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STUDY OF THE ANTIBACTERIAL ACTIVITY OF PLANT-DERIVED MEATABOLITES AGAINST PHYTOBACTERIA IN RICE CULTURE

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ABSTRACT

Bacterial blast of the rice panicle, caused by the Gram-negative bacterium Burkholderia glumae, causes grain rot, generating losses of 15 to 80% of production. Although integrated management methodologies and the use of agrochemicals have been implemented to mitigate this situation, satisfactory results have not been achieved, and the excessive use of oxolinic acid has generated resistance on the part of the bacterial strains and, on the other hand, the safety of the final product for food safety worldwide. The present study aimed to implement new environmentally friendly biocontrol strategies such as the use of essential oils of Lippia alba and Lippia origanoides to reduce losses in rice crops caused by B. glumae, finding significant results of the essential oils, observing antibacterial activity of 100%, Lippia origanoides at a concentration of 90 ppm. Lippia alba had inhibitory activity at 1550 ppm with 80% effectiveness. The chemical profiles of the essential oils showed thymol as the major secondary metabolite with an area percentage of 68% for the essential oil of L. origanoides, while L. alba contains geranial and neral in 35% and 29% respectively and are possibly associated with antibacterial activity against B. glumae and its future use for the biological management of bacterial blast in rice plants.

Keywords: B. glumae, essential oil, chemotypes, secondary metabolites, antibacterial activity.

1. INTRODUCTION

According to the report of the Food and Agriculture Organization of the United Nations año 2017, the world production of rice in 2016 was 751.9 million tonnes (MMt), being 499.2 MMt of milled rice, which increased by 1. 6% over the low level of 2015, it is known that the high production or loss of the crop is affected by climatic conditions, which in some cases favour the proliferation of diseases, likewise the FAO estimates a production of 758.9 MMt for 2017, counting on climatic conditions and normal growth, with 503.8 MMt of milled rice, increasing by 0.9% annually.

In Colombia, rice is the third largest crop after coffee and maize, accounting for 13% of the harvested area and an estimated 30% of the country's transitory crops (DANE-Fedearroz,

2010). It is a highly important cereal for the national agricultural system, but it is worth noting that in recent years there has been an increase in crop diseases (Pérez, 2010).

The disease that causes most damage in these crops is the bacterial panicle blast of rice, a disease that causes seedling and grain rot, caused by the bacterium Burkholderia glumae (Devescovi et al., 2007), transmitted by the seed (Sayler et al., 2006; Nandakumar et al., 2009) causing a decrease from 15% to 80% of the total production (Ham et al., 2011).

Integrated crop management methodologies have been implemented, seeking to minimise the incidence of diseases by establishing lower risk seasons (Pérez and Saavedra, 2011). Using certified seed, resistant rice varieties (Shahjahan et al., 2000; Saichuk et al., 2011) and chemicals. However, satisfactory results have not yet been achieved, and these chemical compounds are harmful to the environment. Therefore, this research seeks to implement environmentally friendly alternatives such as the use of essential plant oils from medicinal plants to counteract the pathogenic effects of B. glumae and the loss of rice crop production.

2. MATERIALS AND METHODS

2.1 Sampling. The plant material was collected in the municipality of Sincelejo, department of Sucre, within the campus of the University of Sucre. Obtaining the plant material. The plant material used was leaves of L. alba and L. origanoides, which were divided into two parts: one part corresponded to fresh leaves that were used to obtain essential oils and the whole plants were sent to the Herbarium of the University of Sucre for taxonomic confirmation.

2.2 Extraction of essential oils. For the extraction of essential oils, fresh leaves of L. alba and L. origanoides were used using a hydrodistillation equipment assisted by microwave radiation, where the plant material was immersed in water and heated to boiling, for three cycles of 15 minutes each and these extracted essential oils were dehydrated with anhydrous sodium sulphate and stored in amber chromatographic vials at low temperature until further use (Pérez et al., 2017).

2.3 Phytopathogen. The bacterium B. glumae was used as phytopathogen, which causes the bacterial blast disease of the panicle of the rice crop, provided by the International Center for Tropical Agriculture (CIAT) - Colombia.

2.4 Antibacterial activity of essential oils against Burkholderia glumae.

Antibacterial activity using the MTT assay. For this test, 96-well Elisa plates were used, in each well 100 µL of a suspension of bacteria adjusted to a concentration of 0.1 to 0.08 OD were added. On the McFarland scale at 1.5x108 CFU/mL (Alviz et al., 2017) and the same volume of treatments, they were incubated at 35±2°C for 24 hours at 160 rpm. After the incubation time, 50 µL of a 5mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) solution was added, based on the methodology of Ruilova, (2007) with modifications; Ramírez and Castaño (2009). The wells containing the treatments and the controls, then the plates are left for 2 hours under the same conditions. To determine the inhibition of B. glumae, the optical density was measured in an Elisa Chromate Awareness microplate reader, model 4300 using a wavelength of 490 nm. The absorbance was taken as a measure of the percentage inhibition of the essential oil using the following formula:

% Bacterial index: 100 –[OD trated cell/ OD control cell] x 100

Controls were included on each plate. The test was performed in triplicate.

2.5 Antibacterial activity by colony forming units (CFU). From the previous test, 5µL of the concentrations and treatments were taken from each well of the Elisa microplate and each was added to a test tube containing 15ml of sterile distilled water to make a dilution, then 10µL of this dilution was taken and deposited in Petri dishes containing Kim B solid agar, prepared the previous day. The sample was spread over the entire Petri dish with the help of a Drigalsky spatula, and finally, each Petri dish was labelled and filled with paper towels and incubated at a temperature between 30°C and 35°C for 48 hours,

after which time the B. glumae colonies were counted using a colony counting technique (Doncel et al., Pérez, 2016).

2.6 Minimum inhibitory concentration (MIC) of B. glumae**.** The MIC of essential oils was defined as the lowest concentration of treatments showing bacterial growth inhibition ≥90% (Consentino et al., 1999; Ramírez and Castaño, 2009). Elisa 96-well plates were used, in each well 100 µL of an adjusted bacterial suspension with a concentration equivalent to an absorbance of 0.1 to 0.08 at 625 nm and the same volume of treatments were added. Serial dilutions of treatments showing near 100% inhibition were used, applying the colony forming unit (CFU) methodology described above.

2.7 Comparison of in vitro inhibition efficiency between the essential oils and the positive control with oxolinic acid. For this test, the methodology proposed by Rana et al., (1997) was followed with modifications, where elisa microplates were used and 100 µl of a suspension of Burkholderia glumae bacteria with an OD around 0.1 and the same volume of the minimum inhibitory concentration (MIC) of each oil was added to each well, after which measurements were taken every hour for 13 hours. Measurements were performed with a Chromate Awareness elisa microplate reader, model 4300, with prior addition of 50µl of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) 0.5 mg/mL.

3. RESULTS AND DISCUSSION

3.1 Antibacterial activity of essential oils against Burkholderia glumae.

Antibacterial activity using the MTT assay. The assay demonstrated the antibacterial activity of essential oils against B. glumae (figure 1). The wells colored with dark blue show bacterial growth in the different concentrations and controls due to the addition of tetrazolium salt (MTT), which when in contact with bacterial enzymes forms a compound called formasan causing a change in the color of the medium, due to this the evaluated concentrations of L. alba did not show 100% effectiveness in inhibiting the bacteria, but L. origanoides showed the presence of bacteria at concentrations of 1 ppm and 50 ppm, and from 100 ppm onwards a light yellow color was observed, which suggests that there is no growth of B. glumae as there was no change in color to dark blue. The presence of bacteria was observed in the negative control, indicating that the solvent does not inhibit B. glumae, in addition to the optimal growth of the control, which shows the growth of the bacteria and finally the inhibition by the oxolinic acid as a positive control.

The absorbance expressed in percentages of inhibition, indicate that the oil essence of L. alba in its different concentrations was not 100% effective giving an inhibition percentage of 63.9%, but L. origanoides in only 50 ppm already had 51% inhibition, reaching the concentration of 100 ppm where the bacterium B. glumae was totally inhibited, from there on all the concentrations showed a total inhibition, as the concentration was increased, the bacterial incidence was decreasing, thus increasing the antimicrobial index. L. alba leaf essential oil showed activity against B. glumae at 1500ppm to 2000ppm, found in consecutive analyses, and the minimum inhibitory concentration was predicted to be in these concentration ranges with a percentage inhibition of 100% against the plant pathogenic bacterium.

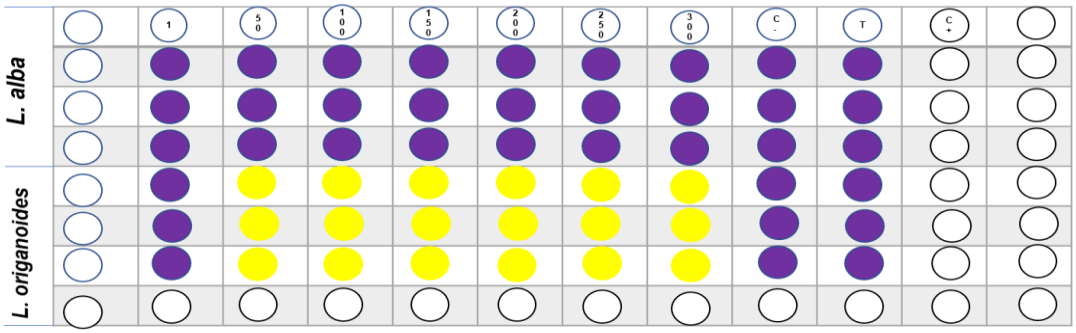

Figure 1. Antibacterial activity of essential oils against B. glumae MTT assay. The two different treatments (the bacterial suspension together with the essential oil concentration) of the two plants L. alba (La) and L. origanoides (Lo) tested are shown. The concentrations range from 1 ppm to 300 ppm each with 3 replicates as shown in the figure, accompanied by the negative control (C-), control (T) and the positive control (C+).

Figure 2 shows the antibacterial activity of the essential oils of L. alba and L. origanoides against B. glumae. The wells coloured with dark blue show bacterial growth in the different concentrations and controls due to the addition of the tetrazolium salt (MTT) which when in contact with enzymes of the bacterium forms a compound called formasan causing a change in the color of the medium, due to this the evaluated concentrations of L. alba did not show 100% effectiveness in inhibiting the bacteria, but L. origanoides showed the presence of bacteria in the concentrations of 1 ppm and 50 ppm, from 100 ppm onwards the presence of a light yellow color was observed in the wells, which infers that there was inhibition of the growth of B. glumae as there was no change in color to dark blue. The presence of bacteria was observed in the negative control, indicating that the solvent does not inhibit B. glumae, in addition to the optimal growth of the control, which shows bacterial growth, and finally the inhibition by oxolinic acid as a positive control.

The results of the absorbance obtained expressed in percentages of inhibition infer that the antibacterial activity of the leaves of the essential oil of L. alba in its different concentrations was not 100% effective, giving a percentage of inhibition of 63.9%. origanoides at only 50 ppm already had 51% inhibition, reaching the concentration of 100 ppm where the bacterium B. glumae was totally inhibited, thereafter all concentrations showed a total inhibition, as the concentration was increased, the bacterial incidence was decreasing, thus increasing the antimicrobial index.

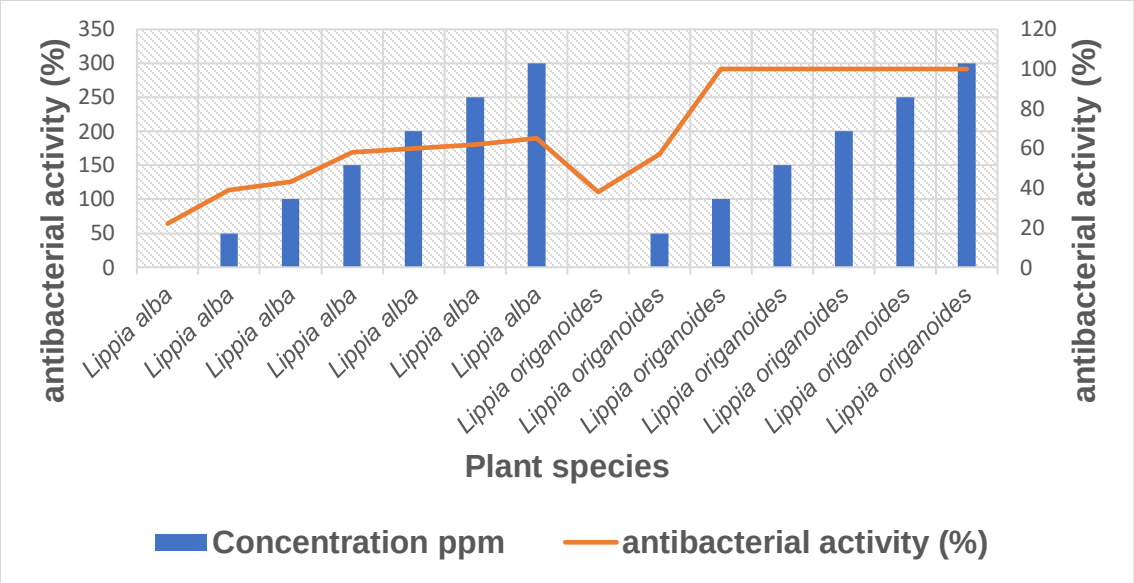

Figure 2. Inhibitory activity of essential oils by plant type. L. alba and L. origanoides in terms of inhibitory activity of essential oils, the latter being more effective at a lower concentration of oil at only 100ppm.

Since Lippia alba did not inhibit within the concentrations evaluated, we proceeded to look for a concentration where it is effective, 9 concentrations were evaluated starting with 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm, 4000 ppm, 4500 ppm and 5000 ppm. It was observed that the concentration that inhibited B. glumae was from 2000 ppm onwards, showing growth at 1000 ppm and 1500 ppm, therefore it can be inferred that the minimum inhibitory concentration (MIC) of L. alba is in the range of 1500ppm to 2000 ppm.

Figure 3, shows the interaction of the treatments (L. alba; L. origanoides and oxolinic acid), where it is shown that from the first hour the essential oil of L. origanoides was effective, inhibiting 100%, while the antibacterial activity of L. alba presented an inhibition of 80%. The evaluation of the kinetics of antibacterial inhibition of L. alba and L. origanoides shows that the activity of these oils starts from the first hours after the addition of these metabolic compounds in contact with the suspension of B. glumae in relation to the treatment with the chemical control with oxolinic acid, which showed inhibitory activity of 55%, with respect to the 80 and 100% shown, respectively, for L. alba and L. origanoides. The essential oils obtained from the leaves of L. origanoides showed in vitro a higher inhibitory efficiency of 100%.

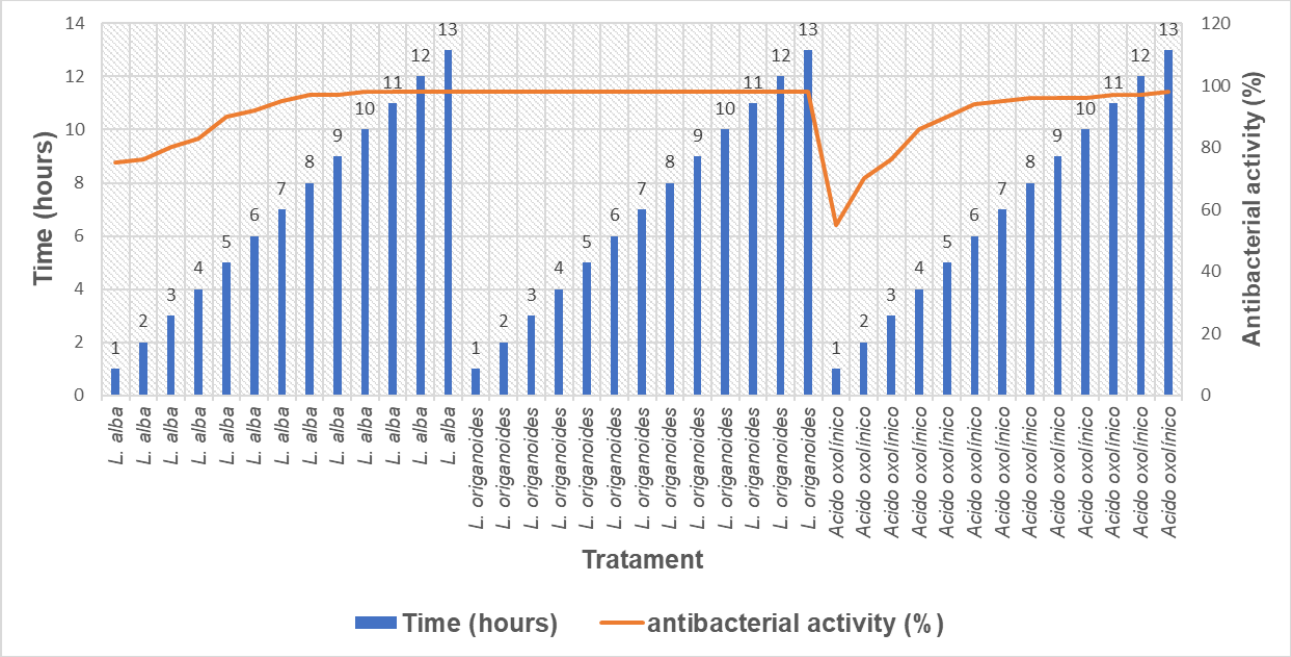

Figure 3. Kinetics of antibacterial inhibition by treatment. The graph of the interaction of the treatments shows that from the first hour the essential oil of L. origanoides was effective, inhibiting 100%, while the essential oil of L. alba presented an average inhibition of 80% in its initial reading and progressively gained more inhibition as the hours passed and finally oxolinic acid, which at the beginning only showed an average inhibition of 55%, showing that it was the worst treatment per hour.

B. glumae, is one of the pathogenic bacteria with more incidence in diseases, because it does this by various mechanisms that this same has acquired, among many the most important is the secretion of a toxin called toxoflavin, is distinguished in Kim B agar medium when the bacterial colonies turn yellowish showing greater pathogenicity as seen in all the figures of this study, This toxin is involved in metabolic and cellular processes of the plant as well as in the synthesis and degradation of proteins (Wan and Liu, 2008), doing the job of electron transporter NADH and oxygen, resulting in a toxic product for cells manifesting tissue damage (Latuasan and Berends, 1961). Toxofavin is synthesised by two groups of genes, the first group in charge of toxofavin biosynthesis by the genes (toxABCDE), the second group in charge of toxofavin transport to the extracellular space by the genes (toxFGHI), together with them operate two more genes which are toxJ and toxR very important since they encode the protein that regulates the expression of the two groups of genes mentioned above (Shingu and Yoneyama, 2004) in addition to the genes the production of toxoflavin is favoured by temperatures between 30 °C and 37 °C where is the optimal growth of B. glumae (Pérez and Saavedra, 2011).

The essential oil of L. alba is abundant in terpenes and terpenoid compounds (Cowann, 1999), among the most common ones found are citral, linalool, carvone and limonene but a great variety of compounds have been found throughout the research in different areas even within the same country, such is the case in Brazil where 3 different chemotypes were detected for 3 areas studied (Tavares et al., 2005). Regardless of this variation, the joint action of the chemotypes found in the essential oil of L. alba has classified it as a bioinput with antimicrobial activity against different pathogenic bacteria such as: Lactobacillus casei, Staphylococcus epidermidis, Enterobacter aerogenes, Candida albicans, Bacillus subtilis among others (Hennebelle et al., 2008).

On the other hand, phenolic compounds such as thymol and carvacrol are the most common and highest proportion in the essential oils of L. origanoides (Celis, 2007) also found in the list of chemical compounds of our study plant annex 10 being a potential antimicrobial against food microorganisms (Lambert et al., 2001). Carvacrol, which has a phenolic group, has been used mainly for its ability to disintegrate the outer membrane of Gram-negative bacteria.

CONCLUSION

Among the essential oils used in the present study, there is a different range of inhibition, with L. origanoides having a higher percentage of inhibition compared to the essential oil obtained from L. alba, and possibly this difference in the antibacterial activity observed in vitro is due to the effect produced by the chemical compounds present in each oil and as has been supported, the phenolic compounds that constitute the essential oil of L. origanoides represent an antimicrobial power of greater potential than the terpenes and terpenoids present in the majority of the oils of L. alba.

AUTHOR CONTRIBUTION.

Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors of the manuscript, declare that there is no conflict of interest related to the article.

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