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RESEARCH ARTICLE

EVALUATION OF THE ANTAGONISTIC ACTIVITY OF INDIGENOUS TRICHODERMA SPECIES AGAINST COLLETOTRICHUM GLOESPORIODES, THE FUNGAL PATHOGEN CAUSING MANGO ANTHRACNOSE IN SENEGAL

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Abstract

Mango is a major crop that represents 63% of fruit production in Senegal. The mango business suffers however from major phytosanitary constraints such as mango anthracnose caused by *Colletotrichum gloeosporioides*. Fungicides are usually sprayed for the control of this fungal pathogen with their downside of negative impacts on human health and on environment. Fungi of the genus *Trichoderma* are reported to have great potential for biological control on pathogens. Their ability to control *C. gloeosporioides* was tested through confrontation with three isolates of *Trichoderma asperellum* (S3M4ZIG, F3GP3 and F3GP3-5), one isolate of *Trichoderma viride* (F2KV3) and a fifth isolate of *Trichoderma sp.* (S5M5ZN). These isolates, obtained from mango tree organs in Senegal, were used in direct confrontation against *C. gloeosporioides in vitro* and further *in vivo* tests on fruits. The incidence and severity of anthracnose were evaluated after 20 days of incubation. In dual culture in the petri dishes, the isolate S5M5ZN inhibited totally the mycelial growth of *C. gloeosporioides*, followed by the strains F3GP3 (*T. asperellum*), F3GP3-5 (*T. asperellum*), F2KV3 (*T. viride*) and S3M4ZIG (*T. asperellum*), that caused respective reduction of mycelial growth of the pathogen by $89.41 \pm 18.33\%$, $89.01 \pm 19.01\%$, $77.25 \pm 19.77\%$, $77.25 \pm 12.95\%$. For the *in vivo* tests, the mangoes soaked with distilled water (negative control) and those inoculated with only *C. gloeosporioides* (positive control) had a disease severity of respectively $20.36 \pm 4.39\%$ and $50.69 \pm 6.58\%$. The *Trichoderma* isolate S3M4ZIG, showed the best efficacy on mango with the lowest disease severity of $5.55 \pm 0.00\%$.

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Introduction:-

Mango is the sixth most produced fruit in the world with more than 50 million tons in 2017, harvested on about 50 million hectares (FAO, 2019). In Senegal, its production is subjected to many constraints related to pests and

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diseases (Diédhiouet *et al.*, 2007). Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc (Arauz, 2000., Zakaria *et al.*, 2015) remains the most important fungal disease of mango with greater losses in the post-harvest stage (Nelson, 2008; Pardo-De la Hoz *et al.*, 2016). In Senegal, anthracnose can cause losses ranging from 42 to 90% depending on climatic conditions (Mbaye, 2006; Diédhiouet *et al.*, 2014). The intensive use of chemicals for the control of pathogens does not completely solve the problem due to the acquisition of resistance of some strains and public health problems. Many cases of skin irritation, headaches, hot flashes or dizziness, endocrine disruption, congenital malformations of the male reproductive system have been noted worldwide through misuse of chemicals (Hileman, 1994; Ahouangninou *et al.*, 2011). Pesticide residues exceeding standards have been found in the groundwater of the Niayes peri-urban area in Senegal (Cissé *et al.*, 2003). In Ivory Coast, organophosphorus compounds have been noted in large quantities in cocoa, coffee, and banana and vegetable plantations (Traoré *et al.*, 2006). Due to these negative environmental and health impacts caused by pesticides, research for alternative options gained popularity and included biocontrol products such as essential oils (Cissé *et al.*, 2020), extracts from medicinal plants and also microbial antagonists. Several microbial antagonists such as species of *Trichoderma*, *Beauveria*, *Metharizium*, *Gliocladium*, etc. have been used against pathogenic fungi in fruit crops (Caron *et al.*, 2002; Kébé *et al.*, 2009; Yan *et al.*, 2018; Guèye, 2020; Dièye *et al.*, 2021). Fungal species from the genus *Trichoderma* are widely used as biocontrol agents for eco-friendly management of plant pathogens. They produce enzymes and antibiotics, induce plant resistance to diseases and inhibit spore germination of pathogens (Samuels, 1996; Almeida *et al.*, 2007; Degenkolb *et al.*, 2008; Schuster *et al.*, 2010; Cheng *et al.*, 2012.; Lopes *et al.*, 2012; Mukherjee *et al.*, 2013). The effectiveness of *Trichoderma spp.* on *Colletotrichum spp.* (Deepak *et al.*, 2020) and on many phytopathogenic agents has already been documented (Kumar *et al.*, 2014; Dièye *et al.*, 2021; Sharma *et al.*, 2021; Matas-Baca *et al.*, 2022). However, when the species of *Trichoderma* are applied to crops other than those from which they were isolated, their efficacy was often reduced (López-López *et al.*, 2022). Therefore, the objective of this research work was to assess the efficacy of five isolates of *Trichoderma* isolated directly from mango tree organs to control mango anthracnose due to *Colletotrichum gloeosporioides*.

Materials and Methods:-

Fungal material

The *Colletotrichum* isolate

The isolate of *Colletotrichum gloeosporioides*, used in this study was isolated, from mango fruit showing typical symptoms of anthracnose in Ziguinchor. It was isolated, cultured and maintained on potato dextrose agar plates in the laboratory.

The *Trichoderma* isolates

The isolates of *Trichoderma spp.* originated from different mango production zones in Senegal, such as the Casamance (Ziguinchor, Sédhiou and Kolda) and the Niayes Zone (coastal area between Dakar, Thiès and Saint-Louis) (Table 1). They were all isolated from different organs of mango trees, identified and kept in the laboratory for further tests.

Table 1:- The five isolates of *Trichoderma spp.* used for antagonistic tests with *C. Gloeosporioides*.

Codes	Origin of isolates	Region of isolates	Species
(MA)S3M4ZIG	mango fruit	Ziguinchor	<i>Trichoderma asperellum</i>
(MB)F3GP3-5	mango leaf	Goudomp (Ziguinchor)	<i>Trichoderma asperellum</i>
(MC)F3GP3	mango leaf		<i>Trichoderma asperellum</i>
(MD)F2KV3	mango leaf	Kounkané (Kolda)	<i>Trichoderma viride</i>
(ME)S5M5ZN	mango fruit	Niayes Zone (Thiès)	<i>Trichoderma sp.</i>

In vitro antagonistic activity of *Trichoderma spp.* on *Colletotrichum gloeosporioides*

The isolates of *Trichoderma spp.* were screened for their antagonistic activity against *Colletotrichum gloeosporioides* by using dual culture technique as described by Gueye (2020). Agar discs (5mm) of seven-day-old of *Trichoderma spp.* and *C. gloeosporioides* cultures were placed at two opposite ends of PDA plates (Fig 1). The control had a culture free disc of PDA and PDA disc colonized by *C. gloeosporioides* at the opposite ends of the PDA plates. Three replicates were used for each treatment. The petri dishes were then sealed with parafilm and incubated in an oven at a temperature of 25°C.

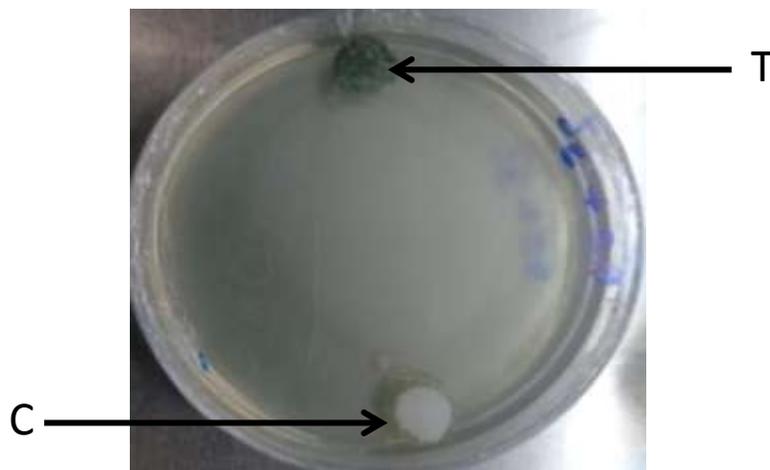


Figure 1:- Direct confrontation set up used for *Trichoderma* spp. (T) and *Colletotrichum gloeosporioides* (C).

The antagonistic effect of the control agent on the pathogenic fungus *Colletotrichum gloeosporioides* was assessed after 10 days of incubation, corresponding to the time taken by the pathogen to completely fill the Petri dish. This was determined by calculating the percentage of inhibition of radial growth of *C. gloeosporioides* by *Trichoderma* spp. using the formula proposed by Begum *et al.* (2008).

$$\text{GIR} = \frac{\text{R1} - \text{R2}}{\text{R1}} * 100$$

Where, **GIR**= Growth inhibition rate,

R1 indicates average diameter of fungal colony in the control treatment without antagonist (in cm)

R2 indicates diameter of fungal colony in treated plates with antagonist (in cm)

In vivo* antagonistic activity of *Trichoderma* spp. on *Colletotrichum gloeosporioides

For the *in vivo* test of the efficacy of *Trichoderma* to control the fungal pathogen, mangoes of kent variety were used. After washing with tap water, the fruits were soaked successively in a 1% household bleach solution for 3 min, then in 70% ethanol for 60s and finally in sterile distilled water for 10 min. The mangoes were further rinsed a second time under the laminar flow hood with sterile distilled water. Each treatment consisted of 9 mangoes with a mango representing the experimental unit. The negative control was made of mangoes treated only with sterile distilled water and the positive control aggregated mangoes soaked with only the spore suspension of the pathogen. The biological control agent was made of a spore suspension of a pure culture of 7 days old *Trichoderma* culture. The same process was applied to *Colletotrichum gloeosporioides* with the only difference that a 10 days old culture were used. For both fungi, the spore suspension was obtained by pouring 30 ml of sterile distilled water into petri dish, then scraping the mycelium together with the water with a sterile scalpel blade into a funnel equipped with multilayer sterile gaze for filtration into an erlenmeyer. A spore suspension free from mycelial fragments was obtained for further experiments.

For the inoculation purposes, the spore suspensions of both the pathogen and antagonists were adjusted at 10^5 spores per ml. Inoculation of mangoes was performed by soaking the fruits one by one in a 1000 ml spore suspension during 5 min. The fruits were thereafter left for drying at room temperature in the laboratory for 30 min. For the treatments involving inoculation with the two fungi, the mangoes were first inoculated with *C. gloeosporioides*. After drying at room temperature in the laboratory for 30 min, the fruits were soaked in the spore suspension of *Trichoderma* for 5 minutes and allowed to dry for another 30 min. For the control treatments, mangoes were soaked in sterile distilled water for 5 min in a volume equal to those used for the treated mangoes with spore filtrate (1000 ml). The fruits were thereafter incubated at room temperature for 20 days.

Evaluation of fruit disease

Fruit disease was evaluated by assessing incidence and severity of anthracnose 20 days after fruit inoculation.

The incidence (I) of the experimental unit was calculated using the formula developed by Ngullie *et al.* (2010):

$$I = \frac{\text{Number of mango showing symptoms of anthracnose}}{\text{Total number of fruits}} * 100$$

The severity (S) of mango anthracnose was evaluated using a visual scale from 0 to 4 where:

- 0 = mango fruit without symptoms of anthracnose,
- 1 = 1 to 25% of mango surface with anthracnose symptoms,
- 2 = 26 à 50% of mango surface with anthracnose symptoms,
- 3 = 51 à 75% of mango surface with anthracnose symptoms,
- 4 = 76% à 100% of mango surface with anthracnose symptoms

The severity of the experimental unit was calculated using the formula developed by Ngullie *et al.* (2010):

$$S = \frac{\sum(N_i * S_i)}{N * Z} * 100$$

Where N = Number of mango fruits with severity i, S_i = Severity value i of anthracnose, N = Total number of mango fruit, Z = Highest severity index

Statistical Analysis

The statistical analysis was performed using the software R version 3.6.2. All the data were first subjected to a normality test. The data were then compared through the Analysis of Variance (ANOVA) when they were normally distributed. The Student-Newman-Keuls (SNK) test was used to compare the means.

Results:-

In vitro antagonistic activity of *Trichoderma spp.* on *Colletotrichum gloeosporioides*

All isolates tested were able to reduce the mycelial growth of the fungal pathogen (Fig2). *Trichoderma sp* (isolate S5M5ZN) showed the greatest efficacy with complete inhibition of mycelial growth of the pathogen. It was followed by the strains F3GP3 (*T. asperellum*), F3GP3-5 (*T. asperellum*), F2KV3 (*T. viride*), S3M4ZIG (*T. asperellum*), which caused respective reduction of mycelial growth of the pathogen by 89.41 ± 18.33%, 89.01 ± 19.01%, 77.25 ± 19, 77%, 77.25 ± 12.95%.

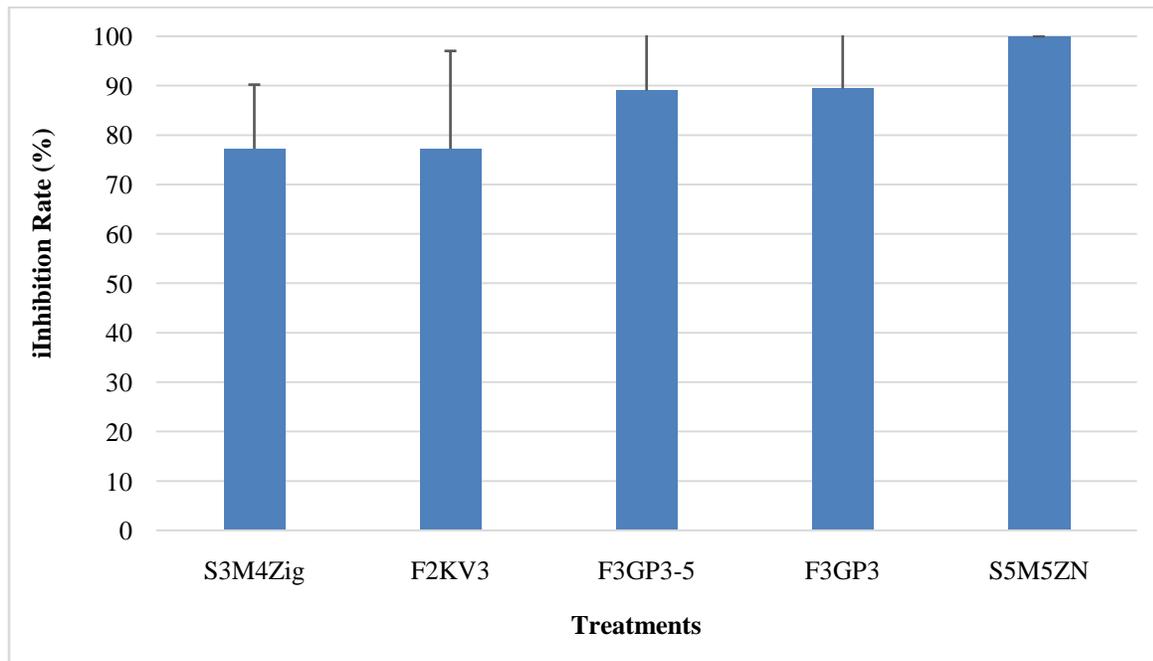


Figure 2:- Performance of different *Trichoderma spp.* at inhibiting mycelial growth of *Colletotrichum gloeosporioides*, in a dual culture confrontation *in vitro*, n=3, p=0,00103; S3M4ZIG, F3GP3 and F3GP3-5= *Trichoderma asperellum*, F2KV3= *Trichoderma viride*, S5M5ZN= *Trichoderma sp.*

Observation of fungal structures of both the antagonistic *Trichoderma* strains and the *C. gloeosporioides* in the confrontation zone under microscope (Gx400) showed mycelial cord transformation (Fig 3a), coiling (Fig 3b) and cell lysis of the pathogen (Fig 3c).

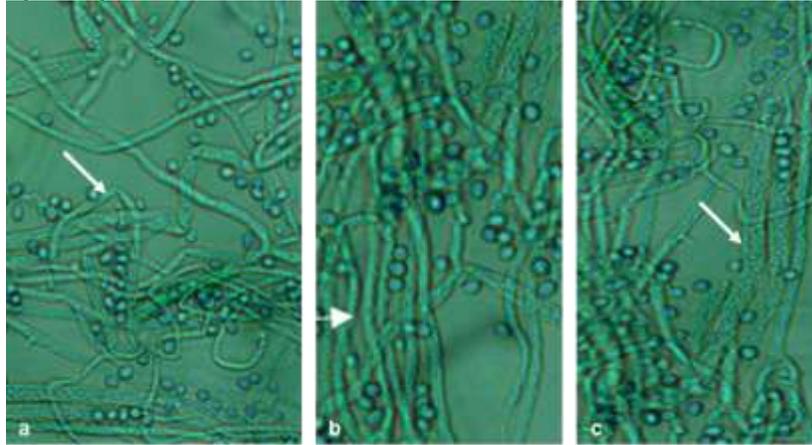


Figure 3:- Modification of the mycelium of *Colletotrichum gloeosporioides*, a= cord transformation (arrow), b= coiling of the mycelium (arrow), c= lysis of the mycelium (arrow) (Gx400).

In vivo antagonistic activity of *Trichoderma* spp. on *Colletotrichum gloeosporioides*

Incidence of mango anthracnose

The evaluation of the efficacy of *Trichoderma* to control mango anthracnose on inoculated mangoes was performed after 20 days of incubation. It showed that fruits that received the isolates F2KV3 (*T. viride*), F3GP3 and S3M4ZIG (*T. asperellum*) after inoculation with *Colletotrichum gloeosporioides* had a low incidence of anthracnose, with a mean value around 22 % (Fig 4). At the same time the negative control treatment (T0) that was soaked in sterile water was displaying $44.44 \pm 00.00\%$ incidence. The positive control (T+), inoculated with the pathogen presented the highest incidence with $88.88 \pm 00.00\%$.

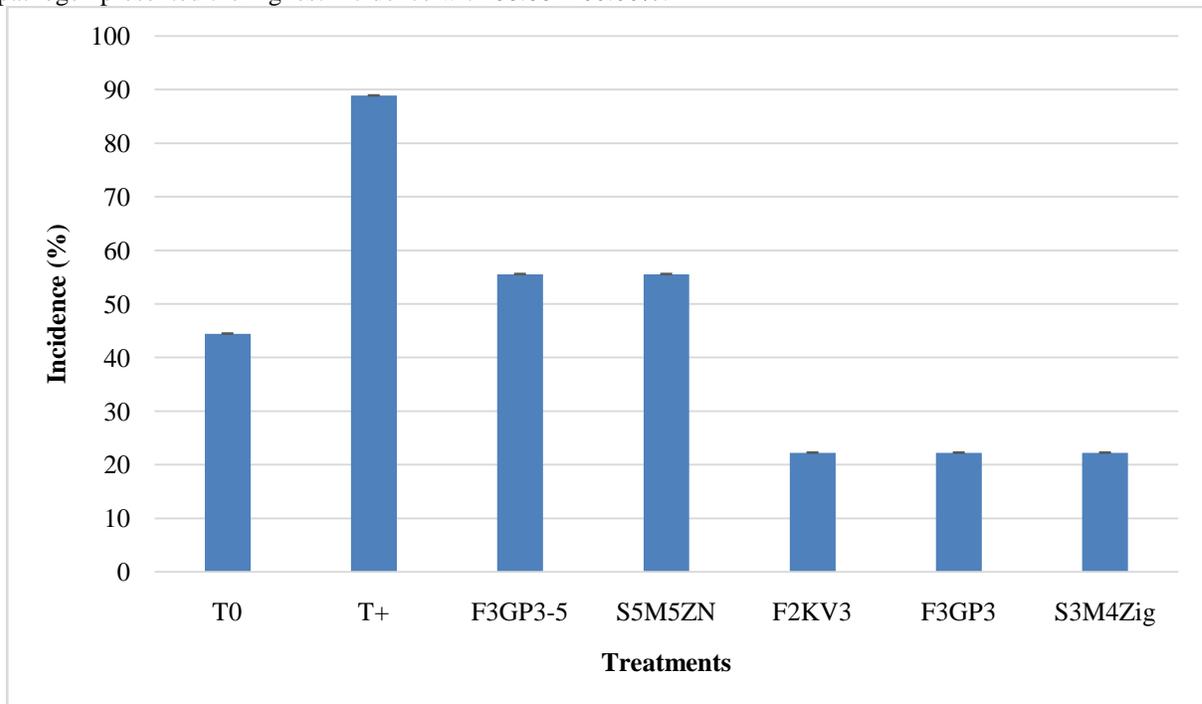


Figure 4:- Incidence of mango anthracnose on fruits inoculated with *Colletotrichum gloeosporioides* and *Trichoderma* spp. alone or in combination after 20 days of incubation, $n = 9$, $p < 2e^{-16}$; T0= mango treated with sterile distilled water, T+= mango treated with filtrate of pathogenic fungus *Colletotrichum gloeosporioides*, S3M4ZIG, F3GP3 and F3GP3-5 = *T. asperellum*, F2KV3 = *T. viride*, S5M5ZN = *Trichoderma* sp.

Severity of mango anthracnose

Mangoes from the negative control treatment soaked only with sterile water presented a severity of $20.36 \pm 4.39\%$ (severity index 1) (figure 5). The positive control treatment with mangoes inoculated with only *Colletotrichum gloeosporioides* showed the highest severity with a percentage of $50.69 \pm 6.58\%$ (severity index 2). At the same time, the fruits treated with the isolate S3M4ZIG (*T. asperellum*) after inoculation with *Colletotrichum gloeosporioides* presented the lowest disease severity with $5.55 \pm 00.00\%$ (severity index 1).

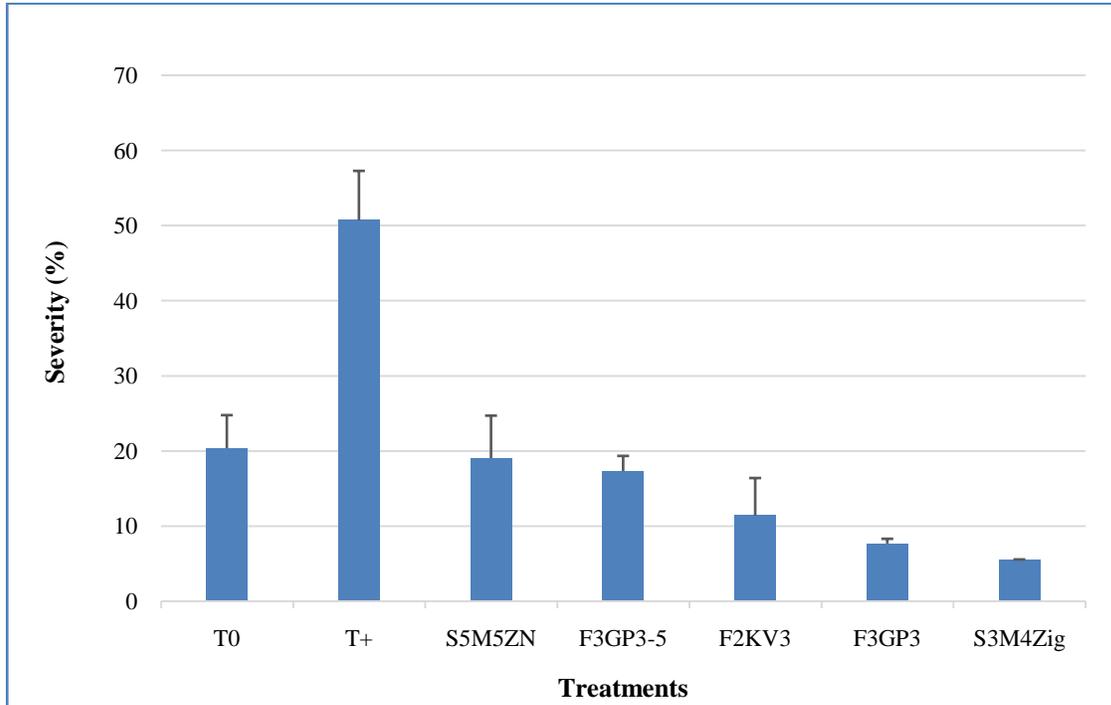


Figure 5:- Severity of mango anthracnose on fruits inoculated with *Colletotrichum gloeosporioides* and *Trichoderma spp.* alone or in combination after 20 days of incubation, $n=9; p < 2e^{-16}$; T0= mango treated with sterile distilled water, T+= mango treated with filtrate of pathogenic fungus *Colletotrichum gloeosporioides*, S3M4ZIG, F3GP3 and F3GP3-5= *Trichoderma asperellum*, F2KV3= *Trichoderma viride*, S5M5ZN = *Trichoderma sp.*

Mango appearance at the end of the experiment was variable depending on treatment. Departing from an apparently healthy status of the mature not yet ripe mangoes (figure 6 a), symptoms of disease were noticed after 20 days of incubation. Anthracnose spots were prevalent on mangoes from all treatments, but affected more those from the positive control fruits (figure 6 d). The mangoes from the negative control, that were not inoculated, were also affected (figure 6 c). The diseases symptoms were less visible with mangoes inoculated with *C. gloeosporioides* then *T. asperellum* isolate S3M4ZIG, with 7 out of 9 mangoes that were still showing a marketable aspect after 20 days.



a = mangoes before inoculation



b=mangoes inoculated with both *C. gloeosporioides* and antagonist fungi *T. asperellum* strain S3M4ZIG after 20 days of incubation



c=mangoes of the negative control treatment, soaked with only sterile distilled water after 20 days of incubation



d=mangoes of the positive control treatment, inoculated with the pathogenic fungi *C. gloeosporioides*, after 20 days of incubation

Figure6:- Aspects of mangoes at time of inoculation and according to different treatments 20 days later, **a**=mangoes before inoculation, **b**=mangoes inoculated with both *C. gloeosporioides* and antagonist fungi *T. asperellum* strain S3M4ZIG, **c**= mangoes of the negative control treatment soaked with only sterile distilled water, **d**= mangoes of the positive control treatment, inoculated with the pathogenic fungi *C. gloeosporioides*

Discussion:-

Anthrachnose is one of the most damaging postharvest diseases of mango worldwide. It is usually controlled with fungicides, the frequent use of which may lead to environmental pollution, appearance of resistance in number of cases and also serious threat to human health (De Curtis *et al.*, 2010; Wong *et al.*, 2015). Alternative control methods based on environment friendly and less risky products to human health are being developed. Among those alternatives, natural products such as essential oils and *Trichoderma spp.* have shown a good efficacy on many pathogenic fungi (Begum *et al.*, 2008). In the present work, the experimentations were carried out to test the *in vitro* and *in vivo* efficacy of native *Trichoderma* isolates for the biological control of *Colletotrichum gloeosporioides*, the causal agent of mango anthracnose. On mycelial growth of *Colletotrichum gloeosporioides*, the isolate S5M5ZN (*Trichoderma sp.*) caused total inhibition. It was followed by isolates F3GP3 (*T. asperellum*), F3GP3-5 (*T. asperellum*), F2KV3 (*T. viride*) and S3M4ZIG (*T. asperellum*), which allowed respective reductions of mycelial growth of $89.41 \pm 18.33\%$, $89.01 \pm 19.01\%$, $77.25 \pm 19.77\%$ and $77.25 \pm 12.95\%$. In previous works, *Trichoderma harzianum* isolate Th-2 was reported to cause 100% inhibition of mycelial growth of *Colletotrichum capsici* (Begum *et al.*, 2015). Dièye *et al.* (2021) observed *in vitro* antagonistic activity of *Trichoderma koningii* Audemas (M35) and *Trichoderma longibrachiatum* Rifai (M37) on thirteen isolates of *Fusarium sp.* isolated from symptoms of mango malformation disease in Southern Senegal.

The ability of *Trichoderma spp.* on the control of fungal pathogens is related to their capacity to act as antagonist against other microorganisms. This efficacy is associated to their ability of growing fast and occupying the space and rapid nutrient use as well as lytic actions against the pathogens (Begum *et al.*, 2008; Begum *et al.*, 2015; Sharma *et al.*, 2021). At the end of the experimentations, observations under optical microscope, showed coiling, mycelial cord transformation and cell lysis of the pathogen in the confrontation zone with the *Trichoderma* isolates. This suggests that a potential combination of both competition for space and nutrients as well as chemical activity leading to cell lysis would be the likely mode of action deployed by the tested *Trichoderma* isolates. It is also known that *Trichoderma spp.* may also secrete volatile compounds such as Trichodermins and Trichodermins to degrade the cells of pathogens (Elad *et al.*, 2000). Previous experimentations also documented that metabolites were involved in the efficacy of fungi of the genus *Trichoderma* on *C. gloeosporioides* (Waterhouse *et al.*, 1968). Enzymes such as xylanases and chitinases, involved in the degradation of the cell walls of phytopathogenic fungi were also demonstrated to be produced by fungi of the genus *Trichoderma* (Shi *et al.*, 2012; Vinale *et al.*, 2014; Pandey *et al.*, 2015).

The *in vivo* tests were conducted to verify the ability of *Trichoderma spp.* to control mango disease during post-harvest stage and preserve the phytosanitary quality of fruits. When the mangoes inoculated with *C. gloeosporioides* in a spore suspension were soaked with the spore suspension of the antagonist, the incidence and severity of anthracnose were highly reduced. In fact, the negative control and the positive control showed respective incidence of $44.44 \pm 00.00\%$ and $88.88 \pm 00.00\%$ and severity means of 20.36 ± 4.39 and $50.69 \pm 6.58\%$. In the most effective treatment that involved *T. asperellum* isolate S3M4ZIG the severity was reduced down to $5.55 \pm 00.00\%$ while the incidence was $22.22 \pm 00.00\%$. In terms of fruit quality 20 days after treatment, 7 out of 9 mangoes treated isolate S3M4ZIG with were still showing a good phytosanitary quality and still had market value. In terms of disease control, this isolate allowed a reduction of incidence by 75% compared to the positive control and 50% with respect to the negative control. The severity was reduced down by 89% for the positive control and 72.7% for the negative control. Likewise, Sharma *et al.* (2021) reported that dipping of mangoes in *Trichoderma harzianum* and *Pseudomonas fluorescens* suspensions reduced anthracnose by 40.0 to 43.3%. *In vivo* efficacy was also reported for *Trichoderma spp.* on mango anthracnose (Rahman *et al.*, 2013).

In addition, application of a culture filtrate of *T. harzianum* on mangoes was reported to reduce significantly the severity of anthracnose (Rahman *et al.*, 2012), pointing towards the involvement of biochemical mechanisms to control pathogens. The ability of *Trichoderma* species to reduce disease severity was also put in relation with their ability to inhibit the conidial germination of *C. gloeosporioides* on mango surfaces (Begum *et al.*, 2015). In the present study, the efficacy of the isolate S3M4ZIG was higher on the positive control (75% reduction of incidence and 89% reduction of severity) compared to negative control (50% reduction of incidence and 72.7% reduction of severity). This suggests that mangoes from the negative control with respective incidence of 44.44% and severity of 20.36% were very likely to harbor natural latent infections of *Colletotrichum*. In fact, developing fruit may be infected in the field, but infections remain quiescent until the onset of ripening, which occurs after harvest. Once the climacteric period of the fruit starts, lesions begin to develop (Arauz, 2000). The eventuality of a latent infection may therefore explain that our inoculation period came late to control the spore germination stage, reducing thereby the efficacy of *Trichoderma* control.

The significant suppression of the mycelial growth of *Colletotrichum gloeosporioides* *in vitro* added to the ability of *T. asperellum* isolate S3M4ZIG to significantly inhibit the development of anthracnose on mangoes inoculated with pathogen suggests a capacity to produce substances with antifungal effects beside the competition for space and nutrients.

The incidence of $88.88 \pm 00.00\%$ and severity of $50.69 \pm 6.58\%$ found for the positive control shows that the inoculation method was effective for *C. gloeosporioides*. This data is another demonstration of the ability of the fungus to create own access to the fruit tissues without any help through wounding as already reported by Diallo *et al.* (2017).

The *in vitro* performance of *T. asperellum* isolate S3M4ZIG on *Colletotrichum gloeosporioides* was about 77% while isolate S5M5ZN (*Trichoderma sp.*) inhibited totally the mycelial growth. In many screening works for microbial antagonists, an efficacy threshold of 85% is often set to qualify to further tests (Hermosa *et al.*, 2012; Sabbagh *et al.*, 2017; López-López *et al.*, 2022). The results of this study corroborated those of Kumar *et al.*

(2014) that the testing environment may play an important role on the performance of living agents to control pests and diseases.

Conclusion:-

The *Trichoderma* isolates tested showed a good control activity against *Colletotrichum gloeosporioides*, the causal agent of mango anthracnose. The isolate S5M5ZN (*Trichoderma sp.*) showed the greatest *in vitro* efficacy against *C. gloeosporioides* with a total inhibition of mycelial growth. While not being among the best performing isolates during the *in vitro* tests, S3M4ZIG (*Trichoderma asperellum*) allowed a better control of mango anthracnose with the lowest incidence and severity on mangoes 20 days after inoculations. The negative control showed anthracnose spots deriving from natural contaminations that were further enhanced through inoculation as shown by the positive control. Differences on control efficacy on the negative and positive control treatments suggest that early inoculation with the antagonist to cover spore germination stage of the pathogen may enhance the efficacy of *Trichoderma* species.

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