SJIF IMPACT FACTOR (2015): 5.416 CRDEEP Journals
International Journal of Environmental Sciences Serawit et. al., Vol. 6 No. 3 ISSN: 2277-1948
International Journal of Environmental Sciences. Vol. 6 No. 3. 2017. Pp. 87-93

Full Length Research Paper

Invitro and *Invivo* Evaluation of Antagonistic Microbes against Pepper Anthracnose(*Colletotrichum capsici*(syd.) Bisby and Butler

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Article history
Received: 18-06-2017
Revised: 22-06-2017
Accepted: 09-07-2017

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Abstract

Pepper (capsicum annum l.) An important tropical and subtropical vegetable crop, is affected by several diseases among which anthracnose caused by colletotrichum spp *is the most important one. This bio-control research was aimed to manage anthracnose by using different antagonists. Isolates of bacillus, trichoderma and actinomycete were arranged in crd experiment. The treatments were replicated thrice. Data on incidence, severity, plant height and dry weight had been collected and analyzed. Anova showed that bacillus-2 (55.5-100%) which was significantly superior to the other antagonists, and followed by bacillus-1 (50-97.5%), bacillus-3 (44.4-100%), trichoderma-2 (33.3-82.3%), trichoderma-1 (44.4-75.3%), actinomycete-1 (11.1-66.7%), actinomycete-2 (6.7-66.7%) and actinomycete-3(2.7-66.7%).. Under in vivo evaluation of biocontrol agents the minimum per cent disease incidence was observed in bacillus + trichoderma isolates (0, 8.33 and 16.67%) at marko fana, oda haro and bako local pepper varieties, respectively. The effect of biocontrol agents on growth parameters of different pepper varieties showed that, among the in vivo evaluation of growth parameters, in treatment of bacillus + trichoderma isolates gave maximum length and dry weight (except in bako local of dry weight). Thus, the use of bacillus, trichoderma and actinomycete are recommended for field use.*

Key words: *anthracnose, bacillus, morpho-physiological variability, pathogencity, in vivo, invitro*

Introduction

Pepper (*Capsicum* spp) is one of the principal spice crops with many economic and culinary advantages that constitutes steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fiber and mineral elements [1, 2]. According to Faisal and Muhammad[3],Ethiopia is world's sixth largest producer of pepper next to India, China, Bangladesh, Peru and Pakistan with total share of 36, 11, 8, 8 and 6%, respectively. Hot pepper covers 67.98% of all the area under vegetables in Ethiopia [4]. EEPA [5],reported that Ethiopian small holder farmers earned 509.44 million Birr from pepper production in 2004/05. This indicates that hot pepper serves as one of the important sources of income to smallholder farmers and as exchange earning commodity in the count ry [6] .The yield of pepper in Ethiopia is very low (0.4 tones fruit yield/ha) [7] , which is very low as compared to 5 metric tons per hectare. The decline of hot pepper production is also attributed to poor varieties, poor cultural practices, the prevalence of fungal diseases [7], Several diseases affect the production and marketability of pepper in Ethiopia [8,9]. Currently, outbreak of anthracnose caused a significant decline in quantity and quality of pepper yield.[8] adding up that yield losses due to anthracnose varied from 10–60% in different parts of India.

According to Ashoka [9], Rocha *et al*. [10] and D'Souza *et al*. [11], biocontrol agents maximize reduction in mycelial growth. *T. harzianum* had the highest performance as the biocontrol agent's *in vitro* conditions by over covering the pathogen within 5-6 days. Antagonistic bacterial strains (DGg13 and BB133) were found to effectively control *C. capsici*, in Thailand [12]. Santha [13] observed that the isolates of *T. harzianum* and the isolates of *Aspergillus niger* were effective in inhibiting the mycelial growth of *C. gloeosporioides* causing anthracnose of black pepper under *in vitro* condition. Wharton and Dieguez-Uribeondo [14] reported that biological control agents, such as *Bacillus subtilis* and *Candidao leophila*, had been effective against *Colletotrichum* spp. *B. subtilis* and *T. viride* strains were reported to have promoted the growth parameters [15]. Suthin Raj *et al*. [16] isolated pathogen from infected pepper fruits collected from the Chidambasam. Conversely, plant growth-promoting rhizobacteria (PGPR) significantly increase crop yield in the greenhouses and fields [17]. Application of antagonists with *Trichoderma* and *Pseudomonas* species against pepper diseases will reduce the disease incidence and severity and also increase the yield and quality of pepper in major pepper growing

regions of the country. For this purpose, the new advances in solid and submerged fermentation technologies can be applied for small and large scale production of *Trichoderma* and *Pseudomonas* species biomass as biocontrol fungicides [18]. Attempt was made to study the most limiting factors for pepper disease management practices in main growing areas of Ethiopia [18]. However, there is a need for practical use of antagonists [19, 20]. Thus, the present study was carried out to evaluate the potential *Trichoderma, Bacillus* and *Actnomycete* species against anthracnose diseases of pepper *invitro* and *invivo*.

Material and methods

Study Area

This experiment was carried out in Addis Ababa University, College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology laboratory in 2014. Some of antagonistic agents such as two *Trichoderma* spp, three *Actinomycets* spp and three *Bacillus* spp were tested against *Colletotrichum* spp. The antagonists and test pathogens were grown on potato dextrose agar in order to get fresh and active growth of each fungus.

Isolation of Trichoderma isolates

Two *Trichoderma* species were isolated from leaves of major *Capsicum* growing areas of Beya and Worita woredas (Kefa Zone). One piece (~2-5 mm) of leaves tissues (5-8) was cut from the edge of the lesion on the collected infected pepper using sterilized sharp scalpel. The tissue was then surface sterilized by dipping the tissue in 70% ethanol for 2 min followed by washing twice with distilled water [21]. Cultures were isolated and maintained on Potato Dextrose Agar (PDA).

Isolation of Bacillus isolates

Three soil samples were taken in sterilized plastic bag from Yeki, Abashegie and Gimbo Woredas from roots of pepper. The pepper land soil used in experiments was collected from the 0-15 cm layer. Each gram of sample was suspended in 9 ml of sterile distilled water and shaken vigorously for 5 min in a shaker. The samples were heated at 60^{o}C for 60 min in a water bath. Then the soil suspensions were serially diluted with sterile distilled water up to 10^{-7} and the dilutions from 10^{-6} to 10^{-7}. 0.1ml of each dilution was taken and spread on nutrient agar medium with composition of(Peptic digest of animal tissue 5.0 gm, Beef extract 3.0 gm, Agar 15.0g/ litre). The plates were incubated at 28^{o}Cfor 72 h according to Watanabe and Hayano[14].

Isolation of Actinomycetesisolates

Three soil samples were taken from Boketa, Aman-shesheko and Ofudo Woredas from roots of pepper. For each collected sample, 1g of the soil were suspended in 9 ml of sterile distilled water then incubated in an orbital shaker incubator, at 28 ^{0}C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to 10^{-5} were prepared using sterile distilled water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10^{-4} to 10^{-5} was taken and spread evenly over the surface of starch casein agar medium (starch 10g; casein 0.3g; KNO_3 2g; NaCl 2g; K_2HPO_4 2g; $MgSO_4$ 7 H_2O 0.05g; $CaCO_3$ 0.02g; $FeSO_4$ $7H_2O$ 0.01g Agar 18 g/l). Rifampicin (2.5 mg/ml) and amphotericin B (75 mg/ml) added to inhibit bacterial and fungal contamination, respectively. Plates were incubated at 28 ^{0}C, and read after 72 h. repeated streaking on starch casein agar plates led to purify bacterial colonies that showed *Actinomycetes* like appearance according to Watanabe and Hayano [14].

Dual culture technique

In dual culture technique, twenty ml of sterilized and cooled potato dextrose agar was poured into sterile Petri plates and allowed to solidify. Fungal antagonists were inoculated at one side of Petri plate and the test pathogen was inoculated at exactly opposite side of the same plate by leaving 3cm gap. In case of evaluation of bacterial antagonist, mycelial discs of pathogen were inoculated in the centre of the plate and bacterial antagonist was streaked opposite side of the same plate. Each treatment was replicated three times. After 72 hrs of incubation, the radial growth of pathogen was measured. Per cent inhibition over control was worked out according to formula given by Sundar *et al.[22]*.

$$\% \text{ Inhibition} = \frac{X-Y}{X} \times 100$$

Where; X= Growth of control; Y= Growth of treated plate

Green house experiments

The experiment was conducted under Greenhouse conditions, at the College of Natural Sciences, at Addis Ababa University. The three pepper varieties were sown in pots from April to March 2014, in the greenhouse, at Addis Ababa University. The three hot pepper varieties which were collected from Bako Agricultural Research Centers, Oromia Region State. The pepper varieties that used were Bako local, Marko fana and Oda haro.

Seeds were surface sterilized for 20 min with 70% ethanol, after which they were air dried on the sieve for 24 hr at room temperature [26]. Three varieties of pepper seeds were then sown in a fifteen clean and sterilized plastic pots (16 cm × 22 cm) containing each nine kilo gram of soil and compost (2:1 ratio) which was sterile in autoclave for 30 minutes at 121^{0}C. The study was arranged in

Randomized Block Design (RBD) with five replications at each variety. Transplanting to the actual pot was done when the seedlings attained 15 to 20 cm height and or at 57 days after sown [26]. In the pots there were fifty pots and six plants per pot with a total of 300 plants containing the same commercial soil for *in vivo* evaluation and testing of the susceptibility of released varieties

Table 1: Pepper varieties used for the evaluation study of the *Colletotrichum* spp.

Pepper variety	Year of Release	Altitude m.a.s.l	Temperature (^{O}C)	Rain Fall (mm)	Seed Source
Mareko Fana	1976	1400-2200	20/29	600-1337	MARC
Bako Local	1976	1400-2120	20/29	600-1237	BARC
Oda Haro	2005	1400-2200	13.3/27.9	830-1559	BARC

Source: [23-25]

Experimental design

The treatment of the pathogen with the test organisms was performed under conditions. The treatments included:

1. *Tricoderma*sp6×10^5 conidia/ml (2ml/plant).
2. *Bacillus* sp 10^8cfu /ml (2ml/plant).
3. A mixture of: *Tricoderma*sp6×10^5 conidia/ml (1ml/plant) + *Bacillus* sp 10^8cfu /ml (1ml/plant).
4. Positive control (received only the recommended pathogen. AUPEP-6 and AUPEP-10 isolates 6×10^5 conidia/ml (2ml/ plant).
5. Negative control (received only distilled water 2ml/ plant).

Inoculation method (In vivo)

Conidia suspensions of test pathogen AUPEP-6 and AUPEP-10 (according to their virulence) and the antagonistic *Trichoderma*-2 spp isolate (AUT-2) were harvested from 14-day-old cultures grown on potato dextrose agar (PDA) at 25°C. Ten replication of each isolate culture plate was flooded with 20ml sterilized distilled water, and the conidia were gently scraped from the culture plate by sterile glass rod. After which they were filtered through four layers of cheese cloth to remove any mycelial debris. Conidial suspensions were generated from the pure culture and conidial suspensions were adjusted to 6×10^5 conidia/ml with sterile distilled water using haemacytometer according to method described by Ambreen and Javed [27]. By the formula:

Cell concentration per ml = n x dilution factor x 10^4 spores/ml

Where, n=Average cell count per square of the four chamber counted.

Number of cells of *Bacillus*-2 sp was adjusted to 10^8cfu /ml with sterile distilled water using spectrophotometer (0.1opaticl density).Each pepper seedling was inoculated with 2ml/per plant by wounding the plant at collar region according to the method described by Oh *et al*. [28]. The control plants were inoculated with sterilized water. The antagonists were inoculated against the test pathogen after 24hrs.

Pathogenicity test (In Vivo)

For the disease assessment, suspensions of conidia and microbial cells were spray on pepper plants (2 ml/seedling). In three varieties of pepper, 2ml of harvested AUPEP-6 and AUPEP-10 were inoculated on all pots except negative control. There were two negative (pots-13 and 14) and four positive controls (pot-9, 10, 11 and 12) for each variety. After 24 hours, harvested biological control *(Bacillus*-2and *Trichoderma* -2 spp) were applied individually (pot 1-8) and in combination of the two (pot15 and 16) for each variety. Experiments were arranged in the greenhouse in a completely randomized block design layout with each treatment bearing six pot ted plants. Observations were made every week for 5 weeks. After development of symptom, re-isolation was done from the artificially infected stem and leaf. Disease incidence, disease severity and disease reduction was recorded by using the following formula as described by Haruna *et al*. [29] :

$$\text{Disease incidence} = \frac{\text{No. of infected plants per pot X 100}}{\text{Total No. of plants per pot}}$$

$$\text{Disease severity} = \frac{\text{Infected tissue area X 100}}{\text{Total tissue area}}$$

$$\text{Disease reduction (\%)} = \frac{\text{Disease severity in control} - \text{Disease severity in treatment} \times 100}{\text{Disease severity in control}}$$

Data Analysis

The experimental data was analyzed by using one way analysis of variance (ANOVA) and comparison of means at 5% level was made by Turkey's test. Statistical analysis was done by using SPSS Version 20.

Results

In vitro evaluation of antagonistic micro-organisms on the test isolates

Eight antagonistic viz;*Trichoderma*-1, *Trichoderma*-2, *Bacillus*-1, *B*acillus-2, *Bacillus*-3, *Actinomycete*-1, *Actinomycete*-2, and *Actinomycete*-3 (Table 2),were evaluated and tested against *Colletotrichum* isolates. The results showed that all the antagonists significantly (p=0.05) reduced the growth of *Colletotrichum* isolates either by over growing or by exhibiting inhibition zones

(appendix-5). After measuring the colony diameter of *Colletotrichum* isolates, it was observed that maximum reduction in colony growth was observed in *Bacillus*-2(55.5-100%) which was significantly superior over all the bioagents tested. Followed by *Bacillus*-1(50-97.5%), *Bacillus*-3 (44.44-100%), *Trichoderma*-2 (33.3-82.3%), *Trichoderma*-1(44.4-75.3%) and *Actinomycete*-1(11.1-66.7%). However, least mycelial reduction was noticed by the isolates of *Actinomycete*-2(6.7-66.7%) and *Actinomycete*-3(2.7-66.7%) (Table-2).

Table 2: *In vitro* Per cent inhibition of mycelial growth of *Colletotrichum* isolates by Bio-control agents of *Trichoderma*, *Bacillus* and *Actinomycete* isolates

***Colletotrichum* isolates**	**Per cent inhibition of mycelia Growth in % of bio control agents**							
	***Trichoderma*-1**	***Trichoderma*-2**	***Bacillus*-1**	***Bacillus*-2**	***Bacillus*-3**	***Actinomycete*-1**	***Actinomycete*-2**	***Actinomycete*-3**
AUPEP-1	55.6	55.6	77.8	97.8	77.8	34.2	34.2	15.8
AUPEP -2	57.3	80	78.1	100	75.3	13.3	6.7	2.7
AUPEP -6	75.3	82.3	50	55.5	58.3	11.1	16.7	22.2
AUPEP -7	60	66.7	83.3	81.1	100	5.5	11.1	11.1
AUPEP -8	73.2	82.1	92.3	100	84.6	66.7	66.7	66.7
AUPEP -9	55.6	33.3	97.5	97.5	97.5	50	25	50
AUPEP -10	44.4	33.3	66.7	72.2	44.44	16.7	16.7	11.1

Pot experiment

In Vivo evaluation of antagonistic activity of Bacillus and Trichoderma isolates against Colletotrichum isolates

The results showed that *Colletotrichum* isolates incidence and severity on three varieties of pepper ranged from 25-100% and 14.4-99.04%, respectively (Table 3). Anthracnose occurrences in pepper were affected by stem treatment of *Bacillus* and *Trichoderma* isolates in the greenhouse (Table 3). The two biocontrol antagonists reduced anthracnose disease symptoms on foliage and stems of pepper plants compared to pathogen-inoculated controls (Table-3). Isolates of *Bacillus* and *Trichoderma* in combination showed the greatest disease reduction of (91- 97%) when compared to pathogen-inoculated (control) plants. Moreover, isolates of *Bacillus* and *Trichoderma* individually showed (45.65-85%) disease reduction (Table-3). The negative control did not show any disease symptom and its disease incidence was zero whereas in the positive control disease incidence ranges from 25 to 100%. Comparing the mean of treatments *Trichoderma*,*Bacillus*, and *Bacillus* + *Trichoderma* have mean value ranges from 16.67-91.67, 8.3-91.67 and 0 to 16.67% disease incidence respectively (Table 3).

Table 3: Percentage of disease incidence, severity and disease reduction on three pepper varieties inoculated with two *Colletotrichum* isolates and two biocontrol agents under greenhouse condition.

Isolates and Treatments	**Pepper Varieties**								
	Bako local			**Marko fana**			**Oda haro**		
	PDI*	PDS	DR	PDI*	PDS	DR	PDI*	PDS	DR
AU-6	100	99.04	-	25	14.4	-	58.3	24.5	-
AU-10	100	92	-	33.33	14.03	-	50	37.03	-
AU-6 + *Trichoderma*	91.67	24.75	75	16.67	6.4	55.56	16.67	22.1	9.8
AU-6 + *Bacillus*	83.3	15.05	84.8	8.3	6.4	55.56	41.67	6.45	51.43
AU-10 + *Trichoderma*	83.3	31.82	65.4	25	5.53	60.6	25	11.9	67.9
AU-10 + *Bacillus*	91.67	50	45.65	16.67	5.5	60.8	16.67	5.56	85
AU-10 + *Bacillus* + *Trichoderma*	16.67	5	94.8	0	1.26	91	8.33	0.93	97

**PDI=Percent disease incidence , PDS= Percent disease incidence ,DR= Disease reduction*

Growth parameters

The result of this experiment showed that low values of growth parameters, (height, and dry weight of plants) were in the positive control treatment in comparison to other treatment (Table14). The growth parameters of pepper plants were significantly increased in the dual inoculation of *Bacillus* + *Trichoderma* spp (except in Bako local dry weight), ranged from height of (37-45.33cm) and dry weight of (16.5-40g/12 plants) compared to the individual one (26.3-33.33cm and 17.4-30.5g/12 plants) and even that of negative control (36-40.17cm and 21.1-38.9g/12 plants). All biocontrol isolates induced a significant increase in growth parameters. Among them, *Bacillus* + *Trichoderma* induced the greatest increase in length and dry biomass.

Discussion

The results of dual culture technique on *Colletotrichum* isolates showed that all the antagonists inhibited colony growth of the isolates by their fast and over growing nature. It was observed that the maximum percent inhibition of mycelial colony growth was observed

by *Bacillus*-2 (55.5-100%) which was significantly superior to the other antagonistic isolates, and followed by *Bacillus*-1(50-97.5%), *Bacillus*-3(44.4-100%), *Trichoderma*-2(33.3-82.3%), *Trichoderma*-1(44.4-75.3%), *Actinomycete*-1(11.1-66.7%), *Actinomycete*-2(6.7-66.7%) and *Actinomycete*-3(2.7-66.7%). On the contrary Santha kumara [13] and Raheja and Thakore[30] in case of *C. gloeosporioides* reported that *T. virens* and *T. koningii* showed more mycelial inhibition compared to bacterial antagonists. Moreover, D'souza *et al*. [11] reported the efficacy of eight isolates of *T. harzianum* against *C. capsici* and noted that *T. harzianum* had the highest antagonistic effect under *in vitro* conditions by within 5-6 days.

Table 4: The effects of two biological control agent individually or in dual application on growth characters of pepper plants

Treatment		Bako local		Marko fana		Oda haro	
		Av. Plant height (cm)	Dry weight (g/12plant)	Av.Plant height (cm)	Dry weight (g/12plant)	Av.Plant height (cm)	Dryweight (g/12plant)
Negative control		36.33	21.1	36	30.15	40.17	38.9
***Bacillus* + *Trichoderma* + AU-10**		37	16.5	37.33	32.16	45.33	40
***Bacillus* + AU-10**		26.33	17.4	31	26.69	33.5	30.05
***Bacillus* + AU-6**		27.5	18.6	33.33	24.2	32.67	28.17
***Trichoderma* + AU-10**		26.83	20.2	30.86	29.15	27.33	29.03
***Trichoderma* + AU-6**		25.67	17.18	32.83	25.17	29.17	29
Postive control	AU-10	23.8	12.1	27	24.14	27.17	29.04
	AU-6	22.83	13.6	28	20.15	28.67	24

Under *in vivo* evaluation of the antagonistic on pepper, the least PDI was detected from *Bacillus*+ *Trichoderma*spp (0 %) on Marko fana, (0.93%) on Oda haro and (16.67%) on Bako local. Followed by *Trichoderma* spp (87.5, 20.83 and 20.83%) on Bako local, Marko fana and Oda haro respectively. *Bacillus* spp (87.5, 29.17 and 12.48%) on Bako local, Oda haro and Marko fana respectively. Percent disease reduction using *Bacillus* and *Trichoderma* spp in the green house followed the pattern of percent disease incidence. The highest disease reduction activity was observed by the combination of *Bacillus* + *Trichoderma* spp with 94.8%, 91% and 97% on Marko fana, Oda haro, and Bako local varieties, respectively. The disease incidence reduction was also observed with *Trichoderma* spp (65.4-84.5%), (55.56-60.6) and (9.8-67.9%) and on *Bacaillus* (45.65-84.6%), (55.56-60.8%), (51.4-85) Bako local, Marko fana and Oda haro varieties, respectively. Sharma *et al*. (2010) also studied *T. harzianum* against *Colletotrichum capsici* with 69.4 % disease incidence reduction.

Freeman *et al*. *[31]* observed that antagonistic reduced disease incidence by up to 45% when three applications were made during the growing season. On pot and field experiments biological control of *Colletotrichum* species was also demonstrated in other studies using *Trichoderma* species and *Bacillus subtilis* applied to strawberries [31]. Kloepper *et al*. [17] suggest that specific strains of *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases (decreasing disease severity up to 73.3%.) on a variety of hosts including greenhouse studies or field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* species, cucumber, loblolly pine, and tropical crops.

The growth parameters of pepper plants were significantly increased in the dual inoculation of *Bacillus*+*Trichoderma* isolates (except in Bako local Dry weight) compared to the individual one. All biocontrol isolates showed a significant increase in growth parameters. Among them, *Bacillus* + *Trichoderma* induced the greatest increase in plant length and dry biomass. Similarly, Morsy [15] reported that the promotion of growth parameters by *B. subtilis* and *T. viride* strains may be due to their abilities to produce phytohormones, vitamins and solubilizing minerals.

Kabir *et al*.[32]reported that the weight of pepper fruit inoculated by strains of different bacteria and fungus such as *Bacillus* and *Trichoderma* induced a significant increase in the weight of pepper fruit compared to the negative control. Overall, strain consistently inhibited the symptoms of anthracnose disease in foliar parts and fruits of pepper. Furthermore, rhizobacteria treated plants showed an increase in fresh weight of red and combined fruit compared to the pathogen-inoculated controls [17]. All other treatments also induced significant increases in plant height and root length.

Conclusion

The dual treatment by *B*acillus+ *Trichoderma* isolatesfor pepper plants showed a significant disease reduction on all varieties in comparison to *Trichoderma* and *Bacillus* individually.All bioagent isolates induced a significant increase in growth of the test except for dry weight in Bako local. Among them, *Trichoderma* + *Bacillus* induced the greatest increase in length and dry biomass. Positive

(+) control plants showed physically retarded growth and pale color when compared to other treatments especially in Bako local pepper.From this study, it could be concluded that the dual treatment with *Bacillus*combined with *Trichoderma* isolateshas a potential to control disease and increased the growth and dry weight of pepper compared with the individual treatments.

Recommendations

The present investigation has given rise to new idea on fungal disease of pepper anthracnose caused by *Colletotrichum* spp. Hence, investigations on the host pathogen interaction with especial emphasis on the fungal and bacterial host pathogen interactions and their management had to be undertaken in light of harmonious integrations of them with fungicides and antagonistic organisms.An in-depth insight to notion of microbial ecology with ultimate goals of safe, sustainable anthracnose disease management should be strengthened.

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