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A novel α-1, 3-glucan elicits plant defense responses in potato and induces protection against *Rhizoctonia solani* AG-3 and *Fusarium solani* f. sp. *eumartii*

Erika A. Wolski[a,*], Sara Maldonado[b], Gustavo R. Daleo[a], Adriana B. Andreu[a]

[a]*Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250 4to nivel, P.O. Box 1245, Mar del Plata, Argentina*
[b]*Laboratorio de Anatomía y Embriología Vegetal, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, P.O. Box. 1428, Buenos Aires, Argentina*

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Abstract

All glucan elicitors from fungal cell walls have been described as ß-glucans. In a previous work, we have isolated and characterized an α-1, 3-glucan from a non-pathogenic *Rhizoctonia* isolate which induced glucanase activity in potato sprouts. In this work we showed that, in addition to eliciting a wide array of defense reactions, the α-1, 3-glucan induces protection against Rhizoctonia canker and dry rot in plants and tubers, respectively. The α-1, 3-glucan strongly induced ß-1, 3-glucanase and chitinase activities. Immunological analysis showed that the level of the pathogenesis-related proteins increased. Histological studies showed an increase in cell wall deposition of callose and lignin, and ultrastructural analysis showed changes in the cytoplasm and the accumulation of electron-dense bodies in vacuoles. Protection assays showed that pre-treatments of potato sprouts and potato tubers with the α-1, 3-glucan results in a 40% of protection against Rhizoctonia canker and 60% protection against dry rot, respectively.
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Keywords: α-1, 3-glucan; Elicitor; Non-pathogenic *Rhizoctonia* isolate; Plant defense responses; Potato sprouts; Protection; *Rhizoctonia solani*

1. Introduction

In the course of evolution, any plant/pathogen interaction has developed a complex array of recognition, attack and defense reactions at the plant/microbe interface. Pathogens have developed offensive strategies and, in turn, plants have developed a wide range of sophisticated defense mechanisms to resist the colonization by microbial pathogens and parasites. Pathogen recognition by the host triggers a variety of early defense responses, such as modification of the ion influxes across the plasma membrane, phosphorylation and dephosphorylation of signaling proteins, and production of reactive oxygen species [1]. Later, these events are followed by the induction of a broad spectrum of defense reactions that confer local resistance against pathogens. These include: (i) cell wall reinforcement through lignin synthesis, callose deposition and cross-linking of macromolecular components, (ii) production of signaling secondary metabolites from the phenylpropanoid and octadecanoid pathways and (iii) accumulation of antimicrobial compounds such as phytoalexins, and synthesis of proteins with hydrolytic or inhibitory activity against microbes such as pathogenesis-related (PR) proteins [2]. These proteins have well-described antimicrobial activity against different pathogens [3,4]. ß-1, 3-glucanase (PR-2 group) hydrolyzes ß-glucans and chitinase (PR-3 group) hydrolyzes chitin, which are major components of fungal cell walls. Hydrolysis of these fungal cell-wall leads to the inhibition of the growth of several fungi [5] and they might also play an important role in the amplification of defense reactions through the release of ß-1, 3-glucans [6] and chitin oligosaccharides [7].

*Corresponding author. Tel.: +54 0223 4753030; fax: +54 0223 4751392.
E-mail address: ewolski@mdp.edu.ar (E.A. Wolski).

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Thaumatin-like proteins (PR-5 group) have showed antifungal activity, particularly against oomycetes [8].

Signal molecules from the pathogen or from the host that are able to trigger defense responses are known as elicitors. Many of these can be surface components released from the cell wall of the microbe or the host. Various types of elicitors have been characterized, including oligosaccharides, lipids, proteins and glycoproteins. ß-glucans, oligogalacturonides and chitin-derived oligomers are well-known elicitors [9–12].

Due to their activity as plant protectants, by triggering a large array of defense responses, elicitors have been considered as alternative tools for disease control in agronomic crops [11–15].

All glucans with elicitor activity characterized so far are formed by ß-linked glucose units [16–18]. However, we recently reported the isolation and characterization of an α-1,3-glucan from the cell wall of a non-pathogenic binucleate *Rhizoctonia* (BNR) isolate which induces ß-1,3-glucanase activity in potato sprouts [19]. BNR isolates have been characterized as effective biocontrol agents against several plant diseases caused by the pathogenic fungus *Rhizoctonia solani* Kühn [20–22], including in potato against Rhizoctonia canker, the disease caused by *R. solani* AG-3. Symptoms and signs of Rhizoctonia canker include canker on stems, sprouts and stolons, death of pre-emerging sprouts, malformation of tubers and dark brown-black sclerotia formed on the surface of mature tubers.

In this work, we characterized at biochemical level the response elicited by the α-1, 3-glucan in potato sprouts, the primary site of infection by *R. solani* AG-3, and we tested the ability of the α-1, 3-glucan to protect potato plants against *R. solani* AG-3 and *Fusarium solani* f. sp. *eumartii*. We report here the analysis of the accumulation of PR-proteins, the reinforcement of the cell wall by cytological studies, the ultrastructural changes in the elicited tissue and the results of biological assays of protection.

2. Materials and methods

2.1. Plant, fungus and growth conditions

Potato tubers (*Solanum tuberosum* cv. Kennebec and *Solanum tuberosum* cv. Pampeana) were obtained from the Experimental Station of the INTA Balcarce, Argentina. Kennebec cultivar was selected because it is moderately susceptible to Rhizoctonia disease and Pampeana cultivar was selected because it is susceptible to dry rot disease caused by *F. solani* f. sp. *eumartii*. Potato tubers from Kennebec cultivar were incubated at 18 °C, on the dark, to allow sprouting.

All fungal isolates were obtained from the Laboratorio de Fitopatología, INTA Balcarce, Argentina. Stock cultures of both, binucleated non-pathogenic *Rhizoctonia* (BNR) isolate (232-CG) and the pathogenic isolate *R. solani* (AG-3), were maintained on potato-2% glucose agar, at 18 °C in the dark. While *F. solani* f. sp. *eumartii* isolate 3122, was maintained on potato-2% glucose agar, at 25 °C in the dark.

For mycelial wall extraction, BNR was grown on potato-2% glucose liquid culture at 18 °C in the dark for 15 days.

For protection assay both *Rhizoctonia* isolates were grown on dried oat grains [23]. Cereal grains were soaked overnight in vials with water supplemented with 250 μg of chloramphenicol. The water was decanted and the grains were autoclaved at 121 °C for 1 h 2 consecutive days. Mycelial disks from the margins of actively growing cultures were placed in the vials and incubated at 18 °C during 7–10 days. After incubation the oat grains colonized by the fungi were used for the protection assay.

2.2. Elicitor preparation

The α-1, 3-glucan from BNR cell wall was prepared following the method described by Ayers et al. [16]. Purification and characterization were carried out as described by Wolski et al. [19]. Neutral sugars were determined by the phenol–sulfuric acid method, using glucose as a standard [24].

2.3. Elicitor treatments

Elicitor activity was determined by measuring the effect of the α-1, 3-glucan from BNR cell walls on potato sprouts.

Potato sprouts were injected with an aqueous solution of the α-1, 3-glucan (5 mg ml^{-1}) in sterile conditions using a needle, and then incubated in sterile conditions at 18 °C during several days. At different times the sprouts were homogenized as we describe below and were used to measure ß-1, 3-glucanase, chitinase and polygalacturonase-inhibitor activity (PGIP activity). Controls included potato sprouts injected with distilled and sterile water.

For the immunoblot analysis of PR-proteins, potato sprouts were infiltrated with a solution of 5 mg ml^{-1} of the α-1, 3-glucan or water (control). The infiltration was carried out by immersing the sprouts in the α-1, 3-glucan solution (5 mg ml^{-1}) and putting them under vacuum three times of 15 s each. Then the sprouts were incubated in sterile conditions at 18 °C. At different times the sprouts were homogenized as described below and used for the immunoblot analysis.

For microscopy studies potato sprouts were immersed in an α-1, 3-glucan solution (5 mg ml^{-1}) or water (control) during 10 min, air-dried at room temperature and then incubated in sterile conditions at 18 °C during 6 days.

2.4. Induction assay with the non-pathogenic BNR isolate

Potato sprouts were inoculated with a disc of agar containing mycelium from BNR on the base of potato sprout and were maintained at 18 °C in the dark. Controls included potato sprouts inoculated with a disc of agar without BNR mycelia. At different times after inoculation,

potato sprouts were first washed with sterile water to eliminate the mycelia and then processed to determine glucanase activity.

2.5. Crude extracts preparation

Potato sprouts were homogenized in 50 mM sodium acetate buffer (pH 5.2), 0.1% sodium metabisulfite using a mortar. The homogenate was centrifuged for 20 min at 12 000 *g* and dialyzed over night against the same buffer. Then the samples were stored at −20 °C. All operations were carried out at 0–4 °C.

2.6. β-1, 3-glucanase activity

Glucanase activity was assayed by measuring the rate of reducing sugar production with laminarin from *Laminaria digitata* (Sigma) as substrate. The reaction mixture consisted of 0.06 ml of 50 mM sodium acetate buffer pH 5.2 containing 1% laminarin, 0.01 ml of enzyme extract and 0.05 ml of sodium acetate buffer pH 5.2. After 2 h of incubation at 37 °C the enzyme reaction was stopped by heating in boiling water for 2 min and the reducing sugar released was measured according to Nelson [25] and Somogyi [26]. Glucose was used as a standard. The activity was expressed in units (U) per gram of fresh weight; 1 U: 1 nmol glucose released h^{-1} ml $enzyme^{-1}$.

2.7. Chitinase activity

Chitinase activity was assayed by measuring the rate of *N*-acetyl-glucosamine release from chitin (crab shells, Sigma). The reaction mixture contained 100 µl of partially hydrolyzed chitin in 50 mM sodium acetate buffer pH 5.2, 80 µl of 50 mM sodium acetate buffer pH 5.2 and 20 µl of enzymatic extract. After 3 h of incubation at 37 °C, the mixture was centrifuged at 15 000*g* for 20 min; 100 µl of the supernatant plus 20 µl of 0.8 M borate buffer pH 9 were heated in boiling water for 3 min. Ehrlich's reagent (0.6 ml of *p*-dimethylaminobenzaldehyde) was added to the mixture and then it was heated at 37 °C for 20 min. The absorbance at 585 nm of the mixture was measured [27].

The activity was expressed in units (U) per gram of fresh weight; 1 U: absorbance at 585 nm h^{-1} ml $enzyme^{-1}$.

2.8. PGIP activity assay

The inhibition of polygalacturonase (PG) activity was determined by measuring reducing end-groups released from polygalacturonic acid (PGA, Sigma, St Louis, MO, USA) in the absence or presence of PGIP. Reducing end-groups were measured by the method described by Nelson [25] and Somogyi [26], using D-galacturonic acid as standard.

An extracellular fungal PG was used as a source of PG activity isolated from liquid culture of *R. solani* AG-3 [28]. PG activity was determined in 0.1 ml reaction mixture containing 0.5% (w/v) PGA as substrate, 50 mM sodium acetate (pH 5.2) and suitable amounts of enzymatic extract from potato sprouts treated or not with the glucan (source of PGIP). Samples were incubated at 37 °C for 30 min and reducing sugars were determined.

Glucose was used as a standard. The activity was expressed in units (U) per gram of fresh weight; 1 U: 1 nmol glucose released h^{-1} ml $enzyme^{-1}$.

2.9. Immunoblot analysis

For immunoblot analysis, proteins were separated on 12% SDS-PAGE and subsequently transferred electrophoretically (15 V, 20 min, 25 mM Tris-HCl (pH 8); 0.192 M glycine, 20% methanol) to nitrocellulose membranes. Membranes were incubated with the following antibodies raised against: (i) a basic β-1, 3-glucanase (36 KDa) purified from intercellular fluid of potato leaves infected with *Phytophthora infestans* (1:10,000 dilution) [3]. (ii) Purified tobacco PR proteins of the following families: PR-2 (glucanase), 1: 3000 dilution; PR-3 (chitinase), 1: 7000 dilution; and PR-5 (thaumathin-like protein), 1: 3000 dilution [29]. Then the membranes were incubated with goat antirabbit IgG alkaline phosphatase conjugate (1: 10 000 dilution). Immunodetection was performed using 5-bromo-4-chloroindolyl-phosphate (BCIP) and nitroblue-tetrazolium (NBT), method described by Turner [30].

2.10. Light and fluorescence microscopy

Samples of about 2 mm^3 from potato sprouts infiltrated with the α-1,3-glucan (5 mg ml^{-1}) or water (control), were fixed in 0.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) (v/v), for 3 h at 4 °C. The tissue was washed three times with phosphate buffer and then dehydrated in an ethanol series with 50%, 60%, 70%, and 90% ethanol (30 min each). The samples were then embedded in LR-White Resin (Polyscience) and stained with toluidine blue O (Sigma T3260 CI 52040), according to Harris et al. [31]. Sections (1 µm thick) were mounted on glass slides, using Poly-L-lysin (P4707, Sigma-Aldrich). For detection of callose and lignin, sections were incubated with Aniline Blue (M6900, Sigma-Aldrich) as a marker for β-(1-3)-D-glucans [31], or acidic phloroglucinol (P6900, Sigma-Aldrich) [31,32], respectively. The Aniline Blue reaction was visualized with a Zeiss epifluorescent microscope provided with an III RS epi-illuminating condenser and a combination of comprising filters 365 and 418 as exciter and barrier filters, respectively (Zeiss, Oberkochen, Germany). The two dyes were used separately or simultaneously.

2.11. Transmission electron microscopy

The samples were fixed as we described above and embedded in LR-White Resin (Polyscience), according to Harris et al. [31]. Ultra thin sections (60–80 nm) were made

with a Reichert-Jung (Vienna) ultramicrotome and mounted on nickel grids. Subsequently the ultra thin sections were stained with uranyl acetate and citrate. Sections were then examined with a Zeiss EM 109 turbo (Germany) transmission electron microscope.

2.12. Protection assays

For Rhizoctonia canker protection assay, the experiments were performed using potato tubers of *Solanum tuberosum*, cv. Kennebec, which is moderately susceptible to the fungus *R. solani* AG-3. Before experimental use, pieces of certified seed tubers containing sprouts were immersed in 0.5% NaOCl for 5 min, rinsed in running tap-water and air-dried at room temperature. Experiments were conducted in a steam-pasteurized substrate potting mix in plastic pots under controlled conditions. Holes of 7 cm deep were made in the mixture and one seed piece with only one sprout was placed in each hole. Fifteen potato sprouts were planted for each treatment.

Protection was determined by eliciting the potato sprouts with the α-1, 3-glucan and then inoculating them with the pathogenic isolate. The sprouts were immersed during 10 min in α-1, 3-glucan solution (5 mg ml^{-1}) or water (control), air-dried at room temperature and then placed in a hole. Ten oat kernels colonized by *R. solani* were placed on the top of the plastic pots, at about 1–2 cm deep. On the other hand, 10 colonized oat kernels of BNR isolates were placed in the bottom of each planting hole with a seed piece containing one potato sprout (without any pretreatment) and then infected as we described above. After planting, pots were placed in a growth chamber (18 ± 2 °C, 16-h daylight) for 5 weeks and irrigated daily to maintain high soil moisture.

Disease severity was determined 5 weeks after planting by evaluating each underground stem on an arbitrary scale described by Escande and Echandi [20] with some modifications. 0 = no lesions, 1 = lesions < 5 mm long, 2 = lesions > 5 mm long, 3 = lesions girdling the stem and 4 = death of the pre-emerging sprout. Protection percentage (PP) was calculated as follows: PP (%) = 100 $(1-x/y)$, where x and y are disease severity values for treated and control plants, respectively.

For dry rot protection assay, the experiments were performed using potato tubers of *Solanum tuberosum*, cv. Pampeana, which is susceptible to the fungus. *F. solani*. f. sp. *eumartii*, isolate 3122, from EEA-Balcarce INTA Collection (Argentina). Before experimental use, pieces of certified seed tubers cv. Pampeana were immersed in 1% (v/v) NaOCl for 5 min, rinsed in running tap-water and air-dried at room temperature.

Tubers were inoculated with a 0.4-cm diameter disk of fungus grown in potato-2% glucose agar and introduced in the potato cortical tissue by the hollow punch method described by Radtke and Escande [33]. As this method causes mechanical injury to the tuber tissue (wounding), control tubers were wounded and inoculated with sterile potato-2% glucose agar. After inoculation, tubers were stored for 7 or 13 days at 25 °C, then cut and analyzed.

For α-1, 3-glucan pre-treatment, 50 µl (0.5 mg) of an aqueous solution of the α-1, 3-glucan were applied on wounded tubers. After 1 h, fungal inoculations were performed as described above. Controls were pre-treated with water. The tubers were kept at 25 °C. Inoculated tubers were transversally cut in the middle of the lesion area. Cross-section areas showing visible rot lesions were recorded by scanning. Surface of rot and control areas were quantified by image analysis (Image J 1.33U (Wayne Rasband, National Institute of Health, USA).

2.13. Statistical analysis

Statistical analysis of the data was performed by analysis of variance (One way ANOVA). Means were compared with Dunnett's Multiple Comparison Test and Bonferroni's Multiple Comparison Test at a significance level of $P = 0.05$.

3. Results

3.1. Kinetics of induction of ß-1, 3-glucanase activity by the α-1, 3-glucan and dose–response assays

In order to test the ability of the α-1, 3-glucan to induce ß-1, 3-glucanase activity, potato sprouts were injected with 250 µg of the glucan or water (control). ß-1, 3-glucanase activity was determined at 0, 4, 6 and 8 days after treatment (Fig. 1). Glucanase activity began to increase significantly at day 4 and maximal increase of ß-1, 3-glucanase activity

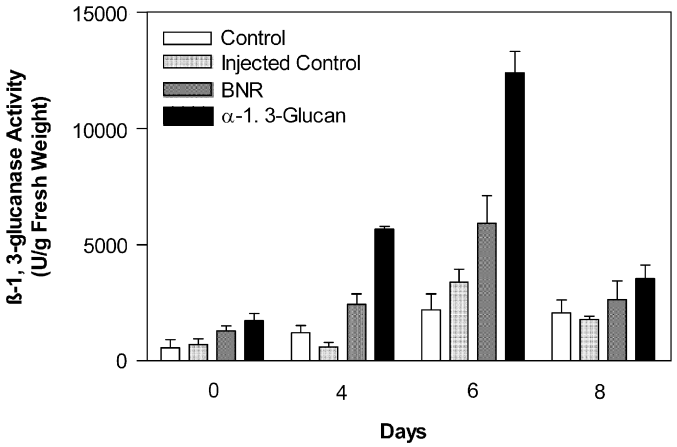

Fig. 1. Induction of β-1, 3-glucanase activity in potato sprouts. Potato sprouts were injected with 250 µg of the α-1, 3-glucan. Another set of potato sprouts were inoculated with a disc of agar containing mycelium of BNR. Then the sprouts were maintained at 18 °C in the dark. Control sprouts were injected with distilled sterile water (Injected control) and another set were inoculated with a disc of agar without mycelia (Control). Glucanase activity was determined as described in Section 2.6 of Materials and methods. Bars represent the mean values ± SE of three independent experiments, with five replicates per experiment.

was detected after 6 days, being 4-fold higher than controls (Fig. 1).

To compare the elicitor activity induced by the α-1, 3-glucan with the non-pathogenic binucleate *Rhizoctonia* (BNR) isolate, a kinetic study of ß-1, 3-glucanase activity of potato sprouts inoculated with agar disks containing BNR was carried out (Fig. 1). The results showed that ß-1, 3-glucanase activity was increased between 4 and 6 days after inoculation, being maximal at day 6 (2.5-fold higher than controls). When comparing these results with the ones obtained with the α-1, 3-glucan (Fig. 1), it was concluded that both, BNR isolate and the α-1, 3-glucan, produced similar kinetics, with the maximal activity at 6 days after inoculation.

On the other hand, we tested the dose dependence of the elicitor activity (Fig. 2). The highest activity was obtained when the sprouts were inoculated with 250 μg of the α-1, 3-glucan. Higher doses (500 μg) did not produce a further increase of activity.

3.2. Kinetics of induction of chitinase activity in potato sprouts treated with the α-1,3-glucan

Potato sprouts were treated with 250 μg of the glucan or water (control). At different times the samples were processed and chitinase activity was measured (Fig. 3). A strong induction of chitinase activity was observed in potato sprouts treated with the α-1, 3-glucan. The highest chitinase activity was attained between 4 and 8 days. At days 4, chitinase activity was three times higher in the sprouts treated with the α-glucan than in controls.

3.3. Immunoblot analysis of induction of PR proteins

Immunological analysis of PR proteins of potato sprouts infiltrated with the α-1, 3-glucan was performed (Fig. 4). The α-1, 3-glucan induced the accumulation of three of the tested families of PR-proteins and a basic isoform of a *β*-1, 3-glucanase (36 KDa) (Fig. 4). Maximal accumulation of PR proteins was detected at 6 days after infiltration.

We also measured a PGIP activity, which is known to interact with extracellular endo-α-1, 4-polygalacturonases (PGs) secreted by phytopathogenic fungi to inhibit their activity [28,34]. However, the PGIP-activity was not significantly altered by the treatment with the α-1, 3-glucan (data not shown).

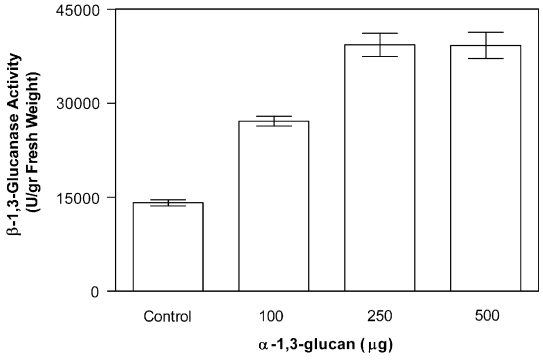

Fig. 2. Dose–response of ß-1,3-glucanase activity in potato sprouts treated with the α-glucan. The sprouts were injected with 50 μl of distilled sterile water (Control) or with 50 μl of glucan solutions of different concentrations. The sprouts were maintained at 18 °C in the dark during 6 days and glucanase activity was determined as described in Section 2.6 of Materials and methods. Bar represent the mean values ± SE of three independent experiments, with five replicates per experiment.

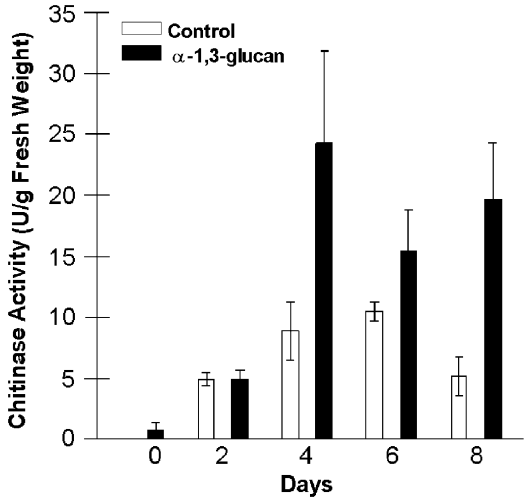

Fig. 3. Kinetics of induction of chitinase activity. The sprouts were injected with 50 μl of the α-glucan solution (5 mg ml^{-1}) or with 50 μl of distilled sterile water (Control). Chitinase activity was determined at different times after inoculation. Bars represent the mean values ± SE of three independent experiments, with five replicates per experiment.

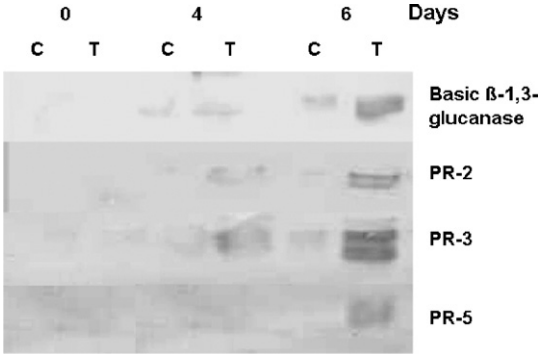

Fig. 4. Immunoblot analysis of PR-proteins induced by the α-1, 3-glucan. Total proteins of potato sprouts infiltrated with an aqueous solution of 5 mg ml^{-1} of the α-1,3-glucan (lanes T) or infiltrated with distilled sterile water (lanes C), were analyzed at different times from the treatment by 12% SDS-PAGE. The proteins were transferred onto nitrocellulose and immunodetected with polyclonal antibodies raised against: (i) a basic *β*-1, 3-glucanase (36 KDa) (ii) the PR proteins from families: PR-2, PR-3 and PR-5. Equivalent amounts of protein (700 μg) were loaded on each lane. The results are representative of at least three identical experiments.

3.4. Histological and ultrastructural features of treated potato sprout tissues

Differences between control (sprouts immersed in water) and induced tissues (sprouts immersed in the glucan

solution) were not detected in sections stained with toluidine blue (Fig. 5a, b).

In induced tissues we observed an increase in callose deposition on cell walls of all sprout tissues, including parenchyma from cortex, pith and xylem, as shown by epifluorescence microscopy when sections were stained with aniline blue (Fig. 5c). Control sections stained with aniline blue did not show callose accumulation (Fig. 5d). On the other hand, the cell walls of the tracheary elements of potato sprouts treated with the α-1,3-glucan showed stronger intensity as compared to the controls, when stained with phloroglucinol for lignin detection (Fig. 6).

Transmission electron microscopy of potato sprout tissue elicited with the α-1, 3-glucan revealed a disorganized and condensed cytoplasm (Fig. 7a, b). As compared to controls (Fig. 7d–f), both the cytoplasm and the cell wall were electronically denser after staining with uranyl acetate and lead citrate. In addition, dense deposits occurred in vacuoles (Fig. 7a, b) and the cell walls showed expansions of fibrillar material (Fig. 7c). In control tissue cytoplasm was electronically lucent, vacuoles were empty and fibrillar expansions of the cell wall were absent (Fig. 7d–f).

3.5. *Effect of the α-1, 3-glucan on the protection of potato plants against R. solani AG-3*

When potato sprouts were pretreated with the α-1, 3-glucan and then infected with the pathogen *R. solani* AG-3, we observed a 40% of protection compared to the control (only infected with the pathogen) (Fig. 8a). In addition, when the sprouts were inoculated with the non-pathogenic isolate (BNR) and then inoculated with the pathogen, the protection was about 60% (Fig. 8a). Fig. 8b shows a reduction of the lesions in the stems of plants that were pretreated with either the glucan or the BNR isolate.

In this assay we also tested other doses of the α-1, 3-glucan (1, 10 and 15 mg ml^{-1}), but better results were obtained with 5 mg ml^{-1}. With lower doses the protection was reduced and higher doses affected the normal plant development.

3.6. *Protection of potato tubers against dry rot disease by the α-1, 3-glucan*

Potato tubers cv. Pampeana were pre-treated with an aqueous solution of the α-1, 3-glucan (0.5 mg ml^{-1}) or water (control), and then artificially infected with *F. solani* f. sp. *eumartii* (Fig. 9). After 8 days at 25 °C, the tubers which were transversally cut in the middle of the lesion area and cross-section areas showing visible rot lesions were recorded by scanning. The surface of rot and control areas was quantified. Fig. 9 shows that treatment with the α-1, 3-glucan reduced the symptoms of dry rot disease by approximately 60%.

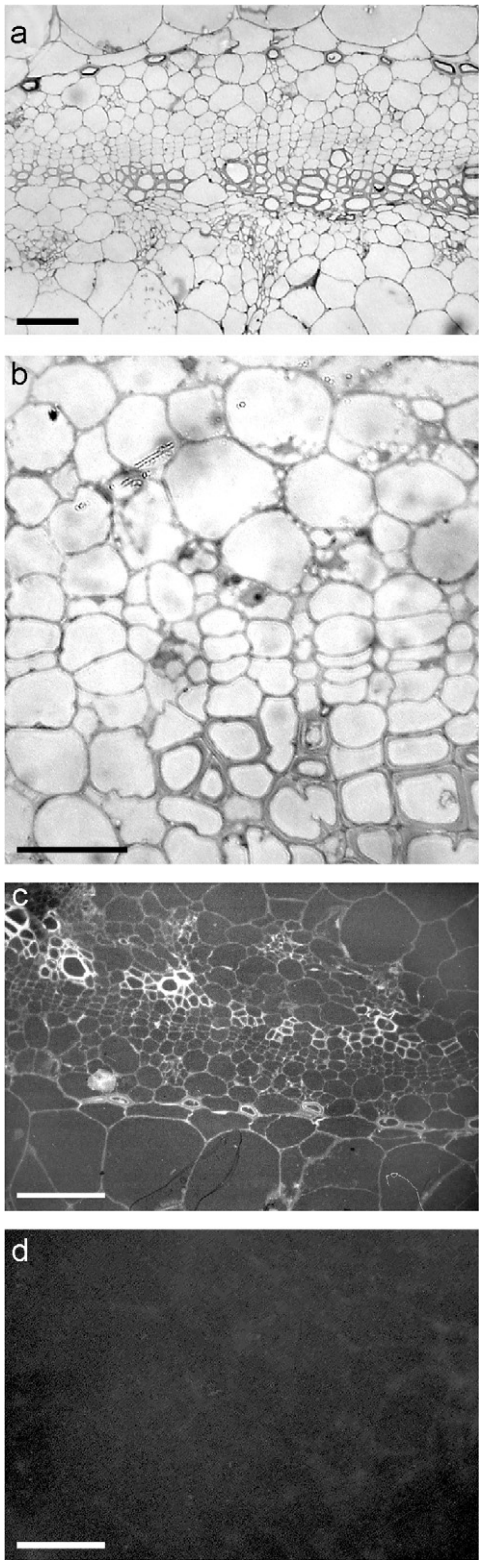

Fig. 5. Histological features of the elicited sprouts. Induced ((a), (c)) and control ((b), (d)) tissues, using toluidine blue/light microscopy ((a), (b)) and aniline blue/fluorescent microscopy for callose staining ((c), (d)). ((a), (b)) Using sections stained with toluidine blue, histological differences are not observed between induced (a) and control tissues (b). ((c), (d)) When sections were stained with aniline blue, callose was detected in the cell walls of parenchyma of cortex, pith and vascular tissues of potato sprouts treated with the α-1, 3-glucan (c). Controls did not show callose accumulation (d). Bar = 20 μm ((a), (b), (c) and (d)).

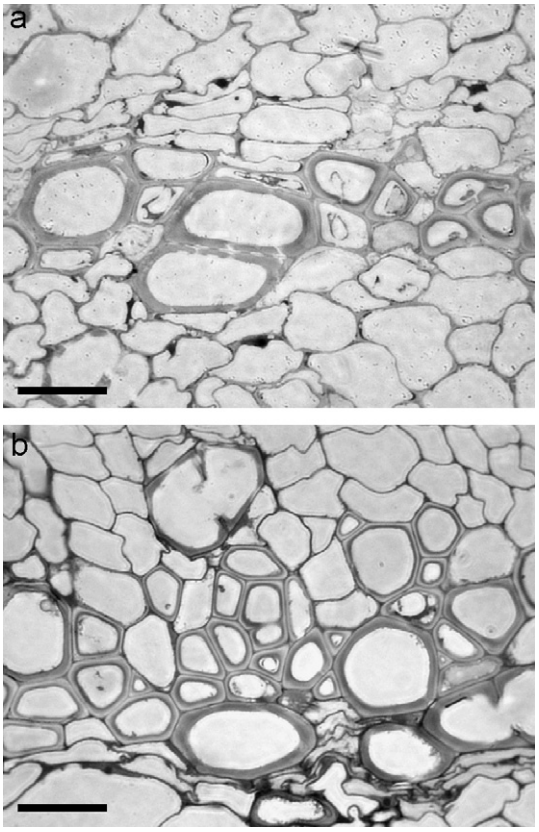

Fig. 6. Detection of lignin on potato sprouts sections stained with phloroglucinol. Staining for lignin results strong in tracheary elements of potato sprouts treated with the α-1, 3-glucan (b), whereas the control samples show only weak intensity (a). Bar = 20 μm ((a), (b)).

4. Discussion

Non-pathogenic *Rhizoctonia* isolates have the ability to protect several plants (potato, bean seedlings, poinsettia) against further infection by virulent isolates of *Rhizoctonia* and it has been proposed that such a protection is due to induced resistance [20,22,35]. Induced resistance can also be triggered by elicitors [12,13,15,36] and the elicitation of defense mechanisms is assumed to be a powerful approach for the management of plant diseases and to be an alternative to environmentally undesirable chemical control.

In a previous work, we have reported that the α-1, 3-glucan was effective in inducing ß-1, 3-glucanase activity [19]. Therefore, in this work we studied the ability of the α-1, 3-glucan elicitor to induce other defense responses in potato sprouts and we also studied the ability to protect potato plants against the disease caused by *R. solani* AG-3. We chose this experimental system because potato sprouts are the primary sites of infection by *R. solani*.

The fungus BNR and the α-1, 3-glucan were both effective in inducing ß-1, 3-glucanase activity. Kinetics of this enzyme was similar in both cases, with a maximal induction at 6 days after treatment (Fig. 1). However, ß-1, 3-glucanase activity was higher in the sprouts treated with the α-glucan than in the sprouts inoculated with BNR. In addition, when the α-1, 3-glucan was treated with amyloglucosidase, the degraded glucans were less active than the original one [19]. The similar kinetic responses could be an evidence to support the idea that ß-1, 3-glucanase may be involved in the mechanism of induced resistance by BNR. On the other hand, the α-1, 3-glucan also induced chitinase activity (Fig. 3).

Maximal accumulation of a basic ß-1, 3-glucanase (36 kDa) and a PR-2 family protein were well correlated. (Fig. 4). Both proteins showed the strongest signal at 6 days after treatment. It is important to point out that the antibody raised against PR-2 is for an acidic glucanase (PR-2b (II)). Therefore, the α-glucan induces both acidic and basic glucanases. Acidic PR proteins are predominantly located in the intercellular spaces and basic PR proteins are mainly located intracellularly in the vacuoles [37]. It has been suggested that in induced plants, the accumulated intercellular proteins constitute the first line of defense to a challenging pathogen, and if this fails and the tissue is disrupted, the release of the vacuolar PRs functions as a second line, engulfing the pathogen with lytic enzymes [38]. In addition, an acidic chitinase (PR-3b (II) family) was accumulated with a maximal induction at 6 days after treatment (Fig. 4). This chitinases may have a role in the observed protection because a significant suppression of disease symptoms caused by the soil-borne fungus *R. solani* was demonstrated in tobacco and canola expressing a chitinase from bean [39].

The immunoblot assay also showed the induction of PR-5 protein. This is interesting because it has been reported that transgenic rice plants, which over-express thaumatin-like proteins, showed enhanced resistance to the pathogen *R. solani* [40], and transgenic potato expressing a tobacco PR-5 protein showed delayed symptoms induced by *Phytophthora infestans* [41]. In addition, the α-1, 3-glucan did not induce PGIP activity, in contrast to the results described by Aziz et al. [11] with laminarin as elicitor.

We considered important to detect cellular events that are induced in pathogen-attacked plants such as the deposition of lignin or callose in cells or tissues where they normally are scarce or absent. Callose deposition (out of phloem tissue) is thought to contribute to disease resistance by reinforcing the plant cell wall beneath fungal penetration sites [42], which is indicative of an enhanced defensive response [43]. In induced tissues, we detected an increase in callose deposition on cell walls of all parenchyma tissues, including cortex, pith and xylem. We also observed that lignin deposits were thicker and extended on the cell walls of the tracheary elements, as compared to controls. Lignin and callose were deposited simultaneously, following the treatment with the α-1, 3-glucan. Therefore, they represent a parallel quick cellular defense response.

The ultrastructural analysis of potato sprout tissue treated with the α-glucan elicitor showed that cell walls became electron-denser and exhibited expansions of fibrillar material. These results are consistent with previous

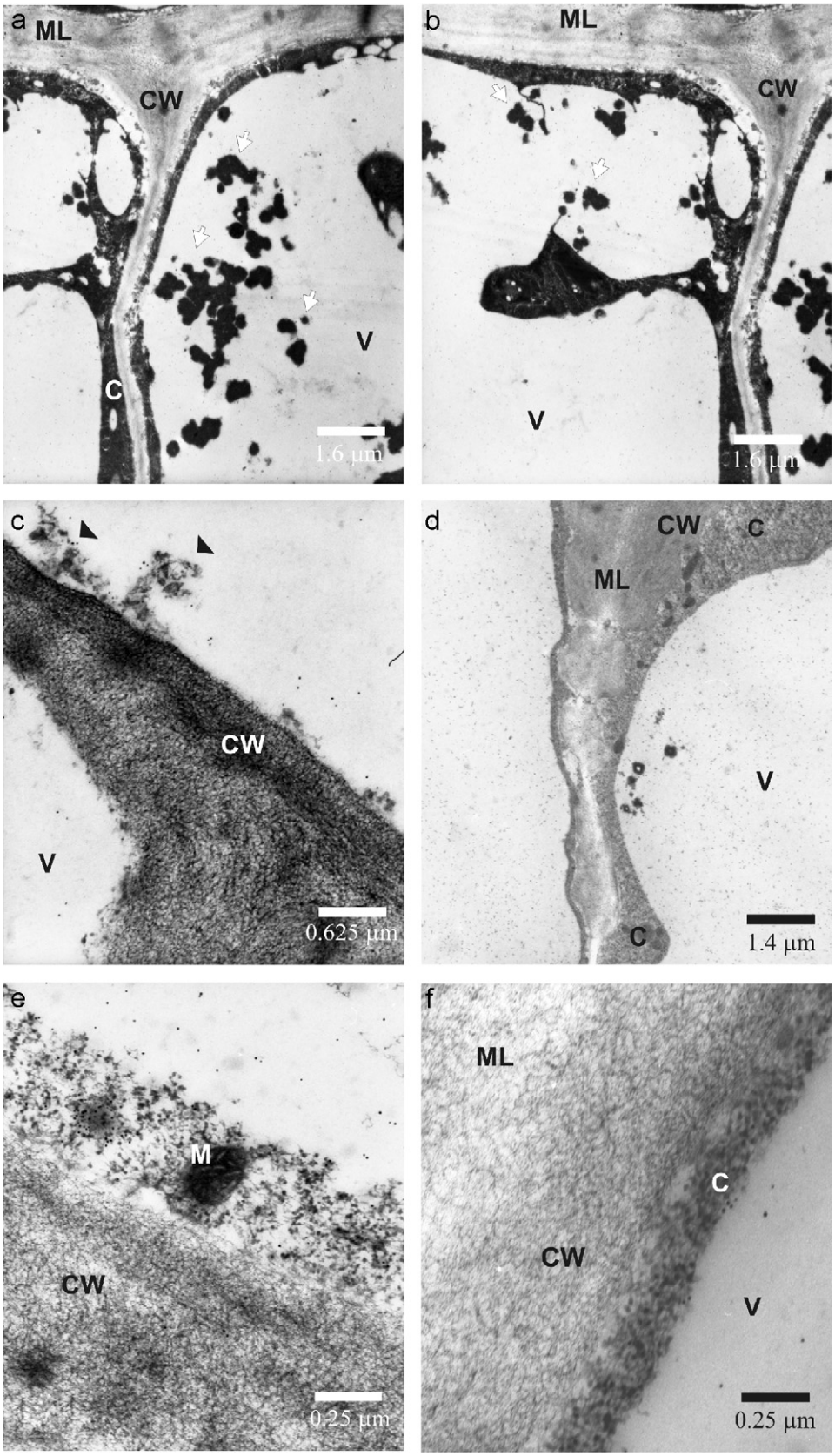

Fig. 7. Transmission electron micrographs of potato sprout tissue elicited with the α-1, 3-glucan. (a)–(c) potato sprouts elicited with 5 mg $ml^{-1}$of glucan after 6 days from treatment time. Potato tissue changes occur in treated tissues: cytoplasm becomes electronically denser, deposits of probably phenols appear in vacuoles (arrows) and fibrillar expansions of the cell wall are developed (arrowheads). (d)–(f), untreated potato sprouts. Cytoplasm is electronically lucent, vacuoles are empty and fibrillar expansions of the cell wall are absent. Abbreviations: C: cytoplasm, CW: cell wall, M: mitochondria, ML: middle lamella, V: vacuole.

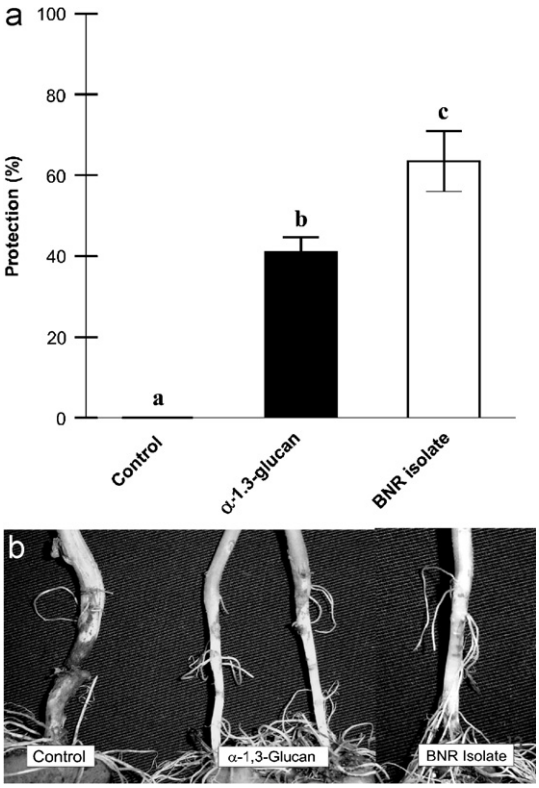

Fig. 8. Protection of potato sprouts against *R. solani* AG-3 by the α-1, 3-glucan. (a) Potato sprouts were pretreated with the α-1, 3-glucan (5 mg ml^{-1}) or water (control) and planted. Immediately, the soil was inoculated with the pathogenic isolate *R. solani* AG-3. A positive control was carried out using potato sprouts inoculated with the non-pathogenic BNR isolate and then with the pathogenic isolate. After 5 weeks disease severity was estimated and protection was calculated as described in Section 2.12 of Materials and methods. Bars are mean ± SE of 15 replicates and the results are from one representative experiment out of three. Bars with different letters are significantly different at $P = 0.05$. (b) Representative photograph of the protection induced by the α-1, 3-glucan.

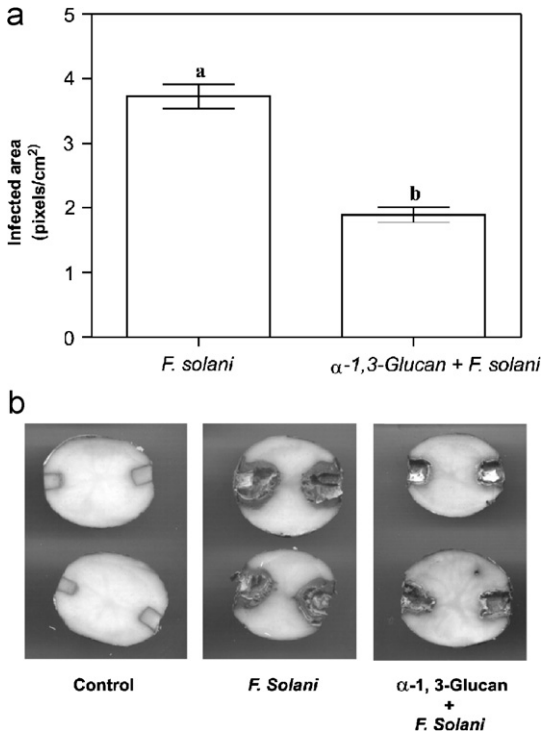

Fig. 9. Protection of potato tubers against dry rot. (a): potato tubers were pre-treated with an aqueous solution of the α-1, 3-glucan (0.5 mg) or water (Control). The tubers were then artificially infected with the pathogenic isolate *F. solani* f. sp. *eumartii*. After 8 days at 25 °C, the protection against dry rot disease was estimated measuring the infected area. Bars are mean ± SE of 15 replicates and the results are from one representative experiment out of three. Bars with different letters are significantly different at $P = 0.05$. (b) Representative photograph of the protection induced by the α-1, 3-glucan.

data reported for cryptogein, a protein elicitor from *Phytopthora cryptogea*, in tobacco plants [44]. Non-cellulosic fibrillar expansions were probably hemicellulosic in nature. In addition, electron-dense deposits, probably phenols, were observed in vacuoles. Phenols are known to be involved in host defense reactions [2]. In potato, suppression of PAL, the key enzyme in phenol production correlated with higher aggressiveness of *Phytophthora infestans* [45].

The impact of many pathogens is mostly controlled by fungicide treatments, but pathogen strains generally developed resistance. The use of bioactive substances constitutes an attractive alternative to reduce the use of chemical methods. For example, chitosan which is an elicitor of the plant defense responses was able to protect wheat, peas and lentils against *F. graminearum* infection [46,47]. In this work we showed that in addition to eliciting a wide array of defense reactions, the α-1, 3-glucan induces protection against the disease caused by *R. solani* in potato plants. Although the percentage of protection was not too high, it was significant and the size of the necrotic lesions was reduced visibly on the stems of plants pre-treated with the α-1, 3-glucan or with the BNR isolate. The dose used (5 mg ml^{-1}) was similar to those previously reported for other elicitors, such as laminarin and oligalacturonides, which were in the range of 0.05–1 mg ml^{-1} [11,12]. Further protection is conceivable by improving the penetration of the elicitor, the method of application, formulation and combination with chemical control methods. These assays are in progress.

On the other hand, preliminary results showed that the α-1, 3-glucan also confers protection of potato tubers against the dry rot disease agent, *F. solani* f. sp. *eumartii* (Fig. 9), indicating that this compound could be effective against others pathogens.

Oligosaccharides of glucose that were reported as elicitors of defense response are present in the *β*-configuration [10,17]. Occurrence of ß-1, 3 linked glucans is common in fungal cell walls [48]. However, the presence of α-glucans in fungal cell walls has been rarely described and their elicitor activity is unknown [49,50]. This is the first report which showed that α-glucan induces plant defense reponses and protects against plant diseases.

The use of new bioactive substances can play an important role in a more complex vision of crop protection, as key elements of the Integrated Management of

Pesticides programs. Our results show that the activation of plant defenses using the α-1, 3-glucan elicitor could be a valuable tool to contribute to new alternative strategies which are developing for plant protection.

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