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Research article

Potassium phosphite increases tolerance to UV-B in potato

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ABSTRACT

The use of biocompatible chemical compounds that enhance plant disease resistance through Induced Resistance (IR) is an innovative strategy to improve the yield and quality of crops. Phosphites (Phi), inorganic salts of phosphorous acid, are environment friendly, and have been described to induce disease control. Phi, similar to other plant inductors, are thought to be effective against different types of biotic and abiotic stress, and it is assumed that the underlying signaling pathways probably overlap and interact. The signaling pathways triggered by UV-B radiation, for instance, are known to crosstalk with other signaling routes that respond that biotic stress. In the present work, the effect of potassium phosphite (KPhi) pre-treatment on UV-B stress tolerance was evaluated in potato leaves. Plants were treated with KPhi and, after 3 days, exposed to 2 h/day of UV-B (1.5 Watt m^{-2}) for 0, 3 and 6 days. KPhi pre-treatment had a beneficial effect on two photosynthetic parameters, specifically chlorophyll content and expression of the *psbA* gene. Oxidative stress caused by UV-B was also prevented by KPhi. A decrease in the accumulation of hydrogen peroxide (H_2O_2) in leaves and an increase in guaiacol peroxidase (POD) and superoxide dismutase (SOD) activities were also observed. In addition, the expression levels of a gene involved in flavonoid synthesis increased in UV-B-stressed plants only when pre-treated with KPhi. Finally, accumulation of glucanases and chitinases was induced by UV-B stress and markedly potentiated by KPhi pre-treatment. Altogether, this is the first report that shows a contribution of KPhi in UV-B stress tolerance in potato plants.

1. Introduction

An innovative strategy within integrated crop management (ICM) is the use of biocompatible chemical compounds that enhance disease control in plants through Induced Resistance (IR) (Gozzo, 2003; Walters et al., 2013). These compounds are proposed to protect plants against disease by activating their inherent defense mechanisms during exposure to specific biotic or abiotic elicitors. Among them, phosphites (Phi), inorganic salts of phosphorous acid, have received particular attention because they are environment friendly and appear to be capable of controlling crop diseases caused by oomycetes and fungi (Deliopoulos et al., 2010). They can act through both a direct effect on the pathogen (Lobato et al., 2010) and an indirect effect on the stimulation of host defense responses. The latter involves the stimulation of plant defense mechanisms, such as enhanced production of phytoalexins and ROS (reactive oxygen species), induction of PRPs (pathogenesis related proteins) and reinforcement of the cell wall (Guest and Grant, 1991; Lobato et al., 2011; Eshraghi et al., 2011; Pilbeam et al., 2011). For potato plants, it has been shown that Phi application to seed tubers and foliage reduced disease symptoms in seed tubers and protected foliage against several pathogens (Lobato et al., 2008). In addition, foliar application of KPhi to field-grown crops resulted in post-harvest tubers with a reduced susceptibility to *Phytophthora infestans*, *Fusarium solani* and *Erwinia carotovora* infection, suggesting that this compound induced a systemic defense response (Lobato et al., 2011). Our group has recently described that KPhi primes the plant for an earlier and more intense response to infection and that salicylic acid (SA) likely mediates this response (Machinandiarena et al., 2012). Priming is the phenomenon that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells. Thus, primed plants show a faster and/or stronger activation of defense responses when subsequently challenged by microbes, insects, or abiotic stress (Conrath, 2011). It has been described that several plant

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inductors could generate IR both to biotic and abiotic stress, as well as prime the plant for further injuries (Vallad and Goodman, 2004). It has become increasingly evident that the underlying stress signaling pathways overlap and interact substantially (Holley et al., 2003). Among them, plant signaling to UV-B (280–315 nm) radiation appears to crosstalk with other types of stress (Schenke et al., 2011).

Depletion of the stratospheric ozone layer and other anthropogenic-related factors are leading to an increase in solar UV-B radiation reaching the Earth at regional or even global scales (Ballaré et al., 2011). UV-B radiation is harmful to living organisms. Numerous studies have demonstrated its detrimental effects on plant growth, development and physiology, particularly related to reduced photosynthesis, biomass reduction, decreased protein synthesis, and DNA damage (Piri et al., 2011; Kataria et al., 2014). UV-B radiation also produces oxidative stress through an increase in ROS production (Hideg et al., 2013). An important mechanism involved in UV-B radiation tolerance is based upon increased activity of the enzymatic antioxidant defense system, including up-regulation of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) (Redha et al., 2013; Majer et al., 2014). The accumulation of flavonoid pigments and lignin deposition on cell walls are also important mechanisms of protection from UV-B (Agati et al., 2013). In addition, the expression of a number of PRP genes, as well as the defencin gene *DF1.2*, have been shown to increase in response to UV-B exposure (Kalbina and Strid, 2006; Kaspar et al., 2010). A rise in the accumulation of ROS leads to increased levels of SA, ethylene and jasmonic acid, which are important secondary messengers that regulate gene expression related to plant resistance/tolerance to both, pathogen infection and UV-B irradiation (Mackerness et al., 2000; Demkura et al., 2010; Bandurska and Cielak, 2013).

In this work, we hypothesized that KPhi protects potato plants against UVB stress by enhancing the general molecular defense responses induced by this stress.

2. Materials and methods

2.1. Plant material and chemical treatment

For foliage assays, *Solanum tuberosum* seed tubers (cv. Shepody) 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. Potassium phosphite: KPhi (Afital Potassium Phosphite, Agro-EMCODI SA) was applied to the foliage at 5 mL per plant (3 L/ha) using an atomizer, 21 days after emergence. The dose utilized was 1% (v/v) of the commercial product. Control plants were sprayed with distilled water.

2.2. UV-B treatment

Three days after KPhi or water treatment, plants were divided in two sets, one batch served as control, the other received white light supplemented with UV-B Philips tubes (TL100W/12; Philips, Amsterdam, the Netherlands) filtered with 0.13 nm thick cellulose diacetate. Plants were exposed to 1.5 Watt m-2 of UV-B (280–315 nm) radiation, 2 h per day, approximately 1.5 Wm-2 of erythemally active radiation. The spectral irradiance was determined with an Ultraviolet Meter Model 3D (Solar Light Co, Glenside, PA, USA). After 0, 3, and 6 day of exposure, leaves were collected and stored at −80 °C for biochemical and molecular studies. To minimize plant-to-plant variation, 10 plants per treatment were sampled and pooled.

2.3. Chlorophyll content

Leaf chlorophyll content was determined in 8 plants per treatment at different times after UV-B stress (3 and 35 days). The average values of ten measurements per leaf were estimated with a non-destructive chlorophyll meter (SPAD 502, Minolta), (Udding et al., 2007). Data were analyzed by means of one-way ANOVA. Means were compared using the Tukey's test at a significance level of 0.05.

2.4. Preparation of soluble leaf extracts

Potato leaves (1 g) were homogenized with a mortar with 1.5 volumes of buffer containing 50 mM sodium phosphate (pH 7.2) and 0.5 M NaCl. Homogenates were filtered through cheesecloth and centrifuged at 10.000 rpm for 10 min at 4 °C. The resulting supernatant was stored at −20 °C. Protein concentration was measured by the Bradford method (1976) using BSA as a standard.

2.5. Detection of superoxide dismutase (SOD) and guaiacol peroxidase (POD) activities by native PAGE

Soluble extracts were precipitated with 5 volumes of cold acetone and resuspended in 50 mM phosphate buffer (pH 7.4). Electrophoresis was carried out under semi-denaturing conditions; samples were SDS-treated and not boiled. Equal amounts of fresh weight (5 mg for SOD and 2 mg for POD) were loaded in 10% SDS-PAGE. SOD isoform (EC 1.15.1.1) activity was visualized as the photochemical reduction of nitrotetrazolium blue (NBT), according to Chen et al. (2001), with the following modifications: gels were first incubated with a solution containing 2.45 mM of nitrobluetetrazolium (NBT) in 0.1 M potassium phosphate buffer (pH 7.5) for 20 min, followed by an incubation with the same solution plus 28 μM of riboflavin and 28 mM N, N, N′, N′-tetramethylethylenediamine (TEMED) for 1 h in dark. Subsequently, the gels were placed in distilled water and exposed to intense light for 1 h. Activity was visualized as white bands on a purple background. For POD (EC 1.11.1.7) activity, isoforms were visualized according to Chen et al. (2000).

2.6. In situ detection of H_2O_2

In situ accumulation of hydrogen peroxide (H_2O_2) was determined macroscopically by the diaminobenzidine staining method (DAB, Sigma), 3 days after UV-B exposure (Thordal-Christensen et al., 1997). Leaf disks from treated plants were stirred in 1 mg/mL DAB (pH 3.8) 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. A minimum of 6 disks were examined for each treatment. The experiment was repeated three times. Quantification of H_2O_2 was performed with Image J software. Data were analyzed by means of one-way ANOVA. Means were compared by Tukey's test at a significance level of 0.05.

2.7. RNA extraction and cDNA synthesis

Total RNAs from each treatment were 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 in 1% agarose gels. Approximately, 1 μg of total RNAs

were further treated with the RNAse-free DNAse I (Invitrogen) and used for first-strand cDNA synthesis using M-MLV Reverse transcriptase (Promega), both according to the manufacturers' instructions.

2.8. qPCR conditions

Real-time PCR was performed using a Real-Time PCR system (Step One model, Applied BioSystems, USA) with Power SYBR Green PCR master mix according to the manufacturer's recommendations (Applied BioSystems, USA). Gene-specific primers were designed using Primer 3 software (Untergrasser et al., 2012; Koressaar et al., 2007) and are listed in Table 1. PCR efficiency for each target gene was checked via the slope of a standard curve constructed from amplification of a dilution series of cDNA. Efficiency was 98% for *psbA*, 89.9% for F3′*H* and 96% for *EF-1*. Quantification of the relative gene transcription was determined by amplifying both genes in duplicates for each treatment, along with a no-template control. Relative transcript levels were determined by normalizing the PCR threshold cycle number of each gene with that of the EF-1 reference gene. The ΔΔCT method was used to determine relative gene induction levels (Livak and Schmittgen, 2001). Data are presented as the average of four independent experiments and analyzed by means of one-way ANOVA. Means were compared by the Tukey's test at a significance level of 0.05.

2.9. Immunoblot analysis

For immunoblotting, protein extracts (4 mg of fresh weight) were separated using SDS-PAGE (12%) under denaturing conditions and transferred onto nitrocellulose, using a semi-dry electrophoretic transfer cell. Immunodetection was performed as described by Turner (1986) using polyclonal antibodies against β-1, 3-glucanase (EC 3.2.1.6) and chitinase (EC 3.2.1.14) (Kombrink et al., 1988). Bound antibodies were visualized with a secondary antibody, goat anti-rabbit conjugated with alkaline phosphatase. PRP accumulation was quantified with Scion Image software (Scion Corporation). Values are the means (±SD) from three independent determinations with the Scion Image software.

3. Results

3.1. Photosynthetic parameters affected by UV-B

The measurement of chlorophyll content is a useful indicator of UV-B tolerance or sensitivity. The effect of UV-B light on leaf green color was followed by measuring chlorophyll content after UV-B treatment (6 days). A decrease in this parameter after exposure to UV-B was observed in KPhi non-treated plants, and this was prevented by KPhi pre-treatment, both 3 and 35 days after UV-B treatment (Fig. 1A).

It is well documented that UV-B exposure has an influence on gene expression, and leads to the down-regulation of gene expression and synthesis of key photosynthetic proteins, such as the chloroplast-encoded D1 polypeptide of photosystem II (*psbA* gene) (reviewed in Frohnmeyer and Staiger, 2003). With that purpose, we tested the protective effect of KPhi on the photosynthetic machinery through the analysis of the expression of the *psbA* gene by qPCR. UV-B treatment resulted in a significant decrease of *psbA* gene relative transcriptional abundance. This reduction was prevented by KPhi pre-treatment, as it was evidenced 3 days after UV-B treatment. Interestingly, *psbA* gene expression also decreased in leaves treated with KPhi but not exposed to UV-B (Fig 1B).

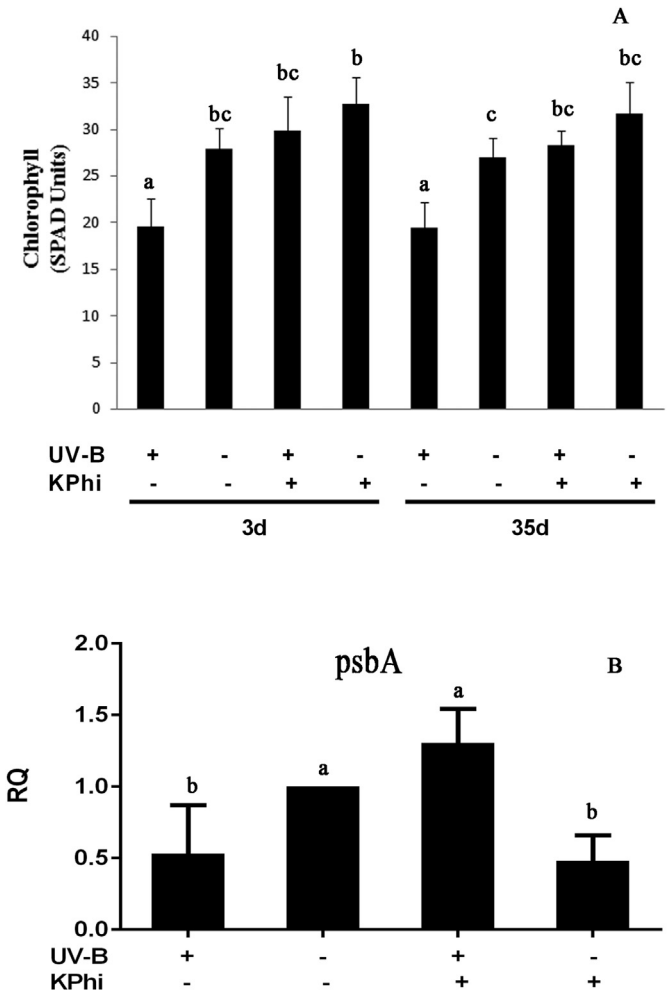

Fig. 1. Effect of KPhi and UV-B radiation on two photosynthetic parameters. A: Chlorophyll content was expressed as SPAD units. SPAD measurements were made in foliage 3 and 35 days after the end of 6-day UV-B treatment period. Pre-treatment with KPhi was applied 3 days before UV-B exposure. B: *psbA* expression. Expression of this gene was quantified in leaves by qRT-PCR, 3 and 6 days after UV-B irradiation on KPhi or water pre-treated plants, as it is described in M&M.

3.2. H_2O_2 accumulation in leaves

UV-B radiation has been reported to lead to the generation of ROS. Therefore, we analyzed the accumulation of H_2O_2 in leaves from KPhi- and water-pre-treated plants exposed to 3 days of UV-B.

Table 1
Primers used for RNA quantification for Real Time-PCR analysis.

Stgene	GenBank accesion number	Reverse primer	Forward primer
StpsbA	XP_006341898	5′-TGTGCTCAGCCTGGAATACA-3′	5′-TTACATGGGTCGTGAGTGGG-3′
StF3′H	HQ_659496	5′-GCTCTGGACACTTTGGGTAAA-3′	5′-CAGAGGCAATGGGCTTAGAG-3′
StEF-1	NM_001288491	5′-ATTGGAAACGGATATGCTCCA-3′	5′-TCCTTACCTGAACGCCTGTCA-3′

As it is shown in Fig. 2, UV-B radiation increased H_2O_2 accumulation in leaves and this was counteracted by the treatment with KPhi before UV-B exposure. KPhi treatment by itself did not significantly modify H_2O_2 accumulation (Fig. 2).

3.3. Antioxidant enzymes: POD and SOD activities

UV-B radiation causes an increase in ROS production and, as a consequence, an up-regulation of antioxidant enzymes that constitute the defense system for alleviating ROS-mediated damage. POD and SOD activities were analyzed in native electrophoresis 3 and 6 days after UV-B treatment on KPhi and water pre-treated plants. Fig. 3 shows that both POD and SOD isoforms changed in response to UV-B, KPhi or both stimuli together. However, different POD and SOD isoforms increased their activities after KPhi or UV-B stimuli (Fig 3 A and B).

As it is shown in Fig 3A, POD isoforms were differentially induced in all treatments. Noteworthy, a lower isoform (L) was specifically induced upon UV-B treatment, and a higher isoform (H), upon KPhi pre-treatment. On KPhi pre-treated plants, the activity of both isoforms increased dramatically 6 days after UV-B irradiation.

Six days after exposure to UV-B radiation, the same two SOD isoforms were identified in all treatments. The higher isoform (H) slightly increased in plants subjected to UV-B radiation, while KPhi treatment enhanced the activity of the smaller isoform (L) (Fig 3B). Exposure to both UV-B stress and KPhi pre-treatment increased the accumulation of both isoforms (Fig. 3B).

3.4. Accumulation of flavone 3′ hydroxylase transcripts

Flavanone 3′ hydroxylase (F3′H) is a key enzyme in the biosynthesis of flavonoids, and it is known to play a key role in plant stress responses. In this work, we have quantified the accumulation of F3′*H* transcripts 6 days after UV-B treatment in KPhi pre-treated plants. Our results show that the expression of this gene increased when both treatments were applied, as compared to their controls (Fig. 4).

3.5. Pathogenesis related proteins (PRPs)

Exposure to UV-B radiation caused an increase on β-1,3-glucanase content. This increment was higher at 6 days than at 3 days of exposure. Pre-treatment with KPhi and further exposure to UV-B radiation caused a greater increase in the enzyme content compared to the non KPhi pre-treated plants. This was more evident 6 days following UV-B exposure (Fig. 5A).

Similar results were observed for chitinases content. In this case, the increase of chitinase expression after 6 days of UV-B exposure in KPhi pre-treated plants was twice compared to UV-B plants without KPhi pre-treatment (Fig 5B). For both enzymes, pre-treatment with KPhi without other stress, did not affect the content of these proteins (Fig 5 A and B).

4. Discussion

The use of Phi to manage foliar potato Late Blight has been well documented (Kromann et al., 2012). Our group has shown the

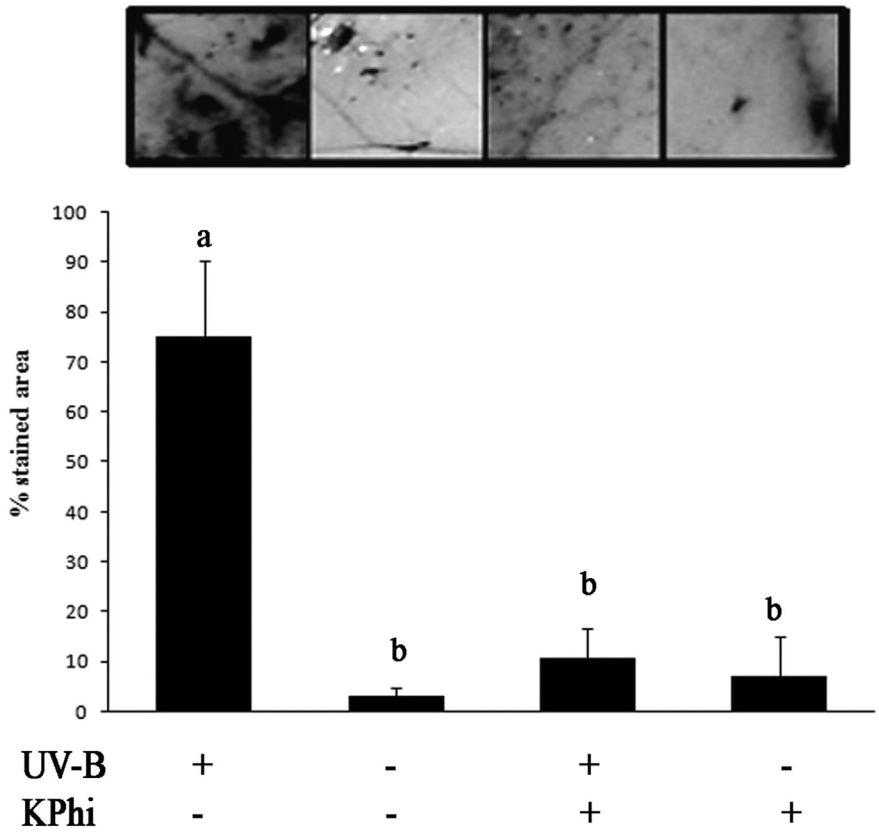

Fig. 2. Effect of KPhi on Hydrogen peroxide (H_2O_2) accumulation in potato leaves upon UV-B treatment. H_2O_2 detection was done using the 3,3- diaminobenzidine (DAB) staining method and visualized macroscopically in leaves from plants exposed to UV-B for 3 days, either pre-treated or not with KPhi. The development of dark color is a measure of H_2O_2 accumulation. Photographs represent 1 cm^2 of leaf area. Quantification of H_2O_2 is shown in a bar graph below each photograph. Each bar represents the mean ± SD. Bars with the same letter do not differ significantly at $P < 0.05$.

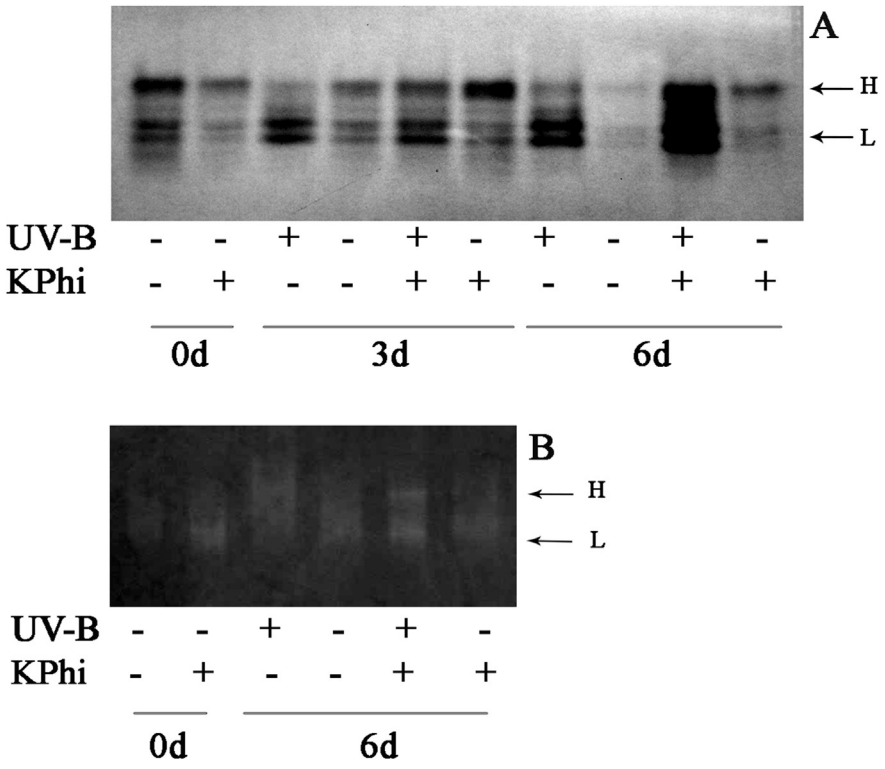

Fig. 3. Effect of KPhi on (A) guaiacol peroxidases (POD) and (B) superoxide dismutases (SOD) after UV-B exposure. Enzymatic activities were measured in leaf extracts after 10% SDS-PAGE, using guaiacol/H_2O_2 or NBT for POD and SOD, respectively. Leaf extracts were prepared from plants exposed to UV-B during 0, 3 or 6 days, either pre-treated or not with KPhi as it is described in M&M. Equal amounts of fresh weight (5 mg for SOD and 2 mg for POD) were loaded in each lane.

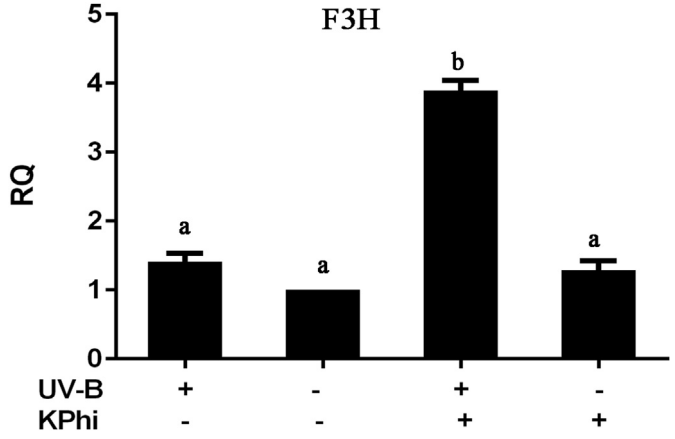

Fig. 4. Effect of KPhi pre-treatment on F3′H gene expression upon UV-B exposure. Expression of this gene was measured in leaves by qRT-PCR 6 days after UV-B treatment, either pre-treated with water or KPhi. Data shown represents the quantification of three independent experiments. Each bar represents the mean ± SD. Bars with the same letter do not differ significantly at $P < 0.05$.

efficacy of Phi to control other potato pathogens (Lobato et al., 2011). Studies on different pathosystems have suggested that Phi can trigger defense responses in plants through a mechanism called "priming" (Massoud et al., 2012; Eshraghi et al., 2011, 2014) In potato, we have studied several components of the priming mechanism involved in the response to biotic stress (Lobato et al., 2008, 2011; Olivieri et al., 2012; Machinandiarena et al., 2012). In the present work, we started with the hypothesis that the responses induced by KPhi in potato plants might also be effective against an abiotic stress such as UV-B radiation.

To test this hypothesis, we have initially evaluated the effect of Phi on a few photosynthetic parameters. The reduction of chlorophyll content in potato leaves caused by UV-B radiation was arrested by pre-treatment with KPhi. The effect of UV-B treatment on leaf green color was still evident 40 days after the first exposure to this stress, and the protective effect of KPhi remained effective until that moment (Fig 1A). This result suggests that KPhi has a protective effect against UV-B radiation. In addition, the analysis of a marker of photosystem integrity, expression of the *psbA* gene, further supports this idea. KPhi pre-treatment prevented UV-B damage on the photosynthetic machinery, since *psbA* expression showed the same levels of expression as its control, after both treatments. A down-regulation of this gene was observed in plants treated with KPhi that were not irradiated with UV-B (Fig 1B). Similar results were described by Lim et al. (2013), where down-regulation of a set of photosynthesis-related proteins was shown to occur on KPhi-pre-treated potato plants, as well as recovery to their initial expression levels after being challenged to a second stimulus.

Oxidative stress is a common feature triggered by UV-B radiation. Plant resistance to UV-B stress was associated with antioxidant capacity, where increased levels of the antioxidant components may prevent stress damage. The increase in H_2O_2 concentration is a typical plant response under UV-B radiation stress (Hideg et al., 2013). In this work, the accumulation of H_2O_2 was measured in irradiated leaves. Our results showed that H_2O_2 levels were significantly reduced in KPhi, compared to non KPhi-pre-treated plants after UV-B stress (Fig 2), supporting the idea that KPhi has a protective role during UV-B radiation stress. Part of the antioxidant mechanism that might participate in this process are the enzymes POD and SOD. Both types of enzymes are induced during UV-B stress (Redha et al., 2013; Majer et al., 2014).

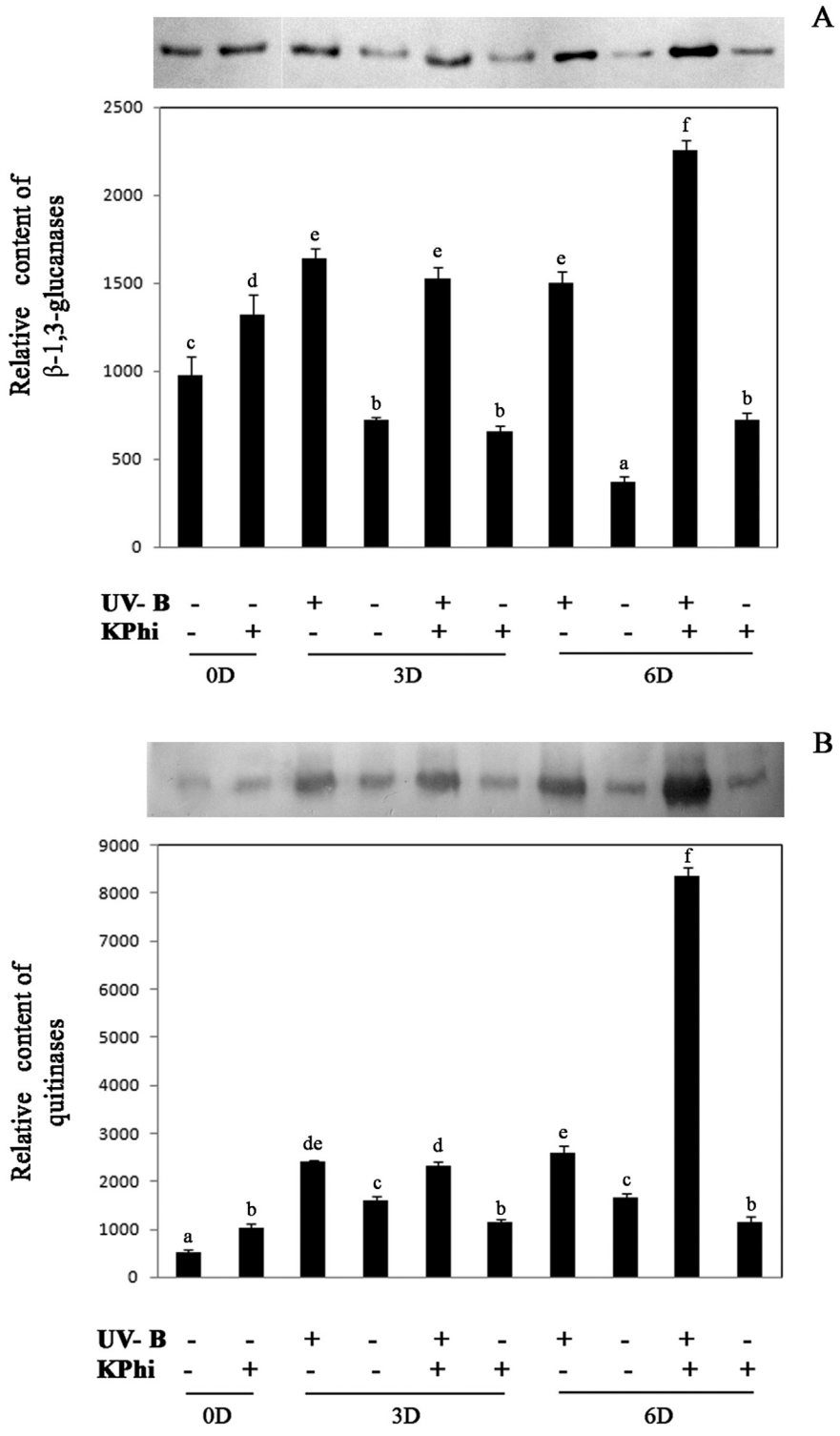

Fig. 5. Effect of KPhi on β-1,3-glucanase (A) and chitinases (B) content after UV-B treatment. For inmunoblots, protein extracts (equal amounts of fresh weight) were fractionated in a SDS-PAGE gel (12%) and transferred onto nitrocellulose. Immunodetection was performed using polyclonal antibodies raised against both PRPs. Chitinases and β-1, 3-glucanases accumulation was quantified with Scion Image software. Values are the means (±SD) from three separate determinations.

Peroxidases have a dual role in the response to UV-B (e.g. they can act as antioxidant enzymes but can also generate ROS as signaling molecules for defense reactions). Their participation in UV-B responses has been reported previously (Mackerness et al., 2001). In the mentioned report, it was shown that peroxidase generates O_2^- which is involved in the up-regulation of *PDF1.2*, a defensin gene. Here we have shown that different POD isoforms are involved in the response to UV-B or to KPhi, while all isoforms appear to increase in leaves treated with both KPhi and UV-B (Fig 3A). This suggests that pre-treatment with Phi potentiates the antioxidant response. A similar response was observed for SOD activity (Fig 3B), since all isoforms increased in UV-B irradiated plants pre-treated

with KPhi. F3′H transcript accumulation was also augmented by KPhi pre-treatment (Fig 4), which might contribute to reduce the damage produced by UV-B stress, together with the induction of antioxidant enzymes such as POD and SOD.

We have described that quitinases and glucanases are induced in response to Phi application after biotic stress (Lobato et al., 2008, 2011). Moreover, PRPs were also described to be induced by UV-B radiation (Kalbina and Strid, 2006; Kaspar et al., 2010). In this work, similar to antioxidant enzymes, the accumulation of both PRPs was increased in leaves pretreated with KPhi and then exposed to UV-B. This induction was higher than for each stimulus applied separately (Fig. 5A and B). These results suggest that KPhi induced PRPs as part of a general mechanism induced upon stress.

Surplus et al. (1998) have shown that the increase in ROS after UV-B treatment result in SA accumulation, which then leads to the up-regulation of PRP transcripts via a ROS- and SA-dependent pathway, as also demonstrated for the response to pathogen infection. UV-B activates an additional pathway that is ROS-dependent but SA-independent, which is known to mediate the down-regulation of photosynthetic genes. Reports by other investigators presented evidence that showed that the exogenous application of SA reduced the damaging effect of UV-B radiation in plants via an up-regulation of the activity of antioxidant enzymes, the accumulation of anthocyanins and the expression of PRPs (Mahdavian et al., 2008; Hayat et al., 2010). In addition, Machinandiarena et al. (2012) and Massoud et al. (2012) suggested that SA might participate in the signaling pathways induced by KPhi in response to biotic stress as part of a priming response dependent on SA. These results led us to suggest that SA might participate in the signaling pathways involved in Phi-induced tolerance to UV-B stress. Further studies will have to be carried out to test this hypothesis.

Studies focused on a comparable system involving treatment with another biocompatible chemical compound, Selenium (Se), showed that this compound has positive effects on photooxidative stress tolerance in potato. These data suggested that Se has synergistic effects on the transcription of antioxidative enzymes such as CuZn-SOD and GPX in plants (Seppänen et al., 2003). Recently, Yao et al. (2013) have shown that Se supply increased the yield and improved the quality of winter wheat exposed to enhanced UV-B. The authors considered that Se supply is an ameliorant or stress alleviant, however the mechanism underlying this phenomenon is not yet known.

In general, stress responses to UV-B appear to be mediated by signaling pathways that are not specific to UV-B, and many of the genes induced can be activated by other sources of stress. In contrast, UV-B-specific signaling is of major importance in mediating photomorphogenic acclimation responses. The results presented in this work allow us to propose that KPhi induces in plants a general signaling pathway (e.g. not specific to UV-B) whose end responses help mitigate this type of stress. This is the first report that suggests the potential agricultural use of KPhi to ameliorate UV-B harmful effects in potato plants. Further studies will be necessary to evaluate the existence of additional KPhi-specific pathways induced during exposure to this abiotic stress.

Contributions

Natalia Oyarburo and Mariana Feldman performed the experiments. Milagros Machinandiarena and Florencia Olivieri designed and instructed the research work. Florencia Olivieri wrote the manuscript. All of the authors revised, discussed and commented on the manuscript. Adriana Andreu and Gustavo Daleo provided funding for this work.

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