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Research Article

Evaluation of the Fungicidal Effect of *Oleifera lam* Leaf Extracts on Anthracnose in *Mangifera indica L.*

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Abstract: The objective of this study was to evaluate the fungicidal effect of *Moringa* on anthracnose in mango (*Mangifera indica* L). Anthracnose is commonly treated by spraying with fungicidal chemicals; continuous use affects the ecosystem due to the residues generated, thus harming the quality of the fruit, its innocuity and the health of humans. This research tried out a process to achieve an extension of the shelf life of mangos by using *Moringa oleifera Lam*. Aqueous Extracts (EA) were obtained by infusion and Ethanol Extracts (EE) by simple maceration of the *Moringa* leaf (*Moringa oleifera* Lam). For the evaluation of the results, chromatic techniques were used through the Image J software. Quantitative data were obtained on the appearance of spots and their radial growth. Each of the extracts obtained was applied to the samples by means of two treatments, immersion and sprinkling, with varying concentrations: 10.0, 15.0 and 25.0%, respectively. It could be observed that the most effective treatment and concentration in the control of the propagation of the spots and the radial growth was EA_2 (Aqueous extract by immersion) at 15.0%. With the use of *Moringa* extracts, a soil propagation percentage of less than 20% was obtained with respect to that of the controlled samples with a propagation percentage above 80%. In addition, a radial growth three times less than that of the control sample was obtained over the same study timeframe.

Keywords: Chromatic, disease, image j, infusion, mango, propagation

INTRODUCTION

Research in España (2014) reports that mango (*Mangifera indica* L) is a highly consumed fruit on the Colombian Atlantic coast, which is the reason many studies have been carried out to achieve the extension of its shelf life in markets with high quality demands. Mango (*Mangifera indica* L) is grown in tropical and subtropical regions, so it is considered an exotic fruit both domestically and abroad.

Research by Páez (2009), offers an account of the serious consequences for humans of the use of chemical fungicides; therefore, it is necessary to look for healthier and friendlier alternatives to the environment, being such an alternative the use of fungicides from plant extracts. Published studies are few, especially those of the extract obtained from the M*oringa* leaf. Taking the great potential of the *Moringa* leaf into account, it is necessary to carry out studies that prove the presence of antifungal activity in it in relation to the target microorganisms, in order to give it a new approach that allows for a potentially natural solution to a problem in this crop, such as anthracnose.

Mango has extensive nutritional and organoleptic properties, which provides it with great commercial value, is very sensitive to pre-harvest and post-harvest diseases. One of the diseases that affects this fruit most is anthracnose, caused by *Colletotrichum gloeosporioides*, which produces conditions of dark coloration that may be watery and sunken in the external tissue, causing for major economic losses in the cultivation of mango. In the department of Atlántico, it was estimated that such losses are within a range of 49.8-53.3% (Aguilar *et al*., 2010).

As reported by Rincón *et al*. (2006), the Colombian Atlantic Coast has a high incidence of anthracnose, which obviously greatly affects production, causing losses of up to 85.0%, which is why this disease is deemed the most important limiting factor in Colombian production. The above researchers also report that the huge losses brought about by this disease lead to excessive use of chemical compounds to inhibit the growth of the fungus, which results in a spike in the cost of the crop and also damages to the environment. In addition to such difficulties, there are too few studies and investigations on this crop and on the biology of its pathogens.

Research by Aguilar *et al*. (2010) claims that anthracnose, caused by *Colletotrichum gloeosporioides*, is the most important pre-harvest, harvest and post-

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harvest disease in mango-producing areas worldwide. This disease is characterized by its lack of visible manifestations, although present in immature fruits; however, after the post-harvest, it causes major damage. The presence of this disease shows up with the appearance of dark colored affections on leaves, flowers and peduncles. Additionally, the fruits may be affected, particularly in the curd stage, which may lead to damage before reaching the maturity stage.

Canett-Romero *et al*. (2014), asserted that there is great concern internationally about the possible harmlessness of the various anatomical parts of *Moringa* and, yet, it is still used as a food supplement, to purify water and treat more than 300 diseases. This possible controversy is of the utmost importance, as it compels the scientific community to do research on the potential adverse effects stemming from its long-term use. Anthracnose is commonly treated by spraying with chemical fungicides and, among the most used compounds, are Benomyl and Prochloraz; nonetheless, their continuous use affects the ecosystem due to the waste generated, which affects the quality of the fruit, its safety and the health of humans (Páez, 2009).

The Moringa tree has been a matter of study in all its parts: leaves, roots, trunk, branches and seed. The leaves have a wide variety of properties, among which is its antimicrobial action, being of great interest in solving an issue such as anthracnose in mango (España, 2014).

In the past few years, a wide array of research has been developed, demonstrating some of the *Moringa* tree's functions in the human system, fundamentally as an antioxidant, anti-inflammatory or antihyperglycemic agent, among others, as well as its bacteriostatic and bactericidal functions. This provides it with potential capacity for the treatment of diabetes, as a liver protector or a guard of endothelia in certain chronic diseases (Doménech Asensi *et al*., 2017).

Research by Kasolo *et al*. (2012), shows that the use of *Moringa* leaves leads to low toxicity levels from the leaf, with an LD50 for ethanol extract of 17.8 and 15.9 g/kg pc for aqueous extract. Even if these results sign off on consumption for medicinal purposes, they are not conclusive and, in higher doses, toxic changes and even death may occur.

According to a review by Villarreal Gómez and Ortega Angulo (2014), many of the characteristics of the *Moringa* leaf meal compare favorably with the nutritional features of milk powder in terms of calcium and protein components as well as its high content of vitamin A. In addition to the above, the *Moringa* leaf is rich in antioxidants, among which we find isothiocyanates, which are endowed with hypotensive, hypoglycemic, antibiotic and anticancer properties. The concentrations of anti-nutritive factors, such as proteases, tannins, tannin inhibitors, lactins and saponins in the leaves, are considered insignificant. Studies that have been carried out on the qualities of *Moringa* have been done *in vitro* or in animals; due to this, there is no exact dose that may lead to any beneficial effect in humans (Olson and Fahey, 2011).

Martín *et al*. (2013), found that there are many properties that make the *Moringa* plant valuable, among which we find its nutritious, antimicrobial, therapeutic and antioxidant features. Currently, *Moringa* leaf flour is used by children, pregnant women and adults as a food supplement. In addition, it is used homeopathically in the treatment of more than 300 diseases, among which are diabetes, pocholesterolemia, anemia, neurodegenerative diseases, hypertension, skin disorders, fertility problems, kidney disease and even cancer.

Two thousand fourteen reports from Spain claim that the use of organic or natural fungicides has seen a great boom due to its sustainability, low cost and positive impact on the environment. Hence the importance of looking for new alternatives to control anthracnose naturally, taking into consideration the fact that the food industry is scrambling for food that is free of toxic traces and controlled by natural elements.

Another research also uses a novel method to evaluate the results through a program called Image J, which focuses on the quantification, filtering and mathematical processing of processed images, as reported by Mateos and Pascau (2013).

Salcedo and Quiroz (2014), determined the action of the avocado seed as an inhibitor of enzymatic browning in avocado (*Perseaamericana*), using chromatic techniques for that analysis, along with the use of a Computerized Vision System (CVS), consisting of a lit-up stage, a CCD digital camera (calibrated) and a computer (Laptop). Images were processed with Photoshop® CS3 and the data thrown by the program were analyzed based on CIE coordinates L*a*b*, polar coordinates (C*) and pitch angle (h°), color parameters L*, a* and b* in a food sample, which denote: L*, luminosity (0 = black and 100 = white); a*, the color red (positive values) or the color green (negative values) and b*, the color yellow (positive values) or the color blue (negative values), resulting from the use of a Computerized Vision System.

MATERIALS AND METHODS

The experimental design of this research is based on varying concentrations of the extracts obtained, in addition to the implementation of two types of treatments, namely immersion and aspersion. Extract solutions were obtained with a ratio of solute: solvent (0.5: 1 P/V) where the solute was the *Moringa* leaf and the solvents were distilled water and ethanol. The Aqueous Extract (EA) and the Ethanolic Extract (EE) were diluted in distilled water at a concentration of 10.0, 15.0, 25.0% v/v, respectively following the Lago tests.

A control sample was taken, which was not subjected to any treatment, but rather kept in the same environmental conditions as the other samples. The control sample served as a standard to compare the differences between the samples that were treated. In total, 12 treatments were performed, each in quadruplicate.

In this research, different *Moringa* leaf extracts were evaluated through the processing of images taken in mangos infected with anthracnose, by analyzing the evolution of the disease over time. In addition, variations in the concentration and methods of application of the extract were shown.

The experimental assembly for the acquisition of spot images on the mangoes was based on the work of Yam and Papadakis (2004), which was comprised of a 60.0 cm long square box, fully closed and lit upon the inside by 4 ENERLUX fluorescent lamps, reference E-LT514W (JTS-Y14-14 W) with a light of 6400.0 K temperature and a power of approximately 14.0 W each, which are located at the top of the box walls. In the center of the box there is a wooden bracket, which serves as support for the samples (mangoes) in the proper position for snapping the pictures. The inside of the box and the bracket are painted black to avoid light reflections that may shed unwanted shadows onto our object of study. In the upper part of the box an observation window has been left which is 30.0 cm from the wooden bracket, where the images of the mangoes inside the box will be taken; also, the angle of incidence of the light on the sample is very important, since, when we take the picture, it is possible that the source of light on the mangoes is reflected. It is then recommended that the correct angle between the light source and our object is approximately 45.0°; accordingly, the angle between the camera and the light source is also 45°, an angle at which the image of the mangoes is totally free of reflections and with appropriate lighting.

Image J software was used to process the images, through which it was possible to determine the evolution of the spots present on the mangoes due to anthracnose. Image J is a public domain multiplatform software (free), encoded in java, which was developed in 1987 and used for image processing. Unlike software such as Adobe Photoshop, GIMP and other graphic editing programs, Image J is more focused on the quantification, filtering and mathematical processing of processed images (Mateos and Pascau, 2013).

Research by Salcedo and Quiroz (2014), recommend that this analysis be done on an average of 3 photographs of a single mango sample. As the number of total mangoes to be analyzed is 52, onto which different treatments will be applied, it is necessary to have the capacity to perform all such tasks in the Image J software in the same manner and under the same parameters, so that it is possible to compare all the images in the same way.

There is a tool in Image J called Macros, which allows writing everything done on an image in the form of computer code, thus enabling that all the images undergo the same processes, as explained by Mateos and Pascau in their book, published in 2013.

Mango samples for the study were collected from a farm located in Ciénaga-Magdalena and then transported in a cardboard box. Fruit that was banged up was discarded; subsequently, fruit was classified by size and degree of maturity and hosed with drinking water in order to remove any adhering particles.

Moringa leaves were collected from trees grown at Universidad del Atlántico, located in the municipality of Abreu *et al*. (2014) (Atlántico). The leaves were stored in *ziploc* bags to be transported to the place of conditioning. The leaves with the best appearance and even sizes were selected, while those that appeared abnormal were discarded. Petioles were removed from the leaf along with any dirt adhered to it, which was done by dunking the leaves in a container with drinking water at room temperature over a maximum time of one minute. The leaves were then passed through a conventional sieve to filter out the excess water.

For the preparation of *Moringa* samples, surface drying was carried out on the leaves at room temperature and outdoors on plastic trays for a timeframe of 72.0 h. Then, *Moringa* leaves were ground by using a conventional chopper in order to reduce their size and increase the contact area between the solvents to be used, thus facilitating the extraction of the secondary metabolites contained in the leaves. As follows, these procedures are briefly described as per the Lago methodology (Lago, 2015).

Aqueous extraction: After drying, an extraction by infusion was carried out, where 500.0 g of previously ground *Moringa* leaf was taken and mixed with 1.0 L of distilled water at a temperature of 100.0°C and left at rest at room temperature. Then, the mixture was separated by decantation and the liquid obtained was passed through a set of Field master sieves of 4000, 2000, 500, 250, 125 and 63 μm, respectively.

Ethanolic extraction: Ethanol was used as solvent to obtain the extract; then a simple maceration was carried out at room temperature, where 500.0 g of *Moringa* leaf, previously dried and ground, was blended with 1.0 L of 96.0% ethanol. The mixture was left in a glass container with a hermetic seal previously sterilized for 24 h, with constant manual shaking for the first 30.0 min, followed by sporadic stirring. The mixture was decanted and filtered with a set of Field master sieves of 4000.0, 2000.0, 500.0, 250.0, 125.0 and 63.0 μm, respectively as described by Mitjans *et al*. (2016).

Treatment of mango samples with ethanol and aqueous extracts: As shown in Table 1, which describes the percentages of the different treatments to

Table 1: Concentrations of ethanol and aqueous extracts

Treatment	$[\,]_1$ v/v (%)	$[\,]_2$ v/v (%)	$[\,]_3$ v/v (%)
EA_1	10	15	25
EA_2	10	15	25
EE_1	10	15	25
EE_2	10	15	25

which the mango samples were subjected with aqueous and ethanol extracts of *Moringa* by means of aspersion, in which a conventional atomizer was used by spraying the entire surface of the mangoes evenly and using 10.0 mL of each dilution at different concentrations, 10.0 15.0 and 25.0% of EA1 (aqueous extract from aspersion treatment), EA_2 (aqueous extract from immersion treatment), EE_1 (Ethanol extract from aspersion treatment) and EE_2 (Ethanolic extract from immersion treatment).

Immersion treatment: In this treatment, mango samples were put in contact with the ethanolic and aqueous extracts of *Moringa* by means of the immersion technique, which consists of dunking the mangoes in a container and allowing the liquid to surpass the samples for 1.0 min, which ensures full contact with the sample.

RESULTS AND DISCUSSION

To determine the effectiveness of the aqueous and ethanol extracts of the *Moringa* leaf on the spots appearing on the mango samples, the images taken were processed and the applied concentrations were assessed at 10.0, 15.0 and 25.0%, respectively for the aqueous and ethanol extracts and the respective target. This procedure was done over the days following the extraction, since, as reported by Pulla Marca (2014), the aqueous extract of *Moringa oleifera*, under the conditions of the study, is not stable for a timeframe not greater than 5 days.

Research by Salcedo and Quiroz (2014) show that the use of a Computerized Vision System allows for the measurement of color variations through time, by means of the evaluation of the CIE L*a*b* coordinates. In our research, photographs were initially taken of all the samples before being subjected to the treatments. After applying the extracts, images were taken periodically for each sample at a defined time, 5:00, 12:00 and 19:00 for 7 days, with a total of 1,092 images over a timeframe of 134.0 h.

To compare the results of the treatments, control samples were taken, which were not subjected to any treatment, but were kept under the same conditions as the others. According to the studies carried out, it was established that for all the cases in which the samples were subjected to the treatments, a positive result was obtained in relation to the control samples. But, in a comparison of the two treatments, the aqueous one was more efficient, since the increase in the number of spots was more constant in such treatment. As for the aspersion and immersion techniques used, it was established that the immersion technique was more effective.

The treatments corresponding to 10.0 and 15.0% do not differ from the behavior shown by the mango used as control. However, the treatment carried out with a 25.0% concentration shows a percentage of appearance of new spots of approximately 26.0%, showing, therefore, that it is more effective in controlling the propagation of new spots.

In ethanol extracts, it is observed that the growth of spots remains constant in relation to time and, furthermore, in comparison with the control sample, between the three concentrations there is a statistically significant decrease, which implies that the 25.0% concentration, was the one that reduced the most growth of spots in the estimated time period by showing a maximum growth percentage of 15.0% spots. In an investigation of Abreu *et al*. (2014), who managed to significantly inhibit the hatching of eggs with leaf extracts of *Moringa olifera*, with an effect that hinged on the doses used. These values were between 41.40 and 67.86% with the best results from the lowest concentration (41.40% at a dose of 12.5 mg/mL). For the aqueous extract by aspersion, at 10.0%, a slight growth in the appearance of new spots is shown with an increase of 30.0%. When the concentration is increased to 15%, such values are maintained. When the concentration is increased to 25%, it is observed how the spots that appear in each sample remain constant over time. In the comparison with the control sample, the samples analyzed differed from the behavior of the control sample. The extracts, in their different concentrations, show a positive effect in the reduction of anthracnose spots.

Puerto, in his research work, determined that the lowest effective concentration found was for *G. sepium* with values that are 50.0% lower in relation to *Moringa oleifera*, which is possibly related to the differences in profiles and concentrations of the secondary metabolites present in each one of them. In the extract treated by immersion at a concentration of 10.0%, a constant propagation of spot growth was observed below 40.0%, with growth rates between 0.08 and 0.3/h. At the 15.0% concentration, a propagation lower than 20.0% was observed. We can, therefore, deduce that the latter concentration is much more effective than the former. In the 25% concentration, the percentage of growth was around 40%. Lastly, we can observe that the concentration of aqueous extract by immersion at 15.0%, brought about less growth and appearance of spots with only approximately 20.0% in spot propagation as compared to 80.0% of the mangoes used as control.

Radial analysis of spots: The analysis of radial growth of the spots was represented by a clear affection of anthracnose and the area of the spot was determined as a function of time. Due to the large number of spots appearing in some mangoes, it was necessary to select a single spot (particular characteristic) per mango unit to be able to perform the analysis. All the curves were normalized on a unit basis, that is, number one represents the maximum area that reached the condition after the time defined for the study. With this, it was possible to make a distinction that could be mutually comparable. Among the three concentrations used, the one that stands out the most is that which presents a concentration of 15.0%, because it was the stain that took longer to grow, as evidenced during the treatment, since, in approximately 80 h, the size of the stain did not grow significantly. The concentrations of 10.0 and 25.0% are very similar to one another, maintaining a constant growth rate slightly below the control sample. Research by Guevara Medina and Rovira Quintero (2012) determined infusions concentrations for direct consumption of (2 and 3 g *Moringa* /240 mL water).

The aqueous treatment by immersion shows fewer spots, approximately 50.0% with respect to the control sample. The concentrations remain constant with the radial growth of the spot; however, we can deduce that the best concentrations to control the growth of the spot are those of 10.0 and 15.0%, as they have similarities in radial growth concentration. The mango used as control had a double growth rate with respect to the other concentrations, which means that the aqueous extracts by this method have a positive effect in reducing the appearance and radial size of the spots. Results from España (2014), on the inhibition of anthracnose with plant extracts from the Eucalyptus species, show anti-fungal capacity, which was measured through the diameter of mycelial growth, which is affected by two factors: the type of plant extract and the concentration in mg/L.

The ethanol extracts applied by aspersion showed an excellent response when compared to the control sample. The three concentrations during the initial 80 h show that the area of affection remains constant, after which time the growth of the spots increases to a ratio that grows exponentially until reaching its top value. The concentrations of 10.0 and 15.0% of such ethanol extracts show constant growth, but the best performance is the concentration of 25.0%, which starts with a large radius with respect to the others, but a low growth range is maintained. In a similar research developed by Costa *et al*. (2002), it was found that mango (*Mangifera indica*) extracts in Brazil showed a hatching inhibition rate of 95.66%, in concentrations of 50 mg/mL, with an effect dependent on the dose used.

CONCLUSION

Most of the treatments applied showed effects on the decrease in anthracnose, with some being more evident while others were less sensitive; those results match those found by Chuang *et al*. (2007), reporting the antifungal activity of essential oils from leaves and alcoholic extracts from *Moringa* seeds and leaves. In the treatment that uses the ethanol extract by aspersion at a concentration of 10.0%, it was clearly observed that there is no effect on anthracnose affections. As the concentration was increased, a slightly greater effect was noted. When applying the alcoholic extract by immersion at a concentration of 10.0%, no significant difference was observed. For the concentrations of 15.0 and 25.0%, a clear effect of the extract on anthracnose was observed, which brings us closer to proving the real effect in the reduction of anthracnose. Clearly, a decrease of approximately 60.0% is observed in terms of the appearance of new spots in the application of the ethanol extract by immersion with respect to the control sample and it was possible to obtain a rate of appearance of new spots of up to 0.08 spots/h, taking into consideration that the rate that was obtained by the control sample was 0.7 spots/h. It is greatly important to take the recommendations of Canett and collaborators in 2010 into account, in that the use of *Moringa* leaf extracts establishes the relative safety derived from the moderate consumption of all the anatomical parts of *Moringa oleifera* and its extracts, especially in the leaves, since the possible toxicity of *Moringa oleifera* is directly proportional to the dose and time of consumption.

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