
- Pilbeam RA, Howard K, Shearer BL, Hardy GESJ. Phosphite stimulated histological responses of *Eucalyptus marginata* to infection by *Phytophthora cinnamomi*. *Trees Struct Funct* 2011;25:1121–31.
- Restrepo S, Myers KL, del Pozo O, Martin GB, Hart AL, Buell CR, et al. Gene profiling of a compatible interaction between *Phytophthora infestans* and *Solanum tuberosum* suggests a role for carbonic anhydrase. *Mol Plant Microbe Interact* 2005;18:913–22.
- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, vol. I, 2nd ed. Cold Spring Harbor Laboratory Press; 1989.
- Shibuya N, Minami E. Oligosaccharide signalling for defence responses in plant. *Physiol Mol Plant Pathol* 2001;59:223–33.
- Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ, et al. NPR1 modulates cross talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 2003;15:760–70.
- Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 2009;137:860–72.
- Subramaniam R, Desveaux D, Spickler C, Michnick S, Brisson N. Direct visualization of protein interactions in plant cells. *Nat Biotechnol* 2001;19:769–72.
- Taheri P, Tarighi S. Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway. *J Plant Physiol* 2010;167:201–8.
- Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J* 1997;11:1187–94.
- Tian ZD, Liu J, Wang BL, Xie CH. Screening and expression analysis of *Phytophthora infestans* induced genes in potato leaves with horizontal resistance. *Plant Cell Rep* 2006;25:1094–103.
- Tonelli ML, Furlan A, Taurian T, Castro S, Fabra A. Peanut priming induced by bio-control agents. *Physiol Mol Plant Pathol* 2011;75:100–5.
- Van der Ent S, Van Hulten M, Pozo MJ, Czechowski T, Udvardi MK, Pieterse CMJ, Ton J. Priming of plant innate immunity by rhizobacteria and β -aminobutyric acid: differences and similarities in regulation. *New Phytol* 2009;183:419–31.
- Vleeshouwers V, Van Doijeweert W, Govers F, Kamoun S, Colon LT. Does basal PR gene expression in *Solanum* species contribute to non-specific resistance to *Phytophthora infestans*? *Physiol Mol Plant Pathol* 2000;57:35–42.
- Vlot AC, Dempsey DMA, Klessig DF. Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol* 2009;47:177–206.
- Wang B, Liu J, Tian Z, Song B, Xie C. Monitoring the expression patterns of potato genes associated with quantitative resistance to late blight during *Phytophthora infestans* infection using cDNA microarrays. *Plant Sci* 2005;169:1155–67.
- Wang D, Amornsiripanitch N, Dong X. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog* 2006;2:1042–50.
- Wilkinson CJ, Shearer BL, Jackson TJ, Hardy GESJ. Variation in sensitivity of western Australian isolates of *Phytophthora cinnamomi* to phosphite in vitro. *Plant Pathol* 2001;50:83–9.
- Yu D, Chen C, Chen Z. Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. *Plant Cell* 2001;13:1527–40.
- Zar JH. Biostatistical analysis. 4th ed. Prentice-Hall: Upper Saddle River; 1999.
- Zimmerli L, Jakab G, Métraux JP, Mauch-Mani B. Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β -aminobutyric acid. *Proc Natl Acad Sci USA* 2000;97:12920–5.