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The reinforcement of potato cell wall as part of the phosphite-induced tolerance to UV-B radiation

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Abstract

Phosphites (Phis), inorganic salts of phosphorous acid, have shown to be effective in protection of plants against biotic stress. Recently, we have described that potassium phosphite (KPhi) induces tolerance to UV-B radiation in potato. To counteract the harmful effect of UV radiation, plants accumulate UV-screening compounds, such as flavonoids, sinapate ester, and lignin. In previous work, we have shown an increase in guaiacol peroxidase (POD) activity in plants pretreated with KPhi and further exposed to UV-B radiation. In order to continue with this study, the expression of different enzymes and components involved in cell wall reinforcement were analyzed. An isoform of POD induced by KPhi was analyzed by isoelectric focusing and further identified as suberization-associated anionic peroxidase (POPA) by mass spectrometry. In addition, other enzymes participating in lignin biosynthesis, like caffeoyl-CoA *O*-methyltransferase (CCoAOMT), determined by accumulation of transcripts, and laccase activity, visualized in zymogrames, were increased by KPhi treatment prior to UV-B exposure. Further, the accumulations of extensin (EXT) transcripts and of conjugated polyamines (PAs) were increased by KPhi treatment prior to UV-B exposure. All these results suggest cell wall reinforcement in leaves due to KPhi pretreatment followed by UV-B exposure.

Additional key words: abiotic stress, extensin, flavonoids, lignin, peroxidase, polyamines, *Solanum tuberosum.*

Introduction

Phosphites (Phis), inorganic salts of phosphorous acid, have shown to be effective in protection of plants against biotic stress and could be used in integrated crop management programs as an alternative to fungicides. They can stimulate plant defense mechanisms such as the enhanced production of phytoalexins and reactive oxygen species (ROS), induction of pathogenesis related (PR) proteins, and reinforcement of the cell wall (Lobato *et al*. 2008, 2011, Eshraghi *et al*. 2011, Pilbeam *et al*. 2011). In potato, we also showed that KPhi causes an earlier and more intense response to infection and that salicylic acid might mediate this response (Machinandiarena *et al*. 2012). Finally, we have described recently that Phi induces tolerance to UV-B radiation in potato (Oyarburo *et al*. 2015).

In plants, UV-B radiation can interfere with plant morphology, biochemistry, physiology, and gene expression (Brosché and Strit 2003, Ulm and Nagy 2005, Li *et al*. 2016). Tolerance to UV-B depends on balance between damaging effects and both repair and acclimation responses. Within the latter, UV-B stimulates accumulation of UV-screening compounds such as flavonoids, sinapate ester, and lignin, which can act as free radical scavengers to counteract the damaging effects of UV-B radiation (Rozema *et al*. 1997, Li *et al*. 2016).

Several reports indicate that the response to UV-B stress significantly overlaps with plant response to some other ROS-involving events, such as pathogen attack, wounding, or drought (Bandurska *et al*. 2013, Araújo *et al*. 2016).

In our previous work, we have shown that an increase in guaiacol peroxidases (POD) and a reduction in H_2O_2 content in leaves occur among the responses induced by KPhi in UV-B exposed potato plants (Oyarburo *et al*. 2015). Class III plant peroxidases are extracellular enzymes present in all land plants and are generally

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Abbreviations: CCoAOMT - caffeoyl-CoA *O*-methyltransferase; EXT - extensin; HPLC-MSMS - high-pressure liquid chromatography-mass spectrometry; IEF - isoelectric focusing; KPhi - potassium phosphite; PAs - polyamines; Phi - phosphite; POD - guaiacol peroxidase; PR - pathogen related; Put - putrescine; ROS - reactive oxygen species; Spd - spermidine; Spm - spermine; TLC - thin layer chromatography.

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secreted into the cell wall or the surrounding medium and into the vacuole. Plant PODs are members of a large multigenic family and this fact suggests the possibility of a functional specialization of each isoform (Cosio and Dunand 2009). For example, PODs could be involved in suberin and lignin formation, auxin metabolism, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS and reactive nitrogen species (Almagro *et al*. 2009). They have been also associated with responses to several stresses and in particular, Jansen *et al*. (2001) reported that an increase in the activity of phenol-oxidizing peroxidases is correlated with an increase in UV tolerance. In addition, PODs also mediate process of extensin (EXT) insolubilization in the cell wall as a rapid response to biotic and abiotic stresses (Merkouropoulos *et al*. 2003, Price *et al*. 2003, Passardi *et al*. 2004, Sujkowska-Rybkowska and Boruki 2014).

Lignin has a dual role, since it reinforces cell wall and also acts as a scavenger of ROS. Increase in lignin content in plants exposed to UV-B has been often reported (Hilal *et al*. 2004, Yamasaki *et al*. 2007, Cabane *et al*. 2012, Choudhary and Agrawal 2016). Among enzymes participating of lignin biosynthesis are POD, caffeoyl-CoA-*O*-methyltransferase (CCoAOMT) and laccases. In particular, CCoAOMT was reported to be induced in grapevine after UV-B irradiation (Pontin *et al*. 2010). Laccases can oxidize phenolic substrates in the presence of oxygen instead of peroxide. The role of these enzymes has not been completely understood; however, it has been suggested to be linked to lignification and a metabolism of other phenolic compounds in the cell wall (Ranocha *et al*. 2002). *In silico* studies of laccase genes of *Arabidopsis* suggest that two genes *LAC1* and *LAC16* are up-regulated by small RNAs after UV-B exposure (Turlapati *et al*. 2011).

Polyamines (PAs) have antioxidant properties and their induction in response to stress is well known. PAs can also be bound to phenolic acids, proteins, nucleic acids, and membrane structures (Martin-Tanguy *et al*. 2001). Several studies suggested that the high amount of bound PAs could help to protect the cells by reinforcing the cell wall and producing a more effective UV screen (Gupta *et al*. 2013). In addition, it was found that PA biosynthesis was significantly up-regulated in response to UV-B, and a proportional increases in soluble conjugated PAs and bound-insoluble PAs were observed (Bassard *et al*. 2010, Todorova *et al*. 2014).

Based on our previous result that showed an increased tolerance to UV-B in potato plants achieved by the application of KPhi, we hypothesize that KPhi produces changes in the expression of gene implicated in cell wall reinforcement, as well as in increases in enzymatic activities and cell wall components related to UV-B tolerance.

Materials and methods

Plants and treatments: The tubers of *Solanum tuberosum* (cv. Shepody) were planted in pots containing a pasteurized mixture of soil:vermiculite (3:1, v/v). Pots were maintained in a greenhouse at a temperature of 18 °C, a 16-h photoperiod, and an irradiance of 160 μmol m^{-2} s^{-1}). A 1 % (v/v) potassium phosphite (KPhi; *Agro-EMCODI SA*, Argentina) was applied to leaves 21 d after emergence, according to Oyarburo *et al*. (2015). Control plants were sprayed with water. For each measured parameter ten plants per treatment were sampled and pooled. All experiments were performed at least three times.

Three days after KPhi or water treatment, plants were divided in two sets, one batch of plants served as control and the other received supplemental 1.5 W m^{-2} of UV-B (280 - 315 nm) radiation, 2 h a day, according to Oyarburo *et al*. (2015) during day 0, 3, and 6. At each time of treatment, leaves were collected immediately after UV-B exposure and stored at -80 °C for later analyses.

Preparation of leaf extracts: Potato leaves (1 g) were homogenized with a mortar and pestle in 1.5 volumes of 50 mM sodium phosphate (pH 7.2), 1 mM EDTA-Na_2, and 1 % (m/v) polyvinyl pyrrolidone (PVP). Homogenates were filtered through cheesecloth and centrifuged at 12 000 *g* and 4 °C for 15 min. The supernatant was stored at -20 °C. Protein content was measured by the Bradford assay (1976) using bovine serum albumin as a standard protein.

Isoelectric focusing (IEF): Soluble leaf extracts were precipitated with 5 volumes of cold acetone and resuspended in deionized water. Polyacrylamide gels were cast on acetate sheet supports 125 × 65 mm (*BioRad*, Hercules, USA) and run using a *Bio-Rad model 111 Mini* IEF cell. The gels containing 5 % ampholytes (pH 4 - 6.5, *Pharmalyte*) were cast according to *BioRad* procedure. The samples (equivalent to 4 mg of fresh mass) containing 20 % of ampholytes, were loaded in the middle of the plate. Bands were cut from IEF for nano high-pressure liquid chromatography - mass spectrometry (HPLC-MSMS) identification, and identical gels were stained for POD activity according to Oyarburo *et al*. (2015).

IEF protein band that exhibited differential expression upon KPhi treatment was excised from gel for its characterization. Protein digestion and mass spectrometry analysis were performed at the proteomics core facility *CEQUIBIEM* at the University of Buenos Aires, Argentina. The proteolytic digestion was performed with trypsin. The digests were analyzed by nano LC-MS/MS in a *Thermo Scientific QExactive* mass spectrometer. Samples were loaded at a constant flow of 33 mm^3 min^{-1} onto an *EASY- Spray* column (C18, 2 mm, 75 μm × 150 mm) (*Thermo Scientific*, USA). Peptides were eluted

with a linear gradient from 5 - 50 % of solvent acetonitrile over 75 min with a constant flow of 0.3 mm^3 min^{-1}. Data dependent *MS2* method, was used to fragment the top 12 peaks in each cycle. Data analysis was performed using *Proteme Discoverer 1.4*.

RNA extraction, cDNA synthesis, and quantitative PCR: Total RNAs from each treatment were isolated using *Tri-reagent* (*Molecular Research Center*, 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 % (m/v) agarose gels. Approximately 1 μg of total RNA was further treated with the RNAse-free DNAse I (*Invitrogen*, USA) and used for first-strand cDNA synthesis using *M-MLV* reverse transcriptase (*Promega*, USA), both according to the manufacturers' instructions.

Real-time qPCR was performed using *StepOne*™ real-time PCR system (*Life Technologies*, USA) with *Power SYBR Green* PCR master mix according to the manufacturer's recommendations. Sequences of *EF-1α*, *CCoAOMT*, and *EXT* genes were obtained from the *GenBank* database (*NCBI*, http://www.ncbi.nlm.nih.gov/). Gene-specific primers were designed using *Primer 3* software (Koressaar *et al*. 2007, Untergrasser *et al*. 2012) and are listed in Table 1. The ΔΔCT method was used to determine relative expression of genes (Livak and Schmittgen 2001) and *EF-1* was used as reference gene (Nicot *et al*. 2005). A final relative expression was estimated from three biological replications, each with three technical replicates.

Laccase activity: Electrophoresis was carried out under semi-denaturing conditions. Leaf extracts were precipitated with 5 volumes of cold acetone and resuspended in 20 mM sodium acetate (pH 5.0). Then, samples were treated with sodium dodecylsulphate. The equivalents to 10 mg (f.m.) were loaded on each lane of 12 % SDS-PAGE. After running, gels were stained with 15 cm^3 of 0.1 mM syringaldazine in ethanol plus 50 cm^3 of 20 mM sodium acetate (pH 5.0) for 10 min, according to Flores *et al*. (2009).

Polyamines (PAs) analysis: The extraction, dansylation, and thin layer chromatography (TLC) analysis were carried out according to Flores and Galston (1982) with some modifications. Fresh leaves were homogenized with 5 % (m/v) cold perchloric acid ($HClO_4$) (250 mg (f.m.) per 1 cm^3 of $HClO_4$), the supernatant phase containing the total free polyamine fraction (free PAs) was stored at -20 °C. For extraction of conjugated PAs, aliquots of the $HClO_4$ supernatant were hydrolyzed with an equal volume of 6 M HCl at 100 °C during 18 h, according to Mapelli *et al*. (2008). The hydrolysates were dried and resuspended in the original volume of $HClO_4$ to obtain soluble conjugated PAs. Aliquots of free and soluble conjugated PAs were dansylated as described by Flores and Galston (1982). Twenty nmol of each standard were dansylated in the same way, and TLC was performed either on TLC silica gel aluminium sheets (*Merck*, USA) or aluminum sheets (*Alumina*, *Fluka*, USA). Approximately 0.05 cm^3 of dansylated extracts (equivalent to 30 mg of f.m.) were loaded, and chromatograms were developed with chloroform: triethylamine 25:2 (v/v) as mobile phase. Identification of the spots was done by comparison of the ratio of front (Rfs) in the two stationary phases.

Statistical analysis: All experiments were repeated at least three times with similar results. The null hypothesis of no differences between treatments was evaluated independently for each variable by *one-way ANOVA*. A posteriori multiple comparison tests (Tukey test) were performed when significant differences between factors were detected.

Table 1. Primers used for real-time qPCR analysis.

Genes	Accesion numbers	Reverse primers 5′ - 3′	Foward primers 5′ - 3′	Efficiency [%]
CCoAOMT	NM_001318579.1	CATCACCAACAGGAAGCTGGCAAA	ATCGACTTGGTGAAGGTTGGTGGA	102
EXT	Z21937	ATGGTGTTGGAGAAGGATGG	CAAATCACCACCAGTGAAGC	97
EF-1α	NM_ 001288491	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	96

Results

We previously reported that potato plants treated with KPhi and then exposed or not to UV-B radiation exhibit differential induction of POD activities (Oyarburo *et al*. 2015). To continue the characterization of the POD isoforms involved in response to UV-B radiation upon KPhi treatment, IEF of leaf extracts were performed in a 4 - 6.5 pH gradient (Fig. 1*A*). Two differentially expressed bands were found. One isoform with pI 4.8 was induced mainly by UV-B radiation and the other isoform with pI 4.5 was induced mainly by KPhi. The activity of both isoforms increased in plants pretreated with KPhi and then exposed to UV-B radiation. The band corresponding to the isoform mainly induced by KPhi treatment (pI 4.5) was excised from the IEF and analyzed

by nano HPLC-MSMS. One of the peptides from tryptic digestion showed the sequence LGGQTYNVALGR and its *Mascot* analysis gave a high homology to a suberization-associated anionic peroxidase named POPA (*Swiss-prot* accession number P12437 with a *Mascot* score 3.00 and identity 100 %). This result was confirmed with a *BLASTP* of the peptide identified against *Solanum tuberosum* database in *NCBI* (identity 100 %, score 3 E-06) (Fig 1*B*) and also in *PeroxiBase* database (data not shown; Fawal *et al*. 2013). In addition, it showed high similarity with other potato peroxidases like suberization-associated anionic peroxidase 2 (XP_006347167.1; identity 92 %, score 9 E-05) and peroxidase 51 (XP_006338701.1; identity 73 %, score 0.29).

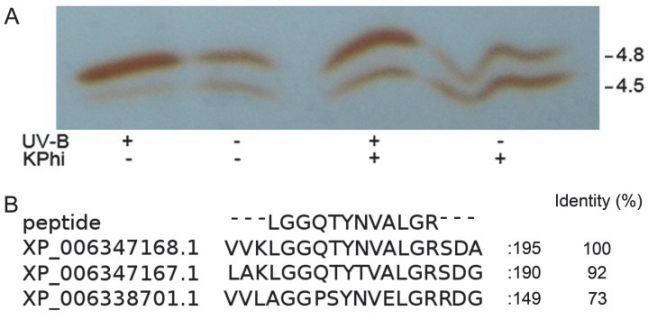

Fig. 1. Characterization of guaiacol peroxidases (POD) in leaves either pre-treated (KPhi +) or not (KPhi -) and after UV-B exposure or not for 3 d. Enzymatic activities were detected in leaf extracts after IEF (pH 4 - 6.5) using guaiacol/H_2O_2. Equal amounts of extract corresponding to fresh mass of 4 mg were loaded in each lane (*A*). The POD isoform induced by KPhi treatment (pI 4.5) was characterized by nano HPLC-MSMS and one peptide was obtained with homology to peroxidase. This peptide was aligned with *Solanum tuberosum* suberization-associated anionic peroxidase (;POPA XP_006347168.1); suberization-associated anionic peroxidase-2 (XP_006347167.1), and peroxidase 51 (XP_006338701.1) using *MAFFT* online server (*B*).

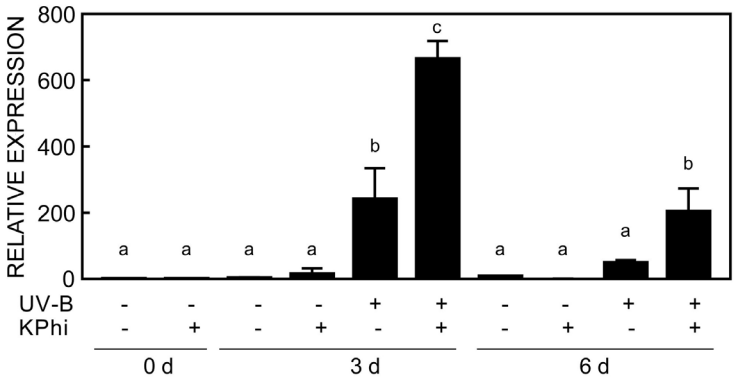

Fig. 2. Effect of KPhi pre-treatment and UV-B exposure on CCoAOMT gene expression. The expression of CCoAOMT gene was quantified by RT-qPCR 0, 3, and 6 d after UV-B treatment (UV-B+) in leaves either pre-treated with water (KPhi-) or with KPhi (KPhi+). Means ± SDs, $n = 3$. *Bars* with different letters differ significantly at $P < 0.05$.

In this study we also investigated the KPhi-induced differential expression of some wall strengthening genes such as *CCoAOMT*, involved in the biosynthesis of lignin, and *EXT* encoding essential proteins in the cell wall structure, after UV-B treatment. A differential expression of *CCoAOMT* gene was observed among treatments. In plants that were only exposed to UV-B during 3 d, the increase in *CCoAOMT* transcripts reached 200-fold; however, when plants were pretreated with KPhi and exposed to UV-B, a 700-fold increase was measured. Six days after UV-B exposure, the transcription was significantly higher only in KPhi pretreated plants. A similar response was observed for the *EXT* transcripts after 3 and 6 d of UV-B exposure (Fig. 3). On the other hand, both *CCoAOMT* and *EXT* did not show any change in expression in KPhi-treated and control plants not exposed to UV-B (Figs. 2 and 3).

It has been proposed that laccase activity could participate in lignin biosynthesis. After 6 d of UV-B exposure, different isoforms from leaf extracts semi-purified by acetone precipitation were detected by zymogram assay. A greater increase in their activity was observed in KPhi pretreated plants (Fig. 4). These results suggest that KPhi potentiates this UV response.

The amount of conjugated PAs in KPhi pretreated potato plants upon 6 d of UV-B exposure changed, at least content of putrecine (Put) and spermidine (Spd) (Fig. 5). The content of these conjugated PAs was higher in plants pretreated with KPhi and exposed to UV-B, than in plants exposed only to UV-B. No differences were observed in soluble free PAs between treatments at this time of treatments (data not shown).

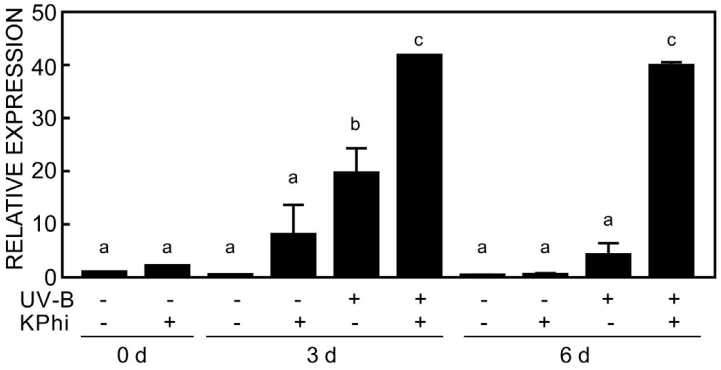

Fig. 3. Effect of KPhi pre-treatment and UV-B exposure on EXT gene expression. The expression of this gene was quantified by RT-qPCR 0, 3, and 6 d after UV-B treatments in leaves either pre-treated with water (KPhi-) or with KPhi (KPhi+). Means ± SD, $n = 3$. *Bars* with different letters differ significantly at $P < 0.05$.

Discussion

In a previous work, we demonstrated that KPhi pre-treatment alleviates UV-B effects in potato plants through an increase of the antioxidant enzymes and prevents UV-B damage to the photosynthetic machinery (Oyarburo *et al*. 2015). In the present work, we show that KPhi treatment induced changes in cell wall components and in genes or enzymes related to cell wall reinforcement.

We reported previously a differential induction of POD isoforms due to UV-B stress or KPhi treatment (Oyarburo *et al*. 2015). Now, we differentiated by IEF isoforms with pIs around 4.8 and 4.5 induced mainly by UV-B or by KPhi, respectively (Fig. 1*A*). The isoform induced by KPhi treatment was identified as an anionic class III peroxidase called POPA, involved in cell wall suberization of wounded potato tubers (Roberts *et al*. 1988). The identified peptide showed also similarity with other *Solanum lycopersicum* class III peroxidases TAP1 and TAP2. The expression of these peroxidases in transgenic tobacco plants was localized at wound site and around the *Fusarium solani* infection site. This correlates with the postulated role for anionic peroxidases in polymerization of phenols in tissues during suberization (Mohan *et al*. 1993).

Among their multiple functions, class III peroxidases can contribute to the building of a rigid cell wall by catalyzing the oxidation of various electron donors like lignin or other phenolic compounds, and by mediating the EXT insolubilization to cell wall polymers. POPA is described to be restricted to suberizing tissue in the immediate vicinity of the wounding site (Espelie and Kolattukudy 1985, Bernards *et al*. 1999). Mutants deficient in this enzyme are more susceptible to late blight caused by *Phytophthora infestans* and show a lower lignin content in areas of infection than wild type plants, but these mutants are not deficient in the phenol compounds necessary for lignin synthesis (Sorokan *et al*. 2014). The present work is the first report of a POD enzyme involved in KPhi induced tolerance to UV-B radiation in potato leaves. In the context of UV-B tolerance, we can suggest that KPhi treatment could induce an increase of POD activity in cell wall that might contributes to the reinforcement response.

The phenylpropanoid and lignin pathways seem to be necessary for acclimation to UV-B stress. Pontin *et al*. (2010) showed that the induction of these pathways regulates the synthesis of UV-B absorbing compounds, such as phenylpropanoids, in grapevine under high

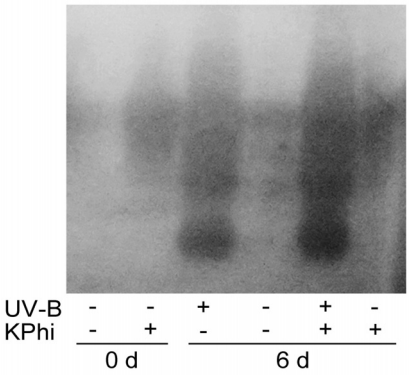

Fig. 4. Effect of KPhi pre-treatment and UV-B exposure on laccase activity. Extracts from leaves were prepared from plants exposed to UV-B (UV-B +) for 0 or 6 d and either pre-treated (KPhi +) or not (KPhi -) with KPhi. Extracts were fractionated on 12 % SDS-PAGE and activity bands were visualized using syrigaldazine as substrate. Examples of three independent experiments.

UV-B radiation. In this pathway, genes involved in lignin biosynthesis, such as *CCoAOMT* were also up-regulated under UV-B stress. Our results showed that the UV-B treatment induced the expression of *CCoAOMT* in potato leaves and that it was even higher after KPhi pretreatment (Fig. 2). Laccases could alternatively mediate the last steps in lignin biosynthesis. Different isoforms of these enzymes increased after UV-B radiation and even more in plants pretreated with KPhi (Fig. 4). The major and

earlier accumulation of lignin in KPhi pretreated plants could be shifting the ROS equilibrium between signaling and toxicity. Due to its scavenging properties, lignin might act as an antioxidant against ROS produced by UV-B stress (Blokhina *et al*. 2003, Karmanov *et al*. 2014). The role of laccases in plants remains still unclear and some efforts were made to elucidate this in *Arabidopsis*

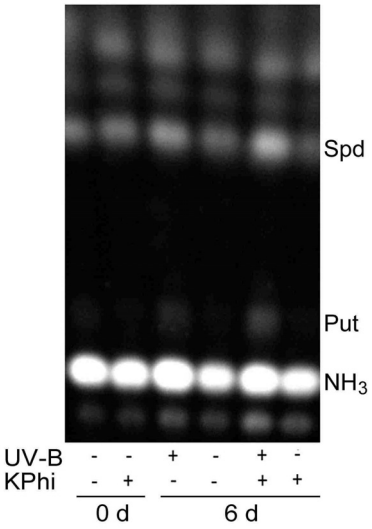

Fig. 5. Effect of KPhi pre-treatment and UV-B exposure on content of conjugated polyamines. Polyamines were separated by TLC in chloroform:triethylamine (25:2, v/v) using extracts from leaves of plants pretreated (KPhi +) or not (KPhi -) and after 0 or 6 d of UV-B exposure (UV-B +). Standards were indicated at right: Spd - spermidine, Put - putrescine, and NH_3.

(Turlapati *et al*. 2011). It has been also reported that several laccase genes are regulated by miRNAs under abiotic stress (Sunkar *et al*. 2010). This regulation cannot be discarded in our study and it could be suggested that the induction of laccase activity by KPhi may be part of a general response regulated by miRNAs.

Another role of POD, related to modifications in the cell wall, is its interaction with EXTs. In the present work, KPhi pretreatment increased the *EXT* expression under UV-B exposure (Fig. 3). Induction of both, POD and EXTs is evidence of their possible common role leading to the reinforcement of cell wall induced by KPhi. There is growing evidence that covalent bonds exist between pectins and EXTs (Passardi *et al*. 2004). Pectins would act as an anchor for peroxidases, which would then cross-link EXTs to generate a solid and dense network in the plant wall. More recently, it has been reported that PAs conjugated to hydroxycinnamic acids also constitute important bridging agents (Bassard *et al*. 2010). In addition, the PAs have both, antioxidant properties and they are involved in tolerance to abiotic stresses (Bouchereau *et al*. 1999). Also, they can generate H_2O_2, necessary for polymerization reactions in cell wall (Bernards *et al*. 1999). In the present study we have shown preliminary evidence that KPhi pretreatment potentiated the increase in the amount of conjugated Put and Spd upon UV-B exposure (Fig. 5).

All the results obtained in this work suggest that KPhi-induced tolerance to UV-B could be explained by a potentiation of the responses of cell wall to the UV-B damage which would involve its thickening and increase in antioxidant activities.

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