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Role of Antagonistic Microbe *Pseudomonas fluorescens* on *Colletotrichum capsici* Infecting *Curcuma longa*

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Abstract

The present study was attempted to find out the efficacy of commercially formulated *Pseudomonas fluorescens* in the form of foliar spray on *Colletotrichum capsici* infecting leaf spot disease in turmeric. The rhizomes dipped in biocontrol/chemical fungicide (bavistin) solution for overnight was planted in their respective triplicate plots for field study. 90-days old plants were sprayed with *C. capsici* spore spray (7-12×10^4 spores/ml) and subsequently on the next day by *Pseudomonas fluorescens*/bavistin spray in their respective plots. The second dosage of biocontrol/bavistin spray was given 15 days after the 1st biocontrol/bavistin spray. The leaf samples were collected from healthy; infected; biocontrol and bavistin sprayed plots on the 10th day after I and II spray schedule were subjected to various biochemical analyses. The result revealed that *P. fluorescens* (2%) in the form of rhizome treatment as well as foliar spray (two times) was found to be best antagonistic microbe in controlling *C. capsici* infecting *Curcuma longa*. The superiority of *Pseudomonas* spray over bavistin was mainly due to rapid cumulative antagonistic and antibiotic action against the pathogen.

Keywords: Turmeric; *Colletotrichum capsici; Pseudomonas fluorescens;* Bavistin

Introduction

Turmeric is one of the major species cultivated for its underground rhizome, which is also called as hidden lily or turmeric of commerce [1]. It has versatile uses in flavoring, dye making, drug preparation, cosmetics and medicine [2,3]. So far, around 100 active constituents have been recorded from turmeric [4,5]. This herbal plant is highly prone to several fungal diseases, one of them is turmeric leaf spot disease caused by *C. capsici* [6-8]. Several works have been conducted to control *C. capsici* by using chemical fungicides [9].

The chemical pesticides are taking to environmental pollution in producing hazardous consequences in terms of soil pollution; reducing the quality of arable land; ground water contamination by leached chemicals (high use areas if persistent products used) [10]. The biopesticides have several distinct advantages over chemical fungicides. Biopesticides are usually inherently less toxic then conventional fungicides [11-13]. They generally affect only the target pathogenic organism, in contrast to broad spectrum, conventional fungicides that may affect organisms as different as birds, insects and mammals. Also they are effective in very small quantities and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional chemical fungicides.

Materials and Methods

Pathogen

The pathogen *Colletotrichum capsici* was isolated from the infected leaves of *Curcuma longa*, sub-cultured and stored in PDA medium with streptomycin.

Field experiment

A field experiment was conducted in sandy loamy soil with the plot size of 4×5 m^2 adopting a standard spacing of 30×20 cm for turmeric plantation. The mother rhizomes soaked in *P. fluorescens* (2%) solution/bavistin solution for overnight were used for plantation in their respective plots. Triplicate plots in randomized block design were maintained for each treatment (healthy; infected; biocontrol and bavistin). 90 days old turmeric plants (in Infected; Biocontrol and Bavistin plots) were sprayed with C. capsici culture spray containing 7-12×10^4 spores/ml. All the plants were immediately covered with polythene bags sprinkled with sterile distilled water on the inner side to maintain the high humidity and kept undisturbed for 24 hrs. The 1st biocontrol agent/bavistin spray was given after 24 hrs. The leaves were collected on the 10th day after 1st spray for biochemical and enzyme assays. The second biocontrol agent/bavistin spray was given 15 days after 1st spray; again the leaves were collected on 10th day after 2nd spray was used for analysis. According to the number of spots seen in the leaf, the disease intensity was measured. The leaves discs were tied in a cheese cloth dipped in 40 ml of double distilled water. The conductivity of bathing solution was recorded on control-dynamics Conductivity Bridge after 5 h.

Biochemical assays

Total chlorophyll content was estimated by the method of Moran and Porath [14], Inskeep and Bloom [15] and carotenoid content by Ikan [16]. The RuBP carboxylase activity were assayed at 30°C by the incorporation of $^{14}CO_2$ into acid stable product, and then measured in the liquid scintillation counter. Total sugar content was found by

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the method of Dubois et al. [17], reducing sugars by Nelson [18], non-reducing sugars by Loomis and Shull [19]. The nucleic acid and protein contents were extracted by the method Sheinder [20], and the DNA content was estimated by Burton [21], RNA content by Rawal et al. [22], total protein content and amino acid content by Furlong et al. [23]. The extraction and estimation of proline was done according to the method of Bates et al. [24]. The total nitrogen content was estimated by Kjeldahl method; nitrate-nitrite content by Wooley et al. [25] and nitrate reductase activity by Jaworksi [26]. The total phenol content was analysed by the method of Bray and Thorpe [27], Ortho di-hydroxy phenol content by Johnson and Shoal [28] and the curcumin content was detected by the method of Manjunath et al. [29].

Enzyme assays

The enzyme SPS activity was assayed by the protocol designed by Sinha et al. [30] and polyphenol oxidase activity by Matta and Dimond [31]; peroxidase activity was assayed by Hampton [32]. The SOD enzyme activity was estimated by the method of Beauchamp and Fridovich [33] and CAT enzyme activity by the method adopted by Luck [34].

Statistical analysis

All values are expressed as mean ± SEM. Statistical analysis was done using SPSS 14 program. The statistical significance of differences between the two means was assessed by the Student t test. P values<0.05 were considered to be significant.

Results and Discussion

The present study brings out the antifungal effects of culture filtrates and formulations of *P. fluorescens* against *C. capsici*, the cause of leaf spot of *Curcuma longa* and the effects of the latter on the rhizome yield of *Curcuma longa* under healthy, infected and treated conditions [35].

Disease intensity study: In the experimental study, the highest disease intensity was recorded in the plants sprayed with only *C. capsici* cultures, which was termed as very severe type of infection. The *P. fluorescens* sprayed plant recorded least infection, this suppression of disease was attributed either to the activity of antifungal compounds produced by the microbe or the hyper parasitism on the pathogen or by ISR in the host plant which combat the pathogen infection. The rapid defense exerted by the treatment at the site of fungal entry delayed the infection process.

Electrolytic conductivity: The study reveals, the infected plant recorded maximum electrolytic leakage and the least was *P. fluorescens*. The action of fungus on the host cell resulted in the disintegration of semipermeable membrane, which in turn leads to higher electrolytic leakage. The leaves treated with the microbe exhibited least leakage due to the action of antibiotics produced by the microbe, which inhibits the establishment and activity of the pathogen or they induce the host cell to develop systemic resistance, which resist the pathogen invasion.

Photosynthetic pigment content: The maximum reduction in the pigments was seen in the infected plants and the least in the treated plants. The reduction in the photosynthetic activity was mainly due to the toxic effect of the pathogen [36]. They initiate the chlorosis and necrosis condition in the phylloplane and reduced the number and size of the chloroplast. The treatment may suppress the chlorophyllase activity or they produce the needed organic acids for the host growth and development. The main reason was that the antibiotic produced by the microbe inhibits the activity of the toxin produced by the pathogen.

Total carbohydrates: The total carbohydrates content was found to be drastically reduced in the infected plants. The pathogen in the infected tissue normally uses the host sugar metabolites for their growth and survival. The least reduction in the biocontrol agents treated plant due to the induction of the treated plant to utilize the sugars for the biosynthesis of phenolic compounds. These phenolic compounds were used for the defense reaction against the pathogen infection. Nucleic acid and protein content: The DNA and RNA content reduced heavily by 68%, the protein content of 72% and the amino acid content of 64% in the infected plants (Table 1). This is due to the pathogen's enzyme

Parameters	Stages	Healthy	Infected	*P. fluorescens*	Bavistin
Total Chlorophyll Content (mg/g)	I	2.1 ± 0.1	0.7 ± 0.01***	2 ± 0.07	1.1 ± 0.02***
	II	2.3 ± 0.15	0.5 ± 0.01***	2.2 ± 0.05	1.2 ± 0.02***
Carotenoid Content (mg/g)	I	0.15 ± 0.008	0.06 ± 0.002**	0.1 ± 0.008*	0.1 ± 0.009*
	II	0.3 ± 0.01	0.4 ± 0.001*	0.3 ± 0.03	0.2 ± 0.002*
RuBP Carboxylase Activity (mol)	I	72 ± 3	31.9 ± 1***	70.3 ± 5	36.9 ± 4***
	II	86 ± 2	28.9 ± 2***	84.2 ± 96	54.7 ± 4**
Total Sugar (mg/g dw)	I	78.4 ± 1.5	32.2 ± 2**	71.9 ± 3	53.7 ± 5**
	II	58.9 ± 3	20.6 ± 1***	48.7 ± 2*	33.9 ± 3**
Reducing Sugar (mg/g dw)	I	47.5 ± 4	16.5 ± 1***	44.7 ± 2	32.9 ± 1**
	II	42 ± 2	13 ± 1***	32.2 ± 2*	23.5 ± 1**
Non-Reducing Sugar (mg/g dw)	I	31 ± 1.3	15.7 ± 1***	27.3 ± 2	20.8 ± 2**
	II	16.9 ± 1	7.6 ± 0.5***	16.5 ± 1	10.6 ± 1*
DNA (mg/g fw)	I	23.9 ± 1	14.3 ± 1***	20.4 ± 1	15.7 ± 1*
	II	36.4 ± 1.1	11.4 ± 1***	32.9 ± 2	24.5 ± 1*
RNA (mg/g fw)	I	37 ± 1.7	21 ± 1*	34.6 ± 2	29.6 ± 2*
	II	51.6 ± 2	15.8 ± 1***	49.8 ± 3	35.5 ± 3**
Total Protein (mg/g fw)	I	9.5 ± 0.1	2.6 ± 0.2***	8.1 ± 0.3	5.8 ± 0.3*
	II	12.3 ± 0.2	3.8 ± 0.1***	10.7 ± 0.2	7.9 ± 0.2**
Amino Acids (mg/g dw)	I	18.7 ± 1	12.1 ± 0.6**	17.8 ± 1	15.4 ± 1*
	II	26.1 ± 1	9.4 ± 0.4***	22.9 ± 2	18.6 ± 1*
Proline (mg/g fw)	I	2 ± 0.1	4.3 ± 0.2***	2.1 ± 0.2	2.7 ± 0.2*
	II	2.3 ± 0.1	10.2 ± 0.2***	2.4 ± 0.2	3.6 ± 0.1**
Total Nitrogen (% dw)	I	2.7 ± 0.2	0.6 ± 0.02***	2.4 ± 0.1	1 ± 0.02****
	II	2.2 ± 0.1	0.4 ± 0.01***	2.1 ± 0.1	0.1 ± 0.03***
Nitrate content (mg/g dw)	I	6 ± 0.1	7.9 ± 0.01*	5.2 ± 0.03	5.9 ± 0.4
	II	22 ± 0.1	4.6 ± 0.02***	2.1 ± 0.02***	4.4 ± 0.02***
Amino Nitrogen Content (mg/g dw)	I	0.8 ± 0.01	0.4 ± 0.01***	0.6 ± 0.02*	0.5 ± 0.03**
	II	0.7 ± 0.01	0.4 ± 0.01**	0.6 ± 0.01	0.4 ± 0.01**
Nitrate Reductase (n mol NO_2/kg fw/s)	I	1148 ± 11	874 ± 15*	1529 ± 14**	1191 ± 11
	II	1136 ± 13	627 ± 12***	1048 ± 12	806 ± 14**
Total Phenol (mg/g dw)	I	10.4 ± 0.1	29.1 ± 1***	11.4 ± 1	12.9 ± 1
	II	10.9 ± 0.3	44.5 ± 2***	12.1 ± 0.1*	14.7 ± 0.3**
Ortho Di Hydroxy Phenol	I	4.5 ± 0.2	16.3 ± 1***	5.1 ± 0.3	6.7 ± 0.3**
	II	4.6 ± 0.2	2.9 ± 0.2***	5.2 ± 0.4	6.9 ± 0.5*
Sucrose Phosphate Synthase (micro mol/g/h)	I	50 ± 3	19 ± 2***	47 ± 4	28.7 ± 2***
	II	72.9 ± 3	13.7 ± 0.1***	66.8 ± 2	42.2 ± 1**
Poly Phenol Synthase (units)	I	43.3 ± 2	140 ± 3***	50.5 ± 2	66.9 ± 1**
	II	59.8 ± 1	178.2 ± 4***	64 ± 3	97 ± 4**
Superoxide Dismutase (mg fw/min)	I	26.4 ± 1	67.6 ± 2***	30.5 ± 1	33.4 ± 1*
	II	28 ± 1	73.8 ± 2***	32.1 ± 1	38.3 ± 2*
Peroxidase (units)	I	28.5 ± 1	83.4 ± 2***	39.9 ± 1	42.6 ± 2**
	II	30 ± 1	120.6 ± 2***	55.5 ± 1**	93.3 ± 2***
Catalase (units)	I	28.7 ± 2	59.4 ± 2***	34.6 ± 1	44.8 ± 1**
	II	34.8 ± 2	87.3 ± 2***	43.6 ± 1*	48.5 ± 1*

Values are mean ± SEM. Statistically significant at *p ≤ 0.05, **p ≤ 0.01 and ***pp ≤ 0.001.

Table 1: The result revealed that P. fluorescens (2%) in the form of rhizome treatment as well as a foliar spray (two times) was found to be a best antagonistic microbe in controlling C. capsici infecting Curcuma longa.

interfered with the cell division process thereby reducing the rate of cell division. The least reduction was noticed in treated plants. The reason might be the treatment induces the host plant to utilize the amino acid of the protein and the sugar of the nucleic acid for the synthesis of phenolic compounds [36].

Nitrogen metabolism: The nitrates that are absorbed by the plants are reduced to nitrites and then immediately to ammonia. Finally, they are converted to amino acid and proteins. The nitrate reductase is the key enzyme for this reaction. There's a maximum reduction of 79% of total nitrogen in the infected plants. The least reduction was seen in *P. fluorescens* due to the induced activity of the enzymes in the nitrogen metabolism.

Phenolic contents: Phenolic compounds are fungi toxic in nature and act as adaptive mechanism in the host plant against the fungal infection [36]. The biocontrol agent sprayed plants exhibited an increase of about 1.5 times in the phenol and proline content over the healthy plants. This might be due to the induction of systematic resistance in the host plant due to treatment. The over production of phenolic compound resists the advancement of the pathogen towards the other healthy cells.

Antioxidant enzymes activity: During the infection process, the pathogen or its activity interfered with the electron transport system thereby resulting in the leakage of electron. These electron leads to the accumulation of AOS within the cell and results in death. The treatment induces the systematic resistance in the host cell, which in turn enhanced activation of these enzymes in the conversion of reactive oxygen species or radicals to water in order to reduce the infection [37].

Cell wall lytic enzyme activity: The fungal plant pathogen produces different types of pectic and cellulose lytic enzymes, which are the main agents for disease development. Maximum pectinolytic and cellulolytic enzyme activity was recorded in the control (infected plant). These enzymes transform the polysaccharides of the host into simple sugars, which are used by the pathogen. The treatment might inhibit the activity of lytic enzymes of the pathogen either by antibiotic action or ISR in the host cell such as thickening the cell wall or it induces the host cell to produce lytic enzymes which are capable to inactivate or inhibit the pathogen lytic enzyme [37].

Yield and its parameter: The higher yield among the treatment was recorded in the *P. fluorescens* treatment. This might be attributed to the control of the disease or due to the production of needed organic acids and growth hormones, which allow the plant to withstand even in pathogenesis. The 2% *Pseudomonas fluorescens* treatment (rhizome treatment and foliar spray) was found to be the best method for the control of leaf spot disease of turmeric.

Conclusion

The treatment inhibited all the cell lytic enzymes of *C. capsici* in *in-vivo* condition. The treatment minimized or reduced the electrolytic leakage and leaf spot disease incidence in the host cells. The treatment enhanced the nitrogen metabolism. The treatment activated the systematic resistance in the host cell to develop different defense mechanism against pathogen infection. The treatment made the plant to withstand even under pathogenesis and fetched higher yield which was equal to that of healthy plants.

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