Biological Forum – An International Journal **14(4a): 781-785(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Biochemical characterization of *Ralstonia solanacearum* causing Chilli Bacterial Wilt

Abhisek Tripathy[1*], Akshaya Kumar Senapati[2] and Satya Narayan Satapathy[3]
[1]Ph.D. Research Scholar, Department of Plant Pathology,
College of Agriculture, O.U.A.T., Bhubaneswar (Odisha), India.
[2]Professor and Head, Department of Plant Pathology,
College of Agriculture, O.U.A.T., Bhubaneswar (Odisha), India.
[3]Assistant Professor, Department of Entomology,
Faculty of Agricultural Sciences, SOADU, Bhubaneswar (Odisha), India.

(Corresponding author: Abhisek Tripathy)*
(Received 16 August 2022, Accepted 10 October, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: In the coastal vegetable belt of Odisha, *Ralstonia solanacearum*-caused chilli bacterial wilt is a fairly well-known disease. It causes around 100 percent yield loss in the farmer's field. The condition is extremely complex, and numerous physical, cultural, chemical, and biological approaches have been tried and failed to control it. The purpose of this study is to determine the diversity of the various isolates and their chances of survival. Five isolates were obtained for this study from the Odisha coastal vegetable region. The isolates were given the numbers RS 1, RS 2, RS 3, RS 4, and RS 5. They were also examined for diversity studies, which take into account biochemical variation among them. The biovar determination test confirmed that *Ralstonia* is a member of Race 1 biovar 3 based on the test results. The bacteria responded positively to the catalase test, the KoH Solubility Test, and the nitrate reduction test, but not to the starch hydrolysis test. From this study, we got an idea about the diversity among various isolates of *Ralstonia* which in turn will help to manage this particular problem efficiently.

Keywords: *Ralstonia solanacearum*, Chilli, Oxidase test, catalase test, Nitrate test, KOH solubility test, Starch hydrolysis.

INTRODUCTION

Several diseases like damping off, Fusarium wilt, Cercospora leaf spot, Alternaria leaf spot and bacterial wilt infect chilli crop worldwide (Dhaliwal, 2015). Moreover, bacterial wilt, one of the most infectious disease which is very widely distributed among chilli growing tracts of the world (Hayward, 1991; Denny, 2006). The said disease is largely seen in the coastal regions of India. The pathogen *Ralstonia solanacearum* (Yabuuchi *et al*., 1995) causing same disease of chilli. This particular disease is ranked one as the most notorious plant diseases worldwide. It is responsible for100 per cent yield loss in almost all solanaceous vegetables (Jyothi *et al*., 2012). First signs of the disease are wilting of new leaves mostly during the hottest part in the day and come back as normal during cooler hours (Ahmed *et al*., 2022). With high temperature as well as rainfall condition, whole plant exhibit wilting and suddenly the plant death occurs (Cerkauskas, 2004). Most of the time, in the starting stage of the disease, plant leaves remain green in most of the cases (Manda *et al*., 2020).

Chilli bacterial wilt caused by *Ralstonia solanacearum* is a very notorious disease in the coastal vegetable belt of Odisha. The disease is highly intricate and many physical, cultural, chemical and biological control methods have been tested to manage it with all in vain. Moreover, the resistant varieties of chilli are failing to manage the disease due to unknown reason specific to the above said part of Odisha. Considering this problem, the present investigation was undertaken. This study is targeted to find out the diversity among the various isolates and its survival on the basis of morphological as well as cultural characteristics.

MATERIAL AND METHODS

A. Isolation of the pathogen

Chilli wilted plants from the field were gathered and subjected to isolation on a specific media. Vascular discolored tissues were cut into small, small pieces and finally put in the beaker containing distilled water which is sterilized earlier. After five minutes, its been observed that a white small thread of fluid was coming down from the cut end of the injured-stem. It acted as a confirmatory test for bacteria presence. 1 ml of the above-said suspension was then discharged into previously sterilized Triphenyl tetrazolium chloride agar (TZC) agar plates. Further, they are perfectly allowed to solidify at room temperature i.e. around 28°C for the next forty-eight hours. After incubation period completed, the media containing petriplates were

observed for the advancement of the colonies of *Ralstonia* and thus been said virulent as well as a virulent bacterial colonies. After this advancement, bacterial virulent colonies were steakd into the above said media containing petripaltes for pure culture development. Slight Creamy white colonies with pink center observed on TZC medium. These colonies were further transferred to five ml of sterile water containing 16 cm^3 Eppendorf tubes and been used as stock suspensions for future use.

B. Biochemical characterization

Detection of the biovars of *R. solanacearum* isolates. Based on their capacity to use different sugar alcohols and disaccharides, *Ralstonia* biovars were classified. To classify the 5 isolates of *R. solanacearum* into biovars, the ability of the isolates to utilize disaccharides such cellobiose, maltose, lactose, and hexahydric alcohols like dulcitol, mannitol, and sorbitol was examined.

Oxidase test. Within 30 seconds of spreading a single *R. solanacearum* colony on the oxidase disc that was detected as oxidase-positive, purple color developed. This will show that an oxidative test will show positive results for all of the tested isolates.

Catalase test. In order for the nutrient agar medium to harden, sterilized plates were filled with it. A brand-new, 48-hour-old bacterial culture was streaked on solid medium on plates and cultured for two days at 28 to 30 degrees Celsius. In addition, a 3% hydrogen peroxide (H_2O_2) solution was poured over them. No bubble formation suggests a catalase activity reaction that is definitely negative, while high bubble formation indicates the opposite. All of the isolates underwent this specific test.

Nitrate test. *Ralstonia* was inoculated into nutrient broth that contained nitrate reagent, and the mixture was then incubated at the ideal temperature of 30-34°C. Nitrate medium's color was altered from blue to pink to red, signalling success in the nitrate reduction test.

KOH solubility test. Test isolates were placed on 50µl of 3% (w/v) KOH on a clean slide. Aseptically transferred bacterial cells from agar plate to the drop of KOH with a sterile tooth pick. The cells were agitated with the tooth pick.

Starch hydrolysis. The isolates streaked nutrient agar plates with 0.2% starch (w/v) and were then incubated at 30°C until complete growth occurred. Iodine, potassium iodide, and distilled water were combined to create the IKI solution (iodine, 1gm; potassium iodide, 2gm; distilled water, 100ml), which was then poured over plates. Positive reactions were noted in the clear zone around the colony.

Table 1: Carbohydrate utilization test of bacterial isolates.

Sr. No.	Carbohydrates	Original colour	Reaction type	
			Positive	Negative
1.	Lactose	Red	Yellow	Red
2.	Maltose	Red	Yellow	Red
3.	Cellobiose	Red	Yellow	Red
4.	Mannitol	Red	Yellow	Red
7.	Sorbitol	Red	Yellow	Red
8.	Dulcitol	Red	Yellow	Red

RESULTS AND DISCUSSION

A. Isolation and purification of R. solanacearum isolates

From the vegetable belts of Odisha, chilli plants showing the typical signs of bacterial wilt, such as loss of turgidity of the leaves, advanced as drooping of the leaves with quick wilting. The roots of the diseased plants have a brownish hue. Ooze testing was used to find the presence of a bacterial pathogen (Fig. 1). The severed ends submerged in sterile water emitted a milky white bacterial slime. On TZC agar medium, bacterial colonies from all five isolates were cultivated. These colonies were irregular and well-separated, with a smooth, white edge and the recognizable pink center. All five of the isolates tested in this investigation displayed the recognizable traits of virulent strains, including being round to irregular, extremely fluidal, and having a white edge with pinkish coloring colonies. The five isolates were chosen for further study based on distinctiveness and were named RS 1, RS 2, RS 3, RS 4, and RS 5 accordingly. They were selected from various survey blocks based on the incidence of wilt.

Characterization of bacterial isolates. The bacteria isolated from different hosts were identified as per the standard procedures on the basis of their biochemical characteristics.

B. Biochemical characteristics of R. solanacearum isolates

All five isolates were subjected to biochemical tests by using standard microbiological procedures. The results of tests carried out are presented below

Oxidase test. Oxidase testing was done using Kovac's technique. All of the isolates' cultures were stilled and patched on filter paper in a petridish on nutrient agar (NA) with 1% glucose. On the filter paper, Kovac's oxidase reagent was wet. Within ten seconds of adding Kovac's oxidase reagent to the culture that was placed on filter paper, purple color emerged. This made it quite evident that the oxidative test was positive for all tested isolates. This was further demonstrated by the soil-borne bacterium *Bacillus licheniformis*, according to Ghaly *et al*. (2007).

Catalase test. On NA medium, all five isolates were grown and cultured for 48 hours. With the aid of an inoculation needle, 3% hydrogen peroxide was applied to a loop-filled bacterial culture on a glass slide. After that, it was observed for the formation of gas bubbles, which indicated a positive catalase test result because the catalase enzyme catalyzed the breakdown of hydrogen peroxide into oxygen and water. All five isolates demonstrated catalase activity. Kumari and Ranjan (2019) also got similar results where he derived that *Ralstonia* got positive on catalase test.

Nitrate reduction test. Nitrate reduction test based on nitrite detection and its ability to react with sulfanilic acid to generate a complex (nitrite-sulfanilic acid), which then combines with a -naphthylamine to produce a precipitate that is a water-soluble azo dye and is deep red in color. After 48 hours, all the isolates were cultivated on NA medium with nitrate. All of the

isolates' colors changed from pink to red, indicating that nitrate is being converted to nitrite.

KOH solubility test. With the aid of a toothpick, a loop full of the five isolates was deposited onto a glass slide containing bacterial culture with 3% KOH. It was then observed that the organisms developed thick, stringy strands within the first 30 seconds. Gram negative bacteria exhibit this. Therefore, it was determined from this test that all five isolates were gram-negative bacteria. According to Suslow *et al*. (1982), the KOH technique is a little bit quicker and easier than the conventional Gram-strain method that uses dyes to distinguish between gram-positive and gram-negative bacteria.

Starch hydrolysis. Each of the five isolates was placed on NA medium with 0.2% starch. Every isolate tested negative for starch hydrolysis. When the plates were inundated with iodine solution, no clear zone was seen around the bacterial growth. That none of the isolates hydrolyzed the starch was amply supported by this observation. Sharma and Singh (2019) concluded after the same experiment conducted where he got starch -vely associated which found similar to this experimental result.

C. Detection of the biovars of R. solanacearum isolates

Based on the ability of the isolates to use various sugar alcohols and disaccharides, *R. solanacearum* biovars were identified.

Based on their capacity to use different sugar alcohols and disaccharides, *R. solanacearum* biovars were classified. To classify the 5 isolates of R. solanacearum into biovars, the ability of the isolates to utilize disaccharides such cellobiose, maltose, lactose, and hexahydric alcohols like dulcitol, mannitol, and sorbitol was examined. All five isolates from various Odisha coastal vegetable belts used disaccharides such lactose, cellobiose, and maltose as well as sugar alcohols like dulcitol, mannitol, and sorbitol. Using carbohydrate fermentation kits purchased from Hi-media, a biovar characterisation test was also conducted. Observations after 48 hours of inoculation revealed that the phenol red agar medium's color changed from light red to yellow surrounding the carbohydrate fermentation disc. This shift in color made it abundantly evident that the bacteria employed in the current experiment were utilizing carbohydrates, and all of the *R. solanacearum* isolates are members of biovar-III. Previous studies by Hayward (1964); He *et al*. (1983); Kumar *et al*. (1993) described the differentiation of *R. solanacearum* biovars based on the use of carbohydrates. They discovered that biovar III oxidizes both disaccharides and sugar alcohols, whereas biovars I, II, and IV only oxidize sugar alcohols and not disaccharides. Razia *et al*. (2021) also get similar results in the biovar determination test for *Ralstonia* where he conducted an experiment involving the above test where he derived that all *Ralstonia* isolates were belong to Race 1 biovar 3.

Table 2: Biochemical Characteristics of *R. Solanacearum*

Sr. No.	Isolates	Gram staining	Oxidase	Catalase	Nitrate Reduction test	Potassium hydroxide Solubility	Starch Hydrolysis
1.	RS 1	-	+	+	+	+	-
2.	RS 2	-	+	+	+	+	-
3.	RS 3	-	+	+	+	+	-
4.	RS 4	-	+	+	+	+	-
5.	RS 5	-	+	+	+	+	-

Note: '+' Positive Reactions '-' Negative Reaction

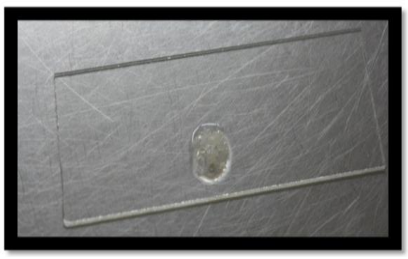

Fig. 1. Catalase test.

Fig. 2. KoH Solubility Test.

Fig. 3. Nitrate Reduction Test.

Fig. 4. Starch Hydrolysis Test.

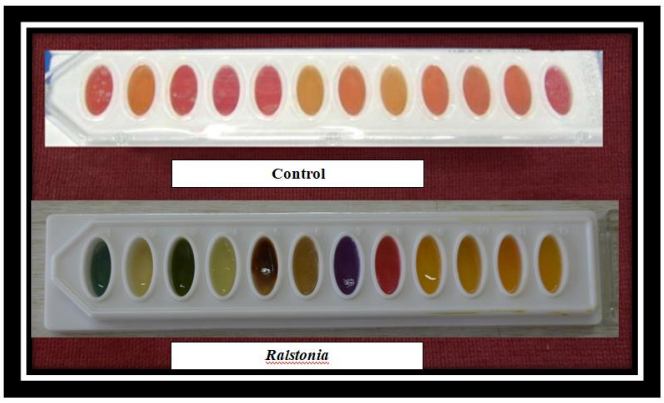

Fig. 5. Carbohydrate utilization by *Ralstonia solanacearum* isolates by HiMivic test kit.

Fig. 6. Carbohydrate utilization by *Ralstonia solanacearum* isolates by manual method.

Table 3: Biovars detection of *R. solanacearum* based on carbohydrate utilization test.

Isolates	Lactose	Maltose	Cellobiose	Mannitol	Sorbitol	Dulcitol	Biovars	Races
RS 1	+	+	+	+	+	+	III	I
RS 2	+	+	+	+	+	+	III	I
RS 3	+	+	+	+	+	+	III	I
RS 4	+	+	+	+	+	+	III	I
RS 5	+	+	+	+	+	+	III	I

CONCLUSIONS

Based on the above test results, it is confirmed that *Ralstonia* belongs to Race 1 biovar 3 through the biovar determination test. Bacterium has showed positive reaction towards catalase test, KoH Solubility Test, nitrate reduction test while showed negative reaction on starch hydrolysis test.

FUTURE SCOPE

On the future scope, Biochemical characterization can also be done upto sixty character in which the bacterium possesses.

Acknowledgement. I extend my sincere thanks to Dr. A. K. Senapati (major advisor) and to my thesis advisory committee members for giving me proper knowledge and guidance throughout the course of study. I also responsibly thank O.U.A.T., Bhubaneswar for supporting the above research.
Conflict of Interest. None.

REFERENCES

Ahmed, W., Yang, J., Tan, Y., Munir, S., Liu, Q., Zhang, J. and Guanghai Ji, Zhao, Z. (2022). *Ralstonia solanacearum*, a deadly pathogen: Revisiting the bacterial wilt biocontrol practices in tobacco and other Solanaceae. *Rhizosphere*, *21*, 137.

Cerkauskas, R. (2004). *Cercospora* leaf spot, in AVRDC Fact Sheet: Pepper Diseases, (*Taiwan: AVRDC–The World Vegetable Center; AVRDC Publication; 4*, 575.

Denny, T. (2006). Plant pathogenic *Ralstonia* species in Plant-Associated Bacteria, *Gnanamanickam (Berlin: Springer*), 573–644.

Dhaliwal, M. (2015). CH-27: a multiple disease resistant chilli hybrid, *Agricultural Research Journal*, *52*(4), 127-129.

Ghaly, A., Mahmoud, N. and Higgins, J. (2007). Production of Levan by *Bacillus licheniformis* for Use as a Soil Sealant in Earthen Manure Storage Structures. *American Journal of Biotechnology and Biochemistry*, *3*(2), 47-54.

Hayward, A. (1964). Characteristics of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology*, *27*(2), 265–277.

He, Y., Sequeira, L. and Kelman, A. (1983). Characteristics of strains of *Pseudomonas solanacearum* from China. *Plant Disease*, *67*, 1357–1361.

Jyothi, K., Santhosha, M. and Basamma. R. (2012). Recent advances in breeding for bacterial wilt (*Ralstonia solanacearum*) resistance in tomato- review, *Current Biotica*, *63*, 70–98.

Kumar, V., Singh, M. and Sugha, S. (1993). Variation in isolates of *Pseudomonas solanacearum* from Himachal Pradesh. *Indian Journal of Mycology and Plant Pathology*, *23*, 232-236.

Kumari, R. and Ranjan, R. (2019). Characterization of *Ralstonia solanacearum* causing bacterial wilt of potato, *Journal of Pharmacognosy and Phytochemistry*, *8*(5), 1762-1767.

Manda, R., Addanki, V. and Srivastava, S. (2020). Bacterial wilt of solanaceous crops. *International Journal of Chemical Studies*, *8*(6), 1048-1057.

Razia, S., Chowdhury, M., Aminuzzaman, F., Sultana, N. and Islam, M. (2021). Morphological, Pathological, Biochemical and Molecular Characterization of *Ralstonia solanacearum* Isolates in Bangladesh. *American Journal of Molecular Biology*, *11*, 142-164.

Sharma, D. and Singh, Y. (2019). Characterization of *Ralstonia solanacearum* isolates using biochemical,

cultural, molecular methods and pathogenicity tests. *Journal of Pharmacognosy and Phytochemistry*, *8*(4), 2884-2889.

Suslow, V., Schroth, N. and Isaka, H. (1982). Application of rapid method for gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathology*, *72*, 917-918.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., and Nishiuchi. Y. (1995). Transfer of two *Burkholderia* and an Alcaligenes species to *Ralstonia* genus, *Microbiology and Immunology*, *39*, 897-904.

How to cite this article: Abhisek Tripathy, Akshaya Kumar Senapati and Satya Narayan Satapathy (2022). Biochemical characterization of *Ralstonia solanacearum* causing Chilli Bacterial Wilt. *Biological Forum – An International Journal*, *14*(4a): 781-785.