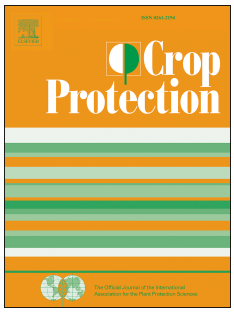

The overexpression of antifungal genes enhances resistance to rhizoctonia solani in transgenic potato plants without affecting arbuscular mycorrhizal symbiosis

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THE OVEREXPRESSION OF ANTIFUNGAL GENES ENHANCES RESISTANCE TO *RHIZOCTONIA SOLANI* IN TRANSGENIC POTATO PLANTS WITHOUT AFFECTING ARBUSCULAR MYCORRHIZAL SYMBIOSIS

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Abstract

The biological control of fungal diseases through the use of genetically modified (GM) plants could decrease the input of chemical pesticides. To overcome possible losses in potato (*Solanum tuberosum*) yield because of susceptibility to soil fungal pathogens, researchers have developed potato transgenic lines expressing antifungal proteins. However, all GM crops must be monitored in their potentially detrimental effects on non-target soil microorganisms. Arbuscular mycorrhizal (AM) fungi are good candidates for this type of analysis, as good indicators of a normal rhizosphere structure and functionality. In this work, we have monitored potato lines with over-expression of genes encoding peptides with antifungal properties on their effects on the soil-borne fungal pathogen *Rhizoctonia solani* and AM fungi.

The six GM potato lines (AG-1, AG-3, RC-1, RC-5, AGRC-8 and AGRC-12) evaluated showed higher reduction in infection indexes in comparison to untransformed plants when challenged with a highly virulent strain of *R. solani*. The growth of RC-1, RC-5 and AGRC-12 lines remained almost unaltered by the pathogen; which evidenced the maximum inhibition of *R. solani* infection. The level of root colonization by the AM fungus *Rizophagus intraradices* (pure *in vitro* isolated) did not significantly differ between transgenic and wild potato lines under *in vitro* and microcosm conditions. An increase in mycorrhization was evident with the addition of potato biomass residues of these GM lines in comparison to the addition of residues of the wild type potato line.

In addition to the *R. intraradices* assays, we performed microcosm assays with soil samples from sites with at least100-year history of potato crop as inoculum source .The roots of AGRC-12 GM line showed significant higher levels of native mycorrhization and arbuscules development. In general, the potato lines apparently were less receptive to *R. intraradices* pure inoculum than to AM species from the natural inoculum. In this work, the selected GM potato lines did not have evident adverse effects on AM fungal colonization.

Key words: Transgenic potato, antifungal genes, *Rhizoctonia solani*, arbuscular mycorrhizal fungi, intraradical variations

1. Introduction

Commercial use of genetically modified (GM) plants, especially those resistant to the attack of insects and pathogenic microorganisms, is increasingly widespread (Clive, 2014). Studies of potential risks associated with these GM plants established their possible impact on the environment. These studies mainly focus on gene transfer from GM to wild plants, the antibiotic resistance transference to natural microbial populations or the impact of transgenic proteins on non-target organisms (Giovannetti, 2003; Turrini et al., 2015). Among these organisms, and within the key functional groups of soil microbial communities, are the arbuscular mycorrhizal (AM) fungi. Despite their importance, a low number of studies have monitored the impact of GM plants on this obligate biotroph group (Liu, 2010; Hannula et al., 2014; Colombo et al., 2016). Some researchers have suggested that unintentional changes in composition of GM plant phenotype (pleiotropic effects) and root exudates could affect the structure and functionality of microbial rhizosphere communities. For example, transgenic proteins expressed by roots could be accumulated into the soil or incorporated through crop residues at the end of harvest (Turrini et al., 2015) and potentially affect non-target microorganisms as AM fungi.

The potato (*Solanum tuberosum*) is the most important tuber crop worldwide, growing in more than 125 countries and daily consumed by more than a billion people. The potato is easy to grow and has great nutritional value; its cultivation is an important food security and cash crop for millions of farmers (Lutaladio et al., 2009). Many production constraints derive from the biological characteristics of the potato itself, including low multiplication rates of seed tubers, high technical difficulties and costs of maintaining seed quality through successive multiplications. Owing to the potato susceptibility to soil and seed-borne insect pests and diseases, major losses in potato production yield are caused by pathogens, especially fungi (Huarte et al., 2013).

Rhizoctonia solani, which was early described on potatoes by Kühn (1858), is a widely spread soil-borne fungal pathogen that comprises several groups pathogenic to different host species. The most common symptom of the disease caused by *Rhizoctonia* is the 'damping-off' of the infected seedlings, which is characterized by an early killing before or after soil emergence (Adam, 1988). The disease control relies mainly on chemical pesticides, which are not always effective because of the development of fungicide-resistant fungal populations. In addition, pesticides are a concern for the environment

and human health. Consequently, the transgenic expression of genes encoding antifungal proteins into commercial cultivars constitutes an interesting approach to reduce losses caused by pathogens. Their potential introduction as agricultural crops would be directly related to a reduction in pesticide application, leading to a more sustainable development of agro-ecosystems and to a consequent benefit to human health and non-target microorganisms.

It has been extensively demonstrated that AM fungal symbiosis improves plant nutrient acquisition and stabilizes soil aggregates through their hyphae; both characteristics are of great interest for low-input agriculture (Koide and Mosse, 2004). Because of their importance within the agroecosystem biodiversity, AM fungi could be considered indicators of possible negative impacts of GM plants with antifungal qualities. In light of the experimental evidence, there is a real need to evaluate the effect of GM plants on AM fungi for each particular transgenic event (Liu, 2010). Little is known about the effectiveness of AM fungi inoculation and host specificities in potato crops (Cesaro et al., 2008). Moreover, to the best of our knowledge, there are no studies on the potential effects of any GM potato line expressing antimicrobial genes on AM fungal development.

All life stages of AM fungi have been studied using *in vitro* culture systems (Declerck et al., 2005). For example, Voets et al. (2005) generated an autotrophic culture system of potato plants *in vitro*, in which roots associates to AM fungi, while the photosynthetic shoot develops under open air conditions. In addition, other studies have assessed the impact of GM plants on AM fungi by assessing root colonization, spore counts and also by molecular methods (Liu, 2010; Turrini et al., 2015). *In vitro* studies would significantly reduce the complexity of soil biological systems, allowing the monitoring of short-term effects of GM plants on AM fungi.

One way to improve or preserve potato yield is through the reduction of the incidence of diseases. Thus, we obtained transgenic plants over expressing selected antifungal genes and assessed their resistance against *R. solani* as well as the potential short-term effects of these potato lines and their biomass residues on AM fungi. For this purpose, we carried out microcosm and *in vitro* experiments with *Rhizophagus intraradices*, commonly used as an AM control species, and with indigenous AM fungal communities sampled from agricultural sites where potatoes had been cultivated for more than 100 years.

2. Materials and Methods

2.1. Transformation of GM potato expressing antifungal genes

Potato (*S. tuberosum* subsp. *tuberosum* cv. Kennebec) plants used for transformation assays were micropropagated under aseptic conditions in complete Murashige and Skoog (MS) medium. They were grown in nurseries at 22 ° C, under a photoperiod of 8/16 h dark/light cycle (5000 lux) for 4 weeks. The RC and AG constructs (Fig. 1) harbor the sequences encoding four antifungal genes under the control of the CaMV (35S) viral origin promoter and contains a cassette that confers resistance to a selective agent (kanamycin or hygromycin). These genes encoded proteins of barley (*Hordeum vulgare*) plants, a ribosome inactivating protein Type I RIP (R) and a class II chitinase hydrolytic enzyme (C) or from tobacco (*Nicotiana tabacum*), osmotin (A) (Ap24 gene) and the β-1, 3 glucanase hydrolytic enzyme (G).

Potato explants were transformed via *Agrobacterium tumefaciens* strains LBA4404 and pAL4404 employing different constructs that combined two genes (RC or AG). Briefly, leaf discs were co-cultured as described by Del Vas (1992) with *A. tumefaciens* carrying the construct of interest, for48 h in MS medium. Explants were sub-cultured in regeneration medium (MS salts and vitamins, 20 g/l sucrose, 7 g/l agar, pH 5.6, plus 2 mg/ml zeatine riboside, 50 mg/ml kanamycin and 300 mg/ml cefotaxime) and transferred to fresh medium every 15 days until distinct shoots appeared. Finally, shoots were grown in micropropagation medium (MS salts, 20 g/l sucrose, 7 g/l agar, pH 5.6) supplemented with kanamycin or hygromicyn.

In addition, co-transformation assays were also performed using the two strains of *A. tumefaciens*, harboring AG or RC construct, simultaneously and employing the two different selective agents. Plants from different transgenic lines were *in vitro* maintained by periodic micropropagation in growing chambers as described above.

Molecular biology techniques were developed according to the protocols of Sambrook et al. (1989), employing commercial kits according to the manufacturer instructions and to Bazzini (2003).

2.2. Challenge inoculation of transgenic plants with *R. solani*

Rooted potato plants (roots about 1 cm) obtained by micropropagation of apexes of selected lines were used. Trays (20 x 35 cm) were filled with soil-vermiculite sand (70-15-15 %) and the substrate mixture was infected by adding six 7-mm diameter discs of potato dextrose agar (PDA) medium with *R. solani* strain AG-3. The infected substrate was left to grow for 48 h at 28°C and eight plantlets of each GM line were then placed in theses trays. The same number of plantlets were transferred into non-inoculated substrate mixture, kept in growing chambers as described above and watered with distilled water at field capacity.

The height of each plantlet was measured from the edge of the tray to the apical apex of the plantlet every 3 days for 30 days. The average height of the plantlets per tray was calculated for both infected and uninfected lines and the individual measure of each infected plantlets was relativized to the average of the uninfected plantlets of the same line. The disease index (DI) (Jach et al., 1995) was calculated by assigning a number from 0 to 4 to these relativized values. The number 0 stands for growth greater than 75 % of the average growth of the same uninfected line, whereas 1 is for growth between 50 and 75 %. The number 2 and 3 are for growth between 25 and 50 % between 2.5 and 25 %, respectively. Finally, 4 stand for growth below 2.5 % of the average growth of the same uninfected line. The average of the disease rates was calculated for each line daily post infection (DPI) (the average value should be between 0 and 4). Finally, the infection reduction index (IRI) of each line for each DPI was calculated as follows: IRI = [(DI plant X - DI Kennebec plant) / DI Kennebec plant] x 100.

2.3. Autotrophic culture system for the *in vitro* AM symbiosis study

Transformed carrot (*Daucus carota*) roots colonized with the GA5 strain of *R. intraradices* (BGIV, http://www.bgiv.com.ar/strains/Rhizophagus-intraradices/ga5) were used to obtain viable pure spores and mycelia as described in Fernández Bidondo et al. (2011). Nodal cuttings of each line (AGRC-12, RC-1, RC-5 and KEN) were placed in sterile Magenta^{TM} culture boxes (77-77-194 mm) containing 40 ml of Minimum medium (MM) without sucrose, solidified with 0.35 % w/v Gel-gro (ICN Biochemicals, Aurora, OH, USA) and adjusted to pH 5.8 before sterilization. The lower section of the boxes (97 mm), where fungus and roots developed, was covered with opaque material. A plug of 3-month-old GA5 monoxenic culture, which contained colonized roots (30 % frequency and 50 % intensity of colonization), approximately 250

spores and abundant extraradical mycelium (ERM), was placed in the proximity to potato plantlets in each culture box. Ten culture boxes (replicate) per potato line (treatments) were made for this experiment. Plantlets were kept for 100 days in a growth chamber at 25 °C with 60 % relative humidity and 16 h photoperiod. After 100 days, the effect of the potato lines on the establishment of AM symbiosis was checked.
Potato plantlets were harvested; roots were cleared in 10 % KOH and then stained with Trypan blue (Phillips and Hayman, 1970). Intraradical colonization was quantified by examination of 50 randomly selected root pieces (1 cm length). Frequency (% F) of mycorrhizal colonization was calculated as the percentage of root segments containing hyphae, arbuscules or vesicles. Measurements were performed under a Nikon light binocular microscope at 40x and 100x magnification.

2.4. Microcosm culture system for the *ex vitro* AM symbiosis study

Potato plantlets of each potato line (AGRC-12, RC-1, RC-5 and KEN) were micropropagated in MS medium from nodal cuttings as described above. Once rooted, plants were removed from medium and hardened in sterile substrate in growing chamber. Six-week-old potato plants were transplanted to pots containing 500 g of autoclaved (100 °C for 1 h, three consecutive days) mixture of 1:1 perlite and soil (pH7.1; total C 12.08 and N 1.1($g.kg^{-1}$); P 34.2$mg.kg^{-1}$; K 0.9, Ca 7.5, Mg 1.7 and Na 0.2($mol.kg^{-1}$)). The roots of each potato line were inoculated at transplanting time with 50 g of natural soil mixture. The soil mixture consisted of mycorrhizal roots, extraradical mycelium and spores. These soils were collected in November (springtime) within the agricultural landscapes of *Balcarce, Buenos Aires* province (37°S 58°W). The sampled fields that were used in this study came from sites that have been used as monoculture cultivation of potato since the early twentieth century (Huarte et al., 2013). AM colonization frequency values were obtained for these soils (F % mean ± SD, 8.56 % ±2.2) by applying the Mean Infection Percentage (MIP) method as described at The International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM, https://invam.wvu.edu//). Sorghum roots cultivated in a 1:10 dilution (inoculum-disinfested growth substrate) (MIP: mean ± SD, 12.33 % ±3.4) were used to obtain these AM colonization values.
Inoculated potato plants were kept for 100 days in a growth chamber at 25 °C with 60 % relative humidity and a 16 h photoperiod. They were watered at field capacity when

necessary and irrigated once a month with Hewitt (1952) nutritive solution without P to avoid influencing mycorrhization levels. Ten pots (replicate) per potato line (treatments) were made in this experiment. At the end of the experiment, entire potato plants were harvested and a portion of roots was cleared and stained as described above. The effect of the potato lines on the establishment of AM symbiosis was assessed under a Nikon light binocular microscope at 40x and 100x magnification.

2.5. Microcosm culture system for the study of biomass residues on AM symbiosis

The clover (*Trifolium repens*), a spontaneous weed, was used as alternative host to analyze the possible impacts of transgenic potato lines on AM colonization levels. Seeds of clover were surface-sterilized with 70 % (v/v) ethanol solution for 2 min, followed with 20 % (v/v) sodium hypochlorite solution for 15 min, then rinsed three times with sterile distilled water and finally germinated on moist filter paper for 2 days. Uniform clover seedlings were selected, in order to obtain a homogeneous batch. These seedlings were transplanted into pots with 250 g of sterilized (100 °C for 1 h on three consecutive days) vermiculite and irrigated once a week with Hewitt (1952) nutritive solution without P to avoid influencing the mycorrhization levels. AM inoculation was performed by placing a 3-month-old GA5 monoxenic culture plug per pot at transplant time. The growing substrate was mixed with 5 g of dry biomass (roots and shots) of each potato line (AGRC-12, RC-1, RC-5 and KEN). Clover plants were kept during 100 days in a growth chamber at 25 °C with 60 % relative humidity and 16 h photoperiod, in ten replicate pots per potato line (treatments). At the end of the experiment, clover plants were harvested and the radical system was cleared and stained as described above. The effect of the different potato lines dry biomass on the establishment of AM symbiosis was assessed under a Nikon light binocular microscope at 40x and 100x magnification.

2.6. Statistical analysis

Significant difference in the infection reduction indexes of GM lines was used as a measure of each line resistance. The analysis was through Chi-squared test at a significance level of 5 %, using Graph Pad Prism 3.0 software. The experiments were arranged in a completely randomized design with equal replications in each treatment.

In the autotrophic culture system and in microcosmos assays the effects of potato lines (factors) on frequency (% F) of mycorrhizal colonization differentiated by kind of AM structure (response variable) were subjected to factorial ANOVA. Comparisons between mean values were made using the least significant difference (LSD) test at $p < 0.05$. Statistical procedures were carried out with the software package STATISTICA 10.0 for Windows.

3. Results

3.1. Characterization of GM potato plants expressing antifungal genes

To obtain transgenic plants resistant to fungal pathogens, we first selected four antifungal genes (A, G, C and R; see 2.1 in Materials and Methods). Potato plants were transformed via *A. tumefaciens* employing different constructs that combined two genes (AG or RC binary vectors). On the other hand, co-transformation assays combining the use of two *A. tumefaciens* strains harboring AG or RC construct and different selective agents (hygromycin and kanamycin respectively) were conducted to achieve simultaneous expression of several transgenes. GM potato lines (events) were rooted in successive passages in selective medium and named as AG (from 1 to 8) or RC (from 1to 7). The plants obtained by co-transformation assays were designated as AG-RC (1 to12) according to the construct. A total of 27 transformants were screened in preliminary molecular and biological characterizations (Bazzini, 2003). According to the preliminary results, we selected six transgenic lines (AG-1, AG-3, RC-1, RC-5, AG-RC8 andAG-RC12) for further molecular and biological characterization.

All selected lines contained the constructions into the plant genome according to PCR analysis (Fig. 2A). In addition, AG-1 and AG-3 lines, followed by AGRC-8 line, displayed high accumulation of Ap24 osmotin, as evidenced by Western blot. In AG-RC12 line, AP24 was undetectable (Fig. 2B). RC-5 potato line showed the highest accumulation of RIP, followed by RC-1and AGRC lines. Anti-RIP serum revealed one apparently unspecific additional band of higher molecular weigh that is also present in the non-transformed control plants.

On the other hand, AG-RC12 line presented high accumulation of chitinase protein according to Western blot analysis revealed with anti-CHI serum. RC-1 and AGRC-8

lines also expressed this protein but at lower levels. In RC5 line, chitinase protein was undetectable (Fig. 2B). Glucanase protein was undetectable in all cases. This result may be due to specific antibody deficiencies.

Altogether, the six selected lines were suitable for evaluation of fungal pathogen resistance.

3.2. Challenge inoculation of transgenic plants with *R. solani*

The six GM potato lines (AG-1, AG-3, RC-1, RC-5, AGRC-8 and AGRC-12) were challenged against a highly virulent strain of *R. solani*. Throughout the challenge, the infection reduction index (IRI) was higher for the six selected GM lines than in control KEN plants (Supplementary material A). At 7 DPI, the AG-1 and AG-3 lines presented the worst results (13 % and 35 % of IRI respectively), whereas RC-1 and RC-5 transformed with the RIP-Chitinase construct showed the highest pathogen inhibition (87 % and 70 % of IRI respectively; Supplementary material A and B). At this same period in time, the co-transformed lines exhibited intermediate IRI values (39 % AGRC-8 and 65 % AGRC-12).

At 18 DPI, no significant differences were observed between the GM line on infected substrate, with IRI values ranging from 75 to 80 %; however, the co-transformed AGRC-12line exhibited an IRI of 100 % (Supplementary material A). Moreover, no significant differences were registered between GM plants grown on infected and uninfected substrates (Supplementary material D). Additionally, *R. solani* typically hyphal development in the substrate was verified under light binocular microscope (Supplementary material C).

RC-1, RC-5 and AGRC-12 lines showed an effective inhibition of *R. solani* and remained almost unaltered when exposed to the pathogen. Thus, we selected these lines for further assays.

3.3. Effect of GM potato plants resistant to *R. solani* on AM colonization

To study the *in vitro* AM symbiosis, we subsequently used an autotrophic culture system. The *in vitro* AM symbiosis was not significantly affected (p=0.75) by GM potato plants with antifungal activity. The mycorrhization values of *R. intraradices* (GA5 strain) were always below 15 % for all potato lines, including the wild type. No

significant differences were apparent between potato lines for AM arbuscules (p=0.86) or vesicles (p=0.09). Arbuscules were especially scarce (2 %); (Fig. 3A).No significant correlations were observed between *in vitro* potato dry biomass and the percentage of colonized roots. However, AGRC-12 line presented the highest dry weight (data not shown) and also had the highest mycorrhizal values.

To study the interaction of potato transgenic lines with native AM fungal biodiversity from fields traditionally cultivated with potato, we conducted a microcosm assay. AM colonization of AGRC-12 roots was significantly higher ($p<0.001$) than that of wild type roots. For all the evaluated potato lines, the mycorrhization values of the native inoculum were higher than those reached by GA5 inoculation. A significantly higher percentage of arbuscules ($p<0.001$) was evident in AGRC-12 line with respect to other potato lines; however, the proportion of vesicles showed no differences (p=0.6689) (Fig. 3B and Fig. 4).

To investigate the effects of transgenic plant residues that could be plowed into the soil after crop harvest, we performed a microcosm assay employing clover as host plant. In clover roots, the addition of RC-5 dry biomass significantly increased AM symbiosis ($p<0.001$), with respect to the control treatment. After 100 days of cultivation, the mycorrhization values reached by *R. intraradices* (GA5 strain) in clover were higher than those registered in potato plants when colonized by the same strain. Moreover, the addition of RC-5dry biomass resulted in a significant ($p<0.001$) increase of vesicles, but not for arbuscules (p=0.823), with respect to the control (Fig. 3C).

4. Discussion

In this work, we developed and evaluated the *in vivo* biological resistance of GM potato lines against a commercially important phytopathogen. Furthermore, we also verified the short-term effect of GM lines on AM fungal colonization. Four antifungal genes were selected to perform transformation assays. The selected genes encoded an osmotin, a glucanase, a chitinase and a ribosome inactivating protein. These pathogenesis-related proteins exhibited antifungal activity, by altering fungal components or degrading the major constituents of fungal cell walls (β-1.3-glucan and chitin) (Datta et al., 1999). Individual genes expression of cDNAs encoding a class-II chitinase, a class-II beta-1,3-glucanase and a type-I ribosome-inactivating protein were expressed in tobacco plants

under the control of the CaMV 35S-promoter as resistance strategy against *R. solani* (Jach et al., 1995). The combined accumulation of chitinase/glucanase and chitinase/RIP showed a significantly enhanced protection against *R. solani* (Zhu et al., 1994).

From our results, the potato GM selected lines, AG-1, AG-3, RC-1, RC-5, AGRC-8 and AGRC-12, exhibited enhanced infection reduction against *R. solani* in comparison to control plants (KEN). At 7 DPI, the lines expressing high levels of RIP proteins (RC-1 RC-5 and AGRC-12) presented the best pathogen resistance results (IRI of 87 %, 70 % and 65 %, respectively). Nevertheless, at 18 DPI, AG-RC12 line exhibited a 100 % of inhibition of infection. The AGRC-12 line also expressed high levels of chitinase protein; thus the expression levels of this transgenic protein may be related to the ability to confer fungal pathogen protection. Concordantly, previous studies have reported that transgenic plants with higher levels of chitinase transcript accumulation showed higher levels of disease resistance (Takahashi et al., 2005). Furthermore, chitinase levels in rice cultivars correlated with resistance to sheath blight pathogen *R. solani* (Shrestha et al., 2008) and the chitinase activity in transgenic grape vines correlated with an inhibition percentage of fungal growth or disease tolerance (Nookaraju et al., 2012). In addition, resistant GM plants exhibited no visual phenotypic differences from wild-type under greenhouse conditions; which suggests that the constitutive expression of these peptides would not alter the physiology of these plants.

Engineered potatoes conferring resistance to nematodes showed a reduction in microbial activity and different physiological profiles of rhizosphere microbial communities (Griffiths et al., 2000). In addition, transgenic potato plants that can control potato-cyst nematode showed a reduction in bacterial and fungal abundance (Cowgill et al., 2002).

On the other hand, the impact of transgenic potato plants expressing a lysozyme gene on bacterial communities was comparable to the effects of plant genotype, vegetation stage, soil type and pathogen infection (Heuer et al., 2002; Rasche et al., 2006).

Here, we confirmed the effectiveness of the antifungal protein strategy for potato plants and evaluated the harmless to the surrounding beneficial organisms. The assays under *in vitro* and microcosm conditions showed that, not only the symbiosis established by the collection isolate GA5 (*R. intraradices*) and a mix of indigenous AM fungi was developed without alterations in the evaluated transgenic potato plants, but also that the AGRC-12 GM line, with high levels of chitinase protein, improved mycorrhization levels. These data are consistent with previous studies in which pathogen-specific

antifungal proteins could interact differently with AM fungi. Kahlon et al. (2017) reported no adverse effects on AM fungi colonization of GM pea (*Pisum sativum***)** expressing endochitinases with antifungal activity (*in vitro* inhibitory activity on spore germination of several pathogens). Mutualistic fungi seem to be adapted to presence of broad-action antifungal chitinases, and they do not suffer their deleterious effects. The authors proposed that the differential targeting of phytopathogens and beneficial microorganisms by the antifungal genes could be attributed to genotype-related recognition specificity. On the other hand, reports of induction of chitinase isoforms during AM symbiosis as specific response (Pozo et al., 1998; Salzer et al., 2000) suggest that chitinase activities are a key point in the establishment and functioning of the AM symbiosis.

Taking into consideration those changes in root exudates composition and presence of transgenic proteins in other plant organs besides roots might affect non-target soil organisms (Griffiths et al., 2000), we expected to detect an effect of the GM potato biomass residues in clover mycorrhization. Previously, Turrini et al. (2015) have demonstrated that GM corn biomass amended to soil negatively affected *Medicago sativa* root colonization by indigenous AM fungal propagules. However, in the present study, clover plants inoculated with *R. intraradices* (GA5 strain) showed an increase in mycorrhization values in the presence of potato biomass residues of GM lines with respect to residues of wild type potato line. Cesaro et al., (2008) have previously reported that two month-old potato plants that were grown in agricultural systems reached AM colonization values of 6% or less, with low colonization frequency, but with high percentage of arbuscules. On the other hand, in greenhouse experiments with potato as trap plant and soil from long term established monocultures of grasses and forbs as inoculum, the AM root colonization registered values of 8 to 41 % (at the 70^{th} day after inoculation) depending on the origin of the initial inoculum. In our study, potato plants inoculated with *R. intraradices* (GA5 strain) displayed low mycorrhization values. By contrast, in previous studies under similar *in vitro* conditions, this fungal strain has produced higher mycorrhization values in roots of transformed carrots (Silvani et al., 2014) and transformed soybeans (Fernández Bidondo et al., 2009), over 40 % and over 30 %, respectively. Even in clover, the values of root colonization by *R. intraradices* (GA5) was high (78 %) in our study. The potato lines used in our assays seems less receptive to *R. intraradices* pure inoculum than to AM species present in the natural inoculum.

The levels of protection reached by the assessed potato GM lines are promising for this crop, since they could reduce the amount of fungicides and pesticides employed and, therefore, the cost and health risks. Even our results support the need of in-depth analysis in order to monitor each transgenic event in the context of its target soil; we present evidence of the protection conferred by over expression of transgenes codifying antifungal proteins against pathogen attack without detrimental effects on non-target soil microorganisms.

In conclusion, our data suggest that the use of antifungal proteins, singly or in combination, is an interesting strategy for engineering plants to confer fungal protection against commercially relevant pathogens, such as *R. solani*, with no adverse effects on colonization by AM fungi and symbiosis relationship.

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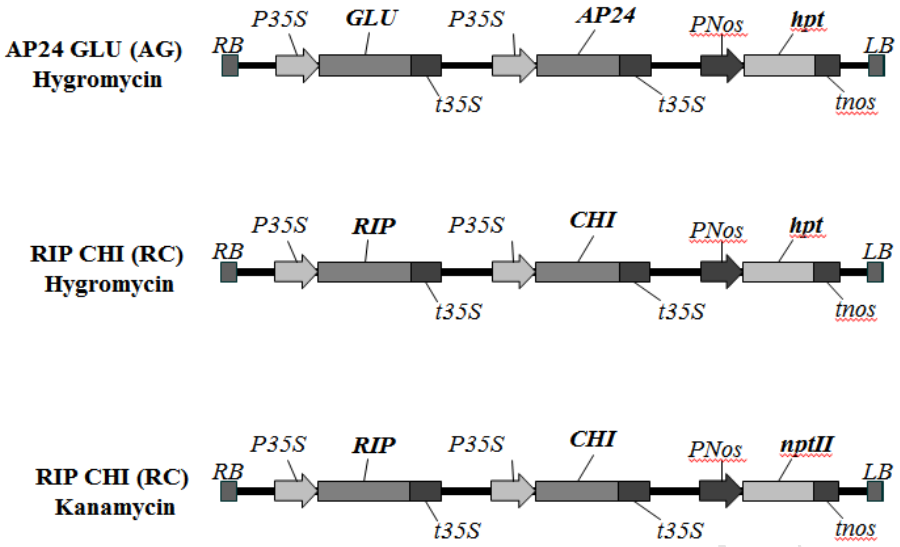
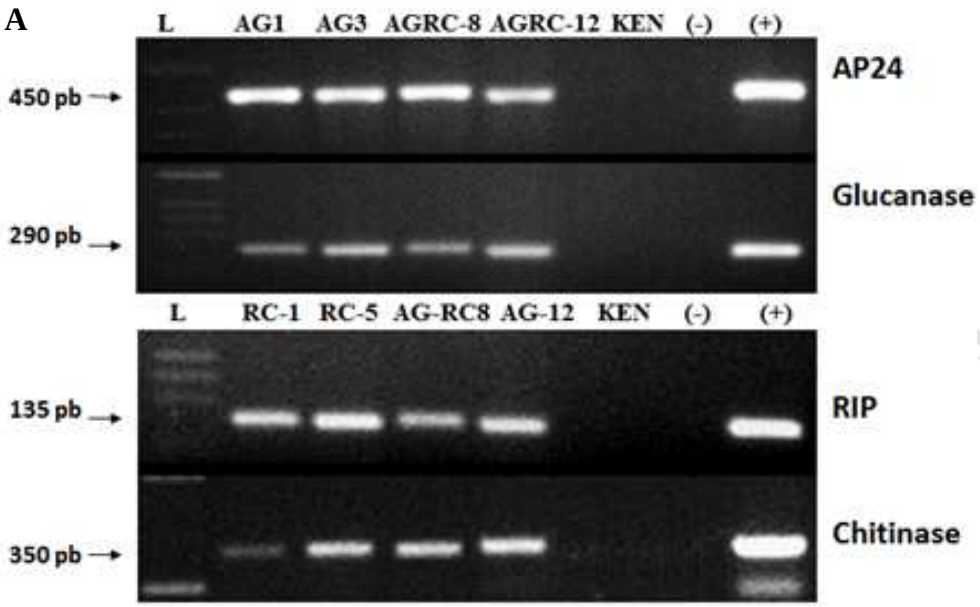
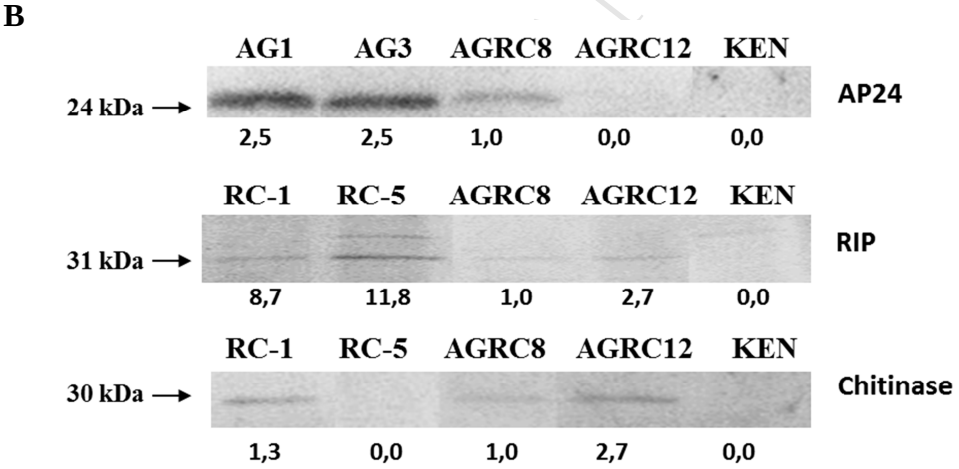
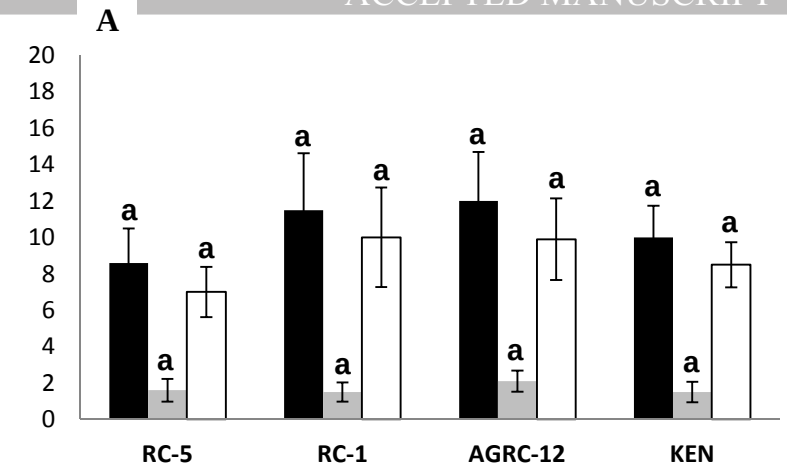
554 **Figure 1.** Schematic representation of the binary vectors used for transformation.
555 AP24: Ap24 gene encoded osmotin; GLU: β-1, 3-glucanase hydrolytic enzyme; CHI:
556 Class II quitinase hydrolytic enzyme; RIP: Type I gene; P35S: CaMV-35S promoter;
557 t35S: CaMV-35S terminator; hpt: hygromycin phosphor transferase; nptII: neomycin
558 phosphor transferase II gene; PNos and tnos: nopaline synthase promoter and
559 terminator; RB and LB, right and left border sequences of the T-DNA region,
560 respectively.
561 Diagrams are not to scale.
562
563 **Figure 2.** Molecular characterization of GM potato lines with antifungal activity. (A)
564 PCR reactions performed with specific primers. Genomic DNAs from GM lines and
565 non-transgenic plants or plasmid vector DNA were used as template. (B) Western blot
566 analysis revealed with specific polyclonal serum. Equal amounts of total protein were
567 loaded on 12.5 % SDS-PAGE. Intensity of the bands was estimated using Image J
568 software. (http://rsb.info.nih.gov/ij/index.html).
569 Designations of the amplified product are shown on the right and product sizes are
570 shown on the left. (–): control reaction without DNA; (+): positive control.
571
572 **Figure 3.** Frequency (%) of mycorrhizal colonization (black bars), arbuscules (grey
573 bars) and vesicles (white bars) in transgenic potato lines (RC-5, RC-1 and AGRC-12)
574 and control line (KEN). Inoculation assays were done with *R. intraradices* (A) or native
575 inoculum (B). Frequency (%) of mycorrhizal colonization (black bars), arbuscules (grey
576 bars) and vesicles (white bars) in clover, as host plant, supplemented with dry biomass
577 of transgenic potato lines (C + RC-5, RC-1 and AGRC-12) and control line (C + KEN).
578 Means of ten replicates ± standard deviation are shown; bars of same color with
579 different letters represent significant differences (LSD test, $p<0.05$) (C).
580
581 **Figure 4.** Active AM intraradical colonization in AGRC-12 potato roots (A) Cortical
582 cell with arbuscules (black arrows). (B) Cortical cell with hyphal coil (black arrow).
583
584 **Supplementary material.** Enhanced resistance of transgenic potato plants to *R. solani*.
585 (A) Percentages of infection inhibition calculated at different days post infection (DPI).
586 (B) Lateral magnified representative view of RC-1 line and non-transgenic control
587 plants growing on non-inoculated (NI) substrate or inoculated (I) substrate, at 7 DPI.

588 Decreased growth exhibited by non-transgenic plants in infected soil is observed. (C)
589 Microscopic diagnostic characteristics of *R. solani*: hyphal septum (S) and 90-degree
590 angle branching with a narrow neck (N) of the hyphae, at 40X magnification. (D)
591 Growth of potato plants in NI or I substrate at 18 DPI.




A
L
AG1
AG3
AGRC-8
AGRC-12
KEN
(-)
(+)
450 pb
AP24
290 pb
Glucanase
L
RC-1
RC-5
AG-RC8
AG-12
KEN
(-)
(+)
135 pb
RIP
350 pb
Chitinase


B
AG1
AG3
AGRC8
AGRC12
KEN
24 kDa
AP24
2,5
2,5
1,0
0,0
0,0
RC-1
RC-5
AGRC8
AGRC12
KEN
31 kDa
RIP
8,7
11,8
1,0
2,7
0,0
RC-1
RC-5
AGRC8
AGRC12
KEN
30 kDa
Chitinase
1,3
0,0
1,0
2,7
0,0


A
20
18
16
14
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10
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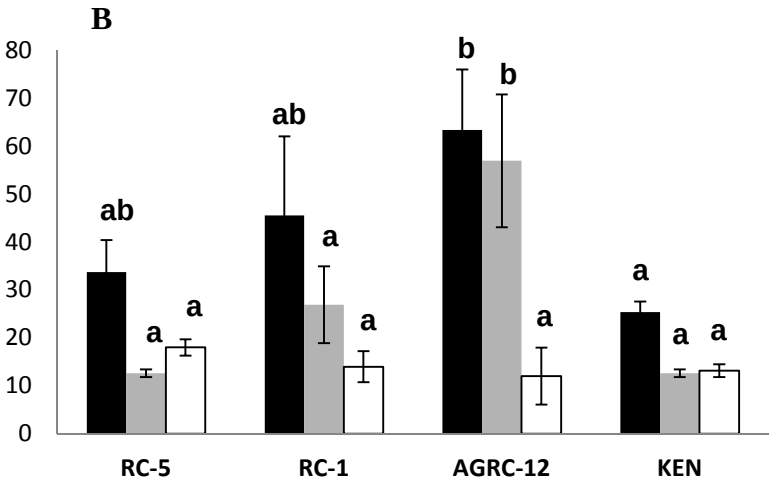
2
0
a a a a a a a a a a a a
RC-5
RC-1
AGRC-12
KEN


B
80
70
60
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40
30
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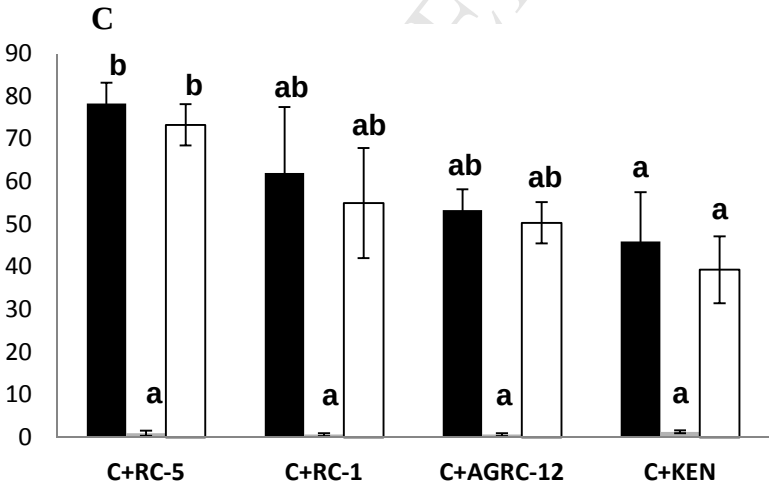
ab a a ab a a b b a a a a
RC-5
RC-1
AGRC-12
KEN


C
90
80
70
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40
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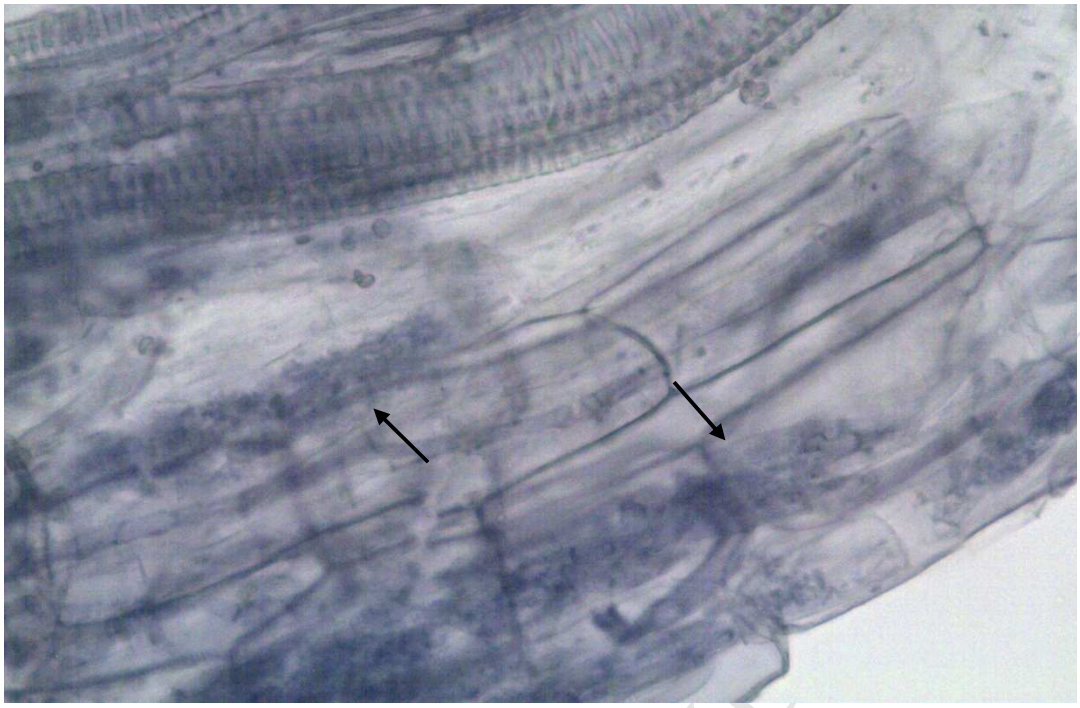
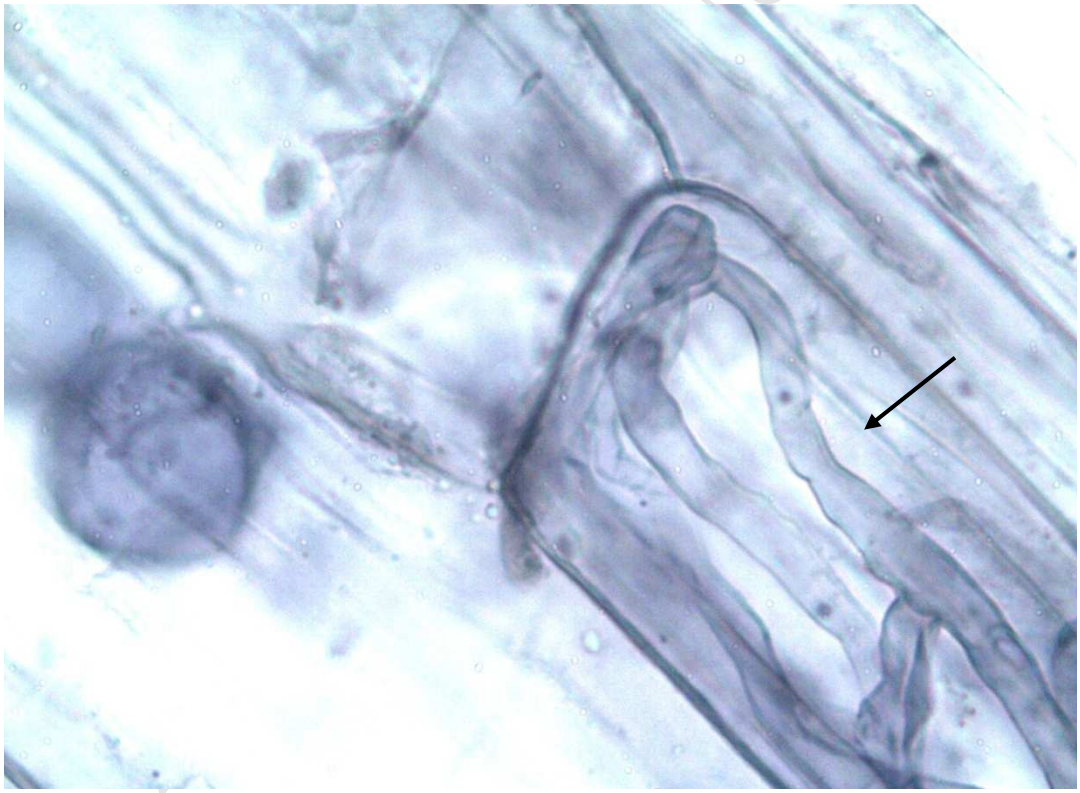
b a b ab a ab ab a ab a a a
C+RC-5
C+RC-1
C+AGRC-12
C+KEN





- Potato transgenic lines expressing genes encoding antifungal proteins has been developed
- These lines showed high reduction in infection indexes against *Rhizoctonia solani*
- *Rizophagus intraradices* colonization did not differ between transgenic and wild potato
- Addition of transgenic potato biomass residues increased mycorrhization
- AGRC-12 transgenic line increased native mycorrhization and arbuscules development