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Prevalence of Fluorescent Pseudomonads in Cruciferous Rhizosphere, Their Characterization and Severity of Black Rot of Cabbage in Northern Karnataka

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Cabbage (*Brassica oleraceae* var. *capitata* (L.)) is one of the most important vegetable crop and cultivated extensively in tropical and temperate regions of the world. Cabbage infected by many diseases among those, black rot of cabbage caused by *Xanthomonas campesrtis* pv. *campestris* is one of the most yield limiting and destructive pathogens of cruciferous crops worldwide. A random survey conducted during *Kharif* 2015 in cabbage growing areas of northern Karnataka revealed that incidence of black rot of cabbage was observed in all district surveyed. The maximum disease index of 23.36% was noticed in Belagavi district, followed by Dharwad (20.17%) and the least disease index was observed in Haveri (18.65%). The severity of black rot was highest at heading stage compared to vegetative stage. Prevalence of fluorescent pseudomonads was observed in all districts surveyed and wherever fluorescent pseudomonads were present in those locations the disease occurrence and severity was less compared to location where pseudomonads were they were absent. Eight fluorescent

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pseudomonads such as GRG- FP, HGK- FP, LKR- FP, KKL- FP, KBG- FP, KBG- FP, SKP- FP, MKH- FP and ARB- FP were identified based on their morphological characters, physiological and biochemical tests.

Keywords: Cabbage; fluorescent pseudomonads; black rot of cabbage; Xanthomonas campestris pv. Campestris.

1. INTRODUCTION

Cabbage (*Brassica oleraceae* var. *capitata* (L.)) is one of the most important vegetable crops and commonly cultivated in winter. It is cultivated extensively in tropical and temperate regions of the world. Cabbage is an excellent source of vitamin A, B, C, K and B_6. It also contains several minerals such as calcium, phosphorous, potassium, sulphur and iron. It is consumed either cooked or raw as salad. It has been used historically as a medicinal herb for a variety of purported health benefits as a laxative and cabbage juice used as an antidote for mushroom poisoning, for eye salves and for liniments used to help bruises heal [1].

China is the world's largest producer with 47 % of the total cultivated area followed by India that accounted for 12% of the total cabbage production. It is cultivated in 0.43 M ha with the total production of 9.03 M mt with an average productivity of 22.6 t/ha. Major cabbage producing states are West Bengal, Odisha, Bihar, Gujarat, Maharashtra, Haryana, Assam and Karnataka. The area of the cabbage production in Karnataka is 0.010 M ha with an estimated average yield of 21.2 t/ha to produce 0.21 M t [2].

The important diseases which infect cabbage are black rot, clubroot, leaf spots, leaf blight, ring spot and downy mildew. Among these, black rot of cabbage caused by *Xanthomonas campesrtis* pv. *campestris* is one of the most yield limiting and destructive pathogens of cruciferous crops worldwide. The continuous and indiscriminate use of chemical pesticides for management of diseases has posed several serious problems such as pesticide residue, development of resistant strains, environmental pollution and adverse effect on beneficial microorganisms and created a greater concern over global food safety and security. Therefore in recent times, biological control has emerged as a key component in Integrated Disease Management.

Symptoms may appear on different growth stages of plants. On young plants the margins of the cotyledons turn black and may drop-off, while on mature leaves, symptoms appear along leaf margins as yellow, V-shaped lesions along the veins. As the lesions expand towards the base of the leaf, the tissue wilts and eventually becomes necrotic later on, heads and curds also get discoloured [3].

Fluorescent pseudomonads are very important bioagent used for the management of plant diseases. The genus *Pseudomonas* was first named by Migula in 1895, it is Gram-negative bacterium belonging to the Phylum Proteobacteria, Class Gammaproteobacteria and family Pseudomonadaceae which includes fluorescent pseudomonads as well as few non-fluorescent species. Fluorescent *Pseudomonas* group consists of phytopathogenic and non-phytopathogenic strains. Among them, non-phytopathogenic strains such as *Pseudomonas fluorescens*, *P. putida, P. chlororaphis*, *P. aureofaciens* and *P. aeruginosa* are present.

Fluorescent pseudomonads play an active role in suppression of pathogenic microorganisms by several mechanisms which include the production of antibiotics as a secondary metabolite at low concentration, competition, HCN, siderophore production and induced systemic resistance [4]. Utilization of these antibiotic-producing microbial antagonists against plant pathogens in agricultural crops has been proposed as an alternative to chemical pesticides [5]. Hence, in the present study prevalence of fluorescent pseudomonad's in cruciferous rhizosphere was studied, prevailing isolates were characterized and correlated with the incidence of black rot of cabbage in northern Karnataka was taken up.

2. MATERIALS AND METHODS

A roving survey was conducted during *Kharif* 2015 to know the prevalence of fluorescent pseudomonads in the rhizosphere of cruciferous in Belgavi, Dharwad and Haveri districts of northern Karnataka, India and soil samples were collected for isolation of fluorescent pseudomonads. Cabbage black rot incidence on

randomly selected plants in each field was recorded. In each field, samples infected with the black rot of cabbage were collected. Disease severity on randomly selected 10 plants in each field was recorded by following 0-9 scale developed by Mayee and Datar [6].

2.1 Disease Rating Scale

Scale	Infection type
0	No infection
1	Minute water soaked lesions on leaf turning brown in colour with yellow halo covering an area of 1 % or less.
3	Small round to irregular lesions covering 2-10 (%) of the leaf area.
5	Round to irregular dark brown lesions covering 11-25 (%) leaf area.
7	Lesions enlarging and coalescing to cover 26-50 (%) of the leaf area and small infection on head and leaf.
9	Lesions enlarging and coalescing to cover > 51(%) area of leaf, infection on head and severe defoliation.

Per cent disease index (PDI) =

$$\frac{\text{Total sum of numerical rating}}{\text{No. of leaves examined} \times \text{Maximum grade}} \times 100$$

2.2 Isolation of Fluorescent Pseudomonads

Initially, 10 g soil sample was transferred to 90 ml sterile water and mixed thoroughly by shaking the flask on rotary shaker for 20 min. After that serial dilution technique was performed up to 10^8 dilutions. An aliquot of 0.1 ml suspension was spread over 90 mm Petri plate containing pre sterilized & cooled down King's B medium. The inoculated plates were incubated at 30 ± 1°C for 24-48 h. The colonies developed were observed and these colonies were picked up and streaked again on fresh plates containing King's B medium and incubated. Three replications were maintained for each location [7].

2.3 Morphological Characterization

Pure cultures of fluorescent pseudomonads isolated from various locations were Gram stained and observed under a microscope. The morphological characteristics of the fluorescent pseudomonads such as cell shape, colour, elevation, margin, transparency and surface of the colony and colour of the fluorescence were studied by following below mentioned methodologies. The fluorescent pseudomonads cultures were identified by following the Bergey's Manual of Systematic Bacteriology [8].

2.4 Gram Staining

For Gram staining, 24 h old culture was used. A loopful of bacterial culture was smeared on to the clean glass slide and then air dried. Further it was exposed to flame for two minutes and then covered with crystal violet for 1 min. The slide was washed with distilled water and covered with iodine solution for 1 min. The iodine solution was washed with 95 per cent ethyl alcohol and subsequently with distilled water, drained and counter stain (safranin) was applied for 1 min. The slide was washed with distilled water, dried with tissue paper, air dried and then subjected for microscopic examination under oil immersion objective lens [9].

2.5 Fluorescence Production

The bacterial culture was streaked on *Pseudomonas* agar for fluorescence production and incubated at room temperature 28 ± 2°C for 48 hr and pigmentation of the colony was observed under the ultraviolet light ranging between 180 nm to 280 nm.

2.6 Biochemical Tests

Oxidase test: A culture grown for 24 h in nutrient agar supplemented with 1 per cent glucose (Schaad, 1980) was used. A loopful of cells were rubbed on a filter paper impregnated with 1 per cent (w/v) aqueous tetra-methyl-p-phenylene diamine dihydrochloride solution. A change in the colour of the cultures to deep purple within 10 seconds was registered as a positive result [10]. The reagent was made fresh once every fortnight and stored at 4°C away from light. Platinum loop was used since traces of iron can catalyse the phenylene diamine compound.

Catalase test: Catalase is a hemi enzyme capable of decomposing hydrogen peroxide to water and oxygen gas. A loopful of 48 h slant grown test bacterium was smeared on a slide and it was covered with few drops of hydrogen peroxide (20 volumes). The reaction was found to be positive based gas bubbles produced [11]. Production of gas bubbles gives a clue for presence of aerobic and facultative anaerobic bacteria.

Starch hydrolysis test: The medium employed is referred to as starch agar and it contains peptone (10.0 g), starch soluble (2.0 g), beef extract (5.0 g), agar (20.0 g) and water (1000 ml) and pH was adjusted to 7.0. Medium was sterilized by autoclaving and poured into sterilized Petri plates. The medium was allowed to solidify and spot inoculated with the test culture in plates. The plates were incubated at 25°C and test for starch hydrolysis was performed on 2nd or 3rd day. Agar surface of the plates with test bacteria were flooded with the Lugol's solution and allowed to act for a few minutes for development of colourless zone around the bacterial growth.

Gelatin liquefaction test: The nutrient gelatin medium contains peptone 10.0 g, beef extract 5.0 g and gelatin 20 g. All the ingredients were suspended in distilled water and volume was made up to 1000 ml and heated over a water bath until the gelatin was dissolved and media was sterilized by autoclaving. The media was cooled and poured to the Petri plates and allowed to solidify. These plates were inoculated with 48 h old cultures of test bacterium and incubated the plates at 20°C. Later on surface of plates was flooded with 0.2 per cent mercuric chloride solution ($HgCl_2$, 12 g; Distilled water, 80 ml; Concentrated HCl, 16 ml) and observed for formation of white precipitation. Based on development of white precipitation, cultures were identified as fluorescent pseudomonads.

2.7 Physiological Tests

Growth at 4°C: The bacterial cultures were streaked on Petri plates containing King's B medium and kept for incubation at 4 ^{0}C. Petri plates were observed after 48 h for pigmentation of colony under the ultraviolet light. Isolates which produced pigmentation found to be grown at 4°C and recorded as positive.

Growth at 42°C: The bacterial cultures were streaked on petriplates containing King's B medium and kept for incubation at 42°C. Petri plates were observed after 48 h for pigmentation of colony under the ultraviolet light. The formation of pigmentation was the indication of the growth of fluorescent pseudomonads at 42°C.

3. RESULTS AND DISCUSSION

Roving survey was undertaken to assess the occurrence of fluorescent pseudomonads in cabbage crop in Belagavi, Dharwad and Haveri districts. Rhizosphere soil samples of cabbage crop were collected and processed to isolate fluorescent pseudomonads. Eight isolates were obtained from the 17 rhizosphere soils collected from the cabbage growing areas of Belagavi, Dharwad and Haveri districts of northern Karnataka.

In Belagavi district, fluorescent pseudomonads were present in Arabhavi village of Gokak taluk and M K Hubli village of Bailhongal taluk. In Dharwad district, fluorescent pseudomonads were observed at three locations *viz.*, Lokur, Garag and Hangaraki villages of Dharwad taluk and in Haveri district, fluorescent pseudomonads were isolated from three locations *viz.,* Shankaripura village of Byadgi taluk, Kakol village of Ranebennur taluk and Kurubagonda village of Haveri taluk. Whereas, fluorescent pseudomonads were not present in other locations surveyed.

The findings of the present study revealed that the fluorescent pseudomonads are more prevalent in Dharwad and Haveri district followed by Belgavi districts and black rot disease index was noticed in all the locations surveyed with a range from 9.33 to 29.75 per cent. The maximum disease index of 23.36 per cent was noticed in Belagavi district, followed by Dharwad (20.17%) and the least disease index was observed in Haveri (18.65%) presented in Table 1.

Similar type of study was made by Sakthivel et al. [12] and they isolated rhizosphere colonizing *Pseudomonas fluorescens* from vegetable crops like tomato, brinjal, cabbage, hot pepper grown in different geographic areas and soil types of Tamilnadu. Jayashree et al. [13] isolated *P. fluorescens* isolates from fresh roots of black gram, carrot, cabbage banana, tapioca, pepper, paddy crops and forest trees grown in several geographic areas of Tamil Nadu.

During the survey maximum severity of black rot of cabbage was observed in irrigated condition. It might be due to development of microclimate, increased soil moisture and prevailing congenial environmental conditions Basur et al. [14]. Maximum severity of black rot was noticed at heading stage and minimum in vegetative stage. The disease index varied from locality to locality because of cropping pattern (Monoculture) leading to build up of inoculums load and heavy rainfall with improper drainage, which has enhanced the relative humidity ultimately favouring the black rot disease development (Table 1a).

Table 1. Collection of cruciferous rhizosphere soil to know the presence of fluorescent pseudomonads and severity of black rot of cabbage in northern Karnataka during *kharif* 2015

District	Taluk	Village	Agro climatic zone	Soil type	Genotype	Stage of the crop	Previous crop	Other disease observed	Per cent Disease Index (PDI)	Fluorescent Pseudomonads
Belagavi	Bailhongal	M K Hubli	8	Medium black	Indu	Vegetative	Soybean	_	21.33	+
		Kittur	3	Black	Saint	Heading	Maize	Alternaria blight	23.10	-
	Gokak	Arabahvi	3	Black	Saint	Heading	Maize	_	14.22	+
		Maldinni	3	Black	Saint	Harvesting	Cabbage	Alternaria blight	28.44	-
	Hukkeri	Kotabagi	8	Black	Saint	Heading	Tomato	Alternaria blight	29.75	-
						Mean			**23.36**	
Dharwad	Dharwad	Garag	8	Medium black	Saint	Harvesting	Soybean	_	14.66	+
		Hangaraki	8	Black	Saint	Vegetative	Soybean	Alternaria blight	12.88	+
		Lokur	8	Black	Ankur manas	Vegetative	Chickpea	_	16.88	+
		Kurubagatti	8	Black	Ankur manas	Heading	Maize	_	23.99	-
		Marewada	8	Black	Sony 50	Heading	Soybean	_	27.33	-
		Tadakod	8	Black	Saint	Heading	Onion	Alternaria blight	25.33	-
						Mean			**20.17**	
Haveri	Byadgi	Shankaripur	8	Medium black	Saint	Harvesting	Sunflower	_	13.76	+
		Teredhalli	8	Black	Sony 50	Heading	Maize	Alternaria blight	22.22	-
		Mallur	8	Black	Saint	Harvesting	Cabbage	Alternaria blight	24.41	-
	Haveri	Kurubagonda	8	Black	Saint	Harvesting	Cabbage	_	27.10	+
	Hirekerur	Kalagonda	8	Black	Saint	Vegetative	Tomato	_	15.10	-
	Ranebennur	Kakol	8	Medium red	Saint	Vegetative	Cabbage	_	9.33	+
						Mean			**18.65**	

Basur [14] revealed that the disease index in surveyed areas *viz.,* Belagavi, Dharwad and Haveri ranged between 4.2 to 35.7 percent with maximum incidence in Belagavi followed by Haveri and least incidence was observed in Dharwad. The results of survey also revealed that there was no much relation between soil types with respect to presence of fluorescent pseudomonads and disease occurrence. Among genotypes all are susceptible to black rot of cabbage, in which Sony -50 recorded maximum severity with (24.77%) and minimum disease severity was observed in saint with (19.84%). Wherever the fluorescent pseudomonads were present on those locations, mean disease severity with (16.27%) and maximum disease severity was recorded on locations where fluorescent pseudomonads were not found with (24.40%) depicted in Table 1b and 1c.

Isolates obtained were identified through standard morphological and biochemical studies following Bergey's Manual of Systematic Bacteriology. Totally eight fluorescent pseudomonad isolates such as GRG- FP, HGK- FP, LKR- FP, KKL- FP, KBG- FP, KBG- FP, SKP- FP, MKH- FP and ARB- FP were identified during the study. The results of the biochemical tests for identification of fluorescent pseudomonads revealed that all the isolates shown positive reaction for fluorescence test, gelatin liquefaction, catalse and oxidase tests and negative reaction for starch hydrolysis. All the isolates exhibited circular shape colonies. Isolate KBG- FP and MKH- FP were showing greenish white colour colonies whereas, rest of isolates were creamy-white in colour. Colonies of KKL- FP and SKP- FP isolates exhibited undulated margin, whereas, other isolates had entire margin. LKR- FP, KKL- FP, SKP- FP and MKH- FP isolates expressed umbonate type of elevation, whereas, GRG- FP, KBG- FP and ARB- FP had convex type of elevation. However, only one isolate GRG- FP had raised elevation. Isolates HGK- FP, LKR- FP, SKP- FP and ARB- FP had smooth glossy colony surface whereas, isolate GRG- FP and KKL- FP were of smooth surface and isolate KBG- FP had rough surface (Table 2a).

Similar type of study was conducted by Sinha and Simon [15] while working with different strains of *P. fluorescens* in cabbage, tomato, tobacco, wheat and pea. They isolated different species of *P. fluorescens* and characterized them based on morphological, physiological and biochemical tests and they also observed variation in colony colour, colony shape , margin, elevations and colony surface observed during the present study. Results of the present study are in tune with results obtained by Soesanto et al. [16] and Sivasakthi et al. [17].

The results of the biochemical tests for identification of fluorescent pseudomonads revealed that all the isolates shown positive reaction for fluorescence test, gelatin liquefaction, catalse and oxidase tests and negative reaction for starch hydrolysis. Growth of all the fluorescent pseudomonad isolates was normal at 30ºC temperature range. Whereas, isolates FP-1, FP-2, FP-4, FP-6 and FP-8 grow at 4ºC and some of the other isolates did not grow at 4ºC. Isolates FP-3, FP-5 and FP-7 exhibited good growth at 42ºC but rest of the isolates did not grow at this temperatures range (Table 2b).

Table 1a. Severity of black rot of cabbage at different growth stages during *kharif* 2015

Growth stages	No. of fields visited	Mean Per cent disease index (PDI)
Vegetative stage	5	15.10
Heading stage	7	24.56
Harvesting stage	5	21.67

Table 1b. Severity of black rot of cabbage in relation to cabbage genotypes

Genotypes	Mean Per cent disease index (PDI)
Indu	21.33
Saint	19.84
Sony 50	24.77
Ankur manas	20.43

Table 1c. Prevalence of fluorescent pseudomonads in diseased and healthy cabbage fields

Prevalence	Mean Per cent disease index (PDI)
Presence	16.27
Absence	24.40

Table 2a. Morphological characterization of fluorescent pseudomonad isolates

Characters	Isolates							
	GRG- FP	HGK- FP	LKR- FP	KKL- FP	KBG- FP	SKP- FP	MKH- FP	ARB- FP
Colony shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colony colour	Creamy white	Creamy white	Creamy white	Creamy white	Greenish white	Creamy white	Greenish white	Creamy white
Margin	Entire	Entire	Entire	Undulated	Entire	Undulated	Entire	Entire
Elevation	Raised	Low convex	Umbonate	Umbonate	Low convex	Umbonate	Umbonate	Low convex
Transparency	Translucent	Opaque	Opaque	Translucent	Opaque	Translucent	Opaque	Opaque
Surface	Smooth	Smooth glossy	Smooth glossy	Smooth	Rough	Smooth glossy	Rough	Smooth glossy
Fluorescence	Greenish	Greenish	Greenish	Greenish	Bluish	Greenish	Bluish	Greenish

Table 2b. Identification of fluorescent pseudomonad isolates from cruciferous rhizosphere in Northern parts of Karnataka

Isolates	Designation of the isolate
Garag (Dharwad)	GRG- FP
Hangaraki (Dharwad)	HGK- FP
Lokur (Dharwad)	LKR- FP
Kakol (Haveri)	KKL- FP
Kurubagonda (Haveri)	KBG- FP
Shankaripura (Haveri)	SKP- FP
M. K. Hubli (Belagavi)	MKH- FP
Arabhavi (Belagavi)	ARB- FP

Table 2c. Physiological and biochemical characterization of fluorescent

Tests	GRG- FP	HGK- FP	LKR- FP	KKL- FP	KBG- FP	SKP- FP	MKH- FP	ARB- FP
Grams reaction	-	-	-	-	-	-	-	-
Fluorescence test	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Growth at 4ºC	+	+	-	+	-	+	-	+
Growth at 30ºC	+	+	+	+	+	+	+	+
Growth at 42ºC	-	-	+	-	+	-	+	-

Chintala and Gundala [9] made similar observations while working with *Pseudomonas fluorescens* isolates which showed positive results for catalase, oxidase activity and citrate utilization. Meera and Balabaskar [7] also studied biochemical characteristics of different isolates of *P. fluorescens.* The isolates showed negative reaction for Gram's staining and starch hydrolysis, it might be due to lack of amylase production by the isolate. Amylase is an exoenzyme that hydrolyses starch into maltose disaccharide and some monosaccharide and positive results for oxidase, catalase and gelatin liquefaction test.

4. CONCLUSION

Totally eight fluorescent pseudomonad strains were isolated from cabbage rhizosphere soil taken from different parts of northern Karnataka, which had circular colonies and the majority of them had creamy-white colonies. All the fluorescent pseudomonad isolates were gram-negative and shown positive reaction for fluorescence pigment production under UV light. All the isolates showed positive reaction for gelatin liquefaction, catalase and oxidase tests and negative reaction for starch hydrolysis. Isolates FP-1, FP-2, FP-4, FP-6 and FP-8 grew

at 4ºC temperature. The severity of black rot of cabbage was observed in all surveyed districts. Irrigated conditions, the disease severity was more with respect to type of soils (black and red soils).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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