Inhibitory Activity Of Secondary Metabolites Isolated From Endophytic Bacteria Associated With The Cultivation Of Yam (Dioscorea Spp.) Against Phytopathogens Of Rice Plants (Oryza Sativa L.)

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ABSTRACT

In search of new effective biological substances of microbial origin for the management of diseases produced by phytopathogens in rice crops, biological control with endophytic bacteria has become a friendly alternative to chemical products, and to answer the question "Do endophytic bacteria have inhibitory activity against Burkholderia glumae, the cause of bacterial blast of panicles in commercial rice varieties? this study was conducted to isolate endophytic bacteria associated to different tissues of yam plants resistant to anthracnose in the department of Sucre and to evaluate in vitro the antibacterial activity against B. glumae. In this study, endophytic bacteria were isolated from different tissues of yam plants (Dioscorea rotundata) and their capacity for inhibition against B. glumae causing rice panicle blast was evaluated in vitro, using vegetative cells and secondary metabolite-type extract from filtered crude culture. The results of this activity show Burkholderia cepacia as an endophytic bacterium with 75.3% antibacterial activity against B. glumae, the cause of rice panicle blast.

Keywords. Bacteria, endophytes, metabolites, inhibition, plant pathogen, rice.

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1. INTRODUCTION

According to Pérez and Saavedra (2011), rice is the main cereal used as a source of food, with more than 50% of the world's population benefiting from this product. One of the main problems is the phytosanitary problems, a limitation in rice cultivation and even more relevant is the increase in the intensity of those caused by bacteria that until some time ago were of little importance. The disease known as bacterial blast of the rice panicle, caused by Burkholderia glumae, has increased its incidence in recent years.

Burkholderia glumae (Kurita & Tabei) (Syn. Pseudomonas glumae Kurita & Tabei) is reported in databases as the causal agent of rice panicle blight (Urakami et al., 1994, Gañán-Betancur, 2011). The phytotoxin toxoflavin, flagellar biogenesis, a type III secretion system and catalase have been reported to be factors involved in the virulence of B. glumae in rice grain and seedling rots (Ham et al., 2011; Chen, 2011, Gañán-Betancur, 2011).

One of the possible causes of this change has not yet been established, but hypotheses such as the recent introduction of aggressive strains, and the presence of conditions for disease development due to climate change have been formulated. B glumae was reported in Colombia at the end of the 1980s and the results of research carried out on it in different rice varieties indicate that most strains are highly aggressive on the susceptible variety Colombia XXI, and that the isolates have a high genetic variability. This disease is considered to be the most important, not only because of the economic losses caused to the crop, but also because of its difficult control and management in the field (Pérez and Saavedra, 2011).

As described by Gañán-Betancur, (2011), the presence of B. glumae in seed was suppressed by seed treatment with benomyl at a concentration of 0.1% (v/v), although the effect was not direct, apparently due to the antagonistic effect of Pseudomonas fluorescens that proliferated in the seeds treated with this fungicide (Goto et al., 1994). Seed treatment with the avirulent strain of B. glumae N7503 showed a high suppressive effect against virulent strains, preventing rice seedling rot (Furuya et al., 1991), similarly, an avirulent strain of B. gladioli prevented disease occurrence when co-inoculated with virulent strains of B. glumae on rice panicles (Miyagawa and Takaya, 2000). However, the efficacy of these potential control agents in the field has not yet been evaluated (Ham et al., 2011).

Biological control of crop diseases and pests using antagonistic bacteria has been an environmentally friendly alternative to the use of chemical pesticides (Moenne-Loccoz et al., 2001) and is being widely studied for many plant diseases, using a diverse group of

antagonistic microorganisms as part of integrated disease management programmes. To answer the question "¿ Do endophytic bacteria have inhibitory activity against Burkholderia glumae, the cause of bacterial blast of panicles in commercial rice varieties?". This study was conducted to isolate endophytic bacteria associated to different tissues of yam plants resistant to anthracnose in the department of Sucre and to evaluate in vitro the antibacterial activity against B. glumae.

2. MATERIALS AND METHODS

2.1. Microorganisms

Three morphotypes isolated from the Hawthorn yam plant variety (D. rotundata) and the rice pathogenic bacterium B. glumae were used. These are part of the strain bank collection of the Bioprospección Agropecuaria group of the University of Sucre. For the activation and purification of the bacteria, R2A culture medium was used for the morphotypes and King B medium for the pathogenic bacteria.

2.2. Isolation of culturable endophytic bacteria.

The roots, stems and leaves of each yam plant were washed with sterile water and cut into fragments of approximately 1 cm in length. The surface disinfection process of each tissue was carried out as follows: washing of each tissue separately in sterile distilled water, followed by shaking for 15 min in potassium phosphate buffer 0.05 mol L-1, pH 7.0; immersion for 1 min in 70% alcohol; shaking for 5 min in potassium phosphate buffer 0.05 mol L-1, pH 7.0; immersion for 1 min in 70% alcohol; agitation for 5 min in 5% sodium hypochlorite solution and immersion in Tween 80%; again immersion for 1 min in 70% alcohol followed by agitation for 15 min in potassium phosphate buffer 0.05 mol L-1, pH 7.0 and, finally, washing four times in sterile distilled water (Pérez et al, 2010).

2.3. In vitro inhibitory activity of endophytic bacterial strains against B. glumae

100 mL of the fermented medium was taken and centrifuged at 7000 rpm for 45 minutes. To each filtrate 80 mL of ethyl acetate was added, then the organic fraction was collected and concentrated using a rotary evaporator. The concentrate was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) using an Agilent Technologies 7820a gc gas chromatograph with an agilent technologies 5977e mass spectrometer, using an hp-5ms column (length: 30 m; diameter: 0. 24 mm stationary phase thickness: 0.25

mm), and the following conditions: oven temperature: initial temperature of 60°C for 5 min rising to 160°c with a heating ramp of 4°c/min up to 240°C/min at a heating ramp of 15°C/min. Carrier gas flow: 1ml/min injection temperature: 250°C vol, injection: 1 µl transfer line temperature: 250°C source temperature: 150°C, carrier gas: helium grade 5.0.

2.4. Antibacterial activity of the extract

Once the bacterial extract was concentrated, its inhibitory activity was evaluated using the agar disc diffusion technique, which consisted of impregnating sterile filter paper discs with 20µL of the concentrated extract and then inoculating them onto the surface of King B medium previously inoculated with B. glumae and B. plantarii. The boxes were incubated for 3 days at a temperature of 34°C (Cuellar and Hussein, 2009).

The percentage inhibition was determined by using the following formula (Barraza et al., 2017):

%IB= 1-(Aai/Acc)*100 (Equation 1)

Where: Aai: average growth of treatment, Acc: average growth of control test.

2.5. Molecular identification of endophytic bacterial strains

Genomic DNA of endophytic bacterial strains was performed according to the protocol proposed by Oliveira et al. (2013). Universal primers that amplify the small subunit of 16S rRNA were used. The amplified products were purified and sent for sequencing to Macrogen. The sequences obtained were compared with those stored in Genbank. Base alignment was performed in the Clustal W program, phylogenetic inferences were obtained by the Neighbor Joining method based on the kimura-2-parameter model with bootstrap 1,000 test in the MEGA X program.

3. RESULTS AND DISCUSSION

Figure 1 shows the results of the in vitro evaluation of the antibacterial activity of the antibiotic and secondary metabolite-type compound obtained from B. cepacia against phytopathogenic bacteria in rice culture.

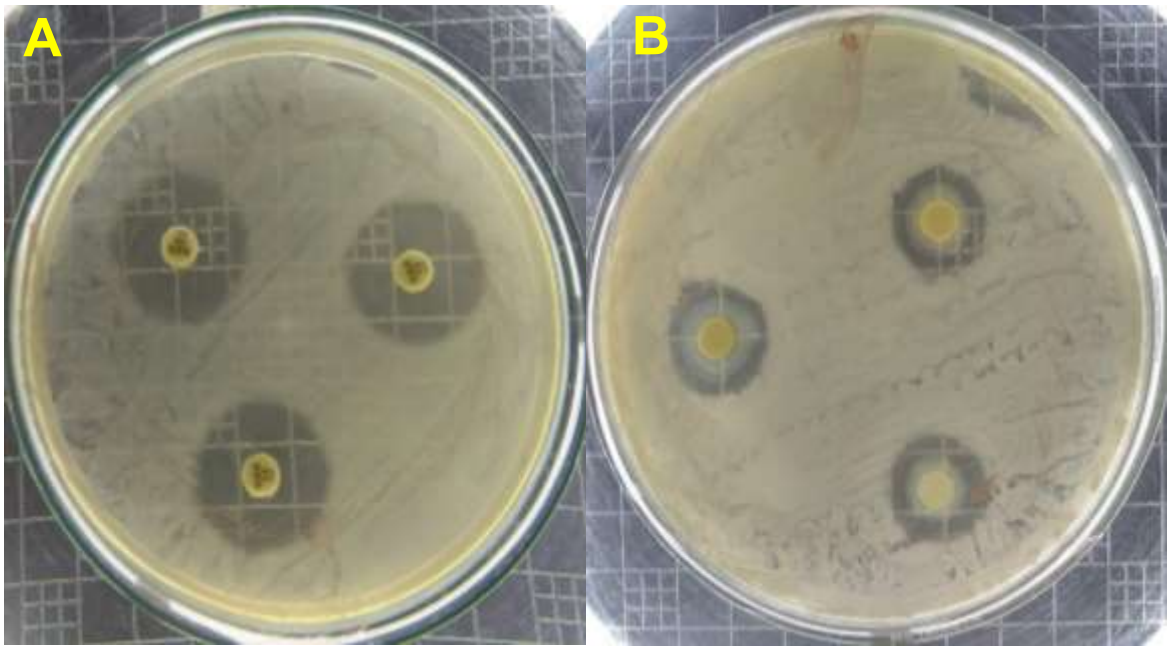

Figure 1. Inhibitory activity assay of chemicals (antibiotics) - Figure 1A and metabolite-like compounds extracted from B. cepacia - Figure 1B against plant pathogenic bacteria of rice crop.

Figure 2 shows the percentage inhibition of six strains of endophytic bacteria against B. glumae. The test results show that the inhibition percentages ranged from 50.1% (C1) to 73.5% (C6). The strain showing the highest inhibitory activity (C6-75.3%) against B. glumae, genomic DNA extraction, subsequent amplification with rDNA sequences using eubacterial specific oligonucleotides and finally sequencing from PCR product was performed.

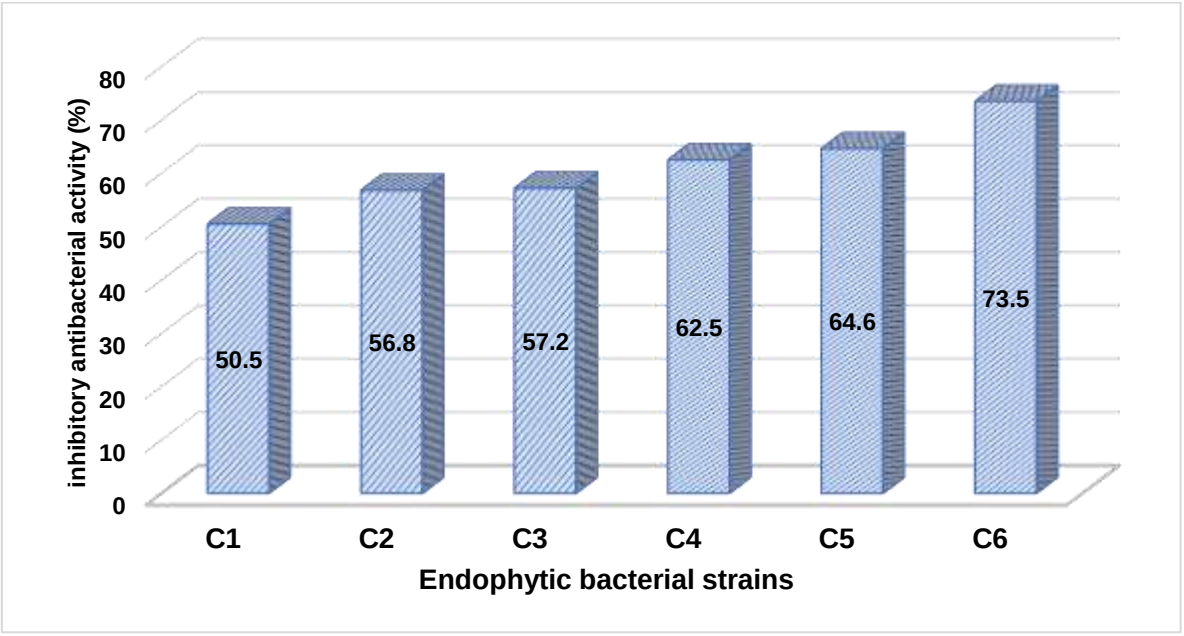

Figure 2. Percentage antibacterial inhibition of endophytic bacterial strains against B. glumae.

Identification of endophytic bacteria with antibacterial activity by PCR product

The results of the phylogenetic analysis using the maximum similarity method of the amplified sequences with primers F948β and R1492, specific for the beta proteobacteria class, show that the isolated bacteria form a clade with bacteria of the Burkholderia cepacia complex, with a branch support of 98%, indicating that the study samples belong to the Burkholderia cepacia complex and show higher homology with the species Burkholderia ambifaria LN889999, Burkholderia diffusa CP013364 and Burkholderia territorio CP013366, taken from the Genkbank database and described in the literature (Coenye et al, 2001; Vanlaere et al, 2008; De Smet et al, 2015) as bacteria of the complex. A wide variety of endophytic bacteria with antagonistic activity against plant pathogenic bacteria and fungi have been reported.

The phylotypes of Burkholderia cepacia and other species of Pseudomonas spp. are examples of two of the most studied strains of endophytic biocontrol bacteria with high potential for field use in the management of plant pathogens in crops of commercial interest. These bacteria produce a variety of antibiotic substances that can inhibit plant pathogens (Kadir et al, 2008).

The results found in this study are in agreement with other studies that have shown that strains belonging to the genus Burkholderia spp. are effective biological control agents. This study showed that strain C6 isolated from yam plants belongs to the Burkholderia spp. complex. with the ability to produce secondary metabolites in vitro that diffused into the surrounding agar and arrested or reduced the growth of Burkholderia glumae, a plant pathogenic bacterium in rice crops that causes panicle blast in commercial rice varieties, which has caused worldwide losses of more than 70%, with a percentage inhibition of the growth from 75.3% for strain B. cepacia (C6).

Identification of metabolites with antibacterial activity against Burkholderia glumae

Chromatographic chemical profiles revealed the presence of pyrazine derivatives such as pyrazine-2, 5-dimethyl; pyrazine 3-ethyl-2, 5-dimethyl pyrazine-2, ethyl-5 methyl and pyrazine 2,5-diethyl as significant chemicals in the bacterial extracts. Pyrazines are a class of compounds that are ubiquitously found in nature in plants, animals and microorganisms and can be synthesized chemically or biologically (Sheoran et al, 2015). The vast majority of pyrazine derivatives

possess diverse pharmacological properties, such as antibacterial, antifungal, antimycobacterial, anti-inflammatory, analgesic, anticancer, antidiabetic and antiviral that attracted increasing attention from researchers (Sheoran et al, 2015). Through GC-MS analysis, the results obtained indicate that most of the compounds found in the extracts consist of organic heterocyclic compounds. These heterocyclic compounds include compounds such as, pyrrolopyrazines, pyrazines, pyrroles and phenols.

Several endophytic bacteria show antimicrobial activity against different plant pathogens. In 2009, Verma et al. studied and evaluated the antimicrobial activity of endophytes (Streptomyces sp., Streptosporangium sp. and Nocardia sp.) associated with Azadirachta indica tree from India. The metabolite-like extracts of these bacterial strains showed activity against Pseudomonas fluorescens, Bacillus subtilis, Streptococcus aureus, Escherichia coli, Candida albicans, Trichophyton sp., Microsporum sp, Pythium sp. and Phytophthora sp. Later in 2014, Machavariani and collaborators isolated endophytic bacteria (Nocardiopsis, Streptomyces and Micromonospora) from tissues of Aloe arborescens, Mentha arvensis, Lysimachia nummularia, Fragaria vesca and Arctium lappa; native to Russia. The results of the well diffusion assays showed activity of these isolates against S. aureus, Micrococcus luteus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and the fungus Sacharomyces. cerevisiae.

Wang et al (2014) reported an endophytic isolate from Polygonum cuspidatum plants collected in China identified as Streptomyces sp. A0916. Metabolic extracts of the tested strains showed inhibitory activity against E. coli, Salmonella sp, B, subtilis, Enterobacter faecium, Staphylococcus aureus and Candida albicans. Significant suppressive activity was also observed against B. subtilis (Liu et al., 2009) and for banana plants pre-inoculated with Pseudomonas spp. and Burkholderia spp. strains (Fishal et al., 2010).

CONCLUSION

In this study, secondary metabolites produced by B. cepacia inhibited B. glumae in vitro which affect rice crop yield. Large-scale production of metabolites from the genus Burkholderia spp can be considered as an alternative for the management of different diseases in the field and to replace agrochemicals.

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