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Potential control of anthracnose in papaya (*Carica papaya*) by treatment with plant extracts

*Controle potencial da antracnose no mamão (*Carica papaya*) por tratamento com extratos de plantas*

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Abstract
Papaya is an economically important plant for Brazil since the country is presented as one of world largest exporters of the fruit. Papaya fruits are often infected by the fungus *Colletotrichum gloeosporioides*, the causal agent of anthracnose, which leads to appearance of lesions in the fruit during postharvest period, resulting in decay and loss of commercial value. In this study, it was demonstrated the *in vitro* fungicide potential of leaf extract from *Phyllostachys aurea* and *Plectranthus barbatus* plants on the *C. gloeosporioides* development. Plant extracts were added at different concentrations (10, 50, 100 ppm) to PDA medium in Petri plates which received the fungal inoculum. As a result, the inhibition of fungal mycelial growth in both treatments at a concentration of 100 ppm as compared to control was observed. *In vivo* assays have shown that 100 ppm of both extracts reduced the incidence of anthracnose symptoms improving the appearance of fruits, compared to symptoms observed in untreated fruits (control). In addition plant extracts used caused no damage to the structure of the papaya seedlings. These data support the idea that plant extracts can be used as an alternative for controlling of agricultural pathogens.
Key-words: Antifungal, *Carica*, Plant Extracts.

Resumo
O mamão papaia é um fruto economicamente importante para o Brasil, desde que o país se destaca como um dos maiores exportadores mundiais. Essa espécie de mamão é frequentemente infectada pelo fungo *Colletotrichum gloeosporioides*, o agente causador da antracnose, que leva ao aparecimento de lesões nos frutos durante o período pós-colheita resultando no apodrecimento e perda de valor comercial. Nesse estudo, foi demonstrado o potencial fungicida *in vitro* do extrato foliar das plantas *Phyllostachys aurea* e *Plectranthus barbatus* sobre o desenvolvimento de *C. gloeosporioides*. Os extratos vegetais foram adicionados em diferentes concentrações (10, 50, 100 ppm) ao meio BDA em placas de Petri, as quais receberam inóculos fúngicos. Como resultado, foi observada a inibição do crescimento micelial fúngico em ambos tratamentos na concentração de 100 ppm quando comparado ao controle. Ensaios *in vivo* mostraram que 100 ppm de ambos os extratos reduziram a incidência dos sintomas de antracnose melhorando a aparência dos frutos, comparada aos sintomas observados nos frutos não tratados (controle). Em adição, os extratos vegetais usados não causaram danos às estruturas das plântulas de papaia. Esses dados suportam a ideia de que extratos vegetais podem ser usados como uma alternativa para o controle de patógenos agrícolas.
Palavras-chave: Antifúngico, *Carica*, Extratos Vegetais.

INTRODUCTION

Papaya plants belong to Caricaceae family. These plants have a great economic importance to Brazil which is the second largest producer of papaya fruits behind only of India (FAOSTAT, 2015). The production is generally affected by pests and pathogens attacks, among which fungus *Colletotrichum gloeosporioides*, that causes anthracnose and stem-end rot on fruit (Ueno et al., 2001; Phoulivong et al., 2010). Losses caused by such plant pathogen, associated with the rapid deterioration of these fruits, compromise the quality of commercial product generating a retarding the development of papaya production industry (Nascimento et al., 2008).

Colletotrichum spp. have been described to be associated with quiescent infections and postharvest diseases in fruits of various plant species besides the papaya, such as strawberry (Akhter et al., 2009), mango (Imtiaj et al., 2005), cucumber (Chen & Dai, 2012), banana (Mirshekari et al., 2012), passion fruit (Benato et al., 2002) as well as cereal crops such as maize, sorghum (Cannon et al., 2008) and dry bean (Pastor-Corrales & Tu, 1989).

Anthracnose control is usually done by application of synthetic fungicides. However, in recent years, it has been observed the increase of pathogens resistance to fungicides. In addition, due to high concentrations applied, it has been demonstrated deleterious effects of these products to consumers and the environment health (Lima et al., 2012). Papaya anthracnose is one of more important diseases of the crop in Brazil (Dantas et al., 2003; Ferreira et al., 2014).

In order to reduce the use of fungicides, alternative techniques have been employed such as heat treatments using spray or immersion in hot water, or even forced hot air (Martins et al., 2010), treatment with gamma irradiation (Cia et al., 2007) and use of biocontrol agents such as bacteria and yeast (de Capdevile et al., 2007; Rahman et al., 2009) has been successful in controlling anthracnose in papaya fruits. More recently, silver-chitosan nanoparticles have been reported to exert strong antifungal activity against *C. gloeosporioides* (Chowdappa et al., 2014).

A novel approach is the use of natural bioactive compounds extracted from plants, which are safe for mammals and other non-target organisms (Ademe et al., 2013), and for control of postharvest disease than synthetics compounds (Barrera-Necha et al., 2008). Several plants have been described to have natural chemicals with antifungal activity against fungi that cause plant diseases. Carpinella et al. (2003) identified natural compounds of *Melia azedarach* that are potential inhibitors of *Fusarium* species. Similarly, Ammar et al. (2013) reported the effectiveness of natural compounds from *Tephrosia apollinea* against *Alternaria alternata* and *Colletotrichum acutatum*.

Hence, the present work was conducted with the objective of determining the effect of plant extracts on development *in vitro* of *C. gloeosporioides* and their efficacy against the postharvest papaya anthracnose.

MATERIAL AND METHODS

Isolation of target pathogen

Colletotrichum gloeosporioides was isolated from papaya fruits showing anthracnose symptoms. An isolate of the pathogen grown in pure culture was maintained in PDA (Potato, Dextrose, Agar) culture tubes at 4 ºC, and used as stock culture throughout the work.

Plants and extraction

Leaves of plants of *Phyllostachys aurea* (Poaceae) and *Plectranthus barbatus* (Lamiaceae) were collected from areas of Campos dos Goytacazes, RJ, Brazil (21º45’15” S and 41º19’28” W), during the period from January to July 2013. The experiments were conducted at the Laboratory of Biochemistry and function of plant proteins of the Federal University of Rio de Janeiro State. Plant tissues (leaves) were shade dried at 40 ºC and milled into a fine powder. Following the procedures employed by Siqueira Junior (2014), 100 g of the pulverized plant leaves were extracted with 500 mL ethanol (99,5%) by slight stirring for 7 days on magnetic stirrer. The extractions were filtered through folded filter paper and reduced to dryness on a rotatory evaporator at 80 ºC temperature in water bath. Dried extracts (50 mg) were dissolved in 1 mL of the extraction solvent (ethanol 99,5%) and then tested for antifungal activities.

In vitro antifungal assay of plant extracts

The antifungal activities of the plant extracts were evaluated via the poisoned food technique as described by Siqueira Junior et al. (2012) with the following modifications: Different dilutions of each extract (10, 50, 100 ppm, v/v) were mixed into each of three replicated plates of PDA performing a total volume of 20 mL/plate, just before it solidified. PDA without extract was used as a negative control. At the center of each plate was added a 5 mm diameter of *C. gloeosporioides* mycelial inoculum. After seven days of incubation at 25 ºC, the mycelia growth in every plate was measured. The experiment was conducted three times.

In vivo antifungal assay of plant extracts

In order to assess the efficiency of plant extracts as fungicide agent the method described by Siqueira Junior et al. (2012) was used with slight modifications. Papaya fruits at color stage 4 (more yellow than green) were purchased in the local market. Fruits were sanitized by dipping in 2% (v/v) sodium hiploclorite for 3 min, 70% ethanol for 3 min, rinsed in sterile distilled water and left to dry naturally. After washing, three uniform wounds, 5 mm deep and 2 mm diameter, were performed on the equator of papaya fruits with a sterile tip. To evaluate the preventive effect of the extracts on infection and development of symptoms of anthracnose, wounded fruits were dipped for 3 minutes into extracts at 100 ppm while control fruits were dipped into sterile distilled water. For each treatment, 20 fruits (i.e. replications) were used and arranged in completely randomized design (CRD). Twenty microliter suspension aliquots containing 1.0 $x10^8$ cell/mL of *C. gloeosporioides* spores were added to injured sites. Following further 7 days incubation at 28 ºC and 95% relative humidity to provide favorable conditions for the postharvest onset of the disease, lesion progress was assessed by measuring the diameter of the damage area relative to initial wound. Subsequently, the percentage of disease symptoms was calculated using the following equation: $n = a-b/a \times 100$ where the percentage of disease reduction is n, the colony area of *C. gloeosporioides* in untreated fruit (control) is a, and the total area of *C. gloeosporioides* in plant extracts-treated fruits is b. Sterile distilled water instead of extracts was used as a negative control. Three replicates of 20 fruits were used for each treatment. The experiment was repeated three times.

Papaya plantlets growth bioassays

Papaya seeds were germinated in vermiculite substrate and five days after germination, seedlings were transferred to pots (3 seedlings per pot) containing fertilized soil. Seedlings were kept under acclimatization at 28 °C with a photoperiod of 12 hours light and 12 hours dark until they complete ten days. As from this period, the plantlets were sprayed with different concentrations of each extract (10, 50 and 100 ppm v/v) daily for another 10 days. Sprayed plantlets with sterile distilled water were used as negative control. After 15 days, all plantlets were assayed for leaf size and total plant size. The presence of allelochemicals was evaluated by comparing the leaves size and total size of treated and untreated (control) plantlets. For each treatment, 18 plants (i.e. replications) were used and arranged in completely randomized design (CRD).

Statistical analysis

Analysis of variance (ANOVA) was carried out with the statistical software ASSISTAT for Microsoft Windows version 7.7 beta (Federal University of Campina Grande [Universidade Federal de Campina Grande]) (Silva & Azevedo, 2016) and statistical significance was set to the level $P< 0.01$. Replication mean with similar results were analyzed together. Means and standard errors were calculated and reported for each experiment. When means were significantly different, a comparison was performed using Turkey's test ($P< 0.01$).

RESULTS AND DISCUSSION

In vitro effect of plant extracts on mycelial growth of Colletotrichum gloeosporioides

The searching for alternative methods due to the serious problems caused by the indiscriminate use of pesticides and fungicides in agriculture has been intensified, especially in developing countries. Under these circumstances, plant extracts as a long-term management method became an adequate solution. In this work the antifungal activity of leaf extracts from two species: *Plectranthus barbatus* and *Phyllostachys aurea* were tested against *C. gloeosporioides* which causes anthracnose in papaya fruits. The results show that the used extracts significantly reduced the fungal mycelial growth compared with the control (p< 0.01) (Table 1). For both plants extracts, antifungal effect increased depending on dose increment. The highest effect on *C. gloeosporioides* mycelium were observed from extract of *P. aurea* at 100 ppm (75.98%). On the other hand, mycelial growth inhibition for *P. barbatus* at 100 ppm was 41% (Table 1). As the data show, at a concentration of 50 ppm, the *P. aurea* extract causes even higher inhibition (44%) than twice concentration of *P. barbatus* (41%). There was not significant effect of the solvent of plant extracts on mycelial growth of *C. gloeosporioides* (results not shown).

Studies showed growth inhibition of *C. gloeosporioides* by a large number of plant extracts. Kalkisim (2012) demonstrated that leaf extracts of *Cornus mas* and *Morus nigra* inhibited the mycelial growth of *C. gloeosporioides* isolated from walnut in 67.95% and 60.04%, respectively. Essential oils and leaf extract from *Lantana camara* were also tested against the radial growth of *C. gloeosporioides* and as result, was found that the plant compounds resulted in high inhibition against the mycelial growth of this pathogen (Richa et al., 2012; Prasad & Anamika, 2015). Thus, the observed data in this study suggest that the *P. aurea* and *P. barbatus* extracts have bioactive components which may be employed as natural fungicide to be used against the *C. gloeosporioides* infections. The isolation and purification of the crude extract of *P. aurea* and *P. barbatus* are in progress.

Table 1 – Effect of plant extracts on mycelial growth of *Colletotrichum gloeosporioides*
Tabela 1 – Efeito dos extratos de planta no crescimento micelial de Colletotrichum gloesporioides

Plant	Concentration (ppm)	Mycelial growth inhibition (%)	Mycelial growth (mm)
	0 (Control)	0.00	70.33[1]±3.60aA
P. aurea	10	12.95	61.22±2.67bA
	50	44.39	39.11±1.89cB
	100	75.98	16.89±2.83dB
	0 (Control)	0.00	65.55±1.57aA
P. barbatus	10	3.21	63.44±2.14aA
	50	20.16	52.33±2.72bA
	100	41.00	38.67±2.22cA

[1]Radial growth after 7 days (mm). All data are expressed as mean (n=72). Different lowercase letter in column denote statistically difference among concentrations compared to positive control group (*P* <0.01). Different uppercase letter in each line denote statistically difference between the two extracts analyzed at the same concentration (*P*< 0.01). Percentage (%) growth inhibition was calculated compared to growth of control (0%)

[1]Crescimento radial após 7 dias (mm). Todos os dados foram expressos como uma média (n=72). Letras minúsculas diferentes na coluna denotam diferenças estatísticas entre as concentrações comparadas ao grupo controle positivo (*P*<0,01). Letras maiúsculas diferentes em cada linha denotam diferenças estatísticas entre os dois extratos analisados na mesma concentração (*P*< 0,01). A percentagem (%) de inibição do crescimento foi calculada comparando ao crescimento do controle (0 %)

In vivo effects of plant extracts on development of anthracnose symptoms on papaya fruits

Based on *in vitro* results, the leaf extracts at 100 ppm for its inhibitory effects were chosen for the tests with the papaya fruits. The results *in vivo* antifungal activity of plant extract against *C. gloeosporioides* are shown in table 2.

Table 2 – Effect of plant extracts on anthracnose symptoms
Tabela 2 – Efeito dos extratos de planta nos sintomas de antracnose

	Percentage (%) of area affected by symptoms of anthracnose	**Inhibition of symptoms in fruits (%)**
Control	41.10[1]±7.11a	0.00
P. aurea	14.20±12.73ab	65.45
P. barbatus	9.43±14.18b	77.05

[1]Onset of symptoms after 7 days. All data are expressed as mean (n=180). Different letter denote statistically difference calculated by comparison to positive control group at $P< 0.01$. Percentage (%) of symptoms inhibition was calculated with comparison with the symptoms in control fruits (0%)
[1]Inicio dos sintomas após 7 dias. Todos os dados foram expressos como uma média (n=180). As letras diferentes denotam diferenças estatísticas comparada ao grupo controle positivo ($P< 0,01$). A percentagem (%) da inibição dos sintomas foi calculada pela comparação com os sintomas nos frutos controle (0%).

Overall, the anthracnose incidence was significantly ($P< 0.01$) lower in fruits treated with plant extracts compared to control after 7 days of ambient storage. The disease incidence and severity in control increased reached 100%. Plant extracts treatment not only delayed the onset of anthracnose disease (table 1) but also maintained the freshness of papaya showing minimal symptoms of anthracnose after 7 days of storage. Despite expectations after analysis of the *in vitro* inhibition, *P. barbatus* extract exhibits a more prominent inhibition (77.05%) when compared to inhibition caused by *P. aurea* extract (65.4%) when used in fruit papaya as protective agents against the development of anthracnose symptoms. Whether such differences between *in vitro* and *in vivo* antifungal activity are influenced by possible interactions between extracts and papaya fruit tissues is not clear. However, some data have shown that isolated compounds have different and reduced activities compared to the crude extract of the same plant (Siqueira Junior et al., 2012).

Thus, the different compounds produced in a plant would differentially affect the development of the pathogen, thus causing different responses according to used extract. Further analyses are needed to elucidate the mechanism. The antimicrobial properties of extracts from various plant species have been demonstrated to affect fungal development *in vivo* (Bautista-Baños et al., 2003; Ogbebor et al., 2007; Ademe et al., 2013). Despite treatment with both extracts did not cause changes in the natural appearance of treated fruits compared to control, a detailed analysis of both nutritional parameters as well as absence of any human health toxicity source must be performed on treated fruits. These data will serve to knowledge about the effect of extracts on nutritional composition as well as a safe utilization of papaya fruits for later application of these extracts as natural fungicides.

In vivo effects of plant extracts on development of papaya seedlings
Some plant extracts have been described to possess phytotoxic effects against development of seeds and seedlings of other plants (Tigre et al., 2012; Oliveira et al., 2014). In this study, phytotoxic effects of plant extracts were investigated at different concentrations on seedling growth of papaya (Figure 1).

Apparently, none of the tested extracts showed relative inhibition on seed germination since the samples had the same germination time that control sample even at highest concentration used (100 ppm) (data not included). In our preliminary experiments, *P. aurea* extract causes no apparent damage to papaya seedlings ($p<0.01$), which showed parameters such as leaves and stem size similar to control seedlings (Figure 1B). However, seedlings treated with *P. barbatus* extract had their size slightly smaller compared to control (Figure 1C), indicating the possible presence of secondary metabolites with phytotoxic potential in this extract.

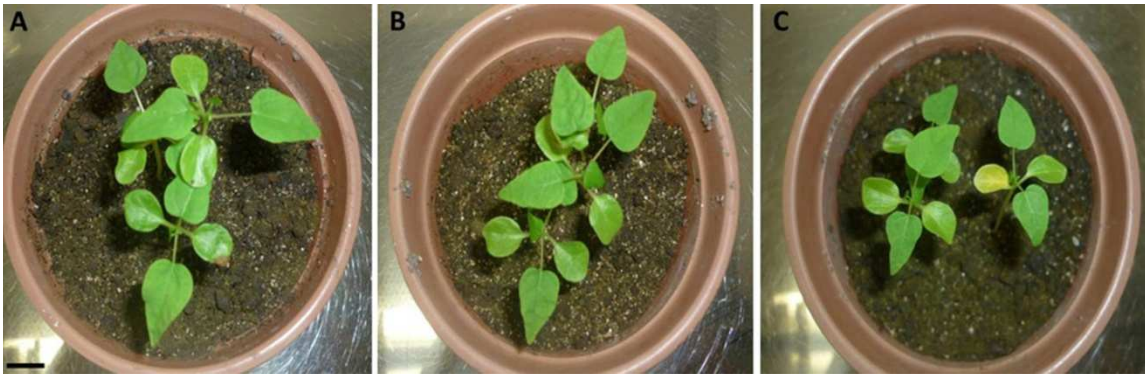

Figure 1 - Appearance of control papaya plantlet (A), treated with *Phyllostachys aurea* extract (B) or *Plectranthus barbatus* extract (C) on the 15th day after treatment. Bar – 1 cm.
Figura 1 - Aparência das plântulas de papaia controle (A), tratada com o extrato de *Phyllostachys aurea* (B) ou com o extrato de *Plectranthus barbatus* (C) no 15º dia após o tratamento. Barra – 1 cm.

It is not doubtful that *P. barbatus* extract could be used as a potential fungicide in the treatment of postharvest papaya fruits because provides significant reduction of the anthracnose symptoms inhibiting the fungal infection, however could not be sprayed directly in papaya plants since the presence of allelochemicals could lead to damages in plants slowing the development and consequently reducing the yield of fruit.

CONCLUSIONS

In the present study, 100 ppm plant extract of *P. aurea* and *P. barbatus* were used as part of an antimicrobial treatment for the postharvest preservation of papaya fruit and showed inhibition of phytopathogenic fungi *in vitro*, as well as a reduction in anthracnose symptoms development in papaya fruit. While *P. aurea* compounds could be used as a microbial agent directly in papaya plants preventing quiescent infections of *C. gloeosporioides*, the *P. barbatus* extract could be employed in papaya fruits protection preventing the onset of symptoms of anthracnose, since this extract could cause phytotoxic effects on papaya plants. Further studies will be planned to identify the bioactive compounds in these plants extracts that could be useful for synthesis and production of natural fungicides.

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