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Cell Wall Reinforcement in the Potato Tuber Periderm After Crop Treatment with Potassium Phosphite

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Abstract The potato periderm is the outer tissue of potato tubers that protects the tuber from pathogen attack, dehydration, and wounding during harvest and storage. It is of secondary origin and replaces the epidermis early during tuber development. The outer layers of the periderm consist of cells with suberized walls. Phosphite compounds (Phi) are inorganic salts from phosphorous acid, and in previous works, we demonstrated that they are effective in protecting potato plants from biotic stress caused by different pathogens and also against UV-B radiation. The aim of the present work was to study the effect of Phi on the post-harvest potato periderm structure, cell wall components, and related enzymes. In three years of field experiments, potassium phosphite (KPhi) applications were compared with an untreated control. KPhi was applied to seed tubers before planting combined with foliage application of a conventional fungicide, to seed tubers and foliage without conventional fungicide, or to seed tubers combined with foliage application of KPhi plus a conventional fungicide. Observations of periderm sections showed increases in suberin and pectin depositions in post-harvest tubers from KPhi-treated plants compared to tubers from non-treated plants. In addition, peroxidase activity increased in the periderm tissue. The activity of laccases and superoxide dismutase was measured in the protein extract of the periderm, and an increase due to KPhi treatment was detected. The results presented here suggest that early KPhi treatment to seed tubers followed by foliage treatment leads to reinforcement of the cell wall of periderm cells, which represents a benefit for future response to stresses.

Keywords Periderm · Potassium phosphite · Potato · Secondary cell wall · Suberization

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Introduction

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world, and therefore, conservation of tubers during and after harvest is of high economic importance. The periderm is a key factor in this issue, because it replaces the epidermis early during tuber development and protects the tubers from pathogen attack, dehydration, and wounding during storage. The potato periderm is composed by three cell types: phellem, phellogen, and phelloderm. The phellem constitutes the outer layers of the periderm and is derived from the unique layer of meristematic cells underneath it, the phellogen. As phellem cells develop, they become suberized and then die, forming a protective layer. The phelloderm, a parenchyma-like tissue, is derived from the inward cell divisions of the phellogen and constitutes the inner layers of the periderm.

The suberin macromolecule is composed of aromatic and aliphatic polyester domains and is synthetized between the plasmalemma and the primary wall (Kolattukudy 1984). The knowledge on the suberin deposition process is incomplete, but there is important evidence supporting the peroxidase-mediated oxidative coupling process in the biosynthesis of the aromatic polyester domain (Bernards et al. 2004). Extracellular peroxidases may modulate the availability of H_2O_2 in the cell wall; H_2O_2 is required for the cross-linking of phenolic groups (Passardi et al. 2004). It has been reported that specific peroxidases (class III) are necessary for terminal processes like lignification and suberization, as well as other processes related to plant defence mechanisms, like extensin and ferulic acid cross-linking (Almagro et al. 2009). Involved in H_2O_2 metabolism are also superoxide dismutases (SODs), antioxidant enzymes that convert O_2^- into H_2O_2 and O_2 (Alscher et al. 2002; Møller 2001). Another polyphenol oxidative enzyme group is constituted by laccases, which are barely studied in plants. The role of these enzymes is not completely understood, but has been suggested to be linked to lignification and the metabolism of other phenolic compounds in the cell wall (Ranocha et al. 2002). Liang et al. (2006) reported that laccases participate in lignin biosynthesis and also suggested their involvement in suberin synthesis. In addition, using the wound healing of potato tubers as a suberization model, laccase, lignin peroxidase, and superoxide dismutase genes were found to be highly represented in this process (Lee 2004).

Phosphites (Phi, inorganic salts from phosphorous acid) have been reported to be able to control crop diseases caused by bacteria and oomycetes through both a direct effect on the pathogen and an indirect effect by inducing host resistance (Bécot et al. 2000; Daniel and Guest 2006; Jackson et al. 2000, 2004; Lobato et al. 2008, 2011; Machinandiarena et al. 2012; Oka et al. 2007; Reuveni et al. 2003). Treatments with Phi could be incorporated in integrated crop management programmes as a strategy to reduce the use of fungicides during the crop cycle and thus minimize toxic effects on humans and environment (Liljeroth et al. 2016). In previous work, it has been demonstrated that Phi might promote plant defence responses through a "priming" mechanism, increasing the level of potato plant resistance against a future pathogen attack (Lobato et al. 2008; Machinandiarena et al. 2012). Among the defence responses, a rapid accumulation of H_2O_2 and O_2^- was measured in leaves from potassium phosphite (KPhi)-treated plants after *Phytophthora infestans* inoculation (Machinandiarena et al. 2012). Taking into account that foliar applications of KPhi increased resistance of postharvest tubers against potato pathogens (Lobato et al. 2011), changes at the periderm

level were studied. Initially, an increase in pectin accumulation and the concentration or activity of pathogenesis-related (PR) proteins was detected in periderm and cortex of *Fusarium solani*-infected tubers coming from KPhi-treated plants, compared with infected tubers coming from non-treated plants (Olivieri et al. 2012).

The aim of the present work was to expand knowledge about phosphite effects on the periderm of post-harvest potato tubers by analysing cell wall components and their related metabolism that may contribute to the resistance acquired by the tuber upon crop treatment.

Materials and Methods

Plant Material and Treatments

Three field experiments were performed at Balcarce (37° 45′ S) during the growing seasons from mid-October to late February of 2008/2009, 2009/2010, and 2010/2011, with cv. Shepody. Crops were grown at the McCain Argentina Experimental Field and irrigation was achieved by a forward advance system. The experiments had a randomized design with four treatments and three replications. Experimental plots were four-row plots (0.85 m between rows), 6 m long, with a plant density of 5.8 plants m^{-2}.

The chemicals and doses used in treatments were (per spray) a 1% (v/v) dilution (3 l ha^{-1}) of potassium phosphite (KPhi, 1.07 kg ha^{-1} of the active ingredient, Agro-EMCODI, Buenos Aires, Argentina) and conventional fungicide (F, 1.5 l ha^{-1} Vitavax 300 (20% Carboxin +20% Captan, Bayer Cropscience) + 2.5 kg ha^{-1} Acrobat MZ® (9% Dimethomorph +60% Mancozeb, BASF Company)). Approximately 5 ml (of either KPhi or conventional fungicide at the doses indicated above) was applied per seed tuber or per plant. The experimental treatments, denoted by letters in square brackets, were as follows: [C] seed tubers and foliage were sprayed with water; [KPhi-F] seed tubers were sprayed with KPhi and foliage was sprayed with conventional fungicide; [KPhi-KPhi+F] seed tubers and foliage were sprayed with KPhi and foliage was also sprayed with conventional fungicide; and [KPhi-KPhi] seed tubers and foliage were sprayed with KPhi. Seed tuber treatments were carried out before planting. KPhi foliar treatment was applied six times every 2 weeks starting at tuber initiation (approximately 25 days after emergence). For the [KPhi-KPhi+F] treatment, conventional fungicide was applied every 2 weeks in between each KPhi foliar treatment. Applications were done with a backpack sprayer SHURflo ProPack™ model SRS600CE. Potato tubers were harvested at approximately 120 days after emergence (when the aerial part of the plants had died) from the two central rows of each replication and stored at 8 °C.

Histochemistry of Periderm Tissue and Microscopy Visualization

Tissue blocks (1 × 0.5 × 0.5 cm) including the periderm were cut from the middle part of the tubers (10 cm length approximately), fixed for 2 h in FAA (50% ethanol, 5% acetic acid, 10% formalin), and then hand-sectioned with a razor blade and stained or directly visualized under a Nikon Eclipse E200 epifluorescence microscope, according to Sabba and Lulai (2005). For suberin

visualization, non-stained periderm sections were examined by autofluorescence. Suberized cells appeared as blue fluorescent cells under the epifluorescence microscope (Ex 365/10, Dichroic 400 LP, Emis 460/50). Relative quantification of phellem thickness was performed with ImageJ software. For pectin visualization, periderm sections were stained with 0.02% ruthenium red. Samples were examined using standard light microscopy. Periderm sections were stained for peroxidase (POD) activity in situ by incubating the sections in 0.05% hydrogen peroxide and 0.2% guaiacol. Samples stained for POD activity were examined using standard light microscopy.

In all cases, a minimum of six sections of at least three different tubers were examined for each treatment and season.

Extraction of Periderm Components

The periderm tissue was separated from the tuber flesh with a scalpel and homogenized with extraction buffer as described by Olivieri et al. (2012). Pieces of periderm were weighed (approximately 1 g fresh weight) and homogenized in a mortar with 1.5 volumes of buffer containing 50 mM sodium acetate at pH 5.2, 0.1% β-mercaptoethanol, and 1.5 M NaCl. The homogenate was stirred for 16 h at 4 °C and then centrifuged for 10 min at 10,000 rpm. The soluble periderm extract was collected and stored at − 20 °C to be used for enzymatic assays.

A minimum of three different tubers from each treatment and season were used for the extraction.

Enzymatic Assays

POD activity (EC 1.11.1.7): Soluble periderm extracts were precipitated with five 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. Two milligrams of fresh weight was loaded in each lane in 10% SDS-PAGE. POD activity isoforms were visualized using guaiacol and H_2O_2 as substrates according to Chen et al. (2000).

Laccase activity (EC 1.10.3.2): Electrophoresis was carried out under semi-denaturing conditions as described for POD activity. Ten milligrams of fresh weight was loaded in each lane in 12% SDS-PAGE. Gels were stained with 15 ml of syringaldazine (0.1 mM in ethanol) plus 50 ml 20 mM acetate buffer (pH 5.0) during 10 min, according to Flores et al. (2009).

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was quantified as the photochemical reduction of nitroblue-tetrazolium (NBT), according to Becana et al. (1986) with some modifications. The reaction mixture consisted in 50 mM Hepes buffer (pH 7.6), containing 0.1 mM Na_2EDTA, 50 mM $NaHCO_3$ (pH 10.4), 13 mM methionine, 0.025% Triton X100, 75 μM NBT, and 2 μM riboflavin, in a final volume of 1 ml. The reaction was initiated by turning the light on (OSRAM VIALX NAVE 400 lamp Phillips) and the reduction of NBT was followed by reading the A_{560} after 7 min. Blanks and controls were run the same way but without illumination and enzyme, respectively. SOD activity was calculated as $\Delta_{Abs560nm}$ min^{-1} mg fresh weight^{-1}.

Data Analysis

Phellem thickness and laccase and SOD activity data were analysed by one-way Kruskal–Wallis test. Phellem layer data was analysed by one-way ANOVA (Zar 1999). A posteriori multiple comparison tests (Tukey tests) were performed when a significant treatment effect was detected ($P < 0.05$).

Results shown in this work correspond to averages of the three independent experiments and images are also representative of them.

Results

Thickening of Periderm

In order to know the effect of KPhi treatments on the reinforcement of the potato periderm, first the skin structure was studied. The number of phellem layers in the periderm of tubers coming from all KPhi treatments was increased compared with the control treatment. The autofluorescence of suberized cells showed that both the number of layers and the thickness of this outer tissue were increased (Fig. 1). In addition, pectin, an important component of the cell wall, was histochemically analysed in the whole periderm. This material was visualized by ruthenium red staining in the inner layers of periderm and a more intense colour was observed in the phelloderm cells of the periderm coming from KPhi-treated plants than in the periderm of tubers coming from untreated plants (Fig. 2).

Enzymatic Activities Associated with Cell Wall Reinforcement and/or Antioxidant Response in Periderm

Enzymatic activities responsible of the cellular changes related to the reinforcement of the cell wall were measured in tuber periderm from KPhi-treated and KPhi-untreated plants. In situ POD activity was higher in tubers coming from all KPhi-treated plants than in control tubers (Fig. 3a). To study POD isoforms, the activity was also analysed in soluble periderm extracts by electrophoresis. Several isoforms were detected and all of them increased in extracts of tubers coming from all KPhi treatments (Fig. 3b), especially in tubers coming from the combined treatment KPhi-KPhi+F (Fig. 3b, line 3).

Laccases, as well as PODs, are involved in polymerization of phenolic substrates present in cell wall matrix. Periderm extracts were also evaluated for laccase activity by electrophoresis using syringaldazine as substrate. This activity was detected in all samples, but it was higher in those tubers coming from KPhi-treated plants than in tubers from the control plants (Fig. 4).

In order to evaluate the peroxide metabolism and/or the oxidative status in periderm, the activity of superoxide dismutase (SOD) was also measured. Figure 5 shows an increase of total SOD activity in all extracts coming from KPhi-treated plants relative to control. This increase was close to 50%; no significant differences were found between KPhi treatments.
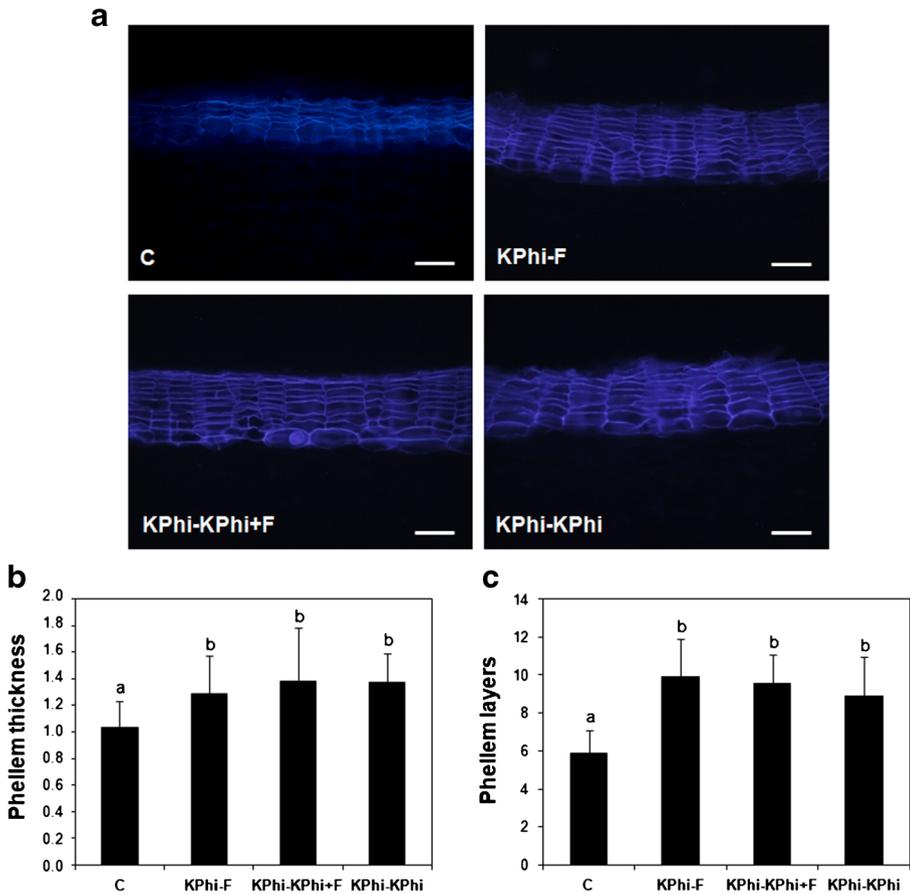
Fig. 1 Periderm thickening of potato tubers coming from plants treated with potassium phosphite (KPhi) alone or combined with conventional fungicide (F) and untreated plants. **a** Photographs of suberin autofluorescence. **b** Phellem thickness relative to the control. **c** Number of phellem layers of the periderm sections. *C* non-treated control; *KPhi-F* KPhi applied to seed tuber and fungicide to foliage; *KPhi-KPhi+F* KPhi applied to seed tuber and KPhi plus fungicide applied to foliage; *KPhi-KPhi* KPhi applied to seed tuber and foliage. Scale bars in **a** represent 50 μm. Each bar in **b** and **c** represents the mean ± SD. Columns with the same letters are not significantly different according to the Kruskal–Wallis (**b**) or Tukey (**c**) tests at a $\alpha = 0.05$

Discussion

In previous work, we have shown that KPhi applications to seed tubers and/or foliage induce molecular modifications in post-harvest potato tuber periderm and cortex that increase resistance to pathogens (Olivieri et al. 2012). In this work, we tested the hypothesis that KPhi alone or combined with fungicide induce biochemical changes leading to periderm reinforcement. The first and notable observation was the increase of the suberized area of the periderm in line with an increase in the number of cell layers of phellem (Fig. 1). These properties suggest that mature tubers coming from KPhi-treated plants would have an improved barrier against pathogens, dehydration, etc., increasing their durability during post-harvest storage. This phellem reinforcement, in addition to previously described changes under biotic stress (Lobato et al. 2011;
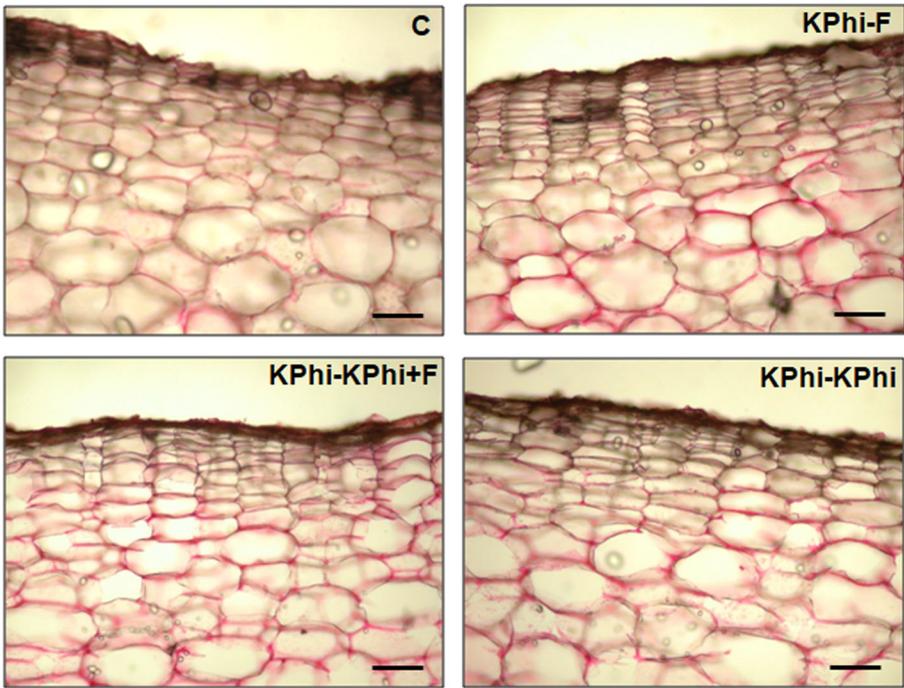
Fig. 2 Pectin detection by ruthenium red staining in periderm sections of potato tubers coming from plants treated with potassium phosphite (KPhi) alone or combined with conventional fungicide (F). *C* non-treated control; *KPhi-F* KPhi applied to seed tuber and fungicide to foliage; *KPhi-KPhi+F* KPhi applied to seed tuber and KPhi plus fungicide applied to foliage; *KPhi-KPhi* KPhi applied to seed tuber and foliage. Scale bars represent 50 μm
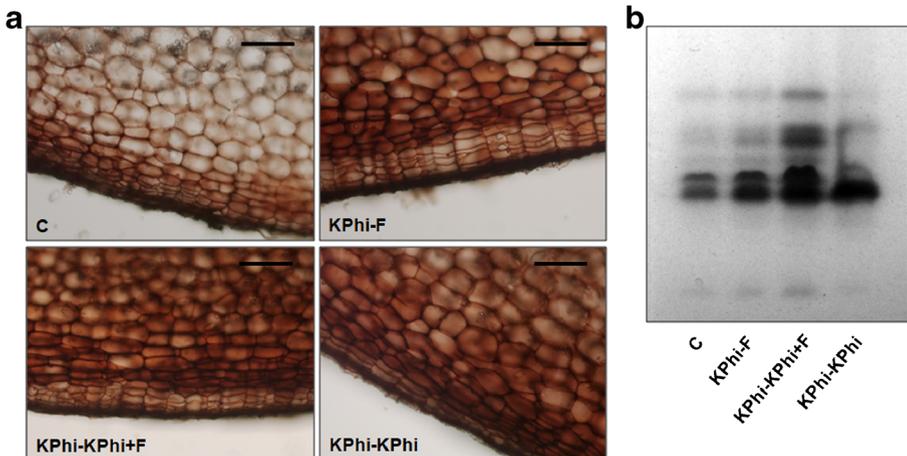

Fig. 3 Peroxidase activity in the periderm of potato tubers coming from plants treated with potassium phosphite (KPhi) alone or combined with conventional fungicide (F). *C* non-treated control; *KPhi-F* KPhi applied to seed tuber and fungicide to foliage; *KPhi-KPhi+F* KPhi applied to seed tuber and KPhi plus fungicide applied to foliage; *KPhi-KPhi* KPhi applied to seed tuber and foliage. **a** In situ POD activity (red-brown colour) in periderm sections of tubers coming from the different treatments. Scale bars represent 100 μm. **b** 10% SDS-PAGE of soluble periderm extracts. POD activity bands were visualized using guaiacol/H_2O_2 as substrates. Equal amounts of periderm fresh weight were loaded in each lane
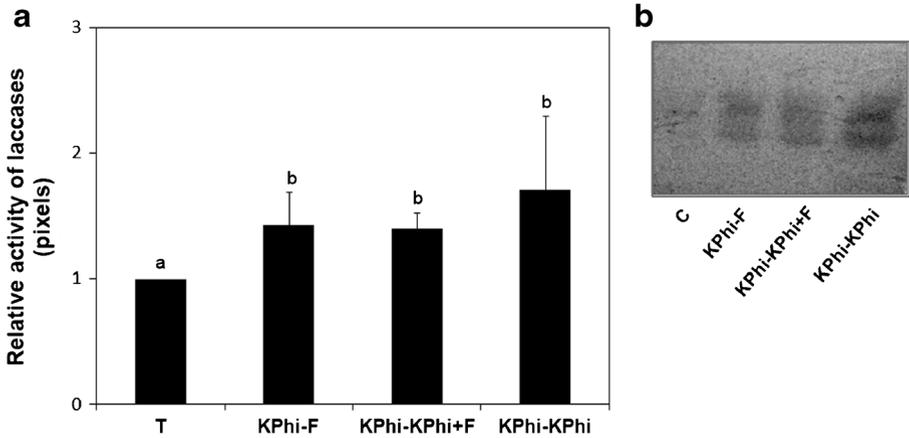
a

b

Fig. 4 Laccase activity in the periderm of tubers coming from plants treated with potassium phosphite (KPhi) alone or combined with conventional fungicide (F). *C* non-treated control; *KPhi-F* KPhi applied to seed tuber and fungicide to foliage; *KPhi-KPhi+F* KPhi applied to seed tuber and KPhi plus fungicide applied to foliage; *KPhi-KPhi* KPhi applied to seed tuber and foliage. **a** The relative laccase activity was estimated by densitometric scanning of the activity gel. Each bar represents the mean ± SD. Columns with the same letters are not significantly different according to the Kruskal–Wallis test at a $\alpha = 0.05$. **b** 12% SDS-PAGE of soluble periderm extracts. Laccase activity bands were visualized by staining using syrigaldazine as substrate. Equal amounts of periderm fresh weight were loaded in each lane
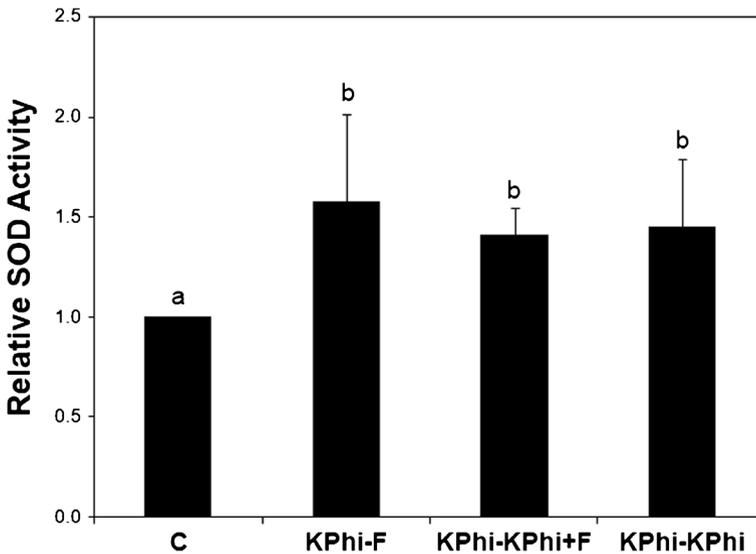

Fig. 5 Relative superoxide dismutase (SOD) activity in soluble periderm extracts of tubers coming from plants treated with potassium phosphite (KPhi) alone or combined with conventional fungicide (F). *C* non-treated control; *KPhi-F* KPhi applied to seed tuber and fungicide to foliage; *KPhi-KPhi+F* KPhi applied to seed tuber and KPhi plus fungicide applied to foliage; *KPhi-KPhi* KPhi applied to seed tuber and foliage. The activity was measured following the change in absorbance at 560 nm of the photochemical reduction of nitroblue-tetrazolium. SOD activity was calculated as $\Delta_{Abs560nm}$ min^{-1} mg fresh weight^{-1} and relativized to control tubers. Each bar represents the mean ± SD. Columns with the same letters are not significantly different according to the Kruskal–Wallis test at a $\alpha = 0.05$

Olivieri et al. 2012), led us to analyse other modifications in the periderm related to cell wall thickening. Pectin deposition and enzymatic activities related to polymerization of cell wall components were analysed. An increase in pectin accumulation was observed in the inner layers of periderm of post-harvest tubers coming from all KPhi-treated plants when compared to control tubers (Fig. 2).

The suberization process in potato is dependent on POD activity, among others (Bernards et al. 2004). POD activity was measured in situ in periderm sections and the extracted isoforms were analysed by electrophoresis. Both analyses showed an increase in POD activity in potatoes coming from all KPhi treatments (Fig. 3). This could be related to the synthesis of suberin and also with the peroxidation of phenolic compounds associated with defence responses and/or with the maintenance of hydrogen peroxide balance in the tissue. Many studies showed that Phi may act indirectly by inducing resistance in plants (Deliopoulos et al. 2010; Pilbeam et al. 2011; Eshraghi et al. 2011; Lim et al. 2013). Some of them reported that Phi induce resistance through a rapid accumulation of reactive oxygen species followed by increased transcription of defence genes (Eshraghi et al. 2011; Goellner and Conrath 2008; Lim et al. 2013; Machinandiarena et al. 2012). This response mechanism includes the activation of the antioxidant system with the participation of superoxide dismutase (SOD) and POD enzymes. The induction of these enzymes by Phi treatment was described in a variety of plants and upon different stresses (Fernandes et al. 2014; Oyarburo et al. 2015).

Other proteins have also been proposed to participate in the assembly of the aromatic monomers in suberin. Soler et al. (2007) showed that no class III peroxidases were found in their cork (*Quercus suber*) library, but three genes coding for laccases were postulated among the candidate genes for suberin biosynthesis. In the present work, the increase in laccase activity after KPhi treatment was an interesting result especially considering the small number of reports regarding these enzymes in plants. The physiological roles of these enzymes are still not well understood, and there are only a few reports that involve them in stress responses. Their direct participation in lignin synthesis was evidenced by polymerization of lignin precursors in vitro in the absence of H_2O_2 (Bao et al. 1993; Ranocha et al. 1999). A more recent evidence of their physiological role was presented by Liang et al. (2006) in maize primary roots exposed to high concentrations of NaCl. They suggested that the maize gene *ZmLAC1* could potentially be involved in the biosynthesis of lignin and of the suberin aromatic domain in the Casparian strip.

It is very interesting to note the anatomical and biochemical changes related to the cell wall reinforcement in harvested tubers produced by plants that were treated with potassium phosphite either at foliage or at seed tubers. We suggest that an early seed tuber treatment followed by foliage treatments before tuberization begins leads to tubers with a thicker periderm and to a faster and higher response of the tubers to future stresses with the consequent benefits for the agricultural production.

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