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AQUEOUS EXTRACTS OF PLANTS IN *Colletotrichum gloeosporioides* INHIBITION *IN VITRO* AND IN POSTHARVEST GUAVA[1]

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ABSTRACT - The effect of plant aqueous extracts in the control of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. the causal agent of guava anthracnose in, was evaluated *in vitro* with 1, 2 and 3% aqueous extracts of *Azadirachta indica*, *Nerium oleander*, *Ocimum gratissimum*, *Syzygium aromaticum*. The experiment was installed in a complete randomized desing in a 3x4 factorial scheme (doses x extracts). For the evaluation, it was calculated the percentage of fungal inhibition. The experiment *in vivo* was conducted by applying *Syzygium aromaticum* and *Azadirachta indica* aqueous extract at 2 and 3%, respectively, in three different storage conditions: refrigerated with and without plastic film (PVC), and at ambient conditions. The experiment was installed in a completely randomized design, in a 2x3 factorial scheme (extracts x storage conditions). We evaluated the external appearance and severity of disease, loss of weight and Brix degrees. *Syzygium aromaticum* extract at 2% provided 100% of fungal mycelial growth inhibition, and *Azadirachta indica* extract at the highest dosage (3%) inhibited 20.22%. In fruits, there was not significant statistical difference between the effect of extracts on the external appearance and severity of disease, loss of weight and Brix degrees. In relation to the storage conditions, the ones with plastic film and refrigerated differed from the other conditions obtaining better external appearance and less severity of disease, lower loss of weight and higher Brix degrees.

Keywords: *Psidium guajava*. Anthracnose. Alternative control.

EXTRATOS AQUOSOS DE PLANTAS NA INIBIÇÃO DO *Colletotrichum gloeosporioides IN VITRO* E EM GOIABA PÓS-COLHEITA

RESUMO - O efeito de extratos aquosos de plantas no controle de *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. o agente causal da antracnose em goiaba, foi avaliado em dois experimentos. No experimento *in vitro* testaram-se três doses (1, 2 e 3%) dos extratos de *Azadirachta indica* L (Nim), *Nerium oleander* (Espirradeira), *Syzygium aromaticum* (Cravo da índia) e *Ocimum gratissimum* L (Alfavaca). O delineamento experimental foi o inteiramente casualizado em esquema fatorial com 3 doses e 4 extratos. A variável usada para avaliação do efeito foi à porcentagem de inibição do fungo. No experimento *in vivo* testaram-se os extratos aquosos de Cravo da Índia a 2% e de Nim a 3% em três condições de armazenamento dos frutos de goiaba (refrigerado com e sem plástico e em condição ambiente). O delineamento experimental foi o inteiramente casualizado em esquema fatorial com 2 extratos e 3 condições de armazenamento. As variáveis avaliadas foram aparência externa, severidade de doença, perda de peso e teor de sólidos solúveis (°Brix). O extrato de Cravo da Índia proporcionou 100% de inibição do crescimento micelial do fungo a partir da dosagem de 2%, enquanto que o extrato de Nim inibiu 20,22% na maior dosagem (3%). Não foi obtida diferença estatística quanto ao efeito dos extratos na aparência externa, severidade de doença, perda de peso e teor de sólidos solúveis dos frutos. Em relação ao armazenamento, a condição refrigerada com plástico diferiu das demais, proporcionando aos frutos melhor aparência externa, menor severidade de doença, menor perda de peso e maior teor de sólidos solúveis.

Palavras-chave: *Psidium guajava*. Antracnose. Controle alternativo.

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INTRODUCTION

Brazil is a leading guava (*Psidium. guajava* L.) producer, along with Mexico, India, China, Pakistan, and South Africa (PEREIRA; KAVATI, 2011). Guava is one of the main raw materials of the processing industries, and it offers raw material and job opportunities along several months (CENTRO DE PRODUÇÕES TECNICAS, 2010). According to IBGE (Brazilian Institute for Geography and Statistics) and IBRAF (Brazilian Fruit Institute), in 2009 Brazil produced 297,377 tons of guava in a 15,048 ha area. Pernambuco, São Paulo, Brasília, Rio de Janeiro, and Bahia are the main Brazilian states producers (PEREIRA; KAVATI, 2011). In 2010, the state of Rio Grande do Norte produced 3,140 tons in a 474 ha area (IBGE, 2012).

Table guava market, however, is limited by low quality of fruits because of inappropriate postharvest procedures and lacking structure for trade. That is complicated by the fact that guava is a climacteric tropical fruit with high respiration rate and too short postharvest life, which also make it less resistant from attack by pathogens (MOURA-NETO et al., 2008).

One of the most common diseases during the rainy season in the pre- and postharvest stage is the anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz.; Sacc, whose sexual stage corresponds to *Glomerella cingulata* (Ston.) Spauld. & Scherenk. (PICININ et al., 2005). The pathogen produces acervula with arrows, conidia hyaline, unicellular protected by an orange-colored mucilaginous matrix (ALVES; DEL PONTE, 2011).

Branches and leaves blight, dark and irregular spots on leaves and fruits are the typical symptoms of the disease. Damage is more severe on ripe and ripening fruits. Lesions show up as low-relief, round, irregular, and dark brown and black spots. It may appear pale-pink conidial mass in the center of the lesions (JUNQUEIRA et al., 2001). The fungus infects fruits at different phenological stages, even without injury, getting quiecentes to maturity (MORAES et al., 2008) The disease-control measures involve treatments improving the crop health, like internal pruning, balanced fertilization, and chemical control with copper products in the preharvest stage (JUNQUEIRA et al., 2001), appropriate fruit handling in the harvest and postharvest stage (CHITARRA; CHITARRA, 1990), along with appropriate packages, wax (JACOMINO et al., 2003), or coating films providing modified atmosphere under refrigeration (OSHIRO et al., 2011) and alternative products, like plant extracts and oils (ROZWALKA et al., 2008). It is due to the lack of accredited fungicides to be used during the postharvest stage in this crop, and also to the need for search of alternative products which represent low risk of toxic waste for consumption and the environment.

This study aims at evaluating the effect of plant aqueous extracts inhibiting *C. gloeosporioides in vitro* and *in vivo*.

MATERIAL AND METHODS

Two experiments were conduct in September 2011 in the laboratories of plant pathology and microbiology of the Departamento de Ciências Vegetais, setor Fitossanidade, in the Universidade Federal Rural do Semi-Árido – FERSA, Mossoró in the state Rio Grande do Norte, Brazil.

C. gloeosporioides was obtained directly from the surface of Paluma guava fruits whose pathogenicity was verified by using Paluma guava fruits sprayed with inoculum suspension at 10^6 conidia.mL^{-1} and then the fruits were kept under ambient conditions at 27 ± 2 ^{o}C in a moist chamber for 12 hours. After 3 days, the fruits presented characteristic symptoms of the disease. The isolation remained in test-tubes with PDA culture medium under refrigeration for preservation.

To produce the inoculum, the fungus was peaked to the center of Petri dishes with PDA culture medium and kept in a B.O.D. incubator at 28 °C ± 2 °C for 7 days.

The extracts were obtained from the following plants: *Azadirachta indica* L (neem), *Nerium oleander* (oleander), *Syzygium aromaticum* (clove) e *Ocimum gratissimum* L (basil). Plants were obtained from non-commercial crops and clover was obtained in the form of dehydrated flower buds from commerce in the city of Mossoró, RN, Brazil. The plants parts used were the leaves and, in the case of clove, the floral bud. The plants leaves were washed with running water and mild detergent, and then, disinfected with sodium hypochloride at 1% for 10 minutes. After, the leaves were put in Kraft paper bags and dried in a semi-open oven under the temperature of 50 °C for three days until they have constant weight. clove floral buds were dehydrated and did not have any treatment for surface disinfection. The dehydrated plants were crushed in a cutting mill and then stored in covered amber-colored glass bottle at a rate of 25% of dry weight and 70% of alcohol for 7 days in ambient conditions (28 ± 2 ^{o}C). After macerated, the extracts were filtered through sterilized filter paper, stored in sterilized flasks with mouths protected with sterile gauze and kept in oven at 40 °C. After alcohol completely evaporated, the volumes were filled with sterilized distilled water, and the extracts were stored in freezer, according to methodology adapted from VIGO et al. (1999).

To evaluate the effect of plant extracts inhibiting the fungus *C. gloeosporioides*, it was used 0.0; 1.0; 2.0; and 3.0% of aqueous extracts. They were added to the culture medium after autoclaving and, as cooled at 50 ± 3 °C, put in Petri dishes. After the solidification culture medium, 7 mm diameter disks were taken from a 7 day fungal growth and peaked to

the middle of the dishes with PDA culture and treatments. The dishes were then kept in a B.O.D. incubator at 28 ± 2 °C to provide fungal growth.

Evaluations were performed 7 days after the fungal incubation – enough time for the culture dishes (control dishes) to be totally covered by the pathogen. The size of the mycelial growth zone was obtained by measuring the radial growth of the colony in two orthogonal axes and, then, averaging the mycelial growth inhibition percentage (MGI %), according to EDGINTON et al. (1971) as follows: MGI % = [(growth of control – growth of treatment) x 100] ÷ growth of control.

The experiment design was entirely randomized in factorial arrangement 4 X 3 (4 treatment x 3 doses) with 5 replications. In order to compare the means, it was used the Tukey test at 5% probability (software SAEG).

The effect of aqueous extracts was evaluated in Paluma guavas in maturation stage 2, light green peel (AZZOLINI et al., 2004) from Alagoinha district in Mossoró, State of Rio Grande do Norte. The fruits were washed with running water and mild detergent and, then, dried naturally on Kraft paper in ambient temperature. After, they received the treatments with 2% of clove aqueous extract and 3% of neem, according to the best results of the in vitro experiment, fruits were manually sprayed until its complete wetness duration. For each treatment, it was used 4 fruits placed on polystyrene, with 5 replications, trays with and without transparent PVC film of 15 µm (self-sticking polyvinyl chloride) in refrigerated storage conditions (8 ± 2°C) and non-refrigerated storage without plastic (partly controlled temperature at 25 ± 2°C). The controls were represented by polystyrene trays with 4 fruits with 5 replications under refrigerated storage conditions with and without plastic film, and ambient condition without plastic film sprayed with distilled water only.

Evaluations were performed 7 days after the storage. However, the fruits on refrigerated storage conditions were removed from storage 2 days before the evaluation and kept in partly controlled conditions (25 ± 2ºC).

The parameters evaluated were: external appearance, of disease – with a scale from 1 to 4 (1 = unstained fruits; 2= from one to two stains smaller than 3 mm; 3 = from two to three stains greater than or equal to 3 mm; 4= more than 3 stains greater than or equal to 3 mm), loss of weight (difference between each fruit's weight before and after the treatment), and Brix degrees (using the DHR-60 digital refractometer, Schmidt Haensch™).

The experiment design was entirely randomized in factorial arrangement 2 X 3 (3 extract and control x 3 storage conditions) with 5 replications. In order to compare the means, it was used the Tukey test at 5% probability (software SAEG).

RESULTS AND DISCUSSION

In vitro experiment had statistic significant interaction between aqueous extracts and doses. The aqueous extract of clove provided 100% of inhibition of mycelial growth of *C. gloeosporioides* according to a dosage of 2% (Table 1). Similar results were noted by Roswalka et al. (2008) using the aqueous extract of clove at 10% to reach 100% of inhibition of mycelial growth of the same fungus. According to Venturoso et al. (2011), the extract of clove at 20% also provided inhibition of 100% of growth of fungi like *Aspergillus*, *Cercospora kikuchii*, *Colletotrichum*, *Fusarium solani*, *Penicillium* e *Phomopsis*. According to Ranasinghe et al. (2002) eugenol is present in clove at 79.2% and it may be the toxic component also in aqueous extract and essential oil, and it is much efficient to inhibit fungi.

Table 1. Effect of aqueous extracts of neem, oleander, basil, and clove in the inhibition percentage of the mycelial growth of *C. gloeosporioides in vitro*.

Extracts	doses %[(a)]		
	1	2	3
Neem	6,67 Bc	13,07 Bb	20,22 Ba
Oleander	0,00 Ca	0,00 Ca	0,00 Ca
Basil	0,00 Cb	16,11 Ba	16,67 Ba
Clove	80,78 Ab	100,00 Aa	100,00 Aa
CV %	8,24%		

[a]Means followed by the same lower case in columns and capital letters in the lines do not differ statistically among them (Tukey 5%).

The treatments with aqueous extracts of neem presented statistic difference in relation to concentrations and the greater the dosage, the greater the inhibition, making 20.22% of fungal growth inhibition with a dosage of 3%. Similar results with extract of neem were also noted by Venturoso et al. (2011), who verified antifungal activity, but less intense in relation to several fungi, including *Colletotrichum* sp. According to Celoto et al. (2008), the aqueous extract of neem at 20% did not inhibit *Colletotrichum gloeosporioides* mycelial growth isolated from papaya fruits.

Basil aqueous extract at 2 and 3% provided inhibition of 16.11 and 16.67%, respectively, which did not represent relevant statistic difference. They only differed statistically in relation to the minor dosage (1%) (Table 1). According to Souza Junior et al. (2009), basil essential oil at 1, 3, 5 and 10 µL/mL of PDA culture medium inhibited 100% mycelial growth and spore germination of *C. gloeosporioides* isolated from yellow passion fruit at all concentrations.

The results obtained with basil aqueous extract may be related to the low amount of active ingredients in the preparation, once the dosages provide fungal growth inhibition according to increase of concentrations. According to Lorenzi et al. (2002), basil leaves presented 3.6% of essential oil, and 73.3% of eugenol, which may be the plant antimicrobial substance.

Oleander aqueous extract, however, did not provide *C. gloeosporioide*s inhibition (Table 1). This plant presents toxic effects to men and animals, and in studies conducted with aqueous extract at 100 and 50% to treat *Peltophorum dubium* seeds, it favored the emergence of fungi, especially *Cladosporium* spp. According to Celoto et al. (2008), oleander aqueous extract at 20% inhibited 41% of mycelial growth of *Colletotrichum gloeosporioides* isolated from papaya fruits.

In vivo experiment had no statistic interaction between aqueous extracts and storage conditions. Was not statistic difference in relation to external appearance, severity of disease, loss of weight, and Brix degree of fruits with treatments using aqueous extracts of neem and clove in all storage conditions (Table 2, 3 and 4).

Table 2. Effect of aqueous extracts of neem and clove in the external appearance and severity of disease of guavas stored for seven days in ambient temperature and refrigerated with and without plastic film.

Extracts	EA and SD[(a)]			
	Storage			
	AT	FPL	FWP	Means[(b)]
Neem	1,45	1,00	1,00	1,15a
Clove	1,95	1,00	1,00	1,31a
Control	1,45	1,00	1,05	1,16a
Means	1,62A	1,00B	1,01B	
CV%		32,13		

[a]EA= external appearance, SD= Severity of disease, AT= ambient temperature (25 ± 2 °C), FPL= refrigerated with plastic, FWP= refrigerated without plastic.
[b]Means followed by the same lower case in columns and capital letters in the lines do not differ statistically among them (Tukey 5%).

Differently, Carvalho et al. (2009) studied the control of diseases in peaches by using clove oil at 0.01% and found the decreased presence of *Monilinia fructicola* (70%) and severity of the disease (42%). The presence of soft rot caused by *Rhizopus* sp. and the severity of the disease decreased 58% and 37%, respectively. According to Alves (2008), clove aqueous extract controlled anthracnose in pepper *in vivo* with a decrease of 30.5% in the severity of the disease. Dias-Arieira et al. (2006), by using neem oil at 0.25%, found 74.4% of *Colletotrichum acutatum* control in strawberry fruits.

In relation to the storage conditions, refrigerated fruits, with and without plastic film, presented less severity of disease and better external appearance. Refrigerated fruits stored with plastic film presented lower loss of weight. There was statistic difference in relation to refrigerated fruits stored without plastic film (Table 2 and 3).

Maintaining fruit quality demands postharvest procedures to reduce fruits respiration rate, delay ripening and senescence (CHITARRA; CHITARRA, 2005), loss of weight (Table 2) (AYUB et al., 2010; DAMATTO JUNIOR et al., 2010; RIBEIRO et al., 2010; LIMA; DURIGAN, 2000), like freezing and modified atmosphere techniques, which make them resistant to pathogens.

Brix degree in refrigerated fruits was greater and differed statistically in relation to the ones in ambient conditions. Regarding the type of package, there was not statistic difference under refrigerated conditions (Table 4). Similar result as the higher degree brix under refrigeration condition was obtained by Silva et al. (2004), using low density polyethylene in Paluma guavas, observed that the amount soluble solids increased during storage in modified atmosphere (refrigerated fruits) in relation ambient condition.

Table 3. Effect of aqueous extracts of neem and clove in the loss of weight (%) of guavas stored for seven days in ambient temperature and refrigerated with and without plastic film.

Extracts	Loss of weight (g)[(a)]			
	Storage			
	AT	FPL	FWP	Means[(b)]
Neem	24,38	5,23	17,12	15,57a
Clove	25,19	4,79	19,16	16,38a
Control	21,95	4,72	18,24	14,97a
Means	23,84A	4,91C	18,17B	
CV%		16,64		

[a]AT= ambient temperature (25 ± 2 °C), FPL= refrigerated with plastic, FWP= refrigerated without plastic.
[b]Means followed by the same lower case in columns and capital letters in the lines do not differ statistically among them (Tukey 5%).

Table 4. Effect of aqueous extracts of neem and clove in the Brix^0 of guavas stored for seven days in ambient temperature and refrigerated with and without plastic film.

Extracts	Brix$^{o\,(a)}$			
	Storage			
	AT	FPL	FWP	Means[(b)]
Neem	6,24	8,78	9,68	8,23a
Clove	6,45	8,57	9,12	8,04a
Control	6,51	7,69	9,37	7,86a
Means	6,40B	8,35A	9,39A	
CV%		26,21		

[a]AT= ambient temperature (25 ± 2 °C), FPL= refrigerated with plastic, FWP= refrigerated without plastic
[b]Means followed by the same lower case in columns and capital letters in the lines do not differ statistically among them (Tukey 5%).

CONCLUSIONS

Clove extract at 2% provided 100% inhibition of fungal mycelial growth;

No statistical difference between the effect of the extracts on the external appearance and severity of disease, loss of weight and Brix degrees;

The storage conditions with plastic film and refrigerated differed from the other conditions obtaining better external appearance and less severity of disease, lower loss of weight and higher Brix degrees.

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