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An alternative biocontrol agent of soil-borne phytopathogens: A new antifungal compound produced by a plant growth promoting bacterium isolated from North Algeria

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ABSTRACT

Bacteria isolated from different environments can be exploited for biocontrol purposes by the identification of the molecules involved in the antifungal activity. The present study was aimed at investigating antifungal protein compounds purified from a previously identified plant growth promoting bacterium, *Pseudomonas protegens* N isolated from agricultural land in northern Algeria. Therefore, a novel protein was purified by chromatographic and ultrafiltration steps and its antifungal activity together with growth-inhibition mechanism was evaluated against different fungi by plate-based assays. In addition, stereomicroscopy and transmission electron microscopy (TEM) was performed to explore the inhibition activity of the compound on spore germination processes. The protein, showing a molecular mass of about 100 kDa under native conditions, was revealed to be in the surface-membrane fraction and displayed an efficient activity against a variety of phytopathogenic fungi, being *Alternaria* the best target towards which it exhibited a marked fungicidal action and inhibition of spore germination. Moreover, the compound was able to significantly decrease fungal infection on tomato fruits producing also morphological aberrations on conidia. The obtained results suggested that the isolated compound could represent a promising agent for eco-friendly management of plant pathogens in agriculture.

1. Introduction

The rhizosphere behavior is the result of the dynamic nature of the soil and its dramatic changes, which affect directly the plant growth. On a large scale, agriculture is subjected to various exogenous factors that can hinder its development, as in the case of abiotic and biotic stresses. The latter represents all plant diseases caused by fungi, bacteria, viruses, and nematodes, which are more aggressive and constitute serious problems, decreasing the final yields of crops (Borges et al., 2014).

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agriculture intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable strategy of crop protection helping with the economic stability of their activities. However, the increasing use of chemical inputs causes several negative effects to the environment and human health besides adding burdens to poor farmer resources (Adesemoye et al., 2009; De Weger et al., 1995; Gerhardson, 2002; Liu et al., 2017). Recently, the use of plant growth promoting bacteria (PGPB) as agents stimulating plant growth and managing plant health, represented a promising alternative (Bensidhoum et al., 2016; Berg, 2009; Saharan and Nehra, 2011). Intensive research on this class of microorganisms is currently underway worldwide for developing new bio-fertilizers and BioControl Agents (BCAs) (Pal and Mc Spadden, 2006). In addition to their direct antagonistic activity, BCAs can indirectly exert their protective effect by sensitizing the plant to microbial attack via the defense mechanisms activation. PGPB are associated with many plant species and are commonly present in several environments. From a microbiological point of view, PGPB living in irrigation well water may constitute a potential

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source of new biological compounds for plant growth enhancement and phytopathogens' biocontrol (Bensidhoum et al., 2016; Handelsman and Stabb, 1996; Ramadan et al., 2015). This phenomenon is the result of multiple interactions between pathogens, plants and antagonist agents. Indeed, sustainable agriculture and the reduction of pesticides are a major concern for farmers to improve soil quality and plant and human health. However, plant stimulated by inducing agents can develop an enhanced defensive state called Induced Systemic Resistance (ISR) (Bakker et al., 2007), which confers to plant more resistance to future attacks by pathogens through endogenous plant signaling molecules (Ramamoorthy et al., 2001) such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Jourdan et al., 2015). Additionally, PGPB may affect indirectly phytopathogens via competition for space and nutrient occupation or through producing antibiotic metabolites (Beneduzi et al., 2012) such as lytic enzymes, antimicrobial peptides or proteins, polyketides, phenolic compounds and bio-surfactants (Fernando et al., 2005). Numerous mechanisms adopted by PGPB to enhance directly plant growth were described, such as biofilm formation (Ravishankar et al., 2013), nutrient uptake, nitrogen fixation (Carvalho et al., 2014), mineral phosphate solubilization (Wang et al., 2017), phytohormones and sidérophores production (Gamalero and Glick, 2015), ACC deaminase synthesis (Saravanakumar and Samiyappan, 2007).

Specifically, many studies reporting on bacteria with potential effects against phytopathogenic fungi and/or insects, i.e. *Pseudomonas*, *Cellulosimicrobium, Bacillus* (Bensidhoum et al., 2016; Mokrani et al., 2018; Nabti et al., 2013; Tabli et al., 2017), were recently conducted in Algeria. These microorganisms isolated from different environments can be exploited for biocontrol purposes by extraction and purification of the specific compounds involved in the antifungal activity.

The present work constitutes the continuation of a previous paper focused on the characterization of PGPB, isolated from irrigation well-water of North-eastern Algeria, and on their inducible antifungal compounds (Tabli et al., 2017). In this context, the purpose of this study was to isolate and analyse the active antifungal protein produced by the previously identified PGPB *Pseudomonas protegens* N strain (Tabli et al., 2017). The antifungal compound was purified through ultrafiltration and chromatographic steps and it was *in vitro* testing for fungistatic and/or fungicidal activity against different phytopathogenic fungi, i.e. *Aspergillus niger, Alternaria* spp, Botrytis cinerea, and Penicillium spp, Moreover, the effects of the isolated molecule were evaluated on *Alternaria* spp spore germination by microscopic analyses and on *A. niger* infection development on a different variety of tomato fruits. Indeed, tomato is one of the most widely grown and extensively consumed vegetables in the world, but it is a very perishable fruit with a short shelf-life and high susceptibility to diseases caused by various pathogenic fungi during harvesting, transportation and prolonged storage (Aktar et al., 2009; Hartman et al., 1995;Snowdon, 1990,1991), producing an enormous impact on plant-food market.

2. Materials and methods

2.1. Pseudomonas protegens strain N growth conditions

Pseudomonas protegens N was isolated from water samples of an irrigation well located in the region of Djebira in Bejaia, northern Algeria (36°41′59.2″N; 5°04′28.8″E). According to Tabli et al. (2017), the strain was initially grown at 30 °C on Plate Count Agar medium (PCA) for 48 h. Therefore, colonies were picked from the plate and pre-inoculated under agitation (180 rpm) in 10 mL of Luria Bertani (LB) broth and grown at 30 °C for 24 h. In the next step, the pre-inoculum was inoculated in 50 mL of LB broth to obtain an Optical Density at 600 nm (OD_{600}) of 0.5 and bacterial growth was monitored using a spectrophotometer (JASCO V-360) at 600 nm for estimating bacterial growth through changes in absorbance (Rodriguez-Valera et al., 1982). The growth curve of *P. protegens* N was obtained by inoculating the bacteria in LB broth at 30 °C, 180 rpm, and collecting culture growth samples at different time intervals.

2.2. Antifungal activity and induction assays

The antifungal activity and the induction assay were performed as described by Tabli et al. (2017). The antifungal activity was evaluated against different fungi strains (*Aspergillus niger, Alternaria* spp, Botrytis cinerea and Penicillium spp) that were plated on PCA plates and incubated at 28 °C until sporulation (2–4 days) (Tabli et al., 2017). The antifungal compound production was induced by pouring 300 µl (1×10^8cells/ml) of the bacterial culture in wells (0.5cm in diameter) aseptically punched on the plates, previously scraped with fungi spores (2×10^4conidia/ml) and by incubating the plates for 48h at 28°C. Therefore, the induced bacterial aliquots were withdrawn from the holes and underwent to the extraction process. The antifungal activity was evaluated measuring the diameter of the inhibition zone, after 2 days of incubation at 28 °C.

2.3. Fungistatic and fungicidal tests

The experimental procedure was carried-out by cutting an agar disk (0.5 mm) from the inhibition zone in PCA plates previously scraped with the above mentioned fungi spores and placing it in the middle of a fresh Malt Extract Agar (MEA) plate (pH 5.5) incubated at 28 °C for 168 h until fungal growth. Agar disks cut from fungal growth zone were transferred on a fresh MEA plate and used as control.

2.4. Preparation of extracellular, intracellular and surface-membrane protein fractions

Induced *P. protegens* N cultures were centrifuged at 8000 rpm, 4 °C, for 45 min. The pellet was used to isolate the membrane protein fractions, accordingly to Gogliettino et al. (2010) with some modifications. The bacterial cell pellet was re-suspended in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 1 mM PMSF, pH 7.5) and lysed by sonication (4 cycles with a 60 s pulse ON and 60 s OFF) using a Soniprep (B. Braun Labsonic U). The unbroken cells were removed by centrifugation (13,000 rpm at 4 °C for 30 min), whereas the supernatant represented the intracellular fraction. The surface-membrane protein fraction was prepared by re-suspending the pellet in 50 mM Tris-HCl, pH 7.5 and solubilizing it by incubation for 15 min at 37 °C in the presence of 1% Triton X-100. Insoluble material was removed by centrifugation at 13,000 rpm at 4 °C for 30 min, and the collected supernatant represented the Surface Membrane Fraction (SMF). All the fractions were sterilized by filtration, using a 0.22 µm pore diameter membrane (Millex GV) and tested for antifungal activity as describe above. Active fractions were stored in 10% glycerol and 0.1% Sodium Deoxycholate (Sigma-Aldrich).

2.5. Purification of antifungal compound

The SMF obtained from *P. protegens* N induced cultures was subjected to ultra-filtration using 100 kDa molecular weight cut-offs filter (Millipore, USA). The resulting retentate, named R100, was loaded onto a DEAE Sepharose Fast Flow column, connected to an AKTA FPLC system (Amersham Biosciences), pre-equilibrated in 25 mM Tris-HCl (pH 8.0) (Buffer A). Proteins were eluted with a linear NaCl gradient (0–1.0 M) in 25 mM Tris/HCl (pH 8.0) (Buffer B) at a flow rate of 1 ml/min. The fractions were collected, pooled and tested for antifungal activity against the indicator strain *Alternaria* spp as previously described. The bioactive fraction was concentrated and then loaded onto a gel filtration YARRA™ SEC-4000 column (Phenomenex) connected to an UFLC system (SHIMADZU), equilibrated in 25 mM Tris-HCl and 50 mM NaCl, pH 7.5 at a flow rate of 0.5 mL/min. The eluted fractions were collected, pooled, dialyzed in 25 mM Tris-HCl, pH 7.5 and then tested by antifungal assay. SDS-PAGE (12% w/v) analysis was performed to

follow the purification of the active compound. Protein concentration was determined with the Bradford assay (Bradford, 1976) with bovine serum albumin used as standard.

2.6. In vitro tomato fruits assay tomato fruits assay

In vitro test on tomato fruits was performed according to Rai et al. (2016), with some modifications. Firstly, tomatoes were disinfected by immersion for 1–2 min in a sodium hypochlorite solution (2% active chlorine), rinsed twice with sterile distilled water and dried at room temperature for about 2 h. The tomatoes were injured near the equatorial zone with three holes (3 mm wide, 3 mm deep), and 10 µL (1.4 mg tot) of the R100 retentate containing the antifungal compound were placed in each wound. The control sets were prepared similarly using equal amounts (10 µL) of sterilized water. After 2 h, wounds were inoculated with 10 µL of *A. niger* spores (1×10^4 spore/ml) suspended in sterile LB broth. Tomato fruits were incubated at room temperature in specific sterile plastic box, covered to defend them from light, and made damp to preserve the sporulation of fungi (Bensidhoum et al., 2016; Rai et al., 2016; Xiao and Kim, 2008). After 6–7 days, the reduction of fungal growth in the R100 treated-tomatoes was evaluated in comparison with control's fruits. The experiments were performed in triplicate on three different preparations of R100 active fractions. The wounds were monitored daily and the data were transformed into a percentage using the formula:

$$\% C = [(n \text{ hole A (or B)/total holes})*100],$$

where A was the contaminated wounds, and B was the not contaminated wounds. Total holes included contaminated and not contaminated wounds. Values ranged between 0 (0% infected wounds) and 100 (max% of contamination).

2.7. Germination test

The germination test was performed in 96-well culture plates according to the method described by Yadav et al. (2007), with some modifications. The wells were prepared in triplicate for each concentration. The R100 retentate containing the antifungal protein compound was diluted at different concentrations in a volume of 100 µL of Tris-HCl 25 mM, pH 7.5, supplemented with Triton (1%) and was inoculated with 100 µL of *Alternaria* spore resuspended in 2xLB broth. As control, 100 µL of *Alternaria* spp spore (1×10^5 conidia/ml), resuspended in 2xLB were diluted with 100 µL of Tris-HCl 25 mM, pH 7.5, containing Triton (1%). The plates were incubated at 28 °C for 7 days and then examined for spore germination. For stereomicroscopic analyses, 12 well flat bottom micro culture plates (Falcon) were used. The wells were prepared in triplicate for each treatment. As control, 500 µl of *Alternaria* spp spore suspension (1×10^5 conidia/ml) in 2xLB were diluted with 500 µL of Tris-HCl 25 mM, pH 7.5. In the treated samples, the wells were inoculated with 500 µL of R100 at different concentrations in Tris-HCl 25 mM, pH 7.5 and 500 µL of *Alternaria* spp spores suspension in 2xLB broth. Plates were incubated at 28 °C and examined for spore germination with a stereoscopic microscope (LEICA MZ 16 F A) at different times of inoculations.

2.8. Ultrastructural characterization by TEM

To test the effects of the antifungal compound on *Alternaria* spp. cell morphology, the fungal spore suspension (1×10^5 conidia/ml) was grown on LB liquid cultures at 28 °C, in a static manner, for 7 days, with or without supplementation of 0.5 µg of antifungal compound obtained from DEAE chromatography. Untreated and treated samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Post-fixation was performed using 1% osmium tetroxide for 1 h. The samples were dehydrated by passing through a graded ethanol series and embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA, USA). Embedded samples were sectioned by a diamond knife on a Leica UCT ultramicrotome (Leica Microsystems, Germany) into thin sections (50 nm thickness), attached to the formwar/carbon copper grids and, to increase the contrast of the samples, an additional staining with uranyl acetate was used. Finally, the samples on the grids were observed under a Jeol JEM-1011 (JEOL, Tokyo, Japan) transmission electron microscope using an accelerating voltage of 100 kV. Images were captured at different magnification by using the iTEM software (Olympus Soft Imaging System, Münster, Germany). The fungus was kindly provided by Professor Houali Karim, Laboratory of analytic biochemistry research and biotechnology (LABAB), Mouloud Mammeri University, Tizi-Ouzou, Algeria.

2.9. Statistical analysis

All measurements were carried out at least in triplicate. The results were presented as mean ± standard deviation (S.D.). Significance differences among means were evaluated by the Student's t test, and $p < 0.05$ was considered as significant.

3. Results and discussion

3.1. Selection of the best fungal target

As previously described (Tabli et al., 2017), a microbiological screening conducted on bacteria isolated from agricultural well-water located in northern Algeria, allowed to select the best strain in terms of antifungal and PGP activities. By the 16S rRNA sequence analysis, the isolated strain was identified as belonging to the genus *Pseudomonas*. Moreover, the phylogenetic analysis revealed that this bacterium, named "N", was very close to the *Pseudomonas protegens* CHAO, with a similarity of 99.93% of their 16S rRNA genes.

In this study, a preliminary screening was performed to select the best fungal target needed to investigate on specific protein compound responsible of the bio-control activities, based on previous observations that it was confined into the Surface-Membrane Fraction (SMF) and its production was induced by the presence of the fungus (Tabli et al., 2017).As shown in Fig. 1A, SMF exhibited a broad spectrum of antifungal activity with a marked inhibitory effect especially against *Alternaria* spp, generating an inhibition halo of about 40 mm of diameter respect to those ranging from 19 to 30 mm, measured with the other three fungi under investigation. Moreover, to investigate the action mechanism of SMF, a more detailed analysis was performed. Interestingly, when a small piece of agar disk from each inhibition zone was transferred to a new plate, only a delay of growth was observed for *Botrytis cinerea*, *Penicillium* spp, and *Aspergillus niger*, while *Alternaria* spp was completely unable to re-growth (Fig. 1B, **1C**). Therefore, a fungicidal effect against *Alternaria* spp can be attributed to SMF, in contrast to the fungistatic activity observed towards the others tested fungal species, possibly due to a different morphology and/or organization of the fungal cell membranes. Based on these data, *Alternaria* spp was chosen as indicator fungus to follow the purification of the antifungal agent by *in vitro* bioassay.

3.2. Time-dependent antifungal compound production from the Pseudomonas protegens N strain

To assess the optimal conditions responsible for the better antifungal compound production by *P. protegens* N, a growth curve of this strain was built (Fig. 2A). For this purpose, samples of *Pseudomonas* cultures collected at 12, 24 and 48 h of growth were tested against *Alternaria* spp. After collection, the induction test was performed on each bacterial sample and the SMFs were prepared and assayed. In Fig. 2B, it was shown the relationship between *P. protegens* N growth and the antifungal compound production, which already started at the

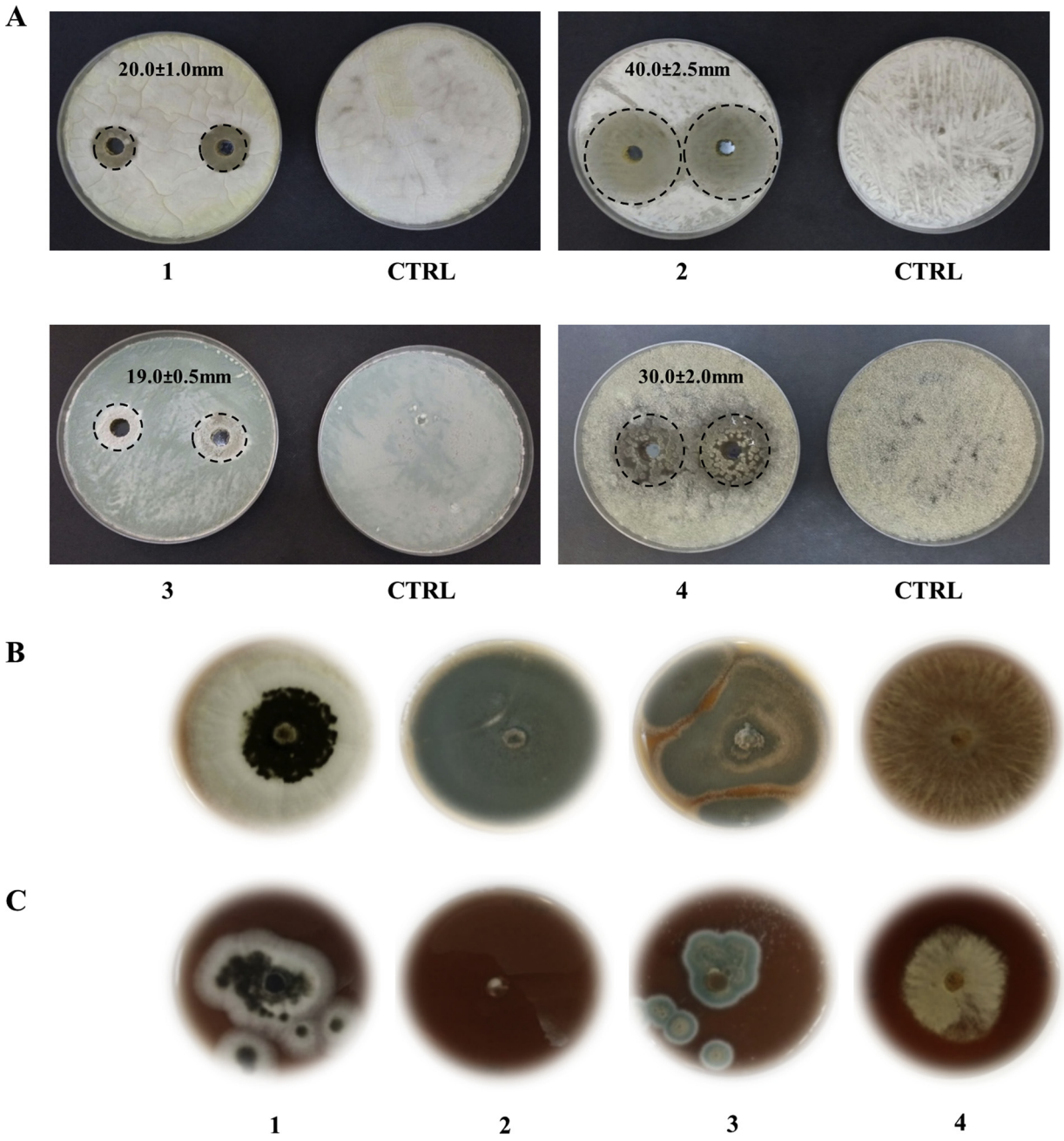

Fig. 1. Antifungal activity of SMF from induced *Pseudomonas protegens* N cells and its mechanism of action against different fungal species. **(A)** Inhibition assay of SMF performed against *Aspergillus niger* (**1**), *Alternaria* spp (**2**), *Penicillium* spp (**3**), *Botrytis cinerea* (**4**). CTRL: control fungi without SMF treatment. The PCA plates were incubated at 28 °C for 48 h until the growth of fungus. Inhibition zone produced by SMF was marked with dashed lines. (**B**) Agar disks cut from fungal growth zone of the SMF treated plates showed in panel (**A**) and transferred on a fresh MEA plate. The plates were incubated at 28 °C for 168 h until growth of fungus. (**C**) Agar disks cut from the inhibition zone of each fungus showed in panel (**A**) and transferred on a fresh MEA plate: *Alternaria* spp (**1**), *Botrytis cinerea* (**2**), *Aspergillus niger* (**3**), *Penicillium* spp (**4**). Results are presented as the mean values ± SD of triplicate analyses from at least three different experiments.

beginning of exponential phase (12 h), reaching the maximum at 24 h of growth, and persisting until the early stationary phase (48 h), as revealed by the inhibition area diameters (Fig. 2B). Based on these results, the 24 h-old *P. protegens* N culture was chosen as the time of growth to set up the antifungal compound purification protocol.

3.3. Purification of an antifungal compound from Pseudomonas protegens N strain

To obtain a sample enriched in the compound with antifungal activity for evaluating its possible use as a bioactive agent to control disease and promote plant growth, the SMF derived from 24 h-old *P. protegens* N cultures was purified by a combination of three different steps. In the first one, SMF was ultra-filtered using 10, 30, 50 or 100 kDa molecular weight cutoff filters, which allowed also to assess the protein/peptide nature of the anti-fungal compound. These analyses clearly demonstrated that only the retentate obtained from all the cutoff filters was active against *Alternaria* spp, being that 100 kDa-ultra-filtered sample (named R100) the most active (Fig. 3**A**) and less complex, as evidenced by SDS-PAGE analysis (Fig. 4). In the second step, R100 was subjected to anion exchange chromatography on a DEAE Sepharose Fast Flow column, from which two main protein fractions (named Peak 1 and Peak 2) were eluted (Supplementary Fig. 1A) and assayed against *Alternaria* spp. A strong antifungal activity was detected with only the

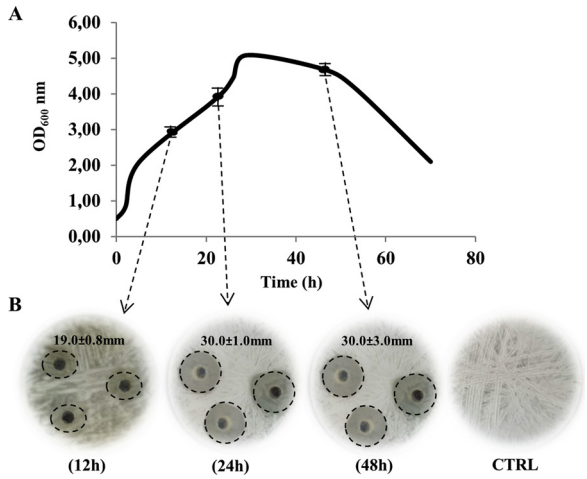

Fig. 2. Time-dependent production of antifungal compound from *Pseudomonas protegens* N strain. **(A)** Growth curve of *P. protegens* N strain. **(B)** Inhibition assay performed at: 12 h, 24 h and 48 h of growth. SMFs were prepared after induction assay and tested against *Alternaria* spp. CTRL: control fungus without SMF treatment. Assays were performed using the same amount of SMFs (1.3 mg). The plates were incubated at 28 °C for 48 h until the growth of the fungus. Results are presented as the mean values ± SD of triplicate analyses from at least three different experiments.

Peak 1 fraction, corresponding to the unbound proteins, by the plate-based agar diffusion assay (Fig. 3B). Further purification of Peak 1 was conducted through the gel filtration chromatography on an SEC-4000 column (**Supplementary** Fig. 1B). The obtained fractions were assayed for the antifungal activity against *Alternaria* spp and the results indicated that only Peak 1 contained the antifungal bioactive component (Fig. 3C). Analysis by SDS-PAGE established that the antifungal compound was isolated following the procedure described above (Fig. 4, **lane 5**). By means of these purification steps, the yield was relatively low but the reported method gave sufficient protein amount to perform

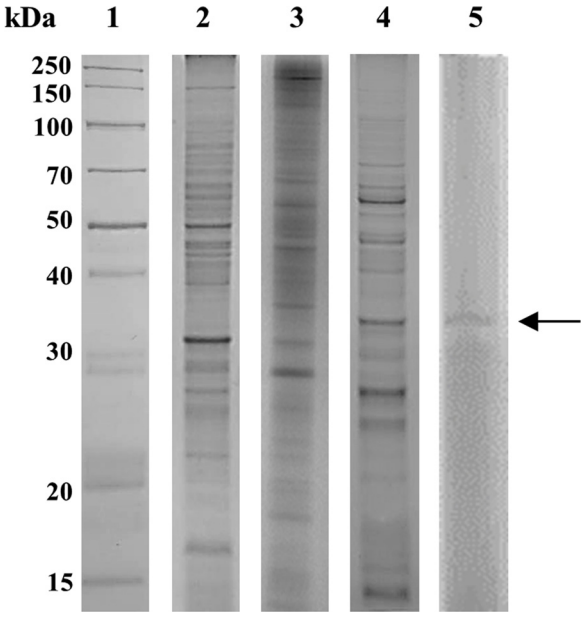

Fig. 4. SDS-PAGE analysis of purified antifungal compound. Lane 1: molecular weight markers; lane 2: SMF prepared from *P. protegens* N cells after induction assay; lane 3: Retentate fraction (R100) obtained by ultrafiltration through 100 kDa cutoff filter; lane 4: Peak 1 obtained from DEAE chromatography; lane 5: Peak 1 obtained from gel filtration chromatography. The image is representative of three different protein preparations. The arrow indicates the purified compound.

the reported functional studies. Gel filtration chromatography allowed to define the molecular mass of the antifungal compound, which was estimated to be approximately 100 kDa as determined by the calibration curve (Supplementary Fig. 1B), suggesting that the protein can be most likely an oligomer. However, further analyses will be done in order to optimize the purification protocol and to perform a more

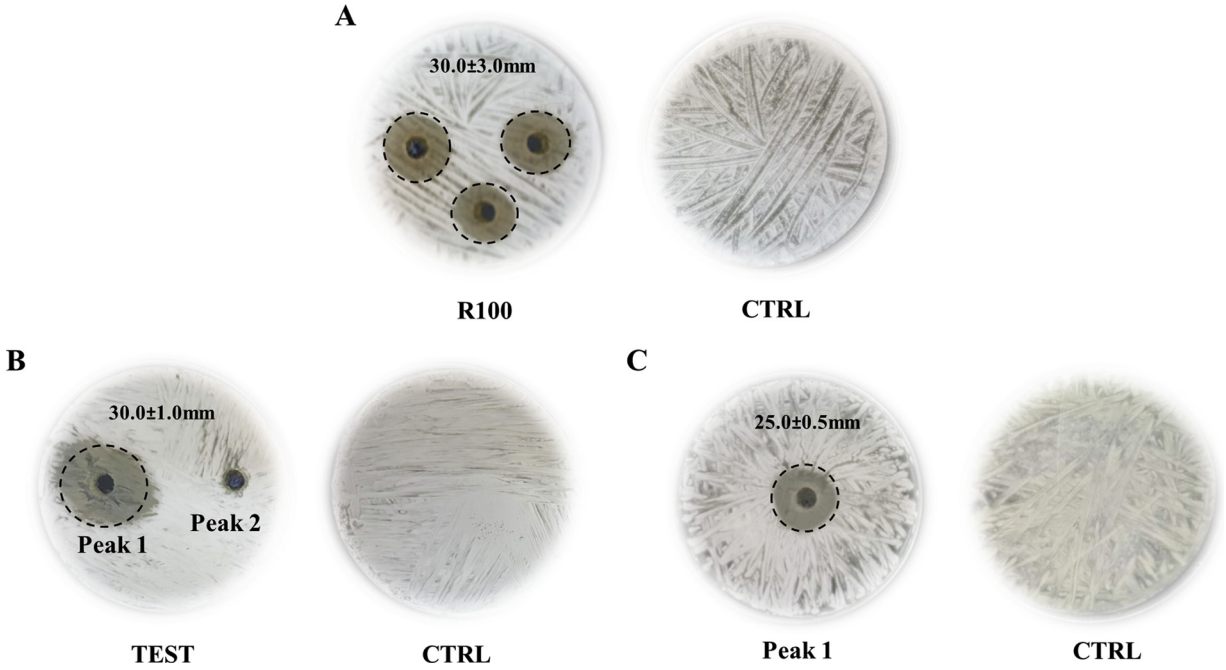

Fig. 3. Plate-based inhibition assay of antifungal compound against *Alternaria* spp after different purification steps. **(A)** Antifungal activity assay of R100 (1.3 mg). **(B)** Antifungal activity of protein fractions eluted from DEAE Sepharose anion exchange chromatography (TEST). **(C)** Antifungal activity of Peak 1 fraction eluted from YARRA™ SEC-4000 gel filtration chromatography. CTRL: control fungus. The plates were incubated at 28 °C for 48 h until the growth of fungus. Results are presented as the mean values ± SD of triplicate analyses from at least three different experiments.

detailed structural characterization aimed at better defining the subunit identity of the oligomeric protein responsible of the antifungal activity.

3.4. Effects of the antifungal compound on fungal infection development on tomato fruits

It has been estimated that plant fungal pathogens are responsible for the majority of postharvest diseases, which can destroy 10–20 % of crop yield (Rai et al., 2016; Tripathi and Dubey, 2004). To date, the application of fungicides to control phytopathogen's effects is one of the most important tools exploited for plant protection and crop improvement (Mahmood et al., 2016; Rai et al., 2016). Nevertheless, their regular use presents a serious risk for the environment and health together with the development of resistance in phytopathogen populations (Aktar et al., 2009). Therefore, in this study *in vitro* experiments were performed to estimate the effects of R100 on reducing the development of fungal infection on tomato fruits, which were selected for uniformity in size, appearance, ripeness and the absence of physical defects. To this aim, *Aspergillus niger,* which represents one of the most locally predominant fungi that are associated with tomato's diseases (Ibrahim and Ebady, 2014; Snowdon, 1990) causing bunch rot (Nair, 1985; Snowdon, 1990) and black mold (Snowdon, 1991), was chosen as the tested fungus. Indeed, the susceptibility to the fungal spoilage of fruits and vegetables is largely due to their differential chemical properties such as pH and moisture contents. As reported in Fig. 5A, tomato fruits, artificially inoculated with *A. niger*, used as control (CTRL) and incubated at room temperature for six days, showed the typical spoilage symptoms generally produced by this fungus. Interestingly, the area of infection was reduced of about 80% in tomatoes treated with R100, evidencing a statistically significant reduction of the infection (Fig. 5A**, 5B** TREATED**)** respect to the control. Similar antifungal effects were obtained using a different variety of tomato fruits with spherical shape (**Supplementary** Fig. 2).

These results suggest a strong antifungal effect of the protein compound produced by the *P. protegens* N strain and a promising prospect for agricultural applications, such as in keeping the quality of fruits during storage and in reducing post-harvest disease caused by fungi. Therefore, the bacterial extract could represent a valid sustainable eco-friendly fungicide and have potential as a biocontrol agent in alternative to chemical pesticides.

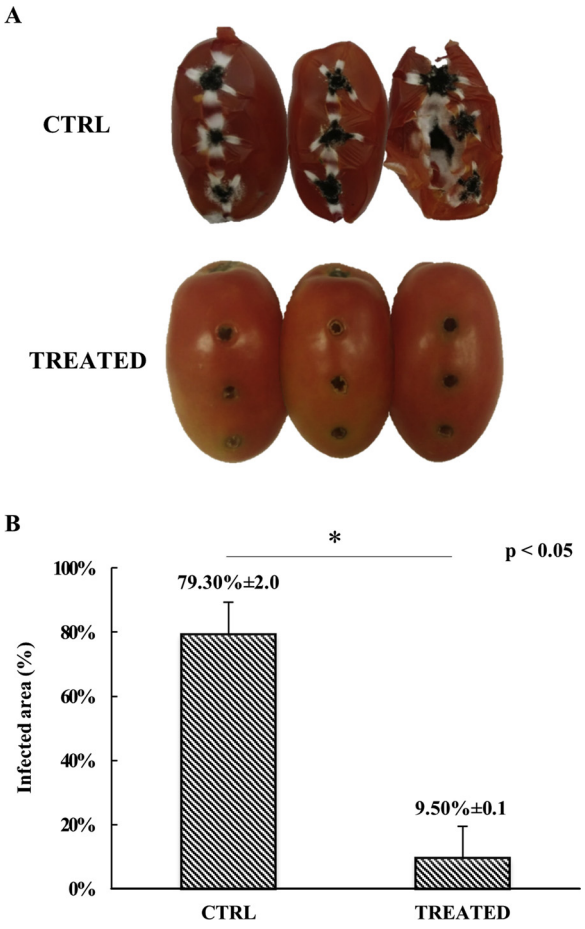

Fig. 5. Effects of R100 on infection development on tomato fruits with oblate shape. (**A**) *In vitro* assay. CTRL: tomato fruits infected by *A. niger*; TREATED: tomato fruits infected by *A. niger* and treated with the antifungal compound R100 (1.4 mg). (**B**) Infected area (%) on tomato fruits in absence (CTRL) or presence (TREATED) of the antifungal compound R100 after 6 days of infection. Statistical data were performed on 21 repeated tests (63 total holes) for control and treated tomato fruits. The results are expressed as the mean ± SD. * indicates significantly different ($p < 0.05$) from the respective control samples.

3.5. Germination test

In fungi, as well as in some prokaryotic and eukaryotic organisms, the spores are resting structures that allow the adaptation and survival for extended intervals of time, in unlucky conditions. For the pathogenic fungi, spore germination is a determining factor at the onset of host colonization (Talbot, 2001). Therefore, inhibition of spore germination measurement is a useful technique for evaluating the sensitivity of filamentous fungi to antifungal compounds.

To assess the inhibitory effects of the antifungal compound on spores' germination of *Alternaria* spp, two different systems were used. In the first approach, the inhibitory effects were analyzed in a qualitative manner by using the liquid culture microdilution assay, in which different concentrations of R100 enriched-fraction in culture medium were tested and inoculated with *Alternaria* spore suspension. As shown in Fig. 6A, fungal samples incubated without R100 clearly revealed the spores' formation with a characteristic green color, whilst a dose-dependent inhibitory activity on *Alternaria* sporulation was observed in the presence of the antifungal compound. The antifungal activity on preventing spore propagation without affecting the mycelium growth was shown already at the lowest concentration used (0.48 mg/ml) (Fig. 6A). To better assess the capability of antifungal protein compound to inhibit spore germination, a scale-up was performed by increasing the assay volume to one-milliliter and using the maximum R100 concentration tested in the 96 multi-wells assays (data not shown). In the second approach, the effects on *Alternaria* spp spores germination in the presence of R100 were observed at different times of germination until 21 days of inoculation by the stereomicroscope. As stated in Fig. 6C, treated sample showed no specific and organized structures ("carpet-like structure"), no conidial formation and no cross of light, in contrast to the fungal growth in the control (Fig. 6B), displaying an enhancement of the 3D structure organization and conidial formation with a resulting much reduced passage of light. A similar approach was adopted by using the purified sample obtained after DEAE chromatography, evaluating its effects at different times of germination (0, 2, 4, 6, 8, 10 and 14 days). The results obtained after 2, 4 and 14 days clearly showed the most marked differences between the treated (TEST) and untreated *Alternaria* (CTRL) in terms of the sporulation process (Fig. 7**)**. Specifically, at time 0 (t = 0), CTRL and TEST were indiscernible. After 2 days of inoculation, it was possible to observe a normal fungal growth and sporulation in the CTRL with the formation of a 3D structure developed in the space, which appears comparable to a nervous system network. This 3D structure can be possibly attributed to a typical organization of growing fungus formed by vegetative and aerial mycelium, which causes a reduction in the crossing of light. On the contrary, an unusual fungal growth was detected in the TEST sample, in which the mycelium aggregation was strongly affected and the sporulation was completely inhibited,

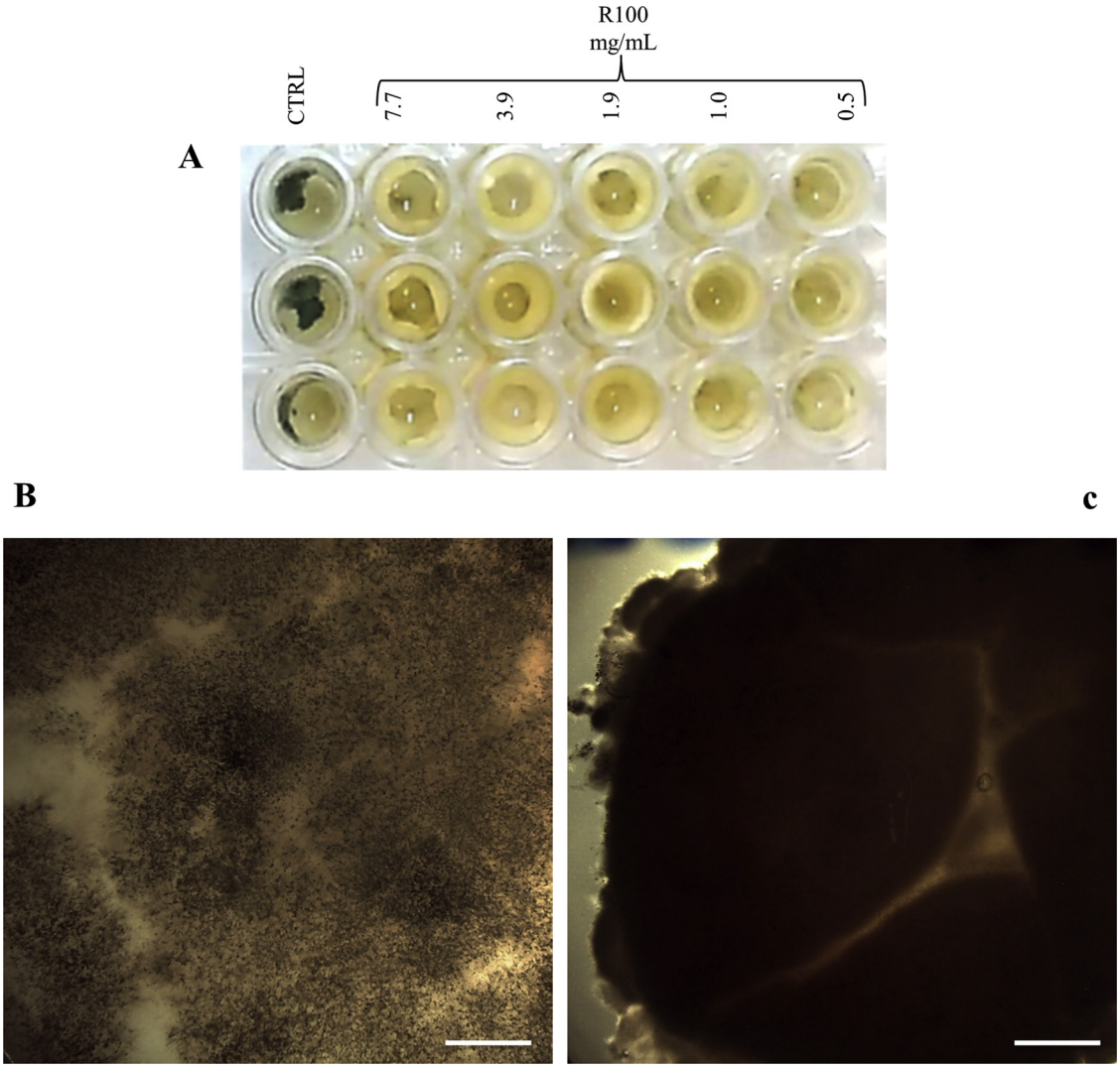

Fig. 6. Inhibitory effects of antifungal compound on *Alternaria* spp spores' germination. **(A)** Spores' germination test in 96 multi-wells plates. Column 1: normal growth of *Alternaria* spp (CTRL); Columns 2-5: *Alternaria* spp treated with different concentrations of R100. Stereomicroscopic analysis of *Alternaria* spp spores' germination at t = 21 days in absence (**B**) or presence (**C**) of the R100 active fraction (0.50 μg) obtained after ultrafiltration step. Scale bar is equal to 2 mm. The images are representative of triplicate analyses from at least three different experiments.

producing an irregular organization of the hyphae, with the absence of compact structures and higher crossing of light. After 4 days, an accurate organization with branched hyphae that obstructed the light's crossing was evidenced in the control fungus (CTRL), while a peculiar "carpet-like structure" morphology was evidenced in the TEST sample. Finally, at 14 days of inoculation, the results were comparable to those obtained with the R100 treatment (Fig. 6B**, C**). These results suggest that the antifungal agent was able to cause the collapse of the normally well-organized 3D structure found in *Alternaria* spp control, forming a "carpet-like structure" possibly due to the development of vegetative hyphae that did not originate the aerial mycelium involved into spores' formation, blocking the asexual spores cycle.

3.6. Ultrastructural characterization of conidia

The effects of antifungal compound/s on *Alternaria* spp conidia germination were estimated at ultrastructural level by using the transmission electron microscopy (TEM). At starting point (t = 0), the conidia of *Alternaria* spp showed well-organized cells, spheroidal in shape, characterized by an undamaged and thick inner and outer conidial cell walls, integrated cell membrane, a homogeneous cytoplasm containing a nucleus almost centralized and proliferated lipid bodies, predominantly form of carbon storage as triacylglycerol in fungi spores (Fig. 8A). After 7 days of incubation at 28 °C in a static way, the untreated *Alternaria* spp conidia usually showed an elongate and ellipsoidal shape, a not homogeneous cytoplasm with a high electron density at the periphery of the cells and, a thinner cell wall (Fig. 8B). In contrast, the *Alternaria* spp conidia, treated with the purified fraction obtained from DEAE chromatography (0.5 μg), after 7 days of incubation at 28 °C in a static way, showed morphological aberrations. Specifically, the spherical and/or ellipsoidal shapes were not well conserved, cell walls appeared very thin and smooth, a low electron-dense cytoplasm was observed predominantly outside of the cells, whereas, only in the central part of them, cellular debris and some membrane ghosts were present, proliferated lipid bodies were absent (Fig. 8**C, D, E**). The alterations of the cell wall and cell membranes could be the cause of the release of cytosol perhaps through the formation of channels in the cell envelopes. These results were consistent with the proposed mechanism of deactivation of fungi by antimicrobial peptides through pore formation (Kim et al., 2017).

4. Conclusions

In the present study, a novel antifungal compound was isolated from

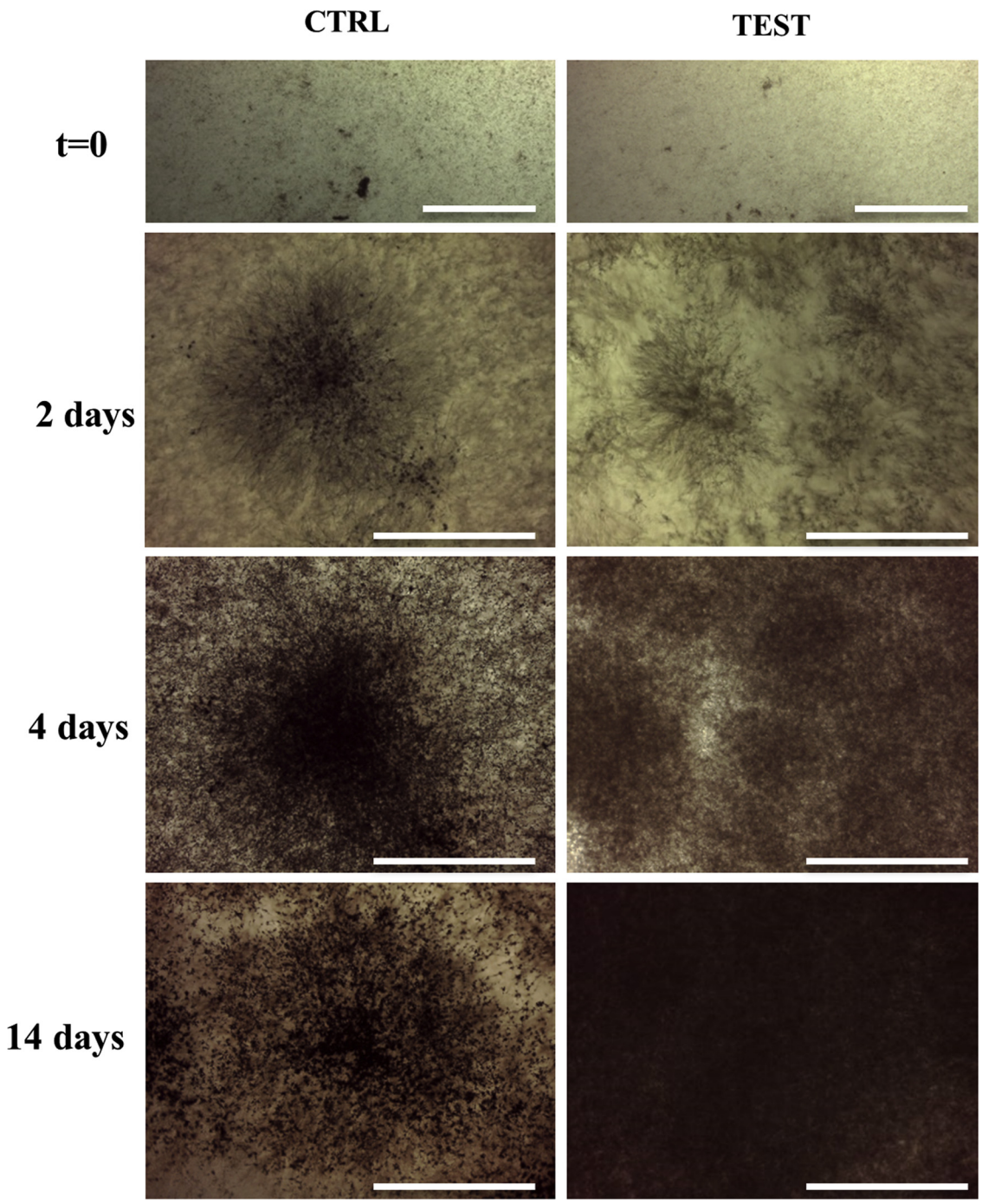

Fig. 7. Inhibitory effects of antifungal compound after DEAE chromatography on *Alternaria* spp spores' germination. Stereomicroscopic analysis of Alternaria spp spores' germination at different times: t = 0, 2 days, 4 days and 14 days in absence (CTRL) or presence (TEST) of active fraction (0.50 μg) obtained after DEAE chromatography. The images are representative of at least three different protein preparations.

the strain *P. protegens* N, selected from agricultural land in northern Algeria, and investigated for its potential antifungal activity. Specifically, the compound turned out to be a large protein oligomer with a high molecular mass of approximately 100 kDa and it was localized in the surface-cell membrane fraction, from which it was purified. Interestingly, the compound displayed an efficient activity against a variety of fungi among the most common phytopathogens. *Alternaria* spp has revealed the best target towards which the compound exhibited a marked fungicidal action and inhibition of spore germination, triggering a concomitant collapse of the normal fungal 3D structure found in the untreated samples. This effect produced a "carpet structure" probably formed by the vegetative hyphae that were no longer able to develop in the aerial mycelium involved into the spores' formation, blocking their asexual cycle. Finally, the antifungal molecule was able to interfere with the conidial germination as revealed by ultrastructural (TEM) observations, causing considerable morphological alterations and severe damage to the cellular structures, which trigger the release of the cytosolic components possibly through the formation of channels in fungal cell envelopes. This is the first report concerning the purification and characterization of a large protein oligomer with antifungal activity produced by a PGPB. However, recently another study has been addressed, concerning the partial purification of an antifungal protein with a molecular mass of about 58 kDa, isolated from *P. protegens* strain XL03 (Wang and Zhang, 2017).

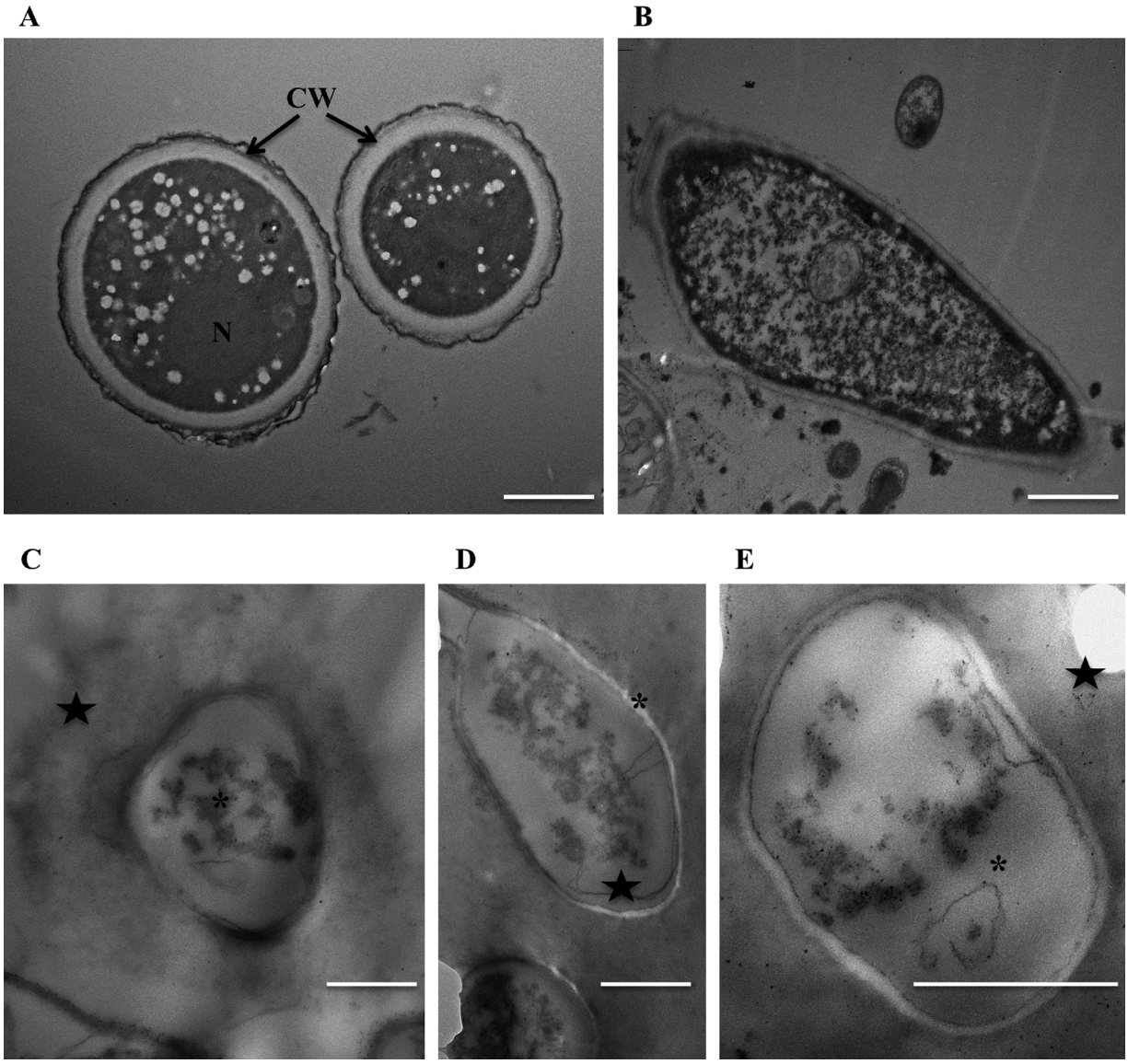

Fig. 8. Transmission electron micrographs of *Alternaria* spp grown on liquid medium. **(A)** *Alternaria* spp conidia at starting time (t = 0). (**B**) Untreated conidia of *Alternaria* spp, grown in static manner at 28 °C on LB medium for 7 days. (**C, D, E**) *Alternaria* spp conidia treated with antifungal compound obtained after DEAE chromatography (0.50 μg), after 7 days of incubation. N, nucleus; CW and arrow, cell walls; asterisks, membranes debris; star, cytoplasm material. Scale bar is equal to 1 μm in all micrographs. The images are representative of at least three different protein preparations.

In conclusion, the antifungal activities exhibited by the isolated compound have given good indications of its potential as promising safe and environmentally-friendly candidate for future applications as a biocontrol agent in minimizing postharvest losses caused by fungal plant pathogens and therefore as a natural alternative to conventional fungicides in agriculture. However, further studies will be necessary to investigate on a possible application of the antifungal compound in other farm practices such as in culture stage.

Declarations of interest

None

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.02.004.

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