Molecular characterization of native pink pigmented facultative methylotrophs of chilli and their induced systemic resistance mechanism in management of anthracnose

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Abstract

Aim: This study aimed to assess the plant growth promotional ability and biocontrol efficiency of native pink pigmented facultative methylotrophs (PPFMs) of chilli for management of chilli anthracnose disease through induced systemic resistance mechanism under pot culture experiments.

Methodology: The selected native PPFMs were identified by analysis of 16S rRNA gene sequence. These isolates along with reference strain (*Methylobacterium extorquens* AM1) were inoculated to healthy chilli plants. The pathogen (*Colletotrichum capsici*) was later inoculated to these chilli plants, and induced systemic resistance (ISR) molecules were estimated at different intervals from one day after challenge inoculation of pathogen (DAI) upto 7 DAI. Influence of PPFM on plant growth parameters, yield, capsaicin content and PPFMs population load on phyllosphere and rhizosphere was studied.

Results: The results of 16S rRNA gene sequence analysis revealed selected isolates as *Methylobacterium populi* (PPFM6) and *Methylobacterium radiotolerans* (PPFM170). The ISR molecules tested were significantly influenced by PPFM isolates. The peroxidase and phenyl alanine ammonia lyase (PALase) activity increased from 1 DAI to 7 DAI whereas chitinase, polyphenol oxidase (PPO) and phenol contents increased upto 5 DAI, after which gradual decrease was noticed. Inoculation of PPFMs to chilli crop significantly improved plant height, dry matter and chlorophyll content. Significantly lesser disease incidence, more yield and more capsaicin content was observed with application of *M. populi* as compared to control.

Interpretation: The present study highlights native *M. populi* (PPFM6) of chilli as an effective plant growth promoter, exhibiting significant biocontrol efficiency against *C. capsici*.

Key words: Anthracnose, Chilli, Facultative methylotrophs, Induced Systemic Resistance, *Methylobacterium*

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Introduction

Chilli (*Capsicum annum*) is one of the most essential spicy vegetable cultivated throughout the world. Its production is affected by various biotic stresses, among which anthracnose caused by *Colletotrichum capsici* (*C. capsici*) is a major constraint affecting quantitative and qualitative chilli production, world-wide. The disease is reported to affect almost all the aerial parts of the plant. The disease is seed and soil borne, which primarily affects the ripe fruits with significant damage at green stage (Agrios, 2005). Although different chemical fungicides are being used to control *Colletotrichum* sp., excessive use of chemicals have resulted in environmental pollution, high human health risk and development of resistant pathogenic strains (Staub, 1991; Komarek *et al.*, 2010) apart from increased cost of cultivation. There is a pressing need for a novel and sustainable alternative which can help in achieving drastic cutback in use of harmful chemical pesticides. In this regard, management of plant diseases through antagonistic microorganisms can be an effective and sustainable alternative (Cook and Baker, 1983).

Development of bio-agents requires extensive study and understanding complex interaction among biocontrol agents and plants. Variety of biocontrol agents like *Bacillus* sp., *Pseudomonas* sp., *Trichoderma* sp. (Trabelsi and Mhamdi, 2013; Cha *et al.*, 2016) have been commercialized for different crops, which are mainly isolated from the rhizosphere soil, and phyllosphere microorganisms were not intensively studied to understand their biocontrol capabilities. The phyllosphere is more hostile for biocontrol agents to establish as it is directly exposed to fluctuating environmental conditions with limited nutrient resources. Phyllosphere microorganisms include both pathogenic and non-pathogenic organisms. Among phyllosphere microbial population, pink pigmented facultative methylotrophs (PPFMs) are of great interest and are distinguished based on formation of pink to red colonies on selective isolation media (Hiraishi *et al.*, 1995). PPFMs comprise mainly the genus *Methylobacterium* and are known to utilize both single and multi-carbon compounds as carbon and energy source. PPFMs are associated with the roots, leaves and seeds of most terrestrial plants and many are known to be phytosymbionts (Trotsenko *et al.*, 2001).

Studies have proven their plant growth promotional abilities directly by producing plant growth promoting hormones like indole 3-acetic acid (IAA), gibberellic acid (GA), cytokinins and showing 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Yim *et al.*, 2012; Savitha *et al.*, 2013, 2019; Priya *et al.*, 2019; Vadivukkarasi and Bhai, 2019). They are known to produce significant amount of IAA (0.14 to 19.77 $\mu\text{g ml}^{-1}$ of culture filtrate) and GA (4.77 to 128.28 $\mu\text{g ml}^{-1}$ of culture filtrate) (Savitha *et al.*, 2013). They assist plants in fighting the phytopathogens by producing siderophores, hydrogen cyanide (HCN) and induced systemic resistance (ISR) mechanisms (Madhaiyan *et al.*, 2005, 2006). ISR is a broad term wherein resistance is triggered in plants by activating defense mechanisms by biological or chemical inducers, which protect

plants from pathogen and insect attack. Induction of defense responses by PPFMs is mainly coupled with the production of pathogenesis-related (PR) proteins like β -1,3-glucanase and chitinase, defense enzyme phenylalanine ammonia lyase (PALase) and oxidative enzymes like peroxidase and polyphenol oxidase (PPO). Mechanism of ISR is well studied in tomato against plant pathogens *Pseudomonas syringae* pv. tomato (Indiragandhi *et al.*, 2008) and *Fusarium oxysporum* f.sp. *lycopersici*, *Sclerotium rolfsii*, *Pythium ultimum* and *Rhizoctonia solani* (Janahiraman *et al.*, 2016). Even though there are few studies on plant growth promotional abilities of PPFMs in chilli, but they have not been explored for management of chilli anthracnose. Hence, the present study was planned to characterize selected native PPFM isolates of chilli through 16S rRNA sequencing and to assess their biocontrol efficiency against chilli anthracnose under pot culture.

Materials and Methods

PPFM strains and inoculum preparation: PPFM strains were isolated from disease-free chilli (*Capsicum annum* L.) plants in anthracnose infected areas of North Karnataka and morphologically characterized (Savitha *et al.*, 2013). Thirty isolates were screened for functional characteristics like IAA, GA (Savitha *et al.*, 2013) cytokinin production, phosphorous solubilisation (Savitha *et al.*, 2019) and biocontrol efficiency against major pathogens of chilli (Savitha *et al.*, 2015). The selected PPFMs bioinoculant was prepared by inoculating 72 hr old log phase culture in ammonium mineral salts (AMS) broth (Whittenbury *et al.*, 1970). The flasks were kept in a temperature controlled shaker at $28 \pm 2^\circ\text{C}$ for five days to get a population of 10^9 cfu ml^{-1} of liquid culture. For seed treatment, culture broth was mixed with sterilized lignite powder at 1:3 ratios. 1 kg carrier based inoculant was mixed with 15 litres of sterilized distilled water for seedling dip. Spraying inoculum was diluted at 1:1 ratio with sterilized distilled water. Fungal pathogen (*C. capsici*) causing anthracnose in chilli was isolated from infected chilli fruits by transferring infected portions aseptically to petriplate containing potato dextrose agar (PDA) medium. Pathogenicity tests were carried out by artificially inoculating pathogen to chilli fruits by pin pricking method. Koch's postulates were confirmed by re-isolating pathogen from diseased tissue. Mycelia and spores of *C. capsici* (2×10^4 conidia ml^{-1}) were harvested from potato dextrose agar grown 10-day-old culture and a suspension was prepared with sterilized distilled water.

Pot culture studies: A pot culture experiment was planned in completely randomized design (CRD) with two efficient native PPFM strains of chilli along with reference strain with eight replications. Seeds treated with PPFMs (20 g kg^{-1} seeds) were sown in nursery bed of size 1m $\times$ 1m. The 45-day-old seedlings were uprooted and dipped in PPFMs inoculum slurry for 30 min before transplanting them to earthen pots. Transplanting was carried out at the rate of two seedlings per earthen pot of 30 cm diameter containing 10 kg sterilized soil. Foliar application of PPFMs was undertaken at the rate of 25 ml per plant at 30, 45 and

60 days after transplanting (DAT). The pathogen was challenge inoculated by placing 15 µl of mycelium and spore suspension of *C. capsici* on the wound punctured by a needle at the middle of chilli leaves. After inoculation, the seedlings were covered with plastic bags for two days to avoid loss of moisture (Oanh *et al.*, 2006). PPFMs uninoculated and plants treated with pathogen alone served as control.

Induction of systemic resistance: The plant leaves were collected at different time intervals viz., 1, 3, 5, 7 and 9 days after pathogen inoculation (DAI) and immediately homogenized with liquid nitrogen. The powder was extracted with 10 ml of chilled 0.1M sodium phosphate buffer (pH 7.0) using sterilized pestle and mortar. The mixture was centrifuged at 10,000 rpm for 30 min and supernatant was used as a crude enzyme extract for assaying peroxidase, polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PALase). Oxidative enzymes like peroxidase and PPO were estimated as per Mahadevan and Shridhar (1986) and Mayer *et al.* (1965), respectively.

The activity of peroxidase and PPO were expressed as change in unit of absorbance at 436 nm and 420 nm, respectively. The enzyme activity was expressed as change in optical density per gram protein per minute. PALase activity was measured using the procedure described by Ross and Sederoff (1992). The absorbance of toluene phase containing *trans*-cinnamic acid was measured at 295 nm against the blank of toluene. Enzyme activity was expressed as µg *trans*-cinnamic acid released min⁻¹ g⁻¹ fresh weight. Chitinase activity was assayed by using 2ml of sodium citrate buffer 0.1M (pH 5.0) for crude enzyme extraction. The release of N-acetyl-glucosamine (GlcNAc) was measured by spectrophotometer at 540 nm (Miller, 1959) and expressed as µg GlcNAc equivalents released min⁻¹ mg⁻¹ of fresh weight. Phenolic compounds were measured by using one gram of oven dried leaf tissue. Leaves were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C and then filtered.

The extracts were pooled and alcohol evaporated on a hot water bath. The methanolic extract was used for estimating total phenol content in leaves by following Folin Cio-calteau method (Sadasivam and Manickam, 1992). The blue colour developed was read at 650nm on a UV visible spectrophotometer. The amount of phenol present in the sample was calculated by referring to a standard curve prepared using catechol and the content was expressed as mg g⁻¹ d. wt.

Plant parameters and microbial population: Plant height, population dynamics of PPFMs (rhizosphere and phyllosphere) and chlorophyll content was recorded at 30, 60, 90 and 130 DAT. Single photoelectric analyzing diode (SPAD) was used for determining chlorophyll content. The population was enumerated by employing serial dilution and plating technique. The colonies appearing on the plates after 72 hr of incubation at 30°C were counted and expressed as number of cfu g⁻¹ sample. The total dry matter was measured at the time of harvesting (130 DAT) by oven drying samples at 70°C till constant weight was achieved.

Disease intensity: Anthracnose incidence was recorded by scoring five plants in each replication using 0-9 scale (Mayee and Datar, 1986). Percent disease index (PDI) was calculated with the disease scales by the formula given by Wheeler (1969).

$$\text{Percent disease index (PDI)} = \frac{\text{Sum of the individual disease ratings}}{\text{Number of fruits/leaves} \times \text{Maximum disease grade}} \times 100$$

$$\text{Percent fruit rot infection (FRI)} = \frac{\text{Number of fruits infected}}{\text{Total number of fruits observed}} \times 100$$

Analysis of 16S rRNA gene sequence: Genomic DNA was prepared from PPFM isolates and PCR amplified 16S rRNA genes were purified and sequenced using both 1F and 1R for forward and reverse reactions, respectively. Sequencing was carried out employing a dideoxy cycle with fluorescent terminators and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer. Comparison of partial 16S rRNA gene sequences was done with the sequences available in NCBI database. 16S rRNA gene sequence similarity of ≥97% was taken for identification of isolates upto species level. Sequence alignment and comparison were performed using multiple sequence alignment tool CLUSTAL W2 (Thompson *et al.*, 1994) with default parameters. The nucleotide sequences were deposited in the GenBank and the accession numbers were obtained.

Statistical analyses: The experimental data were subjected to Completely Randomized Design (CRD) analysis as described by Yates *et al.* (1937). The level of significance used in 'F' and 'T' test was P=0.01. Critical difference values were calculated whenever the 'F' test values were significant.

Results and Discussion

Two efficient isolates (PPFM6 and PPFM170) were selected based on their plant growth promotional abilities like production of IAA, GA (Savitha *et al.*, 2013), Cytokinin, P solubilization (Savitha *et al.*, 2019) and biocontrol efficiency against major pathogens of chilli (Savitha *et al.*, 2015). These isolates showed the highest 16SrRNA gene sequence similarities to strains of *M. populi* z68b (97%) and *M. radiotolerans* PPF61 (99%), respectively, and the nucleotide sequences were deposited in the GenBank and the accession numbers were obtained (Table 1). These two isolates were explored for plant growth promotional ability and biocontrol efficiency against chilli phytopathogen *C. capsici* under pot culture.

The effect of inoculation of PPFMs on plant growth parameters like plant height and chlorophyll content were recorded at various stages of crop growth viz. 30, 60, 90 and 130 DAT whereas dry matter content was recorded at 130 DAT. Significant variation in plant height and chlorophyll content was

Table 1: Potential native PPFM isolates and their phylogenetic relationship

Isolates code	Place	Habitat	Nearest phylogenetic neighbour (degree of similarity)	Gene Bank accession number
PPFM6	Annigeri, Karnataka, India	Phyllosphere	<i>Methylobacterium populi</i> z68b (97%)	KC902788
PPFM170	Savanur, Karnataka, India	Phyllosphere	<i>Methylobacterium radiotolerans</i> PPF1 (99%)	KC902789

Table 2: Influence of native PPFMs on chlorophyll content, plant height and total dry matter production in chilli

Treatments	Chlorophyll content (SPAD value)				Plant height (cm)				Total dry matter (g per plant) at 130 DAT
	30 DAT	60 DAT	90 DAT	130 DAT	30 DAT	60 DAT	90 DAT	130 DAT	
PPFM6 + <i>C. capsici</i>	24.50	35.20	28.90	24.90	28.50	51.80	62.50	80.50	84.50
PPFM170 + <i>C. capsici</i>	23.00	33.60	27.25	23.20	27.40	50.00	62.00	79.80	84.00
<i>M. extorquens</i> AM 1 + <i>C. capsici</i>	23.00	31.50	24.50	20.00	26.00	48.00	57.90	74.00	79.80
Control	22.00	28.00	19.00	13.00	23.50	43.00	50.00	55.00	65.00
S.Em +	0.34	0.30	0.32	0.29	0.26	0.40	0.47	0.40	0.39
CD (0.01)	1.36	1.22	1.29	1.17	1.01	1.57	1.82	1.54	1.54

DAT- days after transplanting; all the values are means of 8 replications

observed between PPFMs treated and control chilli plants. The native isolate *M. populi* inoculated plants recorded significantly higher plant height and chlorophyll content compared to other treatments. Whereas control plants showed least plant height and chlorophyll content at all stages of crop growth (Table 2). Native isolates, *M. populi* (84.5 g per plant) and *M. radiotolerans* (84 g per plant) recorded significantly higher dry matter content compared to AM1 (79.8 g per plant) and control (65 g per plant). This might be attributed to higher production of plant growth regulators like IAA, GA, cytokinin and P solubilisation ability by *M. populi* (Savitha *et al.*, 2013; 2019). Growth promotional ability of PPFMs is widely reported across crops including cotton, sugarcane (Madhaiyan *et al.*, 2005), and their inoculation was found to increase the photosynthetic activity by enhancing the number of stomata, chlorophyll concentration and malic acid content of crops (Cervantes- Martinez *et al.*, 2004).

Estimation of population load of PPFMs on phyllosphere and rhizosphere at various stages of crop growth viz. 30, 60, 90 and 130 DAT revealed higher population PPFMs in phyllosphere than rhizosphere at all crop stages (Table 3). The higher population on phyllosphere indicates their potential in utilizing the gaseous methanol emitted through stomatal openings during leaf expansion by pectin demethylation (Nemecek-Marshall *et al.*, 1995). In addition, foliar spray of PPFMs at different intervals (30, 45 and 60 DAT) might have positively influenced their population on phyllosphere. There was gradual increase in population upto 90 DAT and declined later. In chilli plants inoculated with *M. populi*, the higher PPFMs population was recorded both in rhizosphere and phyllosphere at all crop stages compared to other treatments and diseased control showed very least population (Table 3). Biocontrol efficiency of native PPFMs against *C. capsici* was characterized

based on their ability to produce ISR molecules. The inoculation of *M. populi* and *M. radiotolerans* induced increased level of ISR molecules over reference strain and control (Fig 1A-1E). Among native isolates, chilli plants inoculated with *M. populi* recorded highest activity of peroxidase, PPO, PALase, chitinase and phenol content enzyme compared to *M. radiotolerans*. Significant increase in the activity of ISR molecules was reported when plants were inoculated with different bioagents and challenged with pathogens across crop species (Nielsen *et al.*, 1998; Xue *et al.*, 1998; Nandakumar *et al.*, 2001).

The highest activity of peroxidase and PALase was observed on 7th day after challenge inoculation with pathogen (Fig 1A,B) and in case of PPO, chitinase enzymes and phenol content, the highest activity was recorded on 5th day after challenge inoculation with pathogen (Fig 1C,E) after which there was a gradual decrease. The early and increased expression of peroxidase in this study would have facilitated the biochemical reaction necessary for lignification and production of hydrogen peroxide and other free radicals with antimicrobial effect (Hammerschmidt and Kuc, 1982) which might have restricted the development of *C. capsici*. Another important defense enzyme, PALase showed increased activity after a day of challenge inoculation with *C. capsici* upto 7 days and decreased later. Cinnamic acid, a product of PALase is linked to cell lignification and the highest level of PALase occurred approximately 24 hr after initial infection of *Fusarium udum* in pigeon pea (Podile and Laxmi, 1998). Peroxidase and PPO catalyzes the oxidation of phenolic compounds through PPO-PO-H₂O₂ system (Srivastava, 1987). Induction of PPO activity had resulted in resistance response in this study has been observed earlier by Thipyapong *et al.* (2007) and Jung *et al.* (2011).

Table 3: Population dynamics of PPFMs in rhizosphere soil and phyllosphere at different stages of chilli growth

Treatment details	Rhizosphere soil (10^4 cfu g ⁻¹ soil)				Phyllosphere (10^4 cfu g ⁻¹ leaf)			
	30 DAT	60 DAT	90 DAT	130 DAT	30 DAT	60 DAT	90 DAT	130 DAT
PPFM6 + <i>C. capsici</i>	14.22 (5.15)	16.53 (5.22)	17.50 (5.24)	10.02 (5.00)	35.20 (5.55)	43.50 (5.64)	46.20 (5.66)	38.32 (5.58)
PPFM170 + <i>C. capsici</i>	13.95 (5.14)	16.32 (5.21)	16.90 (5.23)	9.32 (4.97)	34.70 (5.54)	42.00 (5.62)	44.70 (5.65)	37.15 (5.57)
<i>M. extorquens</i> AM 1 + <i>C. capsici</i>	12.95 (5.11)	15.13 (5.18)	15.85 (5.2)	9.00 (4.95)	34.15 (5.53)	41.26 (5.62)	41.21 (5.61)	35.13 (5.55)
Control	5.4 (4.73)	5.7 (4.76)	3.00 (4.48)	NP	14.12 (5.15)	14.23 (5.15)	5.20 (4.72)	NP

Figures in the parentheses are log transformed values; NP - No population of PPFM; DAT- Days after transplanting, all the values are means of 8 replications

Table 4: Impact of PPFMs on anthracnose incidence, yield and capsaicin content of chilli

Treatment details	Percent fruit rot	Percent disease index	Yield (q ha ⁻¹)	Capsaicin content (%)
PPFM6 + <i>C. capsici</i>	58.00 (49.60)	56.50 (48.73)	1.59	0.14 (2.14)
PPFM170 + <i>C. capsici</i>	59.80 (50.65)	60.00 (50.77)	1.50	0.14 (2.14)
<i>M. extorquens</i> AM 1+ <i>C. capsici</i>	63.00 (52.54)	62.00 (51.94)	1.26	0.11 (1.90)
Control (only <i>C. capsici</i>)	70.00 (56.79)	75.00 (60.00)	1.00	0.10 (1.81)
S.Em +	0.32	0.45	0.02	0.002
CD (0.01)	1.01	1.52	0.06	0.007

Values in the parentheses are arc sine transformed, all the values are means of 8 replications

The accumulation of chitinase resulted in acquisition of resistance to *C. capsici*. Plants produce chitinase (Santos *et al.*, 2004) responsible for degradation of chitin, which is a major component of fungal cell and thereby destroy cell wall integrity eventually limiting the growth of pathogen. These enzymes are important determinants of resistance of plants to fungal diseases (Funnell *et al.*, 2004). Our study revealed association of increased phenolic content with disease suppression (or increased resistance) to *C. capsici*. This is in agreement with the previous reports on PGPR *Trichoderma viridae*, *Penicillium chrysogenum* and *Saccharomyces cerevisiae* against blight disease caused by *Botrytis allii* in onion (Hussein *et al.*, 2018). Many phenolic compounds are known for their antimicrobial activities and function as precursors to structural polymers such as lignin or serve as signal molecules to activate plant defense genes (Dakora, 1996).

The intensity of anthracnose disease in chilli was calculated by FRI and PDI. Chilli plants inoculated with *M. populi* and challenged with *C. capsici* recorded 56.50 PDI, followed by 60 PDI observed in *M. radiotolerans*. Plants challenge inoculated with only pathogen showed 75.00 PDI (Table 4). *M. populi* treated chilli plants recorded 58.00% FRI whereas *M. radiotolerans* inoculated chilli plants showed 59.80% FRI. The highest fruit rot (70.00%) was observed in plants challenge inoculated with only pathogen. The direct antagonistic activity of *M. populi* and *M.*

radiotolerans by inhibiting mycelial growth of *C. capsici* (Savitha *et al.*, 2015) and indirect mechanism of *C. capsici* inhibition by induction of increased ISR molecules like peroxidase, PALase, PPO, chitinase and phenol content in case *M. populi* and *M. radiotolerans* compared to other treatments could be the possible reasons for decreased disease incidence. The findings are in congruence with the findings of Lawrence *et al.* (1996), as quantum of accumulation or rapidity of induction of ISR molecules in plants is directly correlated with degree of disease resistance. Previously, several studies have reported the induction of ISR molecules like chitinase, PALase, β -1,3-glucanase, peroxidase and PPO in rice, peanut and tomato plants due to methylobacterial inoculation against various pathogens (Madhaiyan *et al.*, 2004, 2006; Indiragandhi *et al.*, 2008). *M. populi* inoculation showed significant difference with respect to yield of chilli (1.59 q ha⁻¹) followed by *M. radiotolerans* (1.50 q ha⁻¹). The lowest yield (01.00 q ha⁻¹) was recorded in control plants. The export quality of chilli is mainly assessed by their capsaicin content. Inoculation of *M. populi* and *M. radiotolerans* showed significant difference in capsaicin content compared to AM1 and control (Table 4). The improvement in yield and yield parameters of PPFMs treated plants is directly associated to their plant growth promotional ability and disease suppression. The selection of biocontrol agents against any pathogen is based on their antagonistic properties as well as plant growth promoting abilities to achieve suppression of disease and improvement in

Fig. 1: Induction of systemic resistance by native PPFM isolates: (A) Peroxidase activity; (B) PALase activity; (C) Chitinase activity; (D) Phenol content and (E) PPO activity (Note: DAI- Days after challenge inoculation of pathogen).

yield. The present study revealed that inoculation of selected native isolates of PPFM (*M. populi* and *M. radiotolerans*) to chilli plants resulted in significant growth promotion by influencing plant growth parameters like plant height, total biomass and chlorophyll content as compared to uninoculated plants. These native PPFM isolates also exhibited significant biocontrol efficiency against *C. capsici* by producing plant defence molecules like peroxidase, PPO, PALase, chitinase and phenolic content. To the best of our knowledge, this is a first report on exploration of native PPFMs for the sustainable management of chilli anthracnose. This study further highlight, among different native PPFMs of chilli, *M. populi* as an effective plant growth promoter, exhibiting significant biocontrol efficiency against *C. capsici* as compared to other isolates. Exploring beneficial microorganisms is an important component of integrated disease management and sustainable agriculture. The information generated in the present study and identified efficient strain would facilitate sustainable chilli production and effective management of anthracnose disease.

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