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Biological Control

e-Xtra*

A Bacterial Endophyte from Apoplast Fluids Protects Canola Plants from Different Phytopathogens via Antibiosis and Induction of Host Resistance

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ABSTRACT

Endophytic bacteria colonize inner plant tissues and thrive at the apoplast, which is considered its main reservoir. Because this niche is the place where the main molecular events take place between beneficial and pathogenic microorganisms, the aim of this work was to characterize culturable endophytic bacteria from apoplastic fluids obtained from field-grown canola leaves and analyze their potential for biological control of diseases caused by *Xanthomonas campestris, Sclerotinia sclerotiorum*, and *Leptosphaeria maculans*. Dual-culture analysis indicated that three isolates (Apo8, Apo11, and Apo12) were able to inhibit the growth of all three phytopathogens. Sequencing of the 16S ribosomal RNA and *rpoD* genes of these isolates revealed that they are closely related to *Pseudomonas viridiflava*. One of the isolates, Apo11, was able to diminish the propagation of *X. campestris* in whole-plant assays. At the same time, Apo11 inoculation reduced the necrotic lesions provoked by *S. sclerotiorum* on canola leaves. This protective effect might be due to the induction of resistance in the host mediated by salicylic and jasmonic acid signaling pathways or the production of compounds with antimicrobial activity. At the same time, Apo11 inoculation promoted canola plant growth. Thus, the isolate characterized in this work has several desirable characteristics, which make it a potential candidate for the formulation of biotechnological products to control plant diseases or promote plant growth.

Plants and microorganisms are permanently interacting in a continuum ranging from deleterious (pathogens) to beneficial (symbionts) associations. The microorganisms that are able to colonize host inner tissues are known as endophytes. The classic definition of endophytes refers to microorganisms that can be isolated from surface-disinfected plant tissues without visible disease symptoms (Hallmann et al. 1997). There are different degrees of interaction between plants and endophytes. Microorganisms such as mycorrhizal fungi that require plant tissues to complete their life cycle are known as "obligate". On the other extreme are the endophytes that live mainly as epiphytes but are capable of entering into the plant endosphere. Between these extremes we find the majority of endophytes, known as facultative endophytes. It is still unclear whether this class of microorganisms uses the plant as a vector of dissemination or whether the host actively selects them (Hardoim et al. 2015). Regarding the routes of colonization, although bacterial endophytes may enter the plant in several ways, the main entry points are root hairs, where potential endophytes are attracted to root exudates and rhizodeposits. On the other hand, while leaf and stem exudates also attract microorganisms, the microhabitat on the leaf surface is more hostile and only well-adapted strains can survive and enter the plant via stomata, wounds, or hydathodes. In addition to that, it has been shown that endophytes can colonize plants across flowers and fruit (Compant et al. 2011). Once inside the plant, endophytes move through to distant tissues through the xylem vascular system as well as the apoplast (James et al. 2002).

The plant apoplast is believed to be the main reservoir of bacterial endophytes (McCully 2001; Rosenblueth and Martinez-Romero 2006). Apoplast represents the free diffusional space outside the plasma membrane, including plant cell walls, and enables the transport and the interchange of solutes between cells and tissues. Its composition has proven to be complex and dynamic. Many metabolites and proteins are directed to this site and play essential roles in plant physiology by controlling the responses to both biotic and abiotic agents (Hückelhoven 2007; Krause et al. 2013). Importantly, this niche is the main site of interaction between pathogens and beneficial endophytes. Hence, the isolation of beneficial bacterial strains adapted to this environment could be useful to develop natural biotechnological solutions for agriculture.

Beneficial endophytes, commonly known as plant-growth-promoting bacteria (PGPB), induce positive effects such as increased plant growth, reduced susceptibility to diseases, and improved tolerance to abiotic stresses (Bulgarelli et al. 2013). The mechanisms by which PGPB exert these positive effects include different traits that directly affect important processes affecting plant growth such as nutrient solubilization, nitrogen fixation, and production of phytostimulants. On the other hand, there are positive traits that affect plant growth in an indirect way by helping plants to cope with pathogens. Biological control of pathogens mediated by beneficial microorganisms may be due to a variety of mechanisms such as parasitism, competition for niche and nutrients, production of antimicrobial compounds, and the induction of plant defense mechanisms (Whipps 2001). At the same time, it is important to bear in mind that microorganisms associated with a

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specific plant species provide the most effective biocontrol activity due to adaptation to the host environment (Ferrigo et al. 2017).

Canola (*Brassica napus* L.) is an economically important crop grown worldwide that is susceptible to many bacterial and fungal diseases. Such diseases usually require the intense use of agrochemicals and result in huge agronomic losses. Black rot, caused by the Gram-negative bacterium *Xanthomonas campestris* pv. *campestris*, is the most important disease affecting brassica crops worldwide (Vicente and Holub 2013). Black rot is a systemic vascular disease that provokes V-shaped yellow lesions on leaves, starting from the margins, and blackening of veins. The first outbreak of bacterial leaf spot caused by *X. campestris* on canola in Argentina was reported by Gaetán and López (2005). Other fungal pathogens such as *Sclerotinia sclerotiorum*, which causes stem rot, and *Leptosphaeria maculans*, causing blackleg disease, are also responsible for important losses of canola production (Murray and Brennan 2012). *S. sclerotiorum* infects more than 400 plant species belonging to 75 families that include many economically important crops. Typical stem rot symptoms include soft watery lesions or areas of very light-brown discoloration on the leaves, main stems and branches. Stems of infected plants eventually tend to shred and break (Kamal et al. 2015). The black leg disease, caused by a complex of *Leptosphaeria* spp. (the most important of which is *L. maculans*), is even more harmful than stem rot. This disease can potentially cause a complete crop to be lost (Fitt et al. 2006). This fungus initially colonizes the tissue as a biotroph but, behind the front of the hypha, becomes necrotrophic. Following initial infection on the leaf, it moves through intercellular spaces and the xylem to the petiole and finally invades and kills cells from the stem cortex, generating a blackened canker (Howlett et al. 2001). The use of agrochemicals for the management of these diseases has increased over the years but may become impractical when profit margins diminish. Therefore, new strategies are needed for sustainable canola production worldwide (Fitt et al. 2006). In this trend, the use of beneficial endophytic bacteria arouses interest because of its agronomic potential. Over the last few years, there have been several reports on the composition of leaf bacterial communities in different plant species (Bulgarelli et al. 2013; Rathore et al. 2017; Romero et al. 2014), some works assessed the potential of certain rhizospheric microorganisms as biological control agents for the management of canola diseases (Kamal et al. 2015; Simonetti et al. 2012). However, despite the importance of the apoplast as the common niche for both pathogens and beneficial endophytes, the exploitation of apoplast-colonizing bacteria for the biological control of bacterial and fungal diseases is still scant. On these bases, this work aimed to isolate and characterize apoplast-colonizing endophytic bacteria from field-grown canola leaves, and to analyze their potential for biological control of the main canola phytopathogens.

MATERIALS AND METHODS

Strains of phytopathogens, growth conditions, and inoculation. *L. maculans* isolate ME24 was kindly supplied by Dr. Kim Hammond-Kosack (The Sainsbury Laboratory, John Innes Centre) and maintained on potato-dextrose agar (PDA) (Britania S. A.) plates at 4°C. Spores (15 days old) were scraped from PDA culture plates and transferred to a sterile 0.02% (vol/vol) Tween 20 solution. Then, the spore suspension was filtered and the concentration adjusted to 10^5 spores ml^{-1} with sterile water. Inoculation was performed by infiltration of spore suspensions into the cotyledon using a plastic syringe without the needle.

An isolate of *S. sclerotiorum* from the IIB-INTECH Fungal Culture Collection (IFCC 458/02) was used for all the experiments. The fungus was maintained in PDA at 4°C. Before inoculation, mycelium was grown in solid Czapek-Dox medium (glucose at 50 g liter^{-1}, $NaNO_3$ at 2 g liter^{-1}, KH_2PO_4 at 1 g liter^{-1}, $MgSO_4O \cdot 7H_2O$ at 0.5 g liter^{-1}, KCl at 0.5 g liter^{-1}, $FeSO_4O \cdot 7H_2O$ at 0.05 g liter^{-1}, and agar at 20 g liter^{-1}, pH 5.5 to 6.0) at 24°C. Leaf disc inoculation was performed by placing a plug of mycelium (0.25 cm^2) in the center of 18-mm-diameter leaf discs, and subsequently dispensed on Petri dishes containing water agar (0.8% [wt/vol]). Leaf discs were incubated in the plant growth chamber for 48 h. The size of the necrotic area was determined using the Image-ProPlus V 4.1 software (Media Cybernetics).

X. campestris pv. *campestris* strain 8004 was maintained at –80°C in Luria-Bertani (LB) medium (tryptone at 10 g liter^{-1}, yeast extract at 5 g liter^{-1}, and NaCl at 10 g liter^{-1}) supplemented with rifampicin at 50 mg liter^{-1} and 20% (vol/vol) glycerol. For plant inoculation, *X. campestris* was grown in plates with LB medium containing rifampicin at 28°C. Bacterial cells were scrapped off, washed once, and resuspended in sterile $MgCl_2$ at 0.01 mol liter^{-1}, pH 7.0. The suspension was adjusted to 10^6 CFU ml^{-1} (optical density at 600 nm [OD_{600}] = 0.001) and infiltrated with a needless syringe into canola leaves. Leaves infiltrated with sterile $MgCl_2$, at 0.01 mol liter^{-1}, pH 7.0 were used as controls.

Isolation of apoplast-colonizing bacteria. A collection of bacteria was isolated from apoplastic fluids of canola plants cultivated in agriculture-devoted fields in Chascomús, a district of Buenos Aires Province, Argentina. Four samples of canola leaves, consisting of at least three leaves from different plants, were harvested. In order to eliminate the epiphytic microflora, leaves were surface disinfected with a 5% (vol/vol) solution of commercial bleach and 0.01% (wt/vol) Tween 20 for 10 min and finally rinsed three times with sterile distilled water. No bacterial or fungal growth was detected after plating in LB plates aliquots of the water used for the final wash, which served to confirm the efficacy of the disinfection to eliminate cultivable epiphytic bacteria. To obtain apoplastic washing fluids, surface-disinfected leaves were infiltrated with sterile $MgCl_2$ at 0.01 mol liter^{-1} using a 60-ml syringe, as described by O'Leary et al. (2014), then thoroughly blotted with absorbent paper in order to remove surface liquid. After that, leaves were rolled up and inserted into a 20-ml syringe, placed into a sterile 50-ml plastic tube, and centrifuged for 20 min at 1,500 × *g* at 4°C. The apoplastic fluids obtained were maintained on ice. Aliquots (100 µl) were plated on LB plates and incubated at 28°C for 48 h. In total, 105 isolates were obtained, some of which were selected for further analysis. Colonies considered to be morphologically different on the basis of size, shape, and color were selected and subcultured to obtain pure cultures, which were afterward kept at –80°C as glycerol stocks. Ten nonredundant isolates, according to their BOX-polymerase chain reaction (PCR) profiles, were then selected for further analysis. Their ability to endophytically colonize canola plants was confirmed by inoculating them on seed at sowing and their posterior reisolation from seedlings after surface disinfection and grinding of plant tissues.

In vitro assays of antagonism between endophytic bacteria and canola pathogens. The ability of leaf endophytic bacteria to inhibit the growth of *S. sclerotiorum, L. maculans*, and *X. campestris* pv. *campestris* was tested in dual in vitro cultures, as described by Romero et al. (2016). Briefly, to test antagonism against fungi, overnight cultures of each endophytic strain were spotted on the periphery of PDA plates inoculated with a plug of mycelium. Plates inoculated only with the fungi were used as controls. In turn, for the identification of antagonism against *X. campestris*, cells were mixed with LB agar medium at 42°C to a final concentration of 10^6 CFU ml^{-1}. This medium was plated and, once solidified, a 3-µl aliquot of each endophytic bacteria (10^7 CFU ml^{-1}) was spotted in the center of the plate (5.5 cm in diameter). Antagonistic activities were revealed by the inhibition zones of *X. campestris* around the colonies of antagonists after 2 days of incubation at 28°C. In all cases, three biological replicates were used and the experiments were performed twice.

Production of antimicrobial compounds by endophytic bacteria. Antimicrobial activity was also determined in cell-free supernatants of bacterial isolates grown in liquid LB medium. The supernatants were obtained after centrifuging and filtrating through

0.22-μm membrane filters samples of 3-ml bacterial cultures at stationary phase. Production of antibacterial compounds was assessed by growing *X. campestris* in LB liquid medium supplemented with 10 or 50% of the cell-free supernatants from endophytic bacteria. Growth of *X. campestris* was estimated by measuring optical density at 600 nm. In turn, antifungal activity was analyzed based on the inhibition of mycelial growth of *S. sclerotiorum* and *L. maculans* in PDA medium supplemented with 10% of the cell-free supernatant. Three biological replicates were used for each treatment and the experiments were conducted twice independently.

Siderophore production, phosphate solubilization, and indole acetic acid production. Siderophore production was determined on chromo-azurol S medium as described by Romero et al. (2016). Phosphate solubilization was determined as described by Castagno et al. (2011). For indole acetic acid (IAA) production, isolates were cultured in liquid LB medium containing L-tryptophan at 0.0025 mol liter^{-1} for 72 h at 28°C and shaken at 180 r min^{-1}. After centrifugation at 10,000 × *g* for 5 min, supernatants were mixed at a 1:1 ratio with the Salkowski reactive ($FeCl_3$ at 12 g liter^{-1} in H_2SO_4 at 7.9 mol liter^{-1}). After 30 min at room temperature, the OD at 540 nm was measured. The relative IAA production by the isolates was determined using a calibration curve.

Isolation of genomic DNA, PCR amplification, and sequencing of 16S rRNA gene. Total DNA was extracted from 3-ml cultures of bacterial isolates in LB at 28°C and nearly full-length (approximately 1,500 bp) 16S ribosomal RNA (rRNA) genes were amplified using primers 41f and 1488r (Supplementary Table S1) as previously described by Estrella et al. (2009). *rpoD* genes were amplified using primers PsEG30F and PsEG790R as previously described by Mulet et al. (2009). PCR products were purified and sequenced at the Genomic facilities at the Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA). The 16S rRNA and *rpoD* gene sequences determined in this study have been deposited in the GenBank database under accession numbers MG843853 to MG843855 and MH483951 to MH483953, respectively.

Isolates morphologically similar were checked not to be redundant by BOX-PCR fingerprinting using the universal BOXA1R primer. PCR amplification and gel visualization was performed as described by Castagno et al. (2011).

Phylogenetic analysis. Sequence analysis was performed with the ClustalW software from the EMBL server. Aligned sequences were analyzed with the MEGA 6.0 software (Tamura et al. 2007). Phylogenetic analyses were performed using the unweighted pair-group method with arithmetic means and distances were computed by the *p*-distance method. Statistical support for tree nodes was assessed by bootstrap analysis.

Plant growth conditions, inoculation with endophytic bacteria, and growth promotion assays. Canola seed (cultivar Legacy) were disinfected in 70% (vol/vol) ethanol for 2 min, followed by 5% (vol/vol) commercial bleach for 10 min, then were rinsed (five times) with sterile distilled water. Seed were placed in pots containing a mixture of soil-sand-perlite (1:1:1), and were watered with half-strength Hoagland solution (Hoagland and Arnon 1950). Plants were grown for 4 to 5 weeks in a growth chamber with a photoperiod of 16 and 8 h at 24 and 21 ± 2°C and 55 and 75 ± 5% relative humidity during day and night, respectively, with a photon flux density of 200 μmol m^{-2} s^{-1}.

For inoculation with bacterial endophytes, disinfected seed were dipped into bacterial suspensions for 1 h at room temperature with periodical shaking. Bacterial suspensions (OD_{600} of 0.1) were obtained by scraping cells off overnight plates. Inoculated seed were dispensed in pots and cultured as described above. Plants derived from seed immersed in $MgCl_2$ at 0.01 mol liter^{-1} were used as controls. Plant growth promotion by endophytic bacteria was assessed by determining fresh and dry weight of stems and roots after a 5-week growth period. Ten biological replicates were used per treatment. The experiment was performed twice. Experiments intended to reisolate bacteria from leaves were performed on plants inoculated as described above but grown in axenic conditions using solid Murashige and Skoog medium (Sigma-Aldrich).

Biocontrol assays. Canola plants inoculated with bacterial endophytes as described above were used. For *X. campestris* biocontrol assays, two leaves per plant and four plants per treatment were syringe infiltrated at their abaxial side with bacterial suspensions (OD_{600} = 0.001). Three days after inoculation, 100-mg samples of tissue were taken and ground in $MgCl_2$ at 0.01 mol liter^{-1}. Dilutions of the extracts thus obtained were plated on LB agar supplemented with rifampicin at 50 mg liter^{-1}. The titers of *X. campestris* pv. *campestris* were determined as the number of CFU detected after incubating plates at 28°C for 48 h. Previously, endophytic bacteria were confirmed not to be resistant to rifampicin. Four replicate plants were analyzed. For *S. sclerotiorum* bioassays, leaf discs were obtained from the second or third pair of leaves of 12 canola plants inoculated with the endophyte or mock-inoculated control plants. These discs were inoculated with *S. sclerotiorum* mycelium as described above. The necrotic lesions were measured using the Image-ProPlus V 4.1 software (Media Cybernetics). For *L. maculans* biocontrol studies, 7-day-old endophyte-inoculated and control seedlings were infected in the cotyledons as described above. Seven days after infection, cotyledons were frozen in liquid nitrogen and DNA was isolated using the cetyltrimethylammonium bromide method (Ausubel et al. 1987). *L. maculans* was quantified by quantitative (q)PCR with *LmITS1* primers and normalized to plant *ACTIN* as described by Šašek et al. (2012). All experiments were conducted at least twice.

Expression of defense-related genes. Expression of defense-related genes was determined in control plants (without inoculation with endophytic bacteria or pathogens), endophyte-inoculated plants (inoculation as described above with bacterial endophyte), *X. campestris*-inoculated plants (inoculation as described above with *X. campestris*), and plants co-inoculated with the endophyte and *X. campestris*. As markers for the salicylic acid (SA) signaling pathway, we used the genes coding for the *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*), a widely used SA-responsive marker gene, and *WRKY70*, an SA-responsive transcription factor. In turn, *LOX3* was chosen as a marker gene for the jasmonic acid (JA) signaling pathway, whereas *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2* (*ACS2)* and *RD26* were chosen as marker genes for the ethylene and abscisic acid signaling pathways, respectively. Samples were taken 72 h after pathogen inoculation. Plant material was frozen in liquid nitrogen and total RNA was extracted with RNAzol reagent (Sigma Chemicals) according to the manufacturer's instructions. First-strand cDNAs were synthesized using Moloney murine leukemia virus reverse transcription (Promega Corporation). For quantification of mRNA levels by quantitative reverse-transcription PCR, 1 μl of cDNA (1:5 dilution) was further diluted to 5 μl with the primer mix in water, and the same volume of FastStart Universal SYBR Green Master (Rox) was added to a final volume of 10 μl. Reactions were performed in an Mx3005P qPCR system with the aid of MxPro qPCR software 4.0 (Stratagene). Relative quantification was performed by the comparative cycle threshold method with the *ACTIN* gene as endogenous control. For comparative purposes, relative gene expression in control plants was defined as 0. The INFOSTAT software tool was used to calculate the relative expression ratios on the basis of group means for target gene transcripts versus the reference gene transcript (Di Rienzo et al. 2011). Four biological replicates were used for each treatment and the experiment was repeated twice.

RESULTS

Isolation, identification, and antagonistic activity of bacterial endophytes. In order to find isolates with the highest potential to be used as biological control agents against the main canola pathogens such as *S. sclerotiorum*, *L. maculans*, and

X. campestris, we established dual-culture assays with bacterial strains isolated from canola apoplastic fluids. Following this approach, we first isolated 105 bacterial endophytes and, after a first classification based on morphology and BOX-PCR profiles, 10 different isolates were finally selected for evaluation. In all, 9 of the 10 isolates were able to inhibit mycelial growth of *L. maculans* from 11 to 75%. In the case of *S. sclerotiorum*, the isolates Apo8, Apo11, and Apo12 inhibited mycelial growth by 33, 34, and 38%, respectively. In turn, these isolates were also able to inhibit the growth of *X. campestris*, as determined by the size of the halo of inhibition around antagonistic bacteria growing on preinoculated agar plates (Table 1). On these bases, these three isolates were selected for further characterization. With the aim of proving that these isolates are true endophytes of canola, we then tested their ability to recolonize the interior of seedlings by inoculating seed and reisolating bacteria from superficially disinfected seedlings grown under axenic conditions. As expected, the three bacterial strains were isolated from seedlings, demonstrating their endophytic nature (Supplementary Fig. S1). We then compared the 16S rRNA sequences from the isolates with the GenBank database and reference strains. According to this comparison, the three isolates matched with the highest score to bacteria from the genus *Pseudomonas*. Therefore, several 16S rRNA sequences belonging to *Pseudomonas* spp. were chosen to construct a phylogenetic tree. Interestingly, all three isolates (Apo8, Apo11, and Apo12) clustered together and are closely related to the species *Pseudomonas viridiflava* (Fig. 1). The three 16S rRNA gene sequences showed a 99% identity with *P. viridiflava* ATCC 13223 (GenBank accession number NR_114482). In order to confirm the identity of these isolates, we also compared the sequence of their *rpoD* genes with cognate sequences at the GenBank database. This analysis also demonstrated that the three isolates matched with bacteria from the genus *Pseudomonas.* Apo8 and Apo11 showed 99% identity with *P. viridiflava* strain TK11Y (GenBank accession number MG520096.1), while Apo12 showed 99% identity with *P. viridiflava* strain SH (GenBank accession number KY764289.1). Several *rpoD* gene sequences were chosen from different *Pseudomonas* spp. and a phylogenetic tree was constructed. The three isolates clustered together and are closely related to the species *P. viridiflava* (Supplementary Figure S2), confirming the results obtained with the 16S rRNA gene. Because none of these strains showed detrimental effects on canola seedlings, we concluded that they survive in plants as endophytes.

TABLE 1. In vitro growth inhibition of phytopathogens by apoplast-colonizing bacteria from canola leaves

Isolate	Growth inhibition: *Leptosphaeria maculans* (%)[a]	*Sclerotinia sclerotiorum* (%)[a]	*Xanthomonas campestris* (halo area/colony area)[b]
Apo1	53.63 ± 0.03	…	…
Apo2	…	…	…
Apo3	11.31 ± 0.01	…	…
Apo4	47.05 ± 0.04	…	…
Apo5	35.37 ± 0.04	…	…
Apo6	75.51 ± 0.03	…	…
Apo8	40.17 ± 0.03	33.88 ± 0.05	8.01 ± 1.29
Apo10	34.85 ± 0.05	…	…
Apo11	41.07 ± 0.04	34.15 ± 0.10	9.05 ± 0.60
Apo12	38.50 ± 0.05	38.17 ± 0.06	10.13 ± 1.41

[a] Inhibition of mycelial growth was calculated by comparing the diameter of colonies confronted with bacterial antagonists and control colonies. Results are means of three replicate plates ± standard deviation (SD).

[b] Halo area = area of the inhibition zone of *X. campestris* growth around the colonies of antagonistic bacteria and colony area = area of the colony of the antagonistic bacterium. Results are the mean of three replicates ± SD.

Protection of canola plants against *X. campestris* by bacterial endophytes. With the purpose of testing whether these candidates actually have protective effects on plants challenged with pathogens, plants inoculated with these isolates at sowing were challenged with *X. campestris* and the propagation of the pathogen was evaluated at different times by counting CFU per square millimeter of leaf. These experiments demonstrated that inoculation with Apo11 decreased the propagation of the pathogen fivefold at 72 h postinoculation (Fig. 2). On the other hand, inoculation with Apo8 and Apo12 did not show any difference regarding pathogen propagation in comparison with mock-inoculated plants at any postinoculation time.

Expression of defense-related genes in response to Apo11 inoculation. Our analysis described in the previous section suggests that the isolate Apo11 may induce systemic resistance in canola plants (Fig. 2). Therefore, in order to identify whether defense-related hormones are implicated in this process,

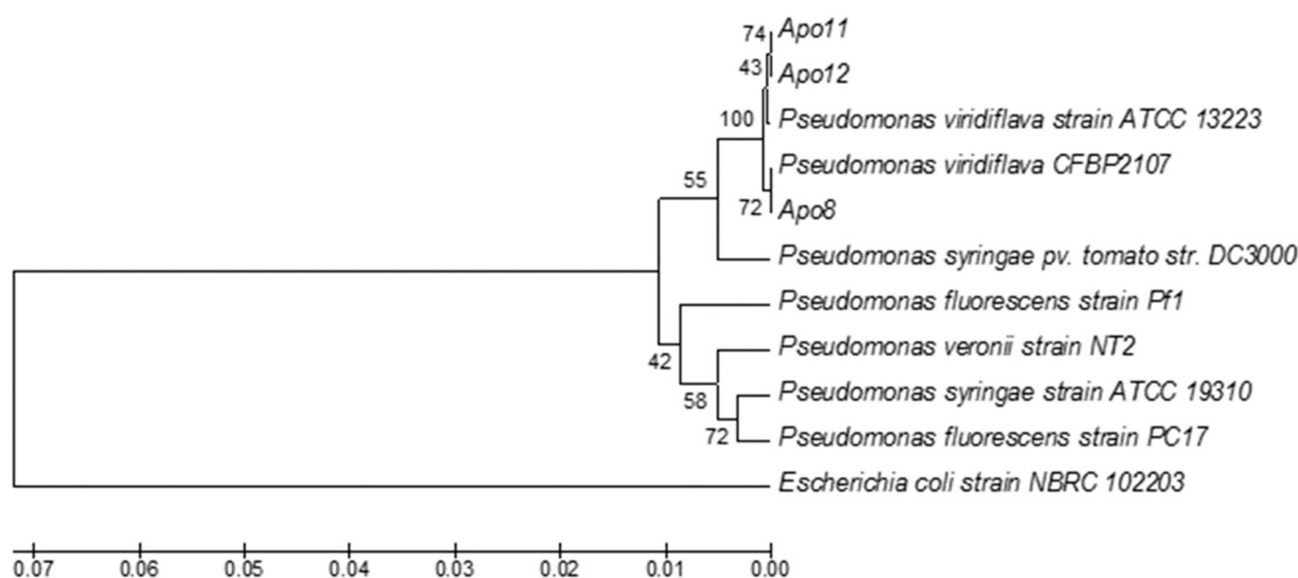

Fig. 1. 16S ribosomal RNA gene phylogeny of endophytic bacteria isolated from apoplast fluids of canola leaves. The tree was constructed from the nucleotide sequence data using the unweighted pair-group method with arithmetic means algorithm, and phylogenetic distances were calculated by the *p*-distance method. Numbers at branch points are the significant bootstrap values (expressed as percentages based on 1,000 replicates). Horizontal branch lines are proportional and indicate the *p*-distances.

we evaluated the expression of marker genes for different defense signaling pathways following endophyte inoculation. Our results indicate that *X. campestris* inoculation induced the expression of all marker genes, with the exception of *LOX3,* which did not change in response to pathogenic infection (Fig. 3), indicating that all except JA are implicated in the defense against this phytopathogen. On the other hand, inoculation with Apo11 induced the expression of *LOX3* and *WRKY70*, indicating that this bacterium is able to induce both SA and JA signaling pathways at the same time. All assessed genes were upregulated in the group of plants inoculated with both Apo11 and *X. campestris* (Fig. 3). In this case, *PR1, WRKY70*, and *ASC2* expression showed levels similar to those in *X. campestris*-inoculated plants.

Production of antimicrobial compounds by Apo11. In order to elucidate the mechanisms by which Apo11 exerts its biocontrol activity against phytopathogens, we evaluated whether this strain is able to excrete compounds with the capability to inhibit the growth of the canola phytopathogens tested in dual-culture assays. First, with the purpose of analyzing its effect on the growth of *X. campestris*, cell-free filtrates from cultures of Apo11 were added to LB medium at final concentrations of 10 and 50%, and bacterial growth was determined by measuring OD at 600 nm. In this case, cell-free supernatants were able to inhibit bacterial growth when used at 50% but no effect was observed at 10% (Fig. 4A). On the other hand, 10% cell-free filtrates were sufficient to significantly decrease the colony radius of *S. sclerotiorum* and *L. maculans*, as determined by colony radius on PDA (Fig. 4B and C).

Apo11 inoculation reduces necrotic lesions provoked by *S. sclerotiorum* in canola leaves. Results from the previous sections suggest that Apo11 could protect plants by direct as well as indirect mechanisms, because this endophyte is able to inhibit mycelia growth of *S. sclerotiorum* in vitro (Table 1) and, at the same time, it activates the defense-related signaling pathway mediated by SA and JA (Fig. 3). Therefore, we next tested whether Apo11 inoculation protects detached canola leaves against *S. sclerotiorum*. Our experiments showed a significant reduction in the area of infection in leaves from plants inoculated with Apo11 24 and 36 h postinoculation (Fig. 5), representing approximately 40 and 21%, respectively.

Plant-growth promotion by Apo11. In order to evaluate the ability of Apo11 to promote plant growth, canola seeds were inoculated and plant dry weight was determined after 5 weeks of culture. Apo11-inoculated plants showed an increase in dry matter of shoots and roots of approximately 30 to 50% when compared with mock-inoculated plants (Fig. 6), and the same increments were observed by measuring fresh weights (data not shown). Our data showed that this isolate was unable to solubilize inorganic phosphate but was able to produce siderophores and hormones such as IAA and their derivatives (Supplementary Table S2).

DISCUSSION

The plant apoplast is the main reservoir of bacterial endophytes and many bacterial phytopathogens (McCully 2001). This is the place where the main molecular events take place between host and microorganisms (Gupta et al. 2015) and, consequently, it is in this niche where the first face-to-face encounter between pathogens and their potential antagonists occurs.

One of the most used methods to screen for antagonists of pathogenic microorganisms is the dual-culture assay (Desai et al. 2002). Using this method, several antagonists of pathogens causing diseases in canola were isolated previously (Etesami and Alikhani 2016; Simonetti et al. 2012). Most of these isolates had been obtained from canola's rhizosphere or from other plant species phylogenetically distant from canola. This is disadvantageous because they may not be effective for the biocontrol of canola leaf pathogens because they might be poor colonizers of these tissues in natural conditions. Even though they might be successful in identifying biocontrol agents, one of the disadvantages associated with these approaches is that they may not be effective in isolating microorganisms that colonize canola inner tissues in natural conditions. Thus, these microorganisms should have reduced potential to control endophytic pathogens surviving at the apoplast. In addition, endophytic biocontrol agents are more protected against environmental stresses and are less restrained by competition with other microbes, which are stressful situations that could affect their biological control ability (Andrews 1992). With this in mind, we isolated bacterial endophytes directly from canola apoplastic fluids, which makes this the first report of the isolation of endophytic leaf-inhabiting bacteria from this crop. A similar approach was used by

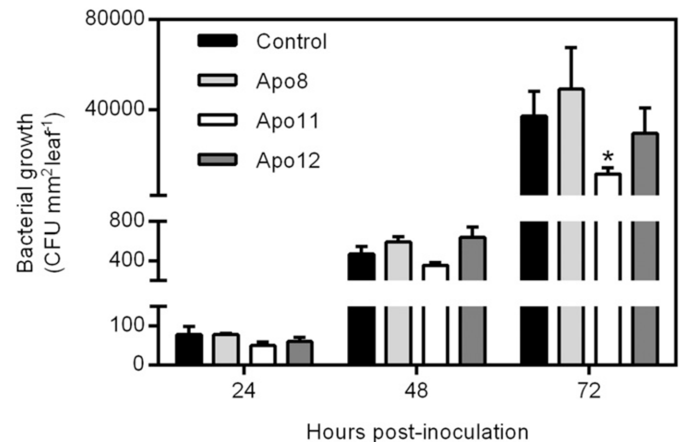

Fig. 2. Propagation of *Xanthomonas campestris* pv. *campestris* in canola leaves. Canola seeds were inoculated with bacterial endophytes suspensions in $MgCl_2$. Seeds treated with sterile $MgCl_2$ were used as controls. Then, 4 to 5 weeks after sowing, two leaves per plant were syringe infiltrated with *X. campestris* suspensions. *X. campestris* titers were determined 24, 48, and 72 h postinoculation. Results are the mean of four replicates ± standard error and statistically significant differences in bacterial titers at each time point between treatments and control according to Student's *t* test are shown by an asterisk: * indicates $P \leq 0.05$.

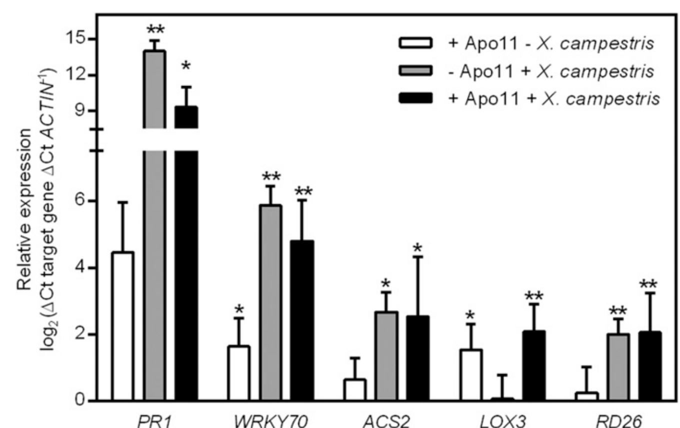

Fig. 3. Expression of defense-related genes on canola. Quantitative real-time polymerase chain reaction was used to analyze the abundance of defense-related genes transcripts in canola plants inoculated with the endophyte Apo11 (white bars), *Xanthomonas campestris* (gray bars), or both microorganisms (black bars). Tissue samples obtained from mock-inoculated plants were used as controls. Transcript levels were normalized to the average signal intensities of controls, which were assigned to 0. Results are means of four replicates ± standard error, and statistically significant differences in gene expression between different treatments and controls, as analyzed with the INFOSTAT software, are shown by asterisks: * indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$. Ct = cycle threshold.

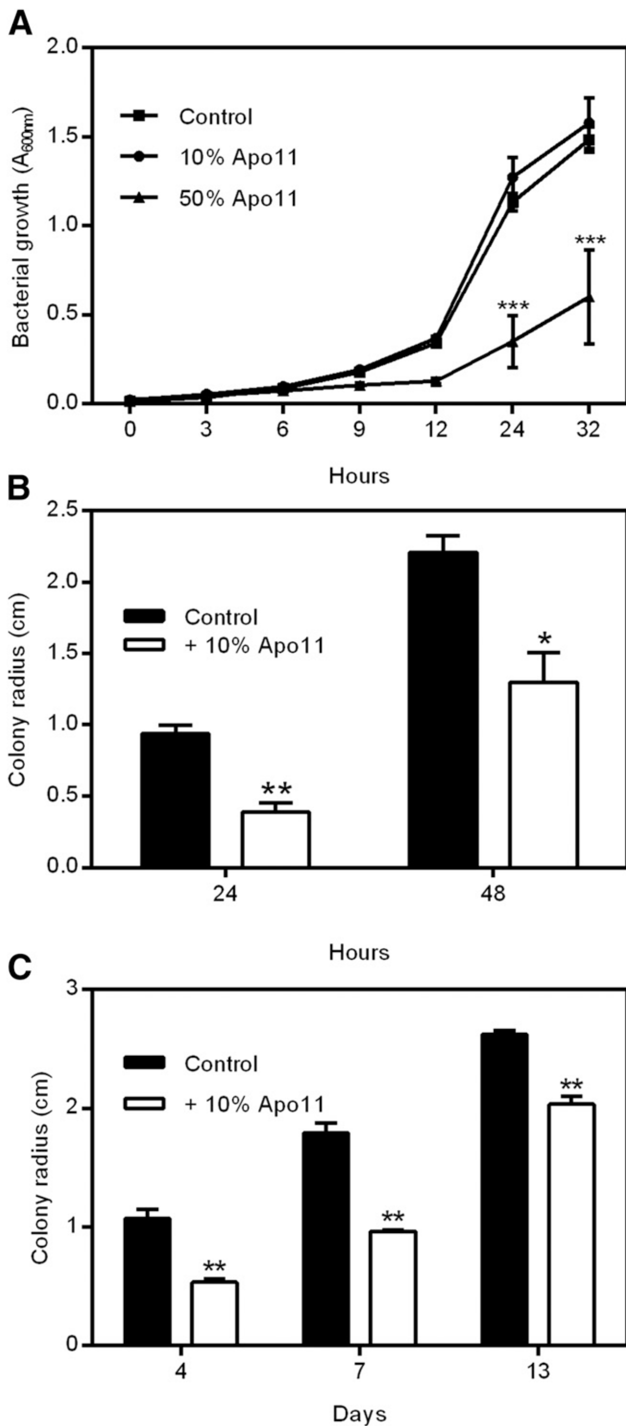

Fig. 4. Antimicrobial activity of cell-free filtrates obtained from cultures of Apo11. **A,** Growth of *Xanthomonas campestris* pv. *campestris* in the presence of cell-free filtrates. Liquid cultures of *X. campestris* pv. *campestris* were mixed with cell-free culture filtrates to a final concentration of 10 or 50% (vol/vol). *X. campestris* cultures in Luria-Bertani (LB) medium without the amendment of cell-free filtrates were used as controls. Growth of *X. campestris* pv. *campestris* was evaluated according to the absorbance at 600 nm for 32 h. Results are means ± standard error of three replicates. Statistical differences between treatments and controls according to one-way analysis of variance and Dunnett's test are shown by asterisks: *** indicates $P \leq 0.001$. Effect of cell-free filtrates in the growth of **B,** *Sclerotinia sclerotiorum* and **C,** *Leptosphaeria maculans*. Melted potato dextrose agar (PDA) was mixed with cell-free culture filtrates to a final concentration of 10% (vol/vol) and poured on plates. PDA mixed with LB medium at the same concentration was used as controls. A plug of mycelium of either *S. sclerotiorum* or *L. maculans* was placed in the center of the plates and the mycelial growth was measured at different times. The radius of the colony was measured using the Image-ProPlus V 4.1 software. Results are means of three replicate plates and statistically significant differences in size of the colony at each time between treatment and control according to Student's *t* test are shown by asterisks: * and ** indicate $P \leq 0.05$ and 0.01, respectively.

Hong et al. (2015), who isolated the antagonist from apoplastic fluids of *Arabidopsis* infected with *P. syringae*. The results by Hong et al. (2015) and those presented here show that apoplastic fluids could be a good reservoir of biological control agents.

According to the in vitro antagonism test, three isolates (Apo8, Apo11, and Apo12) inhibited the growth of the pathogens under analysis. These isolates are closely related to *P. viridiflava* according to their 16S rRNA and *rpoD* gene sequences. Importantly, this species is a member of a group of plant-associated bacteria that includes both pathogenic and saprophytic species. In fact, it belongs to the *P. syringae* species complex, and some strains of *P. viridiflava* have been reported to cause important plant diseases (Bartoli et al. 2014; González et al. 2003), while others were described to show biocontrol activity against various human- and plant-pathogenic fungi (Fleury et al. 2011; Miller et al. 1998).

Although dual-culture assays are useful as a first approach to identify potential antagonists against pathogenic microorganisms, it is important to confirm later if potential candidates have protective effects on plants. The fact that only one of the three isolates was able to reduce the number of *X. campestris* cells on canola leaves reinforces the idea that potential biocontrol agents may behave differentially between in planta and in vitro assays. This was also reported in other systems where endophytic bacteria were able to inhibit the growth of pathogenic microorganisms in vitro but did not show the same effect on plants (Massomo et al. 2004; Romero et al. 2016). One possible explanation is that the strains may have different capability to colonize host tissues or low multiplication rates in inner plant tissues, or even bacterial fitness may be affected by the conditions imposed by the apoplast. We did not see any difference in the level of seedling colonization by any of the endophytic strains being tested, suggesting that the lack of biocontrol activity in planta for Apo8 and Apo12 may be due to a repression of biocontrol-associated traits that are expressed in vitro. Even though the use of bacterial antagonists to control diseases caused by *X. campestris* has been reported before in different plant species, including members of the *Brassica* genus (Massomo et al. 2004; Wulff et al. 2002), this is the first attempt to find biocontrol agents of this pathogen among endophytic bacteria. The research published thus far has isolated promising candidates with a high potential to control different diseases caused by *X. campestris* but failed in exploring the mechanisms of action mediating biocontrol activities. In order to shed more light in this field, in the present work, we explored possible mechanisms of action that could explain the biocontrol activity shown by our *P. viridiflava* isolates.

Induced resistance is a generic term for the induced state of resistance in plants triggered by biological or chemical inducers, which protects nonexposed plant parts against future attack by pathogenic microbes and herbivorous insects. This state is characterized by the activation of latent defense mechanisms that are expressed upon a subsequent challenge from a pathogen and is regulated by a network involving known defense-hormone signaling pathways (Pieterse et al. 2014). Therefore, beneficial microbes may activate plant immunity in two ways. One of them is the priming of defense mechanisms for more efficient activation of cellular defense responses. This physiological condition, in which plants mount more effective or more rapid defense responses to biotic or abiotic stress, is called the "primed state" of the plant (Conrath et al. 2006). During priming, there is usually no expression of *PR* genes unless the primed plant is challenged by pathogens (van Hulten et al. 2006; Verhagen et al. 2004). On the other hand, microbes can directly induce different defense signaling pathways, which is indicated by the overexpression of *PR* genes even before pathogen recognition (Salas-Marina et al. 2011; Timmermann et al. 2017). Our results indicate that *X. campestris* inoculation induced expression of marker genes from all phytohormone signaling pathways tested, with the exception of *LOX3*. This observation agrees with previous works indicating that the infection with

pathogens showing biotrophic or hemibiotrophic lifestyles such as *X. campestris* induces the accumulation of SA both locally and systemically, thus triggering the expression of the genes involved in the SA signaling pathway. In addition, the antagonism between SA and JA may explain why *X. campestris* induces the accumulation of SA-responsive genes while the JA-responsive gene *LOX3* remains unaltered. On the other hand, inoculation with *P. viridiflava* Apo11 induced the expression of *LOX3* and *WRKY70*, which indicates that this bacterium is able to induce both SA and JA signaling pathways at the same time. In turn, all tested defense genes were upregulated in plants inoculated with both Apo11 and *X. campestris*, showing levels of expression similar to those in *X. campestris*-inoculated plants. As a whole, these results could indicate that Apo11 is unable to prime the activation of the defense pathways but it can stimulate the activation of the main defense mechanisms triggered by SA and JA. This is an interesting trait provided by beneficial microorganisms because it could lead to an enhanced resistance to different types of pathogens. Similar results were also found in the interaction between *Arabidopsis* and *Trichoderma atroviride*, where the inoculation with *T. atroviride* enhanced systemic resistance against *P. syringae* and *Botrytis cinerea* through the activation of the JA and SA pathways (Salas-Marina et al. 2011). We also observed that Apo11 reduces necrotic lesions provoked by *S. sclerotiorum* on canola leaves. It has been reported that SA and JA play a crucial role in the defense of canola plants against *S. sclerotiorum*. For instance, Wang et al. (2012) demonstrated that the infections provoked by this fungus lead to the increment in the levels of SA and JA, and that the exogenous application of these hormones reduces disease development. The results obtained in this work are important given the increasing interest in the search for more environmentally friendly technologies to control plant diseases, which has led to the emergence of several works trying to find biological control agents against *S. sclerotiorum.* In this search, a wide variety of microorganisms has been recovered from the rhizosphere, phyllosphere, sclerotia, and other habitats with potential biocontrol ability (Fernando et al. 2007; Kamal et al. 2015; Simonetti et al. 2012), most of them belonging to the genera *Bacillus* and *Pseudomonas*.

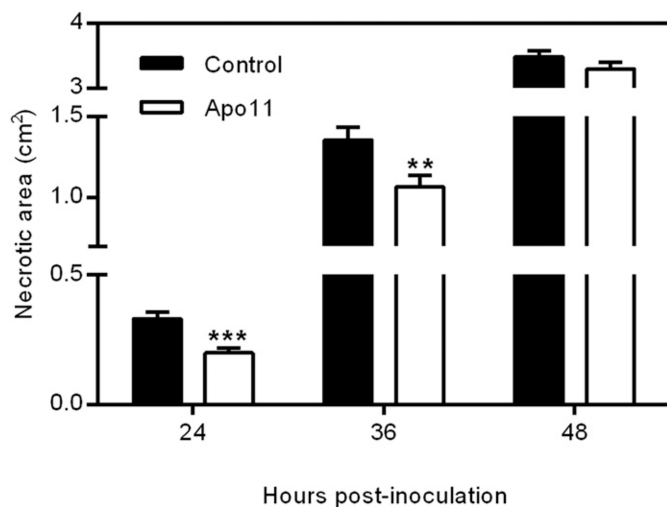

Fig. 5. Necrotic lesions provoked by *Sclerotinia sclerotiorum* infection on leaves of canola plants inoculated with Apo11. Canola leaves were detached from 5-week-old plants previously inoculated with bacterial endophyte at the time of sowing. Twelve plants per treatment and two leaves per plant were used. Leaf discs were dispensed on Petri dishes containing water agar and were inoculated with a plug of mycelium of *S. sclerotiorum*. The size of the necrotic area around the inoculation site was determined 24, 36, and 48 h postinoculation using the Image-ProPlus V 4.1 software. Results are means of 32 replicate discs and statistically significant differences in size of the necrotic area at each time between treatment and control according to Student's *t* test are shown by asterisks: ** and *** indicate $P \leq 0.01$ and 0.001, respectively.

We also verified whether Apo11 could protect canola plants against *L. maculans.* However, despite the fact that the endophyte inhibits the growth of *L. maculans* in vitro (Table 1) and also produces compounds with antifungal activity against this pathogen (Fig. 4C), it was unable to confer protection to canola seedlings under our experimental conditions (data not shown). We assessed this by estimating fungal biomass in planta through the quantification of the gene *ITS1* from *L. maculans*, as described by Šašek et al. (2012). It is worth mentioning that *L. maculans* is a hemibiotrophic pathogen, meaning that, during the first stage of the infection, it colonizes plant tissues and survives on photosynthates produced by living plant cells, then later switches to a necrotrophic stage. Because we only analyzed the period associated with the biotrophic stage of the infection, we cannot rule out the possibility that Apo11 could have a protective effect on more advanced stages of the infection, once the pathogen has adopted a necrotrophic style as *S. clerotiorum*. In this regard, our future research will analyze the effects of Apo11 inoculation on later stages of *L. maculans* infections.

Another mode of action employed by biological control agents is antibiosis due to the secretion of metabolites that are harmful to pathogens (Whipps 1997). In this work, we demonstrated that Apo11 is capable of producing compounds with antibacterial and antifungal activity, which could contribute to the biological control of these pathogens. Most of the bacteria evaluated as biocontrol agents produce multiple antibiotics with different degrees of activity against

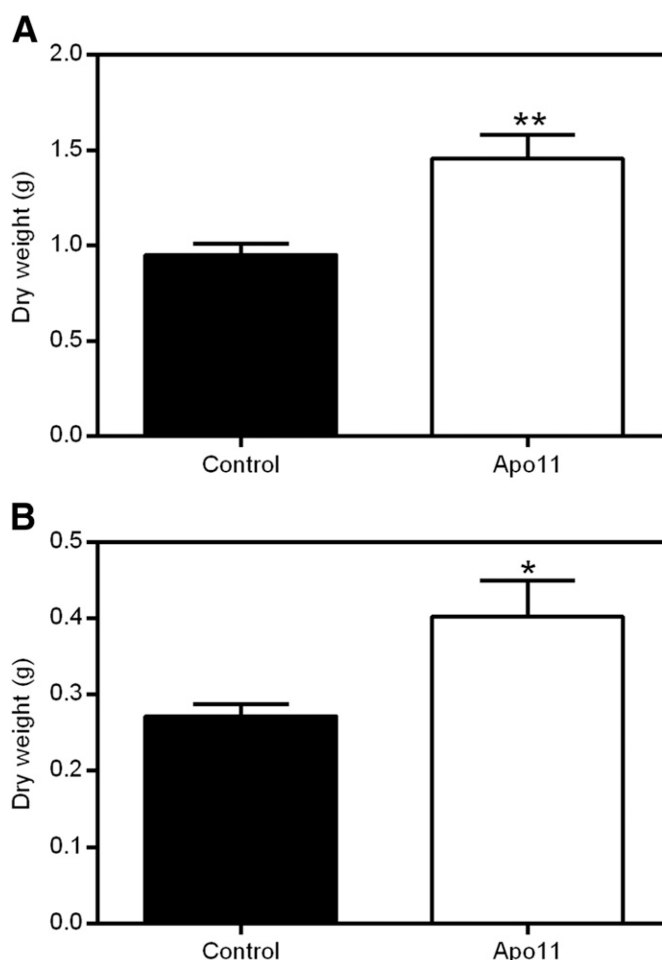

Fig. 6. Plant-growth promotion by Apo11. Dry weight of **A,** shoots and **B,** roots was measured in 5-week-old canola plants inoculated with bacterial endophytes at sowing. Noninoculated plants were used as controls. Results are means of 10 biological replicates ± standard error. Statistical differences between inoculated and control plants were calculated by Student's *t* test and are shown by asterisks: * and ** indicate $P \leq 0.05$ and 0.01, respectively.

pathogenic microorganisms (Raaijmakers and Mazzola 2012). In this trend, the most studied bacteria belong to the genera *Bacillus* and *Pseudomonas*. The genus *Pseudomonas* is also known to produce a large amount of secondary metabolites with antimicrobial activity. One of the best studied is 2,4-diacetylphloroglucinol, which has been demonstrated to have antiviral, antibacterial, antifungal, and antihelminthic activity and is a key determinant for biocontrol activity in several *Pseudomonas* spp. (Weller et al. 2007). Other metabolites with antimicrobial activity produced by members of this genus include pyrrolnitrin, pyoluteorin, phenazines, 2,5-dialkylresorcinol, quinolones, gluconic acid, rhamnolipids, and various lipopeptides (Raaijmakers and Mazzola 2012). *P. viridiflava*, is known to produce ecomycins (lipopeptides with antifungal activity) (Miller et al. 1998), although these compounds have been poorly studied thus far. Therefore, further characterization of the antimicrobial compounds produced by Apo11 would contribute to the identification of novel bioactive molecules involved in biocontrol against pathogenic microorganisms.

In addition to the potential of beneficial endophytes as biological control agents, it is known that they may also contribute to plant growth by diverse mechanisms, which may include associative nitrogen fixation, lowering of ethylene levels, production of siderophores and phytohormones, and solubilization of nutrients (Bhattacharyya and Jha 2012). It has been demonstrated that a combination of these mechanisms usually explains the full effect on plant growth (Bashan and de-Bashan 2010). Our results demonstrated that Apo11 is able to promote growth of canola plants. It is worth pointing out that this analysis was performed by using plants watered with a nutrient solution with no limitation in any nutrient. Thus, growth promotion mediated by Apo11 as observed in this work probably resulted from the production of plant growth regulators rather than the increase in the availability of nutrients. This could indicate that the endophyte promotes plant growth due to the production of auxins. Further work needs to be done in order to obtain a better view of other mechanisms that may be involved in this effect.

In conclusion, the present study describes, for the first time, the potential of bacterial endophytes isolated from apoplastic fluids obtained from field-grown canola leaves as biological control agents and plant-growth-promoting microorganisms. Our results indicate that Apo11, closely related to *P. viridiflava*, is a promising candidate to control the development of the main diseases affecting *Brassica* spp. The evidence shown in this work suggests that the biological control ability is due not only to the ability to antagonize the growth of phytopathogens through the production of compounds with antimicrobial activity but also to the induction of plant defense signaling pathways controlled by SA and JA.

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