Short communication

Biocontrol of the fungal pathogen *Colletotrichum acutatum* in strawberry plants mediated by *Azospirillum argentinense* REC3 and its flagellar protein AzFlap

Biocontrol del patógeno fúngico *Colletotrichum acutatum* en plantas de frutilla mediado por *Azospirillum argentinense* REC3 y su proteína flagelar AzFlap

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Abstract

Strawberry (*Fragaria ananassa* Duch.) is an intensive crop demanding high quantities of fertilizers and pesticides, mainly to prevent yield loss due to pathogens caused diseases. When applied in excess, agrochemicals could damage the environment. Therefore, looking for alternatives to reduce partially the amounts of agrochemicals applied is of great interest nowadays. In this work, we demonstrate that the application of *Azospirillum argentinense* REC3 and its flagelline AzFlap on strawberry plants allow bicontrol of the disease Anthracnose, caused by the fungus *Colletotrichum acutatum* M11. This was confirmed by the reduction of the disease severity index and the area under disease progress curve excerted by REC3 and AzFlap on strawberry plants.

Keywords: Anthracnose; Disease Severity Index; MAMP; PGPB.

Resumen

La frutilla (*Fragaria ananassa* Duch.) es un cultivo intensivo que demanda altas cantidades de fertilizantes y pesticidas, principalmente para evitar la pérdida de rendimiento debido a enfermedades causadas por patógenos. Por lo tanto, es de gran interés en la actualidad buscar alternativas para reducir parcialmente las cantidades de agroquímicos aplicados. En este trabajo demostramos que la aplicación de *Azospirillum argentinense* REC3 y su flagelina AzFlap en plantas de frutilla permiten el bicontrol de la enfermedad Antracnosis, causada por el hongo *Colletotrichum acutatum* M11. Esto fue confirmado por la reducción del índice de severidad de la enfermedad y el área bajo la curva de progreso de la enfermedad ejercida por REC3 y AzFlap en plantas de frutilla.

Palabras clave: Antracnosis; Índice de severidad de enfermedad; MAMP; PGPB.

Plant growth promoting bacteria (PGPB) are beneficial microorganisms that colonize plants and promote growth due to different mechanisms. PGPB provide plants with nutrients, such as nitrogen and phosphorous, synthesize siderophores to increase iron uptake and produce phytohormones involved in growth and development. They also improve plant resistance to biotic and abiotic stresses by different strategies, allowing the plant to use its resources for growth and a better physiological state (Bashan and de Bashan, 2010). These bacteria can produce toxic substances, antibiotic, and hydrolytic enzymes that degrade fungal cell wall. They also compete with the pathogens for nutrients and colonization surface, and provoke changes in plant metabolism inducing systemic resistance immunizing plants against pathogens (Bashan and de Bashan, 2005). All these mechanisms allow PGPB to control pathogens affecting yield in crops.

On the other hand, plants are able to recognize certain microbe-associated molecular patterns (MAMPs) that activate an effective immune response in distal tissues (Pieterse *et al.*, 2009).

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These are conserved molecules among different organisms, recognized by specific plant receptors. The peptide flg22 obtained from bacterial flagellin is a well-known MAMP recognized by a specific receptor in plants (Zipfel, 2009) capable of inducing defense responses (Felix *et al.*, 1999).

It was reported that *Azospirillum* has a polar flagellum responsible for bacterial movement in a liquid medium and is involved in the adsorption of *Azospirillum* on the roots (Croes *et al.*, 1993) constituting a fundamental step in the bacterial root colonization and subsequent mechanisms of plant growth promotion (Steenhoudt and Vanderleyden, 2000). The filament of the flagella is composed by a protein flagellin, which in *A. brasilense* Sp7 is a glycoprotein of approximately 100 kDa (Moens *et al.*, 1995).

The PGPB *Azospirillum brasilense* REC3, currently reclassified as *A. argentinense* REC3 (dos Santos Ferreira *et al.*, 2022), was isolated from strawberry plants in Tucumán, Argentina (Pedraza *et al.*, 2007) and was demonstrated that is capable of protecting strawberry plants from two main fungal pathogens: *Colletotrichum acutatum* (Tortora *et al.*, 2012) and *Macrophomina phaseolina* (Elías *et al.*, 2021). Nevertheless, when applying beneficial bacteria to plants for providing biocontrol it is a main condition to maintain its viability, which can be difficult due to the sensibility of bacterial cells to different factors, such as dehydration, UV radiation, etc.

Recently, REC3 flagellin named AzFlap, was purified and evaluated as a MAMP (Elías *et al.*, 2021). This protein is capable of inducing different defense responses in strawberry plants and it was demonstrated that its application on strawberry leaves diminish crown and root rot caused by *M. phaseolina* (Elías *et al.*, 2021).

Application of a beneficial bacterium as *Azospirillum* or bioproducts such as MAMPS provides not only a way to improve yields but to protect plants from infections and diseases. In this work, we propose that the application of AzFlap and REC3 on strawberry plants allow bicontrol of the disease Anthracnose, caused by the fungus *C. acutatum* M11.

To confirm this hypothesis, a biocontrol assay was carried out on strawberry plants cv. Camarosa. AzFlap was obtained as described by Elías *et al.* (2021) and *A. argentinense* REC3 was grown on NFb liquid medium (Baldani *et al.*, 2014) 24 h, at 30 °C and agitation.

Strawberry plants cv. Camarosa were treated with i) sterile H_2Od; ii) *A. argentinense* REC3 (~10^6 UFC/ml) inoculated by irrigation 15 days before inoculation of the pathogen; and ii) AzFlap (200 nM) applied by spraying the leaves 3 days before fungal inoculation. Three independent assays were carried out, with 5 plants per treatment.

The strain M11 of *C. acutatum* (Salazar *et al.*, 2007) was grown on potato glucose agar medium (PGA) for 7 days under continuous fluorescent light at 28 °C to induce conidial formation (Smith and Black 1990). The culture surface was scraped to remove conidia and then resuspended in sterile distilled water. The conidial suspension was filtered through sterile gauze to remove mycelial debris and diluted with sterile distilled water to a final concentration of ~1.5×10^6 conidia/ml and applied to plants by spraying the leaves up to runoff (Tortora *et al.*, 2012).

Infected plants were put into an infection chamber at 28 °C, with 100% relative humidity (RH) and under continuous light for 48 h. Then, conditions changed to 28 °C, 70% RH with a light cycle of 16 h/day (250 µmol photons m^2/s).

For evaluating Anthracnose severity, the severity scale of Delp and Milholand (1980) was used, based on the length of the lesions in the petioles (Figure 1).

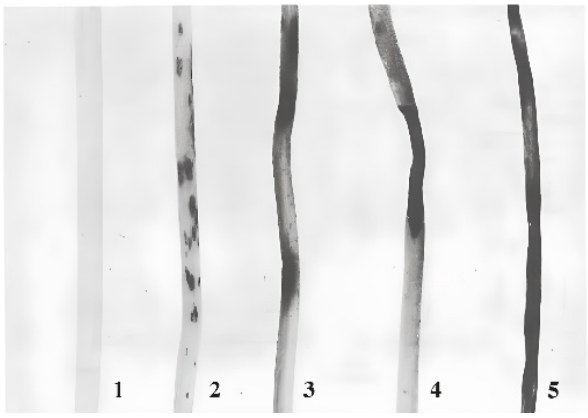

Figure 1. Severity scale of Anthracnosis disease showing lesions on strawberry petioles: 1(without symptoms), 2 (≤3 mm), 3 (3–10 mm), 4 (10–30 mm) or 5 (30 mm), adapted from Delp and Milholland (1980).

The effect of biocontrol was determined according to Chalfoun *et al.* (2018), calculating Anthracnose disease severity index (DSI) according to the severity scale:

$$DSI\% = \frac{\sum(A \times 0 + B \times 3 + C \times 10 + D \times 30 + E \times 50)}{T \times 50} \times 100$$

A, B, C, D, and E are the number of petioles corresponding to the numerical grade 1, 2, 3, 4, and 5, respectively.

T is the total number of petioles multiplied by the maximum severity grade 5, where T = A + B + C + D + E.

An Anthracnose severity of 0% was given to plants where no disease was present and 100% to plants where all petioles were assigned a score of 5.

With the Anthracnose DSI values determined at 14 and 21 days post inoculation (dpi), we calculated the Area Under Disease Progress Curve (AUDPC) (Madden *et al.*, 2007) for each plant and treatment according to the following formula:

$$AUDPC = \sum \frac{(X_i + X_{i+1})}{2} \times (t_{i+1} - t_i)$$

where X_i corresponds to disease severity (%) at assessment i, X_{i+1} corresponds to the severity (%) at subsequent assessment $i + 1$, and $(t_{i+1} - t_i)$ corresponds to the number of days between the two consecutive assessments.

A comparison was made between the AUDPC for Anthracnose DSI for each treatment and the AUDPC for disease control plants treated with H_2Od (AUDPC/P). If AUDPC/P < 1, it indicates biocontrol or resistance induction, whereas an AUDPC/P ≥ 1 indicates that there weren´t disease control.

The statistical analysis was performed using InfoStat (Di Rienzo *et al.*, 2013). The biocontrol effect of the treatment was analyzed with analysis of variance ANOVA. AUDPC/P values were compared with Tukey Test ($p < 0.05$). Different letters indicate significant differences among treatments.

As a result, we observed a biocontrol effect of Anthracnose disease in strawberry plants treated with the bacteria and its protein AzFlap. Plants inoculated with REC3 showed an AUDPC/P of 0.43, indicating a protection effect against M11, as well as AzFlap, which AUDPC/P was 0.45 (Table 1). These results demonstrate that both the bacteria and the protein diminish the deleterious effect of *C. acutatum* M11 in strawberry plants, constituting an alternative to the use of pesticides.

Strawberry is an intensive crop demanding high quantities of fertilizers and pesticides, mainly to prevent yield loss due to pathogens caused diseases. When applied in excess, agrochemicals could damage the environment (Ajwa *et al.*, 2003). Therefore, looking for alternatives, such as the use of bio-inputs to reduce partially the amounts of agrochemicals, is of great interest nowadays. It was demonstrated in previous works that *A. argentinense* REC3 and its flagellin AzFlap are capable of inducing defense responses such as phytohormones production like ethylene and salicylic acid, cell wall reinforcement by callose and lignin depositions, stomatal closure, oxidative burst, lipidic peroxidation, and differential expression of defense-related genes (Tortora *et al.*, 2011 y 2012; Guerrero-Molina *et al.*, 2015; Elías *et al.*, 2018 y 2021). Combined, all these immune responses might contribute to *C. acutatum* M11 biocontrol, as observed in this work, diminishing the incidence of Anthracnose. Even though the bacteria nor the protein wouldn't replace totally the amount of agrochemicals used, they can replace part of them, contributing to the environmental protection.

Table 1. Biocontrol of Anthracnose disease in strawberry plants cv. Camarosa, expressed as Area Under Disease Progress Curve per plant values.

Treatment	AUDPC/P	
H_2Od	1.00 ± 0.00	b
***A. argentinense* REC3**	0.43 ± 0.12	a
AzFlap 200 nM	0.45 ± 0.13	a

Different letters indicate significant differences (Tukey Test, $p < 0.05$).

As conclusion, it was confirmed that AzFlap acts as a MAMP and, as well as *A. argentinense* REC3, they are capable to control the pathogen *C. acutatum* in strawberry plants by reducing the disease severity index and the area under disease progress curve. However, it will be necessary to carry out further studies under field conditions to corroborate these results, and not only in strawberry plants, but also in other crops of agronomic interest.

References

Ajwa H.A., Klose S., Nelson S.D., Minuto A., Gullino M.L., Lamberti F., López-Aranda J.M. (2003). Alternatives to methyl bromide in strawberry production in the United States of America and the Mediterranean region. Phytopathologia Mediterranea 42: 220-244.

Baldani J.I., Reis V.M., Videira S.S., Boddey L.H., Divan Baldani V.L. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for

microbiologists. Plant Soil 384: 413-431.

Bashan Y., De-Bashan L.E. (2005). Plant growth-promoting. In: Encyclopedia of soils in the environment, 1st edn. Hillel D. (ed.). Elsevier, Oxford, United Kingdom. Pp 103-115.

Bashan Y., De-Bashan L.E. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. In: Advances in agronomy, 1st edn. Elsevier Inc., Amsterdam, Netherlands. Pp 77-136.

Chalfoun N.R., Durman S.B., Budeguer F., Caro M.D.P., Bertani R.P., Di Peto P., Castagnaro, A.P. (2018). Development of PSP1, a biostimulant based on the elicitor AsES for disease management in monocot and dicot crops. Frontiers in Plant Science 9: 844.

Croes C., Moens S., Bastelaere E., Vanderleyden J., Michiels K.W. (1993). The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. Journal of General Microbiology 139: 2261-2269.

Delp B.R., Milholland R.D. (1980). Evaluating strawberry plants for resistance to *Colletotrichum fragariae*. Plant Disease 64: 1071-1073.

Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., Robledo C.W. (2013). InfoStat version 2018 Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. http:// www.infostat.com.ar

dos Santos Ferreira N., Coniglio A., Puente M., Sant'Anna F.H., Maroniche G., García J., Molina R., Nievas S., Gazolla Volpiano C., Ambrosini A., Passaglia L.M.P., Pedraza R.O., Massena Reis V., Zilli J.E., Cassán F. (2022). Genome-based reclassification of *Azospirillum brasilense* Az39 as the type strain of *Azospirillum argentinense* sp. nov. International Journal of Systematic and Evolutionary Microbiology 72: 005475.

Elías J.M., Guerrero-Molina M.F., Martínez-Zamora M.G., Díaz-Ricci J.C., Pedraza R.O. (2018). Role of ethylene and related gene expression in the interaction between strawberry plants and the plant growth-promoting bacterium *Azospirillum brasilense*. Plant Biology 20 (3): 490-496.

Elías J.M., Ramirez-Mata A., Albornóz P.L., Baca B.E., Diaz-Ricci J.C., Pedraza R.O. (2021). The polar flagellin of *Azospirillum brasilense* REC3 induces a defense response in strawberry plants against the fungus *Macrophomina phaseolina*. Journal of Plant Growth Regulation 41 (7): 2992-3008.

Felix G., Duran J.D., Volko S., Boller T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 18 (3): 265-276.

Guerrero-Molina M.F., Lovaisa N.C., Salazar S.M., Martinez-Zamora M.G., Diaz-Ricci J.C., Pedraza R.O. (2015). Physiological, structural and molecular traits activated in strawberry plants after inoculation with the plant growth-promoting bacterium *Azospirillum brasilense* REC3. Plant Biology 17: 766-773.

Madden L.V., Hughes G., Bosch F.V.D. (2007). The study of plant disease epidemics. St. Paul, MN: The American Phytopathological Society Press, USA.

Moens S., Michiels K., Vanderleyden J. (1995). Glycosylation of the flagellin of the polar flagellum of *Azospirillum brasilense*, a gramnegative nitrogen-fixing bacterium. Microbiology 141: 2651-2657.

Pedraza R.O., Motok J., Tortora M.L., Salazar S.M., Diaz-Ricci J.C. (2007). Natural occurrence of *Azospirillum brasilense* in strawberry plants. Plant Soil 295: 169-178.

Pieterse C.M.J., Leon-Reyes A., Van Der Ent S., Van Wees S.C.M. (2009). Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308-316.

Salazar S.M., Castagnaro A.P., Arias M.E., Chalfoun N., Tonello U., Diaz-Ricci J.C. (2007). Induction of a defense response in strawberry mediated by an avirulent strain of *Colletotrichum*. European Journal of Plant Pathology 117: 109-122.

Smith B.J., Black L.L. (1990). Morphological, cultural and pathogenic variation among *Colletotrichum* species isolated from strawberry. Plant Disease 74: 69-76.

Steenhoudt O., Vanderleyden J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiology Reviews 24: 487-506.

Tortora M.L., Diaz-Ricci J.C., Pedraza R.O. (2011). *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum*. Archives of Microbiology 193: 275-286.

Tortora M.L., Diaz-Ricci J.C., Pedraza R.O. (2012). Protection of strawberry plants (*Fragaria ananassa* Duch.) against anthracnose disease induced by *Azospirillum brasilense*. Plant Soil 356: 279-290.

Zipfel C. (2009). Early molecular events in PAMP-triggered immunity. Current Opinion in Plant Biology 12: 414-420.