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Biocontrol potential of *Bacillus subtilis* Lb22 against fruit rot of King chilli, *Capsicum chinense* Jacq.

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ABSTRACT: Fruit rot is one of the most destructive diseases of *Capsicum chinense* Jacq., popularly known as *Bhoot jolokia* or King Chilli an export oriented crop in North East India. Morpho-cultural characterization of the isolated fungal pathogen from diseased fruit identified the pathogen as *Colletotrichum gloeosporioides* (Penz.) *Penz*. and *Sacc*. Pathogenicity test further confirmed its association with the disease. A few bacterial and fungal microbial biocontrol agents (MBCAs) were screened *in vitro* against *C. gloeosporioides* as a potential solution in organic and integrated crop management practices. Among all the MBCAs *Bacillus subtilis* LB22 showed highest inhibition (85.00%) on mycelial growth of the pathogen followed by *P. fluorescens* (84.33%) and *B. vallismortis* (83.22%). *Trichoderma pseudokoningii* were recorded the lowest inhibition (53.22%). Our study put forth further exploration *B. subtilis* LB22 (NCBI accession no. ON386193 and NBAIM accession no. NAIMCC-B-03226) *in planta* and their response in disease suppression as well growth promotion in *Bhut jolokia* as a component of sustainable crop health management program.

Keywords: Bioagent, fruit rot, organic production, pathogenicity

INTRODUCTION

Capsicum chinense Jacq., an important cash crop of the Solanaceae family grown extensively in the North Eastern region of India predominantly in the states of Assam, Nagaland, Manipur and Mizoram has huge commercial value (Bora and Bora, 2008). This chilli pepper known by different local names such as Ghost pepper, *Naga chilli* in Nagaland, *Bhoot jolokia* in Assam and *U-Morok* in Manipur (Sanatombi *et al*., 2010 and Verma*et al*., 2013) was ranked as the world's hottest chilli having a rating of 1,013,004 Scoville Heat Units with Guinness World Records (Bosland and Borral, 2007).

King Chilli being one of the hottest chillies with medicinal values has been cultivated commercially in Assam and other NE regions as a major export oriented crop. However, there are various constraints in its cultivation of which die back and fruit rot disease caused by *Colletotrichum gloeosporioides* assumes alarming proportions in the state which cause considerable quantitative and qualitative losses of the produce, warranting effective management measures (Bora and Bora, 2020). In India, a calculated loss of 10-54% has been reported in yield of the crop due to the fruit rot disease (Lakshmesha *et al.,* 2005; Ramachandran and Ramachandran and Rathnamma, 2006). Significant losses have been reported from the other parts of the world as well, with 20-80% loss has been accounted from Vietnam (Don *et al.,* 2007) and about 10% from korea (Byung, 2007). The loss is high owing to the post and pre harvested involvement of the pathogen causing a loss of 10-80% of the marketable yield of chilli fruits (Than *et al.,* 2008). The disease is more conspicuous as it causes severe damage to matured fruits in the field as well as in harvested fruits during transit and storage. Fruit rot is a major constraint in chilli production as even small lesions on the fruit may reduce their market value thereby, affecting profitable yield of the crop (Manandhar *et al*., 1995). Disease management through non chemical methods are the need of the organic growers for export oriented production system considering the NE regions as organic hub of the country. The MBCAs is gaining importance in the light of the hazard caused by chemical pesticides, as a distinct alternative and an eco-friendly approach against many pathogens of horticultural crops (Bora *et al.,* 2020). The presence of naturally occurring microorganisms with antifungal property has been well recognized, documented and these have been tested against an array of *Colletotrichum* spp. infecting many commercially important crop plants (Anand *et al*., 2009 and Ngullie *et al*., 2010).

MATERIALS AND METHODS

Isolation and characterization of pathogen

Diseased fruits of King Chilli(Plate 1)showing typical symptoms of fruit rot disease were collected from the Horticultural Orchard, AAU, Jorhat (26º43′41″ N and 94º12′05″ E).

Plate 1. Typical symptoms of fruit rot of king chilli

The pathogen was isolated by the tissue isolation technique as described by Ricker and Ricker (1936). The infected King Chilli fruits were brought to the laboratory and washed thoroughly with sterile water. A small portion of the progressing diseased tissue with a portion of healthy tissue was cut with the help of sterilized blade. The bits were surface sterilized by dipping in 1 per cent Sodium hypochlorite (NaOCl) solution for 30 seconds. The plant tissues were washed three times with double sterilized distilled water to remove all traces of NaOCl. Excess water was decanted and dried by soaking with sterilized blotting paper. The cut pieces were aseptically transferred to potato dextrose agar (PDA) in petri plate (20 mL/Petri plate) and incubated at 28±1ºC for three days for mycelia formation.

Morpho-cultural identification

The cultural characters of the fungus were studied on PDA medium. The Petri plates having 20 ml sterile media were inoculated aseptically with 5 mm mycelial disc of the fungus from the periphery of actively growing culture and incubated at 28±1°C and cultural features were recorded. The slide culture method was followed for microscopic morphological studies. The fungus was inoculated with a sterile needle on a sterile 2cm² block of PDA and placed over a glass slide present inside the moistened Petri dish. Over the block, one sterilized cover slip was placed and incubation was carried out at 20-25°C for three days (Rosana *et al*., 2014).

Pathogenicity test

The pathogenicity test of the fungus was performed by pin prick injury method (Naik and Rawal, 2002) and Mycelial bit inoculation technique (Rocha *et al.*, 1998). Healthy and uniform sized fruits of King Chilli (10 nos.) were surface sterilized with 4.0 percent sodium hypochlorite and fruits were injured with the help of a sterilized pin. A 5 mm diameter inoculum of mycelial disc was cut using a cork borer from a 7 day old culture in PDA and was transferred on the wounded fruit surface (10 nos. of fruits) using a sterile inoculating needle. Fruits were placed in the Petri plates with moistened blotting paper and incubated at room temperature. A set of another 10 healthy fruits were used as control and were observed for development of symptoms and pathogen was re-isolated.

Source of microbial biocontrol agents (MBCA)

Bacterial and fungal bioagents with NCBI accession number viz**.,** *Trichoderma harzianum* (NCBI-KF439052), *Trichoderma viride* (KF439055)*, T. pseudokoningii* (ON364136)*, Pseudomonas fluorescens* (KT258013), *Bacillus vallismortis* (OM585584), *Bacillus amyloliquiefaciens* (OM232770) and *Bacillus subtilis* LB22 (ON386193) were collected from the author's laboratory (Biocontrol Laboratory, Programme on Biopesticide, DBT-NE Centre of Agricultural Biotechnology, AAU, Jorhat) for the study.

In vitro bioefficacy of MBCAs

*In vitro*bioefficacy of fungal bioagents was performed using dual culture technique (Dennis and Webster, 1971). Briefly, a culture disc (5 mm diam.) of the pathogen was inoculated at one end and culture disc (5 mm diam.) of the fungal bioagent was placed equidistantly exactly at the opposite end of the petri plate. For the bacterial antagonist poisoned food technique of Nene and Thapliyal (2000) was used. A loopful of 48 hrs old bacterial inoculum was aseptically added into molten PDA. The media was poured in 90 mm Petri plates (20 mL per plate) and pathogen was a 5 mm culture disc of the pathogen was placed aseptically at the centre of the plate. Control treatment was maintained with the pathogen alone and plates were incubated in BOD incubator at 28±1° C. The experiment was conducted in CRD with each treatments replicated three times and these till full growth observed in the control.

The per cent inhibition of the mycelial growth was calculated by the following formula Vincent (1927)

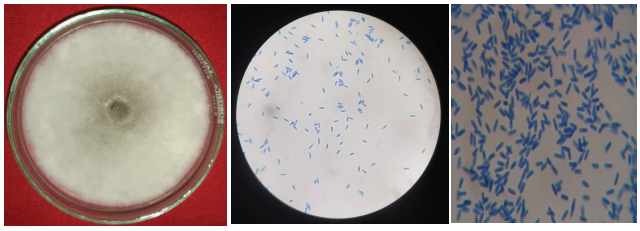
$$I = \left(\frac{C-T}{C}\right) \times 100$$

Where, I= Inhibition of mycelial growth, C= Growth in control, T= Growth in treatment

RESULTS AND DISCUSSION

Isolation and characterization of the pathogen

The pathogen isolated from diseased King chilli were subjected to morpho-cultural characterization and the morphological and cultural characters and microscopic observations are summarized in the Table I and Plate 2. Based on these characters, the pathogen causing fruit rot of *Capsicum chinense* was identified as *Colletotrichum gloeosporioides* in the Mycology laboratory of the Department of Plant Pathology, AAU, Jorhat. Dev *et al*. (2017) isolated *C. gloeosporioides* from symptomatic tissues of anthracnose of pomegranate on PDA producing flat mycelium with regular margin and zonation. Similarly, Papade *et al*. (2019) observed dull white to greyish colour of colony when *C. gloeosporioides* was grown in PDA. However, Kimaru *et al*. (2018) reported that the colony colour of the fungus varied from white to dark grey and growth varied from flat, raised fluffy to sparse in different media. In the present study, the conidial size and morphology of the fungus were also studied. Papade*et al*. (2019) observed cylindrical, hyaline conidia with oil globules at centre where the highest conidial length was of 12.57µm and breadth of conidia ranged between 2.75-7.5µm. On the other hand, Argawy (2012) observed that conidia of *C. gloeosporioides* were mostly monomorphic and exhibited cylindrical, hyaline conidia with size which ranged between 14.5-19.1 µm for length and 4.4-6.5 µm for width. Based on the cultural, morphological and microscopic observations the fungus isolated from the infected fruit of king chilli was identified as *C. gloeosporioides* which was pathogenic and responsible for causing fruit rot disease of the crop.



(A) **(B)** **(C)**

Plate 2 (A-C): Cultural and Morphological characters of *C. Gloeosporioides*

(A) Pure culture of the pathogen, (B) Spore at 10X magnification, (C) Conidia at 40X magnification

Pathogenicity manifestation

C. gloeosporioides produced disease symptoms characteristic to fruit rot with first appearance of symptoms at 5th days after inoculation (Table II and Plate3) while the control fruits inoculated with PDA media bit without pathogen did not develop any symptom. Symptoms appearing at 5 days after inoculation (DAI) progressively developed as angular lesion which enlarged to circular , sunken lesion. The black lesion with concentric ring developed black condia over the lesion. The pathogen was re isolated and checked for morphological details which established C. gleosporioides as the causal agent. The pathogenic nature of the fungus based on pathogenicity test was also supported by earlier reports on *C. chinense* (Sangnunmawia, 2018; Ngullie *et al.,* 2010), on chilli (Manandhar *et al*., 1995; Oanh *et al*., 2004). Talukdar *et al.* (2015) observed the initial symptoms of fruit rot disease of king chilli as shrinkage of the tissue irregularly at different portions of the fruit which were rough and straw coloured and on green fruits, they were pale and dirty green. Both green and ripe fruits were also affected (Kim *et al*., 1999; Sangchote *et al*., 1998).

(A) Healthy B) Control C) Inoculated fruit with symptoms at 5 DAI

Plate 3 (A-C): Pathogenicity test

Efficacy of different biocontrol agents against *C. gloeosporioides*

The antagonistic effects of different biocontrol agents were evaluated against *C.gloeosporioides* in *in vitro* condition.The results presented in Table 3 showed that all the biocontrol agents significantly inhibited the mycelial growth of the pathogen over control with different magnitude. However, among the biocontrol agents treatment *B.Subtilis* LB22 (T2) showed highest inhibition (85.00%) of mycelial growth of the pathogen which was followed by *P. fluorescens*(T5) and *B.vallismortis* (T3) with inhibition of 84.33% and 83.22%, respectively , however which were statistically *at par*. The lowest inhibition (53.22%) on mycelial growth of the pathogen was recorded in *T. pseudokoningii* (T8). Earlier Ashwini and Srividya (2014) recorded that *Bacillus subtilis* isolated from the rhizosphere of Chilli, showed high antagonistic activity against *C. gloeosporioides* OGC1 where the BCA inhibited *C. gloeosporioides* up to 100% in terms of dry weight. *Bacillus amyloliquefaciens* was also found effective against *C. gloeosporioides* isolates infecting fruit crops. *B. amyloliquefaciens* demonstarted 87% inhibition against mango isolate and 67% in case of ornage isolate (Alvindia and Acda, 2015; Arrebola *et al*., 2010) while *B. amyloliquefaciens* in loquat fruit showed 84% reduction of *C. acutatum* (Wang *et al*., 2020). *Bacillus amyloliquefaciens* was reported to produce

Table 1. Morpho - Cultural characters of *Colletotrichum gloeosporioides*

Parameter	Particular
Type of growth	Flate with cottony colonies
Colony colour (top view)	Dull white
Colony colour (Reverse view)	Yellowish
Shape of conidia	Cylindrical
Colour of conidia	Hyaline
Presence of oil globules	Singly at the centre
Size of conidia (Length)*	11.50 µm to 17.45 µm.
Size of conidia (Width)*	2.05 µm to 4.92 µm

Calculated as mean of 10

Table 2. Results of pathogenicity test

Days after inoculation	Symptom development on inoculated fruit
5 Days	Small black circular spots appeared on the skin of the fruit and spread along the long axis of the fruit and thus becoming more or less elliptical
7 Days	Sunken spots with black margin and fruit turning straw colored. Sunken spots are covered with pinkish mass of fungal spores
9 Days	Decaying of the fruits

Table 3. *In vitro* efficacy of different biocontrol agents against *Colletotrichum gloeosporioides*

Treatment	Mycelial growth* (cm)	Per cent inhibition over control
T1 : Control (*Colletotrichum gloeosporioides*)	9.00	0.00
T2 : *C. gloeosporioides* + *Bacillus subtilis* LB22	1.35	85.00 (75.92)**
T3 : *C. gloeosporioides* + *Bacillus vallismortis*	1.51	83.22 (69.25)
T4 : *C. gloeosporioides* + *Bacillus amyloliquefaciens*	1.54	82.88 (70.21)
T5 : *C. gloeosporioides* + *Pseudomonas fluorescens*	1.41	84.33 (73.15)
T6 : *C. gloeosporioides* +*Trichoderma harzianum*	2.00	77.78 (61.89)
T7 : *C. gloeosporioides* + *Trichoderma viride*	2.33	74.11 (59.25)
T8 : *C. gloeosporioides* + *Trichoderma pseudokoningii*	4.21	53.22 (38.35)
SE.(d)	0.150	
$C.D_{(P=0.05)}$	0.321	

** Mean of three replications and ** Data in the parentheses are angular transformed value*

lipopeptideiturin A, which was identified as the main agent producing the antifungal activity (Yan *et al*., 2020). Patel and Joshi (2001), reported maximum per cent inhibition of the colony growth of *C. gloeosporioides* (60.87%) with *T. koningii* . This can be attributed to higher competitive ability of the *Trichoderma* spp. and the antagonism could be due to production of wide range of metabolites as well as parasitism (Bora *et. al.,* 2022). *Trichodermin* and *dermadin* are the major volatile antibiotics produced by *Trichoderma* spp. which suppress several plant pathogens (Dennis and Webster, 1971). Kothikarand Koche (2017) reported that *T. viride* inhibited maximum mycelial growth *i.e*. 80.11 per cent followed by *T. harzianum* which inhibited mycelial growth upto 71.51 per cent. *Trichoderma* species have been reported to effectively control *Colletotrichum* species in chilli with concomitant disease reduction (Boonnratkwang *et al*., 2007). Amongst the antagonists, fungal isolates of *T. viride* and bacterial isolate *Pseudomonas fluorescens* were found effective in inhibiting the growth of *Colletotrichum capsici* (Anand and Bhaskaran, 2009). In contrast, Ngullie *et al*. (2010) reported that the mycelial growth of *C. gloeosporioides* causing anthracnose of Naga chilli fruit rot was inhibited by *P. fluorescens* and *T. viride* to an extent of 67.40 and

63.30 per cent respectively, as against the bavistin (83.40 per cent). This inhibition of mycelial growth of the pathogen might be due to the principle of mycoparasitism with the antagonist for nutrition (carbon source) by secreting cell wall degrading enzymes.

CONCLUSION

Our study demonstrated the *in vitro* bioefficacy of different bacterial and fungal bioagents with different magnitude against *C. gleosporioides,* a major threat to king chilli cultivation in NE India. The most efficient strain *B. subtilis* LB22 in terms of mycelia inhibition needs further screening on *in planta* response against C. *gleosporioides* as well as other pathogens of the crop. Moreover, the genus Bacillus being a endospore former can withstand diverse environmental extremities also known to produce wide range of antimicrobial metabolites. Isolation and purification of such compounds as an alternative to antibiotics may open up a new frontier in plant disease management.

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