SCIENTIFIC NOTE

CHILEANJAR
CHILEAN JOURNAL of AGRICULTURAL RESEARCH

Cysts and alkylresorcinols of *Azotobacter vinelandii* inhibit the growth of phytopathogenic fungi

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Received: 26 January 2022; Accepted: 18 May 2022; doi:10.4067/S0718-58392022000400658

ABSTRACT

Azotobacter vinelandii is a Gram negative that undergo a morphological and physiological differentiation process to form cysts resistant to desiccation. The main components of a cyst are alkylresorcinols (ARs), phenolic lipids of 21 to 23 C. These lipids are homologous to plant ARs, where they have been reported to have antifungal properties. The objective of this study was to evaluate the antifungal capacities of vegetative cells and cysts of *A. vinelandii* AEIV. The cysts were visualized by optical microscope and the detection of ARs was performed by thin layer chromatography. Antagonism bioassay were carried out between vegetative cells and cysts against *Fusarium brachygibbosum*, *Aspergillus niger* and *Colletotrichum gloeosporioides*. We confirmed the induction of cysts and the presence of ARs of *A. vinelandii* AEIV. We observed that using 1×10^3 cysts reduced up to 76.3% ($p < 0.05$) the growth of the three phytopathogenic fungi and this result was not observed when using vegetative cells or the strain blocked in the synthesis of ARs. This shows that the cysts exert an antagonistic effect on phytopathogenic fungi and do so through the ARs, considering the development of an effective strategy to control pests in fungi that affect postharvest fruits.

Key words: Antifungical, *Aspergillus niger*, *Azotobacter vinelandii*, *Colletotrichum gloeosporioides*, *Fusarium brachygibbosum*.

INTRODUCTION

Most plant diseases are caused by fungi, which affect internal tissues such as roots, leaves or fruits, therefore extensively affects crop performance (Jain et al., 2019). *Fusarium brachygibbosum* is a causal agent of stem rot in maize, that causes a decrease in the absorption and translocation of water and nutrients (Shan et al., 2017; EFSA PLH Panel et al., 2021), it was recently reported as a causal agent of basal rot of onion, which causes wilting without leaf yellowing and stunting of plants (Tirado-Ramírez et al., 2019). In addition, it affects crops such as watermelon (Renteria-Martinez et al., 2015) and sunflower (Xia et al., 2018). Although it is not a phytopathogenic fungus, *Aspergillus niger* is considered the main microorganism that deteriorates postharvest fruits (Sun et al., 2020). *Colletotrichum gloeosporioides* is one of the causative agents of anthracnose disease in *Mangifera indica*, *Persea americana* and *Capsicum annuum* (Jin et al., 2020; Liang et al., 2021). In the constant search for strategies to combat these fungi, various antagonistic bacteria have been tried. *Azotobacter vinelandii* Lipman 1903 is a Gram-negative bacterium, which has a characteristic life cycle, with a vegetative stage in which the cell is metabolically active, and a cystic stage, where the differentiation of *A. vinelandii*

takes place, which produces cysts resistant to desiccation (Segura et al., 2020). The cell of a cyst of the wild strain *A. vinelandii* AEIV (Svein Valla, Norwegian University of Science and Technology, Torgarden, Norway) it is surrounded by a two-layer capsule, an external one called exine and an internal layer called intin. The main components of both layers are the alginate polysaccharide and the alkylresorcinols (ARs) and alkylpyrone phenolic lipids, with side chains between 21 and 23 C (Reusch and Sadoff, 1983). Plant ARs are the most studied. In a previous study, they were reported to have various effects: cytotoxic, anticancer, antiproliferative, antioxidant, antiparasitic, and antimicrobial properties (Luís et al., 2016). The 5-*n*-alkylresorcinols of plants were used to inhibit the growth of the fungi *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum* and *Fusarium culmorum* (Patzke and Schieber, 2018). Antifungal activity has not been reported for bacterial ARs, therefore antagonism assays were performed in this work.

MATERIALS AND METHODS

Induction of *Azotobacter vinelandii* cyst formation

To induce cyst formation, strains of *Azotobacter vinelandii* AEIV were grown in Burk's medium with 0.2% butanol as sole C source for 5 d incubation (Romero et al., 2016). The presence of cyst was evaluated by staining with solution of Fast Blue B 0.5% in 5% acetic acid and observation at 100X with an optical microscope (Romero et al., 2016).

Detection of alkylresorcinols by thin layer chromatography

The detection of alkylresorcinols (ARs) was carried out from samples of ARs previously obtained, where the concentrated organic fraction was reconstituted in 200 µL acetone (Romero et al., 2016). This technique was performed on thin layer plates made with high-performance thin layer chromatography (HPTLC) Silicagel 60 F254 0.25 mm thick as a fluorescence indicator (Merck, Darmstadt, Germany); 10 µL ARs extract from each sample were added to the thin layer. For running, the chloroform-methanol system was developed in an 85:15 ratio, respectively. Finally, the development of the plate was then sprayed with a solution of Fast Blue B 0.5% in 5% acetic acid (Sigma-Aldrich, St. Louis, Missouri, USA; Romero et al., 2016).

Determination of the antagonist effect

To determine the antagonistic effect of vegetative cells and cysts of *A. vinelandii* AEIV, in vitro bioassays were performed following the methodology described by Chen et al. (2014). For this, a fragment of the previously grown fungus was extracted using a mycological loop and placed in the center of the Petri dish with PDA medium, wells were made in the PDA medium where 10^3, 10^6 and 10^9 vegetative cells or cysts were placed of *A. vinelandii* AEIV, as negative control magnesium sulfate was used. They were incubated at room temperature (30 °C) with daily monitoring. Experiments were conducted in a completely randomized design; with three replicates for each antagonism bioassay. Data were analyzed with ANOVA and means were compared with Tukey's test ($P < 0.05$) using Prism 9.0 (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

We induced the differentiation process to visualize cysts (Figure 1a) and detected alkylresorcinols (ARs) (Figure 1b) of *Azotobacter vinelandii* AEIV. We were able to observe the formation of characteristic cysts with the exine and intine layers reported by Chowdhury-Paul et al. (2018) and Martínez-Ortiz et al. (2020). When *A. vinelandii* differentiates, it is known to replace the characteristic phospholipids of vegetative cells with ARs (Trejo et al., 2017). Alkylresorcinols are one of the main components of the outer layer called the exine and the inner layer called the intine of the cyst, can be extracted and the production can be visualized by staining with Fast Blue B, developed a red color (Romero et al., 2016).

Vegetative cells of *A. vinelandii* AEIV were found to be unable to inhibit the growth of *Fusarium brachygibbosum* (Figure 2a), while the cysts inhibited their growth (Figure 2b), 1×10^3 cysts inhibited growth by 76.3%, 1×10^6 cysts inhibited growth by 70.7% and 1×10^9 cysts inhibited growth by 71.2% ($p < 0.05$, data not shown). The same inhibition effect of *A. vinelandii* AEIV differentiated cells was observed on *Aspergillus niger* (Figure 2c), 1×10^3 cysts inhibited growth by 75%, 1×10^6 cysts inhibited growth by 66% and 1×10^9 cysts inhibited growth by 71.6% ($p < 0.05$, data not

Figure 1. Cyst (a) and alkylresorcinols (ARs) (b) of *Azotobacter vinelandii* AEIV.

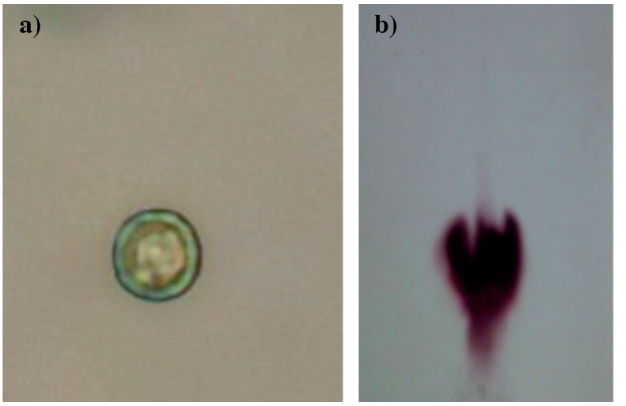
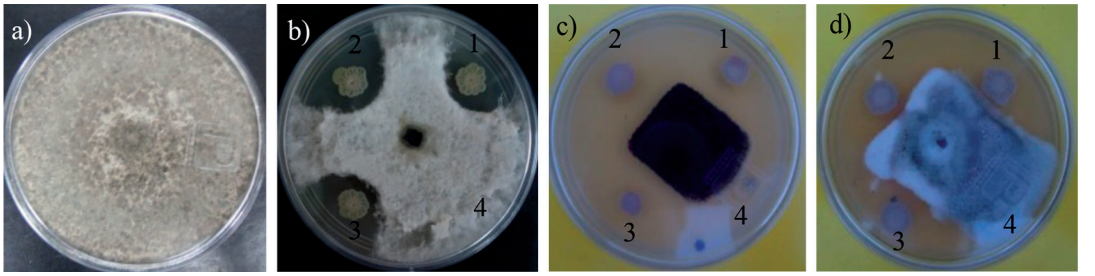


Figure 2. Antagonism assays with vegetative cell of *Azotobacter vinelandii* AEIV against *Fusarium brachygibbosum* (a), and cysts of *A. vinelandii* AEIV on *F. brachygibbosum* (b); *Aspergillus niger* (c), *Colletotrichum gloeosporioides* (d). 1) 1×10^3 cysts, 2) 1×10^6 cysts, 3) 1×10^9 cysts and 4) 16 mM magnesium sulfate.



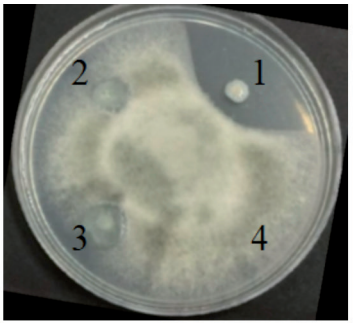
shown) and *Colletotrichum gloeosporioides* (Figure 2d) 1×10^3 cysts inhibited growth by 75%, 1×10^6 cysts inhibited growth by 66% and 1×10^9 cysts inhibited growth by 68.6% ($p < 0.05$, data not shown).

The *A. vinelandii* AEIV cysts were able to inhibit the growth of these phytopathogenic fungi, so it could be a potential biocontrol agent.

To be able to study the role of ARs in inhibiting the growth of phytopathogenic fungi, we performed the antagonism assay using cysts from a strain of *A. vinelandii* affected in ARs synthesis (OV8 strain) (Segura et al., 2009).

We observed that cysts of *A. vinelandii* OV8, no inhibition on growth of *C. gloeosporioides* was observed unlike the cysts of wild type strain (Figure 3). It is known that one of the main components of the exine and intine layers of the cysts are ARs (Zabolotneva et al., 2022), therefore, this result demonstrates that the effect of *A. vinelandii* AEIV cysts on the growth inhibition of the fungi used in this work it is through ARs, since the strain blocked in the synthesis of these phenolic lipids (*A. vinelandii* OV8), was unable to inhibit the growth of phytopathogenic fungi. There are many reports on the biological effects of ARs, but mainly chemically synthesized or plant-synthesized ARs, among the effects, are antimicrobial properties (Marentes-Culma et al., 2022). 4-Hexylresorcinol, 5-methylresorcinol, 2,4- and 2,6-dialkylhydroxybenzen and others were reported as an antibiotic adjuvant, evaluated in *Bacillus subtilis*, *Lactococcus lactis*, *Mycobacterium smegmatis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis* bacteria, and in the fungi *A. niger* and *Saccharomyces cerevisiae*. Of these, the one with the best antimicrobial activity was 4-hexylresorcinol (Nikolaev et al., 2020). It has also been reported that ARs and phytohormones actively participate in the defense system of wheat against *Fusarium* (Chrpová et al., 2021). There are very few reports on the activity of ARs in bacteria, therefore, this is the first report that shows that *A. vinelandii* cysts presented antagonism and antifungal activity on *F. brachygibbosum*, *A. niger* and *C. gloeosporioides*. This research contributes to propose possible strategies for effective control of diseases caused by phytopathogenic fungi used in this work, using *Azotobacter vinelandii* cysts.

Figure 3. Antagonism assay of *Azotobacter vinelandii* against *Colletotrichum gloeosporioides* using cysts (1) and vegetative cells of *A. vinelandii* AEIV (2), and cysts of the non-alkylresorcinol-producing OV8 mutant (3). Magnesium sulfate was used as a negative control (4).



CONCLUSIONS

Azotobacter vinelandii AEIV cysts caused growth inhibition of all tested phytopathogenic fungi, and this effect is through alkylresorcinols. Thus, demonstrating its potential as biocontrol of phytopathogenic fungi.

ACKNOWLEDGEMENTS

This work was supported by grants 858492 from CONACyT. Alejandro Bolaños Dircio thanks CONACyT for M.Sc. scholarships.

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