Original Research Article

Antagonic Agents and Hydrothermal Treatment in the Control of Anthracnosis in Banana cv. 'Pacovan'

ABSTRACT

The objective of this work was to evaluate the thermal and biological treatment efficiency of anthracnose (*Colletotrichum musae*) in banana cv.'Pacovan'. Three experiments were set up in the laboratory, the first "in vitro" to select the isolate of *Trichoderma spp.* which had greater mycelial inhibition on plaque, the 2nd "in vivo" was biological control using 2 races of *Trichoderma spp.* and the 3rd in vivo varying temperatures and exposure times of banana fruits contaminated with *Colletotrichum musae.* Positive treatments (using fungicide) and negative treatments were done with the application of distilled water only for experiment 2 and 3. The antagonistic biological control agents T2 and T9 were efficient in inhibiting the growth of *Colletotrichum musae* "in vitro", when analyzed "in vivo" did not have efficiency in the inhibition of the growth of the pathogen. Thermotherapy is a promising technique for the treatment of postharvest rot in banana fruits of 'Pacovan' cultivar, with the best efficiencies observed at 47ºC for 3 and 9 min, and 51º for 3 and 9 min.

Keywords: Colletotrichum musae, hydrothermal treatment, biological treatment.

1. INTRODUCTION

The banana crop (Musa sp.) Originates in Southeast Asia, grown in most tropical countries, and stands out as the most consumed fruit in the world due to its characteristics as a flavor, aroma and high nutritional value. Brazil is considered the fourth largest banana producer in the world, with an average of 7 million tons and a planted area of 487 thousand hectares [1]. The population of South America is the largest consumer, with 21.13 kg per capita per year, followed by Central America, with 13.9 kg and Oceania, with 11.26 kg [2]. In spite of its importance, the culture can be affected by several pathogens, with negative repercussions in the production, damaging the development of the banana, being worth highlighting in the post-harvest the fungus *Colletotricum musae*, causal agent of the anthracnose, postharvest disease more important in the banana producing regions of the world, which hinders the commercialization of the product in natura, with losses of up to 40% of production [3].

The anthracnose is caused by different physiological races of the fungus *Colletotrichum musae* (Berk and Curtis) [4], whose symptoms are dark and depressed lesions, which with the disease progress and favorable environmental conditions, such as high humidity, cover rose fruit.

The most used disease control is chemical control (pre and post harvest) and cultural practices to try to reduce the amount of inoculum in the field. Finally, to try to minimize the effects on chemicals and to increase production and good quality in the product, prolonging the post-harvest period, new alternative methods have been pursued in the control of the disease, including thermal treatment and biological treatment which have shown to be promising in the practice of control against several pathogens. Control of this disease in banana is an essential component of fruit quality after harvest [5].
Biological control with the use of *Trichoderma* spp. has been identified as a viable alternative for ecologically and economically sustainable agricultural production systems [6]. The genus Trichoderma is considered non-toxic and rapidly biodegradable, thus becoming a good strategy as a biocontrol agent for plant diseases.

In addition to the production of cell wall degrading enzymes and the production and release of toxins, *Trichoderma* spp. competes for sites of infection and nutrients available with other microorganisms. This action has an important role in inhibiting the development of different pathogens, preventing the germination of propagules or the infection itself [7].

Heat treatment is a control method that has been used for several years to control post-harvest fungal diseases [8]. Thermotherapy is able to eradicate or weaken the pathogen. The immersion of fruits in heated water of 50 to 55 ºC for 10 min is considered standard method for post-harvest control of several fungal diseases [9]. Short exposure times at higher temperatures are more effective in altering the surface temperature of the fruits; thus, they can eradicate the pathogen present inside the fruit peel [10].

In view of the above, the objective of this work is to evaluate the efficiency of the thermal and biological treatment in the control of anthracnose (*Colletotrichum musae*) in the banana crop.

2. MATERIAL AND METHODS

The present work was developed in the Laboratory of Phytopathology (CECA / UFAL), BR 104N, Km 87, Municipality of Rio Largo-AL, from October to November 2018.

The *C. musae* was obtained from the banana fruit of the 'pacovan' cultivar with anthracnose symptom in the city of Maceió - AL, at the stage of green maturation. The pathogen was maintained on successive replication in potato-Dextrose-Agar-PDA culture medium. The isolates of Trichoderma spp. were obtained from monosporic cultures of the Phytopathology Laboratory (CECA-UFAL). The isolates were preserved by successive replication method in potato-Dextrose-Agar-BDA culture medium.

Experiment 1 - Effect of *Trichoderma* spp. on the in vitro growth of *Colletotrichum musae*

Paired culture was performed on petri dishes containing PDA culture medium. For this purpose, 5 mm diameter mycelium discs were taken from pure culture of *Colletotrichum musae* and *Trichoderma* spp. and placed one disc of each fungus per plate, which was deposited diametrically on opposite sides 1 cm away from the edge of the plate for the direct confrontation of organisms, treatments were done at room temperature.

Four different breeds of *Trichoderma* spp. (Races: 2, 5, 9 and 15). The control potential of Trichoderma spp. on *Colletotrichum musae* was determined at 7 days of cultivation. The evaluation of the test was done by the measurement of the colonies diameters, which were calculated the mean values of percentage of inhibition, in relation to the control, also were

assigned notes based on the scale of [11] adapted by [12]. For the calculation of the percentage of inhibition of mycelial growth was used the formula of Abbot (1925).

$$\text{PIC}\ (\%) = \frac{(\text{T} - \text{t})}{\text{T}} \text{x}\ 100$$

In what: PIC - percent inhibition of mycelial growth, %; T - control treatment, mm; t - treatment evaluated, mm.

The design was completely randomized, with 5 treatments and 4 replicates (Figure 1). The statistical analyzes of the variables established in the pairings were done by the Turkey test at 5%, with the assistance of ASSISTAT Version 7.7 beta.

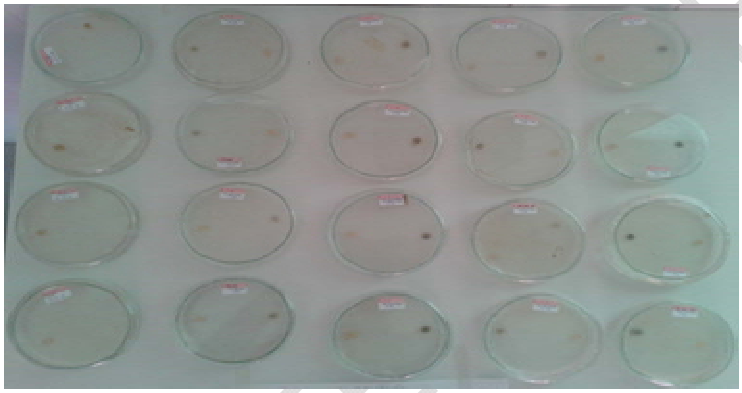

Figure 1. Demonstration of the installation to evaluate the effect of *Trichoderma* spp. on the in vitro growth of *Colletotrichum musae* by culture pairing method. Source: The authors.

Experiment 2 - Biological control of anthracnose in bananas of 'pacovan'

For this experiment, two breeds of Trichoderma spp. with greater inhibitory potential of the development of mycelium of *C. musae* evaluated in experiment 1, which were races 2 and 9. First, the bananas were inoculated with C. musae at a concentration of 107, 24 hours after the spraying of the races of Trichoderma spp. At concentration 106. In addition, two control treatments were done, one positive where the bananas were sprayed in suspension of the fungicide (Q) from the dithiocarbamate ®Mancozeb chemical family at 0.09 g. / 100 mL-1 of distilled water (Fungicide not indicated for control of anthracnose in banana in the post-harvest) and a negative control without the use of any type of treatment, sprayed only with distilled water (T) the same one used for the others treatments.

Experiment 3 - Hydrothermal treatment for control of anthracnose in banana of 'pacovan'

For this experiment, 11 treatments with 5 replicates were used in a completely randomized design, each replicate corresponding to a banana fruit. The bananas were inoculated 24 hours before the application of the treatments, aiming at the fixation and the greater possibility of the presence of the fungus Colletotrichum musae in the fruits that would be treated, the concentration of 107 was used for spraying in the bananas. The spores were counted using a Neubauer chamber under a stereoscopic microscope.

The treatments used consisted of 3 different temperatures in 3 different times: T1T1 (47 ºC for 3 min); T1T2 (47 ° C for 9 min); T1T3 (47 ° C for 9 min); T2T1 (51 ° C for 3 min); T2T2 (51 ° C for 6 min); T2T3 (51 ° C for 9 min); T3T1 (55 ° C for 3 min); T3T2 (55 ° C for 6 min); T3T3 (55 ° C for 9 min). And two control treatments, the same ones used for experiment 2.

Evaluations of data from experiments 2 and 3

The treatments were evaluated at 3 days after application (DAA), 7 DAA and 10 DAA, regarding the incidence and severity of the disease. Regarding incidence, the following formula was used:

$$\%\, I = \frac{NFL}{NTF} \times 100$$

Being: NFL = number of injured fruits; NTF = number of total fruits

The severity of rot was determined using a diagrammatic scale proposed by [13], with variations ranging from 0 to 64% of the area damaged by fruit and the incidence rate (% I) (Figure 2).

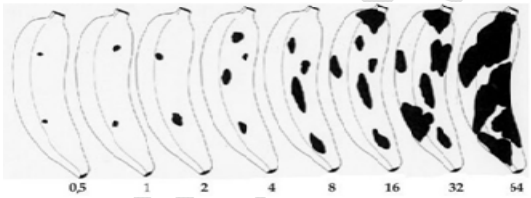

Figure 2. Diagrammatic scale to evaluate the severity of rot in banana fruits, whose values correspond to the percentage of injured area / fruit. Source: Moraes et al., 2008.

The data were submitted to analysis of variance and the means were compared by Turkey test, at the 5% probability level, using the statistical program Sisvar version 5.6.

3. RESULTS AND DISCUSSION

Experiment 1 - Effect of Trichoderma spp. on the in vitro growth of *Colletotrichum musae*

In the evaluation done by the test of crop pairing with the isolates of *Trichoderma* spp. of the T2, T5, T9 and T15 races versus *Colletotrichum musae*, it was observed that there was a significant difference in the mycelial growth of the T9 colony in relation to the other isolates and mainly the control (Table 2). As shown in Table 1, the T9 isolate presented higher antagonism (60.06%), followed by T2 isolates (39.11%), T5 isolates (28.8%) and T15 isolates (26.02%) in the development of *C. musae* (Figure 3).

However, the evaluation done by the scale of [11] adapted by [12], showed that the isolates of *Trichoderma* spp. T2 and T9 showed Note 1, with growth throughout the petri dish and on the pathogen disc, T5 and T15 Note 2, with growth throughout the petri dish, but not on the

pathogen (Table 1). All isolates of Trichoderma spp. evaluated are antagonistic to C. musae, but the T2 and T9 isolates were the most efficient in competition and parasitism.

Table 1. Antagonistic potential of *Trichoderma* spp on *Colletotrichum musae* in culture paired by the [11] scale adapted by [12] to the 7th day of paired cultivation.

Treatments	Bell scale notes	(PIC) % Inhibition
C. musae x Isolated T2	1	39,11%
C. musae x Isolated T5	2	28,8%
C. musae x Isolated T9	1	60,06%
C. musae x Isolated T15	2	26,02%
Attestant	5	---------

In paired cultivation, the efficiency of Trichoderma sp. (TF1) and B. subtilis in the alternative control of *C. musae* in banana fruits, found inhibition indices of 84% and 74% respectively [13].

There are also reports of the use of culture filtrates from four isolates of T. asperellum in the biological control of *Colletotrichum musae*, *Fusarium oxysporum* [14].

Table 2. Effect of *Trichoderma* spp. on the in vitro mycelial growth of *Colletotrichum musae* on the 7th day of matched cultivation.

Treatments	Diameter of the colony (mm)
C. musae x Isolated T2	3.72 ab
C. musae x Isolated T5	4.52 ab
C. musae x Isolated T9	2.44 b
C. musae x Isolated T15	4.35 ab
attestant	A

Means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test at 5% probability. Coefficient of Variation (CV%) = 27.76.

The T2 and T9 isolates showed greater inhibition in the development of *C. musae*. The reduction in mycelial growth of *C. musae* can be attributed to the release of metabolites by the antagonists, competition of nutrients in the culture medium or by mycoparasitism.
In the present study, the hyphae of the hyphae were isolated from the hyphae of the host hyphae [15]. According to [11], in vitro antagonism is a form used only for mass selection of candidates for biocontrol agents, since not all those with in vitro inhibitory effects can exert the mechanism of antagonism "in vivo".

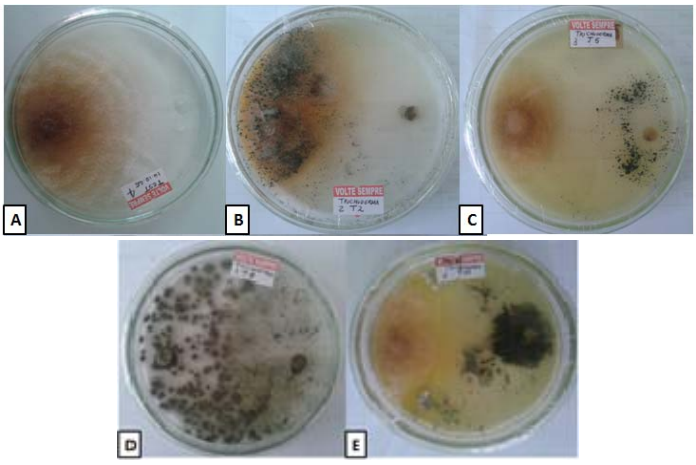

Figure 3. Effect of *Trichoderma* spp. (B - E) on the mycelial growth of *Colletotrichum musae* in the test of crop pairing in PDA medium. The witness; B: Isolated T2; C: Isolated T5; D: Isolated T9; E: Isolated T15. Source: Authors.

This study suggests that the isolates of Trichoderma spp., especially of the T9 and T2 races, can be exploited as biological control of anthracnose in banana plants. However, it is suggested to carry out tests to evaluate the antagonistic potential "in vivo".

Experiment 2 - Biological control of anthracnose in bananas of 'pacovan'

Analyzing the incidence data of the disease in bananas of the 'pacovan' cultivar according to the evaluated days, it was observed that at 3 days after the application of the control types, the treatment with *Trichoderma* spp. of the T2 race was less incident than the other treatments. And the positive control with which it had the use of fungicide had an incidence increased gradually according to the evaluated days after the application. Most treatments had a total incidence at 7 and 10 days of evaluation.

With the data observed for incidence it can be inferred that the pathogen that caused the anthracnose could develop in the bananas even after the treatments.

For the severity data observed according to the visual scale of notes developed by [14] for the growth of the symptoms of the pathogen Colletotrichum musae in banana it can be inferred that the 3 DAA T2 and T9 had low severity compared to the chemical treatment (Q) and the control (T). At 7 and 10 DAA, all treatments presented severity greater than 50% (Table 3).

With this experiment "in vivo" we observed that the use of Trichoderma spp. of the T2 and T9 races are not viable for use with the purpose of inhibiting the development of the fungus Colletotrichum musae in bananas of the 'pacovan' cultivar. It was noted that after the first days of spraying the biological agents it was possible to note that there was a decrease in symptoms soon after the biological agents did not inhibit the growth of the fruits, including these treatments had their cycle of maturation.

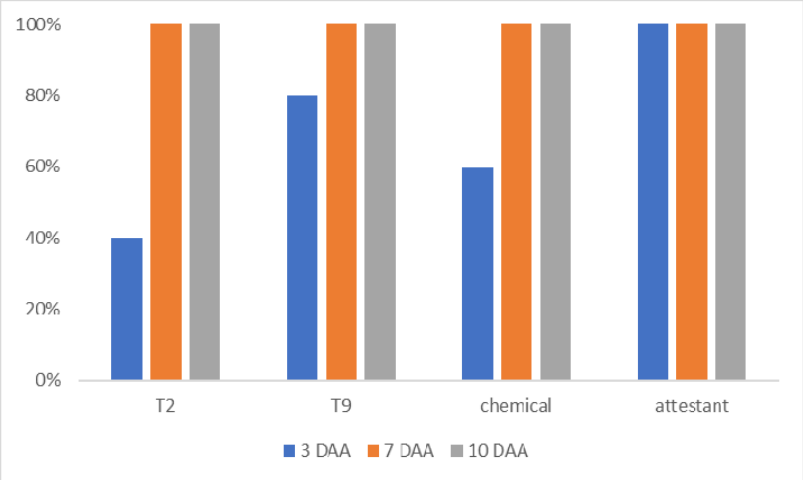

Figure 4. Incidence of anthracnose at 3, 7 and 10 days after application of Trichoderma spp. of the T2 and T9 races for control of Colletotrichum musae in 'pacovan' bananas.

Table 3. Severity (%) of *Colletotrichum musae* in bananas of the 'pacovan' cultivar at 3, 7 and 10 days after application of Trichoderma spp. of the T2 and T9 races.

Treatments	3 DAA	7 DAA	10 DAA
T2	2,9 c	62,4 a	64 a
T9	4,6 c	56,8 ab	64 a
Q	14,8 b	50,6 b	64 a
T	30,0 a	64,0 a	64 a
C.V. (%)	12,42	9,82	0,00

Means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test at 5% probability.

In research using strains of Trichoderma spp. for the inhibition of growth of *Colletotrichum gloeosporioides* in papaya fruits [16] observed that in the in vitro experiments all the strains used characterized antagonistic activity and that in the in vivo experiments it was not possible to decrease growth of the pathogen when inoculated after the pathogen is installed and that there was a decrease when Trichoderma spp. was inoculated 24 hours before contact with the pathogen. Based on this research we can relate that a preventive and non-curative application as used in this research could be that there was greater antagonistic action.

[17] observing the antagonistic effect of Trichoderma spp. against phytopathogens *Sclerotium rolsfii* and *Verticullium dahliae* in ornamental aster and strawberry observed an inhibitory effect on the mycelial growth of the pathogens and also an antagonistic effect by the production of secondary metabolites of *Trichoderma* spp. *Trichoderma* is able to synthesize different compounds, such as proteins, enzymes, and antibiotics, which increase its ability to control phytopathogenic fungi [18].

According to [17] Trichoderma species can act in direct biocontrol, infecting a series of phytopathogenic fungi through the secretion of lysing enzymes, such as cellulases, chitinases, glycanases and proteases, during the microparasitism process [19]

Experiment 3 - Hydrothermal treatment for control of anthracnose in banana of 'pacovan'

At 3 DAA, it was observed that the majority of treatments had a low incidence, with only treatments T1T3 (47 ° for 9 min), chemical (Q) and control (T) presented values above 60% of incidence. At 7 DAA, four treatments remained with incidences below 50%, which were T1T1 (47º by 3min), T2T1 (51º by 3min), T2T3 (51º by 9min) and T3T3 (55º by 9min) above 60%. At 10 DAA, treatments T1T1 (47 ° for 3 min) and T3T3 (55 ° for 9 min) presented respectively 60 and 80% incidence, with the other treatments values of 100% (Figure 5).

The T3T3 treatment was observed to have the lowest incidence of Colletotrichum musae inoculum among the evaluated treatments, but it is important to infer that this treatment made the banana commercialization impossible to burn the fruit, the fruits after this treatment showed black coloration resulting from dry tissue by using a high temperature for a longer time. These aspects were also observed by [20] in 'silver-ãna' bananas, treated at 56ºC for 9 minutes.

For [21], injuries caused by thermotherapy include increased weight loss, peeling of the peel, increased susceptibility to fungi and reduced post-harvest life, and are characterized by a lack of normal development of pigmentation, abnormal softening and decline in ethylene production. Thus, respiration rate and ethylene synthesis are affected by exposure to high temperatures.

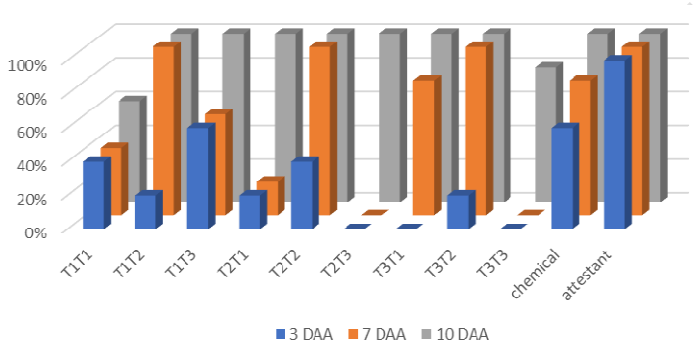

Figure 5. Incidence of anthracnose at 3, 7 and 10 days after application of hydrothermal treatments for control of *Colletotrichum musae* in bananas of 'pacovan' cultivar.

According to the observed for incidence we can indicate the treatments T1T1 and T2T3 as being promising for diminishing the growth of anthracnose symptoms in banana fruits of 'Pacovan' cultivar.

The severity for the hydrothermal treatments, which varied in 3 temperatures and 3 times, can be expressed that for 3 DAA all the hydrothermal treatments obtained a low percentage of severity in relation to the positive and negative controls. At 7 DAA it was also observed for most hydrothermal treatments average severity below 20% and at 10 DAA it was observed that 3 hydrothermal treatments were efficient in decreasing the severity of *Colletotrichum musae* in bananas of the 'pacovan' cultivar that were T1T1, T1T3 and T2T1 (Table 4).

Table 4. Severity (%) of anthracnose at 3, 7 and 10 days after application of hydrothermal treatments for control of *Colletotrichum musae* in 'pacovan'

Treatments	3 DAA	7 DAA	10 DAA
T1T1	0,79 c	1,27 e	1,19 c
T1T2	0,95 c	18,20 b	64,0 a
T1T3	1,50 c	1,37 e	3,50 c
T2T1	0,85 c	0,99 e	8,20 b
T2T2	1,35 c	50,0 b	64,0 a
T2T3	0,77 c	0,69 e	8,0 b
T3T1	0,80 c	15,60 b	64,0 a
T3T2	1,04 c	13,40 de	64,0 a

T3T3	0,52 c	32,50 c	64,0 a
Q	14,80 b	50,60 b	64,0 a
T	30,0 a	64,0 a	64,0 a
C. V. (%)	19,90	26,90	4,25

Means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test at 5% probability.

The heat treatment is based on the effect of elevated temperatures on the cellular activity of the pathogen. Most phytopathogenic organisms present a lethal thermal point at temperatures between 45 and 60 ºC.

[22] observed that from the 53 °C combinations for 9 mi and 56 °C for 3 min the spore germination was reduced to 4% and 0%, respectively and the combination 56 °C for 12 min reduced but did not paralyze mycelial growth, treatment 56 ° C for 6 min delayed but did not paralyze mycelial growth "in vitro", but was effective in complete control of in vivo rot. These results observed by the authors are similar to those observed in this study, but in the temperature of 55º for 9 min there were problems related to fruit quality.
[23] performing work using hydrotherapy for the control of anthracnose in bananas of the cultivar 'Prata' observed that at 50º for 20 min and 53º for 15 and 20 min it reduced the injured area in 85 and 97% respectively.
With this work and relating it to the works that involve hydrothermal research to control anthracnose in bananas we can observe that there is a range of 47º by up to 9 minutes and 51º to 55º by up to 9 to 3 minutes, respectively, that can be explored to decrease lesions caused by *Colletotrichum musae* on banana.

4. CONCLUSION

The antagonistic biological control agents T2 and T9 were efficient in inhibiting the mycelial growth of *Colletotrichum musae* "in vitro", when analyzed "in vivo" did not have efficiency in the inhibition of the growth of the pathogen. And thermotherapy is a promising technique for the treatment of postharvest rot in banana fruits of 'Pacovan' cultivar.

REFERENCES

1. Fao. Food and Agriculture Organization of the United Nations. Available in: <http://www.faostat.fao.org/site/340/default.aspx>. Accessed on: 10 Dec. 2012.

2. Vieira, L.M. Banana: production and world market. 2011. Available in: http://cepa.epagri.sc.gov.br/. Accessed on: Dec 09. 2015.

3. Silva, J. R.; Cordeiro, Z. J. M. Phytosanitary export of bananas. In: CORDEIRO, Z. J. M. (Org) Phytosanitary banana. Brasília, EMBRAPA-SPI, p. 9-14, 2000.

4. Coelho, A.F.S.; Dias, M.S.C.; Rodrigues, M.L.M.; Leal, P.A.M. Post-harvest control of anthracnose of banana-silver dwarf treated with fungicides and kept under refrigeration. Science and agro-technology, v.34, n.4, p.1004-1008, 2010.

5. Sagoua, W. et al. Effect of lactoperoxidase system on the control of *Colletotrichum musae* on bananas. Food Technology Biotechnology, v.49, n.2, p.244-248, 2011.

6. Compant, S. et al. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future perspectives. Applied and Environmental Microbiology, v.71, n.9, p.495-499, 2005.

7. Punja, Z. K .; Utkhede, R. S. Using fungi and yeasts to manage vegetable crop diseases. Trends in Biotechnology, Burnaby, v.21, n.9, p.400-407, 2003.

8. Couey, M.H. Heat treatment for control post-harvest disease and inset pest of fruits. HortScience, v. 24, p. 98-202, 1989.

9. Liu, X.; Guo. G.; Huang, S.; Liu, X.J.; Huang, S.M.; Guo, G. The research and utilization of post-harvest heat treatment for fruit storage. South China Fruits, v. 26, p. 46-48, 1997.

10. Golan, R. B.; Phillips, D.J. Postharvest heat treatment of fresh fruits and vegetables for decay control. Plant Disease, v. 75, p. 1085-1089, 1991.

11. Bell, D. K.; Wells, H. D.; Markham, C. R. *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. Phytopathology, v. 72, p. 379-382, 1982.

12 Rodrigues, Johnathan. *Trichoderma spp.* associated with NPK fertilization levels in the *Sclerotinia sclerotiorum* - common bean. 2010. 84 f. Dissertation (Master's Degree in Agronomy) - Postgraduate Course in Agronomy, Federal University of Santa Maria, 2010.

13. Moraes, W.S.; Zambolim, L.; Lima, J.D. 'Dwarf silver' banana chemotherapy to control postharvest rot. *Archives of the Biological Institute*, São Paulo, v. 75, n.01, p. 79-84, 2008.

14. Oliveira, E.S. Extracts and plant essential oils, antagonistic microorganisms, resistance inducers and antiseptic products in the control of banana anthracnose. Fortaleza: UFC, 2009.

15. Adebesin, A.A.; Odebode, C.A.; Ayodele, A.M. Control of post harvest rots of banana fruits by conidia and culture filtrates of *Trichoderma asperellum*. Journal of Proteome Research., v.49, n.3, p.303-307, 2009.
.

16. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* species - opportunistic, avirulent plant symbionts. Natural Reviews Microbiology, v.2, p.43-56, 2004.

17. Valenzuela, N. L. et al. Biological control of anthracnose by postharvest application of *Trichoderma* spp. on maradol papaya fruit. Biological Control, v. 91, p. 88–93, 2015.

18. Isaias, C. O.; Martins, I.; Silva, J. B. T.; Silva, J. P.; Mello, S. C. M. Antagonistic action and bioactive metabolites of *Trichoderma* spp. against the pathogens *Sclerotium rolfsii* and *Verticillium dahlia*. Summa Phytopathologica, v. 40, n. 1, p. 34-41, 2014.

19. Al-Taweil, H.I.; Osman, M.B.; Aidil, A.H.; Wan-Yussof, W.M. Optimizing of *Trichoderma viride* cultivation in submerged state fermentation. American Journal of Applied Sciences, v. 6**,** p. 1277–1281, 2009.

20. Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R.S., Woo, L., Lorito, M. *Trichoderma*–plant–pathogen interactions. Soil Biology & Biochemistry, v. 40, p. 1–10, 2008.

21. Moraes, W.S., Zambolim, L., Lima, J.D., Salomão, L.C.C. & Cecon, P.'Prata-Anã' banana thermotherapy for post-harvest rot control. Brazilian Phytopathology, v.30, p. 603-608, 2005.

22. Shiffmann-Nadel, M. & Cohen, E. Influence of growth temperatures on the effectiveness of heat treatment of Phytophthora- infected citrus fruits. Plant Disease Reporter, v. 50, p. 867-868, 1966.

23. Sponholz, C.; Batista, U.G.; Zambolim, L.; Salomão, L.C.C.; Cardoso, A.A. Effect of the hydrothermal and chemical treatment of 'Prata' banana fruits on post-harvest anthracnose control. Brazilian Phytopathology, v. 29, p. 480-485, 2004.