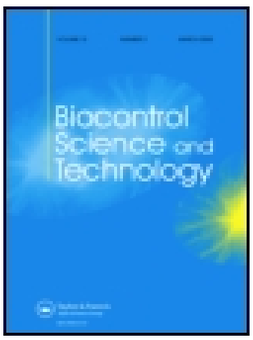

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RESEARCH ARTICLE

Biocontrol of *Macrophomina phaseolina* (Tassi) Goid: differential production of H_2O_2 and $O_2^{\cdot-}$ in the relationship pathogen – PGPR in soybean seedling

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ABSTRACT

We examined the biocontrol and the differential production of H_2O_2 and $O_2^{\cdot-}$ in the relationship *Macrophomina phaseolina* – PGPR in soybean seedling. Fungal colonisation was efficiently prevented by inoculation with *Pseudomonas fluorescens* 9. Its ability to improve ROS production and gene expression of antioxidant enzymes could be related to its capacity to control the disease.

Abbreviations: DAB: 3,3′-Diaminobenzidine; CAT: catalase (EC 1.11.1.6); MDA: malondialdehyde; NBT: nitroblue tetrazolium; PGPR: plant growth-promoting rhizobacteria; ROS: reactive oxygen species; SOD: superoxide dismutase (EC 1.15.1.1); TBARS: thiobarbituric acid reactive substances; TCA: trichloroacetic acid

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Antioxidant defense; *Macrophomina phaseolina;* oxidative stress; PGPR; ROS

Macrophomina phaseolina (Tassi) Goid, the causal agent of charcoal rot, is a soilborne fungus that causes significant yield losses in soybean in Argentina and other soybean-growing regions of the world (Gupta, Sharma, & Ramteke, 2012; Pérez Brandan, Díaz, Carmona, & March, 2009). The fungus affects more than 500 plant species, including soybeans, on which it causes seed rot, root and stem rot, senescence, and death of seedlings and adult plants. Several situations, such as the lack of cultivars with complete resistance, the successful survival of *M. phaseolina* in soil by microsclerotia, and the lack of residuality of the classical fungicide seed treatments, have hindered the development of effective disease management practices. Therefore, the search for alternative control methods, for instance, the use of plant growth-promoting rhizobacteria (PGPR) as biological control agents, could be a feasible measure to reduce the impact of charcoal rot (van Loon, 2007). In a previous report (Simonetti et al., 2015), *Pseudomonas fluorescens* 9 and *Bacillus subtilis* 54, collected from fields in Argentina, were isolated, identified,

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characterised, and evaluated for their antagonistic ability on *M. phaseolina* both in seeds and plants.

Reactive oxygen species (ROS) are widely related to oxidative stress and tissue damage. However, ROS are also key signalling molecules involved in the control of plant growth and development (Foyer & Shigeoka, 2011). Also, ROS have been associated with plant defense due to their antimicrobial activity or their contribution to the lignification of host cell walls that avoid pathogen colonisation of the host tissue (Daub, Herrero, & Chung, 2013; Grant & Loake, 2000). Superoxide dismutase (SOD; EC 1.15.1.1) constitutes the primary step of cellular defense and dismutates $O_2^{\cdot -}$ to H_2O_2 and O_2. The accumulation of H_2O_2 is then prevented by the action of enzyme catalase (CAT; EC 1.11.1.6) or by ascorbic acid-glutathione cycle.

In order to better understand the plant host responses to the pathogen–PGPR interaction, in this work, we carried out *in vitro* assays to explore ROS production and antioxidant system in soybean seedlings preinoculated with *P. fluorescens* 9 and *B. subtilis* 54 and then infected by *M. phaseolina*.

The isolate of *M. phaseolina* and the two strains of bacteria used (*P. fluorescens* 9 and *B. subtilis* 54) that are stored in the Fungi Bank of Plant Pathology Department of the Faculty of Agronomy UBA were selected on the basis of the results previously reported by Simonetti et al. (2015). The soybean cultivar used in the experiments was NIDERA A 4990RG, which proved susceptible to *M. phaseolina* in the previous report (Simonetti et al., 2015). To study the effects of the inoculation of the seeds with the bacteria, the seeds were first surface-disinfected and then submerged in either sterile water (negative control) or in a suspension of strains 9 or 54 (10^7 CFU mL^{-1} in sterile water) for 15 min. Control and treated seeds were plated on Petri dish with PDA (Merck) fully covered by mycelia and microsclerotia of *M. phaseolina*. Plates without the fungus were maintained as uninfected controls. The following treatments were performed: untreated seeds (C); *M. phaseolina* infected seeds (M); *P. fluorescens* (9) and *B. subtilis* 54 (54) inoculated seeds; 9 and 54 preinoculated and infected with the fungus (9M and 54M, respectively). Thus, after incubation at 28°C for 4–7 days (Beas-Fernández, De Santiago, Hernández-Delgado, & Pérez-Mayek, 2006), the symptoms caused by the pathogen were evaluated according to a previously published disease scale (Simonetti et al., 2015). Control efficiency of the disease was assessed as a percentage using Abbott's formula (1925): $[(X - Y)/X] \times 100$, where $X = \%$ infection of *M. phaseolina* in control seeds and $Y = \%$ infection in treated seeds.

Each treatment consisted of six PDA plates/treatment, with six seeds per plate. The experiments were repeated three times.

Lipid peroxidation was measured as the amount of TBARS determined by the TBA reaction as described by Heath and Packer (1968). Roots after germination were homogenised in 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $3500 \times g$ for 20 min. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Extracts for the determination of SOD and CAT activities were prepared from 0.3 g of roots homogenised under ice-cold conditions in 3 mL of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP and 0.5% (v/v) Triton X-100 at 4°C. The homogenates were centrifuged at $10{,}000 \times g$ for 20 min and the supernatant fraction was used for the assays. CAT activity was determined by measuring the decrease

in absorption at 240 nm (Chance, Sies, & Boveris, 1979). Total SOD activity was assayed as described by Becana, Aparicio-Tejo, Irigoyen, and Sánchez-Díaz (1986). The reduction in NBT was followed by reading absorbance at 560 nm. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions.

In order to analyse *in situ* H_2O_2 generation and $O_2^{\cdot-}$ production, roots were immersed in a 1% solution of 3,3′-Diaminobenzidine (DAB) and a 0.1% solution of NBT, respectively, until the appearance of characteristic spots and were quantified according to Santa-Cruz et al. (2014).

Total RNA was isolated using Trizol reagent (Invitrogen), treated with RNase-free DNase I (Promega), and reverse transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen). Quantitative RT-PCR was performed using soybean-specific primers for Cu/Zn SOD (sense primer: 5′-GGTTGTGAGGATTCTCGGTTCA-3′, antisense primer: 5′-GTCTGCTCCAAAGCTTCAGTCA-3′) and CAT (sense primer: 5′-GGAGCTAGCGCAAAGGGTTT-3′, antisense primer: 5′-CGGCACATGTGAGGTGAGAA-3′). Samples were assayed in triplicate using Power SYBR Green master mix on a StepOne real-time PCR system (Applied Biosystems). The threshold cycle (Ct) values were normalised against the reference gene for 18S rRNA. Results were calculated using the Relative Quantification ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001) and are presented as the fold change in gene expression normalised and relative to the untreated control.

Protein concentration was quantified by the method of Bradford (1976) using bovine serum albumin as a standard.

All values reported in this work represent the means of three independent experiments. The mean values ± standard deviation (SD) is given in figures. A one-way analysis of variance test was used to confirm the significance of the data. Comparison with control and treatments was performed using the Tukey test. Statistical analysis was performed using Infostat software (Balzarini et al., 2008).

The percentage of disease control was determined according to the ability of the PGPR to avoid or reduce fungal colonisation on seeds. The results showed that disease severity on microbiolised seeds was decreased significantly compared to non-microbiolised control seeds. Inoculation with *P. fluorescens* 9 was the most effective treatment to control the infection (54%), while *B. subtilis* 54 allowed a partial colonisation of rootlets by *M. phaseolina* (30%).

With the aim to evaluate the oxidative stress generated by *M. phaseolina* infection, lipid peroxidation was quantified as malondialdehyde (MDA) content in 4-day-old soybean seedlings (Figure 1). MDA levels were twofold increased by fungal colonisation. Preinoculation with both PGPR strains reverted TBARS formation to control levels in infected seedlings. Despite this result, *P. fluorescens* 9 and *B. subtilis* 54 induced MDA content in uninfected radicles (40% and 69%, respectively).

To identify those ROS that were implicated in TBARS formation, *in situ* staining were performed on 4-day-old rootlets. The content of $O_2^{\cdot-}$ was evaluated by NBT staining. As can be seen in Figure 2(A), *M phaseolina* suppressed $O_2^{\cdot-}$ production in all treatments. In contrast, in non-infected rootlets, the $O_2^{\cdot-}$ content varied according to the applied treatment: 9 and 54 strains produced an increase of 28% and 76%, respectively. H_2O_2 production was estimated by DAB staining (Figure 2(B)). Rootlets infected with

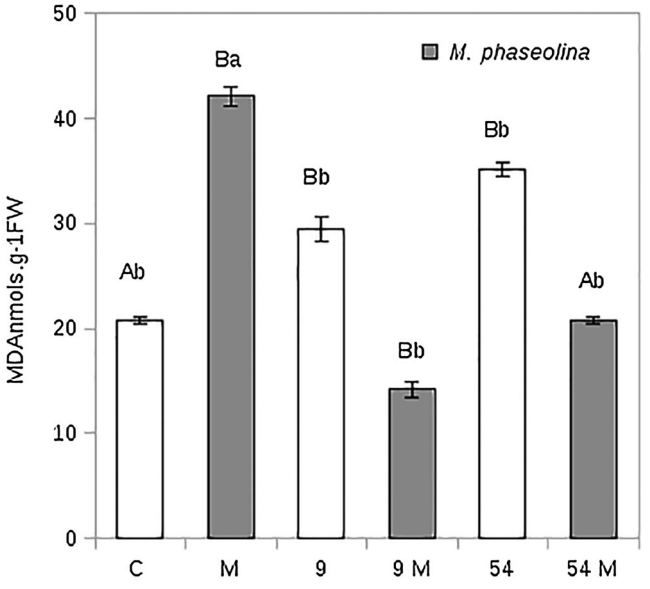

Figure 1. Lipid peroxidation in soybean embryo axes. Mean values for the three independent experiments. Grey bars indicate fungal infected seedlings. Error bars refer to one standard deviation.

Note: Different lowercase letters denote statistical differences ($P < 0.05$) with respect to untreated controls (C); different capital letters denote statistical differences ($P < 0.05$) with respect to infected controls (M) according to Tukey's multiple range test.

M. phaseolina showed a 2.3-fold increase in H_2O_2 levels. Both strains, *P. fluorescens* 9 and *B. subtilis* 54, improved partially H_2O_2 level.

In order to evaluate whether plant host antioxidant response was activated in this context, enzymatic activity and gene expression of two main antioxidant enzymes, SOD and CAT, were assayed. SOD activity decreased in all treatments (between 20% and 49%), except for the 9M case (Figure 2(C)). In contrast, CAT activity was affected only by the fungus (54% lower) while the rest of the treatments remained close to control value (Figure 2(D)). The gene expression of SOD and CAT was determined by real-time PCR. Interestingly, both enzyme transcripts were downregulated in the presence of *M. phaseolina* (between 20% and 60% with respect to uninfected roots). The pre-treatment with *B. subtilis* 54 failed to revert this inhibition, while the two transcripts were significantly upregulated by *P. fluorescens* 9: 3.2-fold for CAT and 3.4-fold for SOD, in comparison to infected seedlings.

Both isolates of bacteria confirmed the antifungal activity against *M. phaseolina* previously demonstrated by Simonetti et al. (2015). Ebtehag, Nemat, Azza, and Hoda (2009) also reported the effect of definite PGPR such as *Bacillus megaterium, Bacillus cereus*, and *Pseudomonas* for their biocontrol efficacy against *M. phaseolina* for soybean seedlings.

The role of antioxidant responses in soybean plants infected by *Corynespora cassiicola* has been studied by Fortunato, Debona, Bernardeli, and Rodrigues (2015), while Kumari, Sharma, Alam, and Sharma (2015) reported an oxidative stress induced by *M. phaseolina* in two sorghum genotypes. The results presented in this study show that *M. phaseolina* and the two PGPR strains trigger lipid peroxidation. This behaviour seems to be related

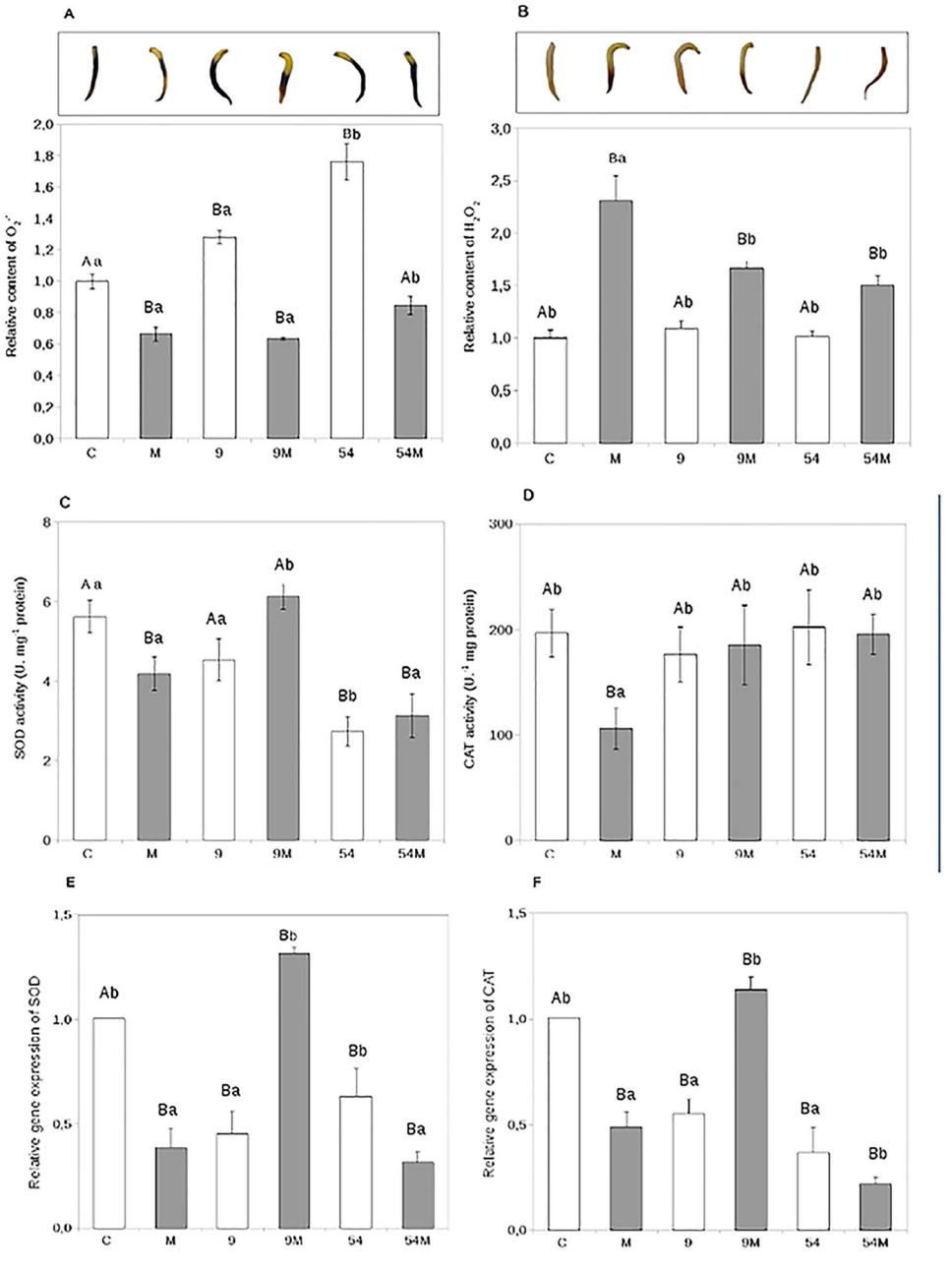

Figure 2. Differential ROS production and antioxidant enzymatic host response against fungal colonisation and PGPR pre inoculation. Histochemical detection of $O_2^{\cdot-}$ (A) and H_2O_2 (B), SOD activity (C), CAT activity (D) and relative gene expression of SOD (E) and CAT (F) transcripts of soybean embryo axes. Values are the mean of three independent experiments. Gray bars indicate fungal-infected seedlings. Error bars refer to one standard deviation. (A,B) are representative of four different experiments. Hydrogen peroxide and superoxide anion deposits were quantified by measuring the number of pixels of spots area versus total root area using the NIH Image program (National Institutes of Health, USA).

Note: Different lowercase letters denote statistical differences ($P < 0.05$) with respect to untreated controls (C); different capital letters denote statistical differences ($P < 0.05$) with respect to infected controls (M) according to Tukey's multiple range test.

to a differential accumulation of ROS. The fungal pathogen provokes an overproduction of H_2O_2, and a similar result was reported by Morkunas, Bednarski, and Kozlowska (2004). In contrast, *P. fluorescens* 9 and *B. subtilis* 54 induce $O_2^{\cdot-}$ production. However, in the two combined PGPR-fungus treatments, 9M and 54M, TBARS and ROS levels were reduced. It should be noted the antagonistic effects observed in both combined treatments: PGPRs prevents H_2O_2 triggered by fungus and *M. phaseolina* suppresses $O_2^{\cdot-}$ induced by PGPRs. A low SOD activity was measured in all treatments, with the exception of 9M that remained in control values. SOD gene expression was consistent with its activity. Catalase activity was also inhibited by *M. phaseolina* and this could explain the high H_2O_2 levels. Interestingly, 9M and 54M maintained the CAT activity close to control values and partially prevented H_2O_2 formation. The CAT transcript was downregulated in all treatments but overexpressed in 9M. These results would indicate that the improvement of the oxidative stress parameters could be related to a lower ROS production rather than a more activated antioxidant defense.

The present study was successful in demonstrating that although seedlings responded similarly to both PGPR preinoculations, there was a differential behaviour when fungal colonisation started. Only 9M induced the host antioxidant defense transcripts. This is in concordance with a better disease control. In the *B. subtilis* 54 case, the partially achieved disease control is obtained through other mechanisms which cannot be associated with an active antioxidant system. Further trials are needed to determine the behaviour and effectiveness of these isolates under different soybean genotypes and field conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*, 265–267.

Balzarini, M., Gonzalez, L., Tablada, M., Casanoves, F., Di Rienzo, J., & Robledo, C. (2008). *Infostat: Manual de ususario*. Córdoba: Edición Electrónica.

Beas-Fernández, R., De Santiago, A., Hernández-Delgado, S., & Pérez-Mayek, N. (2006). Characterization of Mexican and no-Mexican isolates of *Macrophomina phaseolina* base on morphological characteristics, pathogenicity on bean seeds an endoglucanase genes. *Journal of Plant Pathology*, *88*, 53–60.

Becana, M., Aparicio-Tejo, P., Irigoyen, J. J., & Sánchez-Díaz, M. (1986). Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. *Plant Physiology*, *82*, 1169–1171.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Review*, *59*, 527–605.

Daub, M. E., Herrero, S., & Chung, K. R. (2013). Reactive oxygen species in plant pathogenesis: The role of perylenequinone photosensitizers. *Antioxidants & Redox Signaling*, *19*, 970–989.

Ebtehag, E.-B., Nemat, M. A., Azza, S. T., & Hoda, A. H. (2009). Antagonistic activity of selected strains of rhizobacteria against *Macrophomina phaseolina* of soybean plants. *American-Eurasian Journal Agricultural Environmental Science*, *5*, 337–347.

Fortunato, A. A., Debona, D., Bernardeli, A. M. A., & Rodrigues, F. A. (2015). Changes in the antioxidant system in soybean leaves infected by *Corynespora cassiicola*. *Phytopathology*, *105*, 1050–1058.

Foyer, C. H., & Shigeoka, S. (2011). Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*, *155*, 93–100.

Grant, J. J., & Loake, G. J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology*, *124*, 21–30.

Gupta, G. K., Sharma, S. K., & Ramteke, R. (2012). Biology, epidemiology and management of the pathogenic fungus Macrophomina phaseolina (Tassi) Goid with special reference to charcoal rot of soybean (Glycine max (L.) Merr.). *Journal of Phytopathology*, *160*, 167–180.

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives Biochemistry Biophysics*, *125*, 189–198.

Kumari, N., Sharma, I., Alam, A., & Sharma, V. (2015). Oxidative stress and role of antioxidant machinery in two cultivars of sorghum (*Sorghum bicolor* L.) to combat the damage induced by *Macrophomina phaseolina*. *European Journal of Biotechnology and Bioscience*, *3* (4), 5–13.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)). *Methods*, *25*, 402–408.

Morkunas, I., Bednarski, W., & Kozlowska, M. (2004). Response of embryo axes of germinating seeds of yellow lupine to *Fusarium oxysporum*. *Plant Physiology Biochemistry*, *42*, 493–499.

Pérez Brandan, C., Díaz, C., Carmona, M., & March, G. (2009). Comportamiento de variedades de soja frente a infecciones naturales de la podredumbre carbonosa de la soja (*Macrophomina phaseolina*) en Salta (Argentina). EEAA Marcos Juárez Ed: INTA (pp. 41–45), ISSN 1851-9245.

Santa-Cruz, D. M., Pacienza, N., Zilli, C. G., Tomaro, M. L., Balestrasse, K. B., & Yannarelli, G. G. (2014). Nitric oxide induces specific isoforms of antioxidant enzymes in soybean leaves subjected to enhanced ultraviolet-B radiation. *Journal of Photochemistry and Photobiology B: Biology*, *141*, 202–209.

Simonetti, E., Pin Viso, N., Montecchia, M., Zilli, C. G., Balestrasse, K. B., & Carmona, M. (2015). Evaluation of native bacteria and manganese phosphite for alternative control of charcoal root rot of soybean. *Microbiological Research*, *180*, 40–48.

van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *European Journal Plant Pathology*, *119*, 243–254.