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Biochemical Characterization of *Ralstonia solanacerum* Causing Bacterial Wilt of Brinjal in The Hilly District of Assam

Madhusmita Kataky*, Ajit Kr. Tamuli[1], Robindra Teron[1] and Ramen Kr. Sarma[2]

*Krishi Vigyan Kendra, Kamrup

[1]Department of Life Science & Bioinformatics, Assam University, Assam

[2]Assam Agricultural University, Jorhat, Assam

*Corresponding Author E-mail: madhusmitakataky@gmail.com

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ABSTRACT

Ralstonia solanacearum causes bacterial wilt of solanaceous crop plants including brinjal, a most devastating disease in humid tropic. A survey was conducted to study the the status of bacterial wilt incidence in major brinjal growing areas and to characterize the isolates of R. solanacearum causing bacterial wilt of brinjal in the Hilly District Karbi Anglong of Assam. The survey results reveals that the incidence of bacterial wilt disease on Brinjal varied from its lowest 24.28 per cent at Rongkhoi area in Hamren subdivision to the highest 51.87 percent at Taralangso in Diphu subdivision. Among the three different sub division the intensity of damage caused by the bacterial wilt disease of Brinjal was found the highest in Diphu Sub Division (36.54%) followed by Bokajan Sub-Division (34.01%).

The isolates of R. solanacearum were obtained from different locations.Gram's staining and Potassium hydroxide solubility test revealed that all groups of R. solanacearum isolates are gram negative. The isolates of R. solanacearum fermented four basic sugars (Dextrose, sucrose, manitol and lactose). These results of all biochemical tests in combination with the pathogenicity test confirmed the isolates were R. solanacearum causing bacterial wilt of brinjal. All groups of R. Solanacearum isolates were found virulent producing pink or light red color or characteristic red center and whitish margin on TZC medium after 24 hours of incubation. On the biovar test clearly revealed that all groups of R. solanacearum isolates oxidized disaccharides (Sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5days and confirmed biovar as III. Pathogencity test on tomato and chilli indicating wide host range of R. solanacearum isolates and categorized them in race 1. Therefore, it may be confirmed that R. solanacearum causing bacterial wilt of brinjal in Hilly District of Assam belong to Biovar III and Race 1.

Key words: *Bacterial wilt, Brinjal, Characterization, Ralstonia solanacerum, Karbi, Anglong*

INTRODUCTION

Brinjal or eggplant (*Solanum melongena L.*) is an important solanaceous crop of sub-tropics and tropics. The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit. The brinjal is of much importance in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China and the Philippines.

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It is also popular in Egypt, France, Italy and United States. In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except higher altitudes. It is a versatile crop adapted to different agro-climatic regions and can be grown throughout the year.

In Assam, the Brinjal *(Solanum melongena L*) also known as Egg Plant is a common and popular vegetable crop grown extensively and is a major source of income for the small and marginal farmers of the state. The fruit is primarily used as cooking vegetable for the various local dishes. The nutritive values per 100 g of raw eggplant include carbohydrates (5.7 g), fat (0.19 g), protein (1.01 g), thiamine (0.039 mg), riboflavin (0.037 mg), niacin (0.649 mg), pantothenic acid (0.281 mg), vitamin B6 (0.084 mg), folate (22 µg), vitamin C (2.2 mg), Calcium (9 mg), iron (0.24 mg), magnesium (14mg), phosphorus (25 mg), potassium (230 mg), zinc (0.16mg) and manganese (0.25mg) (USDA Nutrient database). It is also reported to posse's medicinal properties.

Bacterial wilt caused by soil borne pathogen *Ralstonia solanacearum*[13] is one of the most devastating bacterial plant diseases in the tropical and subtropical regions of the world causing yield losses up to 90 per cent. *Ralstonia solanacearum* is a serious plant pathogen causing bacterial wilt in solanaceous vegetables in India. *Ralstonia solanacearum* gained its importance in the world scenery of pathology due to its severe destructive nature, wide host range and geographical distribution. *Ralstonia solanacearum* mostly persists in the soil and crop residues[4]. The race 1 as reported by Denny[2] is widely distributed in tropical and subtropical regions and it infects over 50 families including those in the Solanaceae i.e., eggplant (*Solanum melongena* (L.), pepper (*Capsicum* sp.), potato (*S. tuberosum* (L.) and tomato (*S. lycopersicum* (L.). Losses caused by the disease vary from 20-100%.

The bacterium *R. solanacearum* has been reported to be primarily a soil borne and water borne pathogen. However, in crops such as Tomato and egg plant the pathogen is carried in seed. It is a gram-negative, rod-shaped, largely aerobic bacterium that is 0.5-0.7 x 1.5-2.0 µm in size. Liquid and solid agar growth media are commonly used for culture. For most strains optimal growth temperature is between 30 and 35°C[1]. Once inside roots or stems, the bacterium colonizes the intercellular spaces of the root cortex and vascular parenchyma, eventually entering the xylem vessels[12]. In the xylem vessels, the pathogen dissolves the cell walls and produce highly polymerized polysaccharides that increase the viscosity of the xylem resulting plugging of the vessels finally occurrence of wilt of the plant. High atmospheric humidity further favours the development of the disease.

MATERIALS AND METHOD

Survey Site

The survey on disease incidence of Brinjal has been conducted in two seasons, *i.e. Kharif and rabi* in the three subdivision of the Karbi Anglong District of Assam viz. Diphu, Bokajan and Hamren during October 2013 to March 2014.

Sampling Technique:

The survey was conducted using multistage purposive and random sampling procedure. In the first stage, 24 different localities, 8 localities each from Diphu, Bokajan and Hamren agricultural Subdivision having Brinjal growing areas were purposively selected for the survey. In second stage, 4 Brinjal growing fields from each of the selected localities were randomly selected which resulted a total sample of 96 number of brinjal growing fields for observation of incidence on bacterial wilt diseases of Brinjal.

Data collection

The survey was conducted through farmer's participatory approaches like Transit walk, Focus Group Discussion (FGD) and personal interview method. Disease occurrence and per cent infection ie. Disease incidence on the selected crops were observed by eyeball method through the transit walk in the sampled fields. Besides, information was also collected

from the brinjal growing farmers and traders through the personal interview method using specially design pretested questionnaires, group discussion with the representative farmers.

Collection of Diseased samples

At least 10 samples of the diseased plants were collected from each of the surveyed area and were brought into the laboratory for the isolation of different groups of isolate of *R.solanacearum*.

Symptomatological Studies:

The characteristic disease symptoms of the collected samples were studied.

Ooze Test of the suspected plant samples:

Infected stem of target plants was cut obliquely at the base and placed in sterile distilled water for preliminary confirmation of the bacterial disease.

Isolation, Purification and maintenance of Isolates:

Section of plant samples showing bacterial ooze along with some healthy tissues were cut into small bits (0.25mm) and surface sterilized with 1% sodium hypochloride solution for one minute. The surface sterilized bits of both brinjal and bhut Jolokia were later placed on Petriplates containing Nutrient Agar (NA) medium aseptically.

Isolation of the pathogen from the diseased Brinjal was done on Triphenyl tetrazolium chloride (TTC) Agar medium[7] using streak plate technique. The inoculated plates were incubated at 28 ± 1^0C. Individual colonies of bacteria were observed under 48hr of incubation. These were picked up and purified twice following dilution plate technique and finally purified single colony cultures were maintained on Yeast Glucose Chalk Agar Slants(YGCA) at $\pm4^0$C in the refrigerator.

Pure culture preservation was done in Nutrient agar slants to maintain the viability of preserved bacteria for further study.

Pathogenicity test:

After isolation of the causal organisms the pathgenicity test was conducted on specific host through Koch's postulates (1882) by root inoculation technique under two different conditions viz., potted and field conditions for confirmation of the actual pathogen. A set of three seedlings were inoculated with sterile distilled water to serve as control. The plants were observed for the symptoms. Pathogenicity test of the pathogen was confirmed after Koch's postulation.

Tobacco hypersensitive reaction(HR)test

In order to study the hypersensitive reaction on tobacco leaves, a dilute suspension (10^7 cells /ml) of the test bacterium was prepared and inoculated by Klement's[9] injection infiltration method. The observation of the leaf tissues was recorded within 24 hr.

Identification and characterization of the isolates:

Identification of bacterial isolate of Brinjal and their cultural and biochemical tests were performed routinely by following the guidelines described in the Bergey's Manual of systematic Bacteriology[3].

Studies on morphological characters:

In order to study the morphological characters of each isolate, particularly capsule staining, Anthony's method with Tyler's modification was used and Bartholomew and Mittwer's"Cold" method was used to study the sporulation.

Electron Microscopy of bacteria:

Electron microscopy of the bacterium was done for determination of size, shape and flagellation at the Center for Advance Virological Laboratory, Division of Plant Pathology, Indian Agricultural Research Institute(IARI), New Delhi. The actual size of each bacterium was calculated by using the following formula:

$$\text{Size of bacteria (nm)} = \frac{\text{Measured size in mm}}{\text{Magnification}} \times 10{,}00{,}000$$

Cultural Characteristics of the isolate:

Colony characters of the bacterium on Nutrient Sucrose Agar (NSA) medium in plates:

Forty eight hour old growth of each isolate was mixed with sterile distilled water in culture tube.Serial dilutions were made upto 10^7 cfu/ml and finally, 0.1 ml of the final dilution was poured in petriplates containing NSA medium. The plates were incubated at 28+ 1^0C for 72 hr and the colony characters of each isolate were measured in terms of shape, size, elevation, surface, edge, colour, opacity and solubility in water were recorded. Laskin, *et.al*[10].

Gram Staining:

Hucker's modification of Gram Stain was used to determine gram reaction (1922)

KOH solubility Test (3%):

KOH solubility test was performed for further confirmation of gram reaction of the test bacteria.

Oxygen requirement:

Oxygen requirement was tested by stab inoculation in quadruplicate tubes of NSA with 0.005 per cent bromocresol purple.

Pigment production:

Pigment production by the test bacteria was studied in both YGCA and KING's media and observation was made using UV radiation.

Growth characters on stab culture:

Growth on stab culture were observed and recorded.

Growth characters on agar slant:

The growth characters were studied on the slants of same medium used for colony characters.

Studies on Biochemical characters of the Isolates:

The following biochemical tests were performed to study the biochemical nature of each isolates:

a) Catalase production
b) Reduction of Nitrate
c) Production of ammonia
d) Production of H_2S
e) Gelatin liquefication
f) Starch hydrolysis
g) Mode of utilization of Glucose
h) Gas production
i) 3 ketolactose test
j) Arginine hydrolase
k) Levan production
l) Growth on 0.6 per cent NaCl
m) Growth on 0.1per cent
n) Tryphenyl Tetrazolium Chloride

Determination of biovars

The isolates of R. Solanacearum were differentiated into biovars based on their ability to utilize disaccharides (Sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) as described previously by Hayward[5] and He *et al*[6]. The biovars were determined in the mineral medium (NH4H2PO4 1.0g, KCl 0.2g, MgSO4.7H2O 0.2g, Difco bacto peptone 1.0g, Agar 3.0g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 200 µl of the melted medium is dispensed into the wells of microtitre plate. Inoculums for each group of isolates was prepared by adding several loopful of bacteria from 24-48h old cultures to distilled water to make suspension containing about 10^8 CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28-32^{0}C. The tubes were then examined at 3 days after inoculation for change in pH by a color change[11].

Races identification

The races of *R. solanacearum* were identified by pathogenicity test on wide host range[11]. Seedlings of tomato and chilli were raised in tray and one month old seedlings (tomato & chili) were inoculated by soil inoculation method[8]. The incubated plants were then kept in the net house until symptoms development.

Assessment of disease incidence

The per cent wilt incidence was calculated by the following formula:

$$\% \text{ Wilt incidence } = \frac{\text{Number of wilted plants in each field}}{\text{Total number of plants in each field}} \text{x } 100 -$$

RESULTS AND DISCUSSION

Incidence of bacterial wilt

To study the natural incidence of Bacterial wilt diseases in Brinjal in the Hill District District Karbi Anglong, of Assam, a survey was conducted through the farmers participatory approaches covering the three subdivision of the District viz., Diphu , Bokajan and Hamren. The survey results reveals that the incidence of bacterial wilt disease on Brinjal varied from its lowest 24.28 per cent at Rongkhoi area in Hamren subdivision to the highest 51.87 percent at Taralangso in Diphu subdivision. Among the three different sub division the intensity of damage caused by the bacterial wilt disease of Brinjal was found the highest in Diphu Sub Division (36.54%) followed by Bokajan Sub-Division (34.01%). Incidence of occurrence of bacterial wilt disease was observed lower in Hamren Sub Division. For the district as a whole the incidence of bacterial wilt disease was 33.48 per cent on Brinjal.

The survey results indicated a locationl variation in bacterial wilt incidence.Differences of wilt incidence and severity were also reported due to the great diversity of host plants affected by this pathogen, phenotype and genotype of *R. solanacearum*, its wide geographical distribution, and the range of environmental conditions conducive tobacterial wilt.

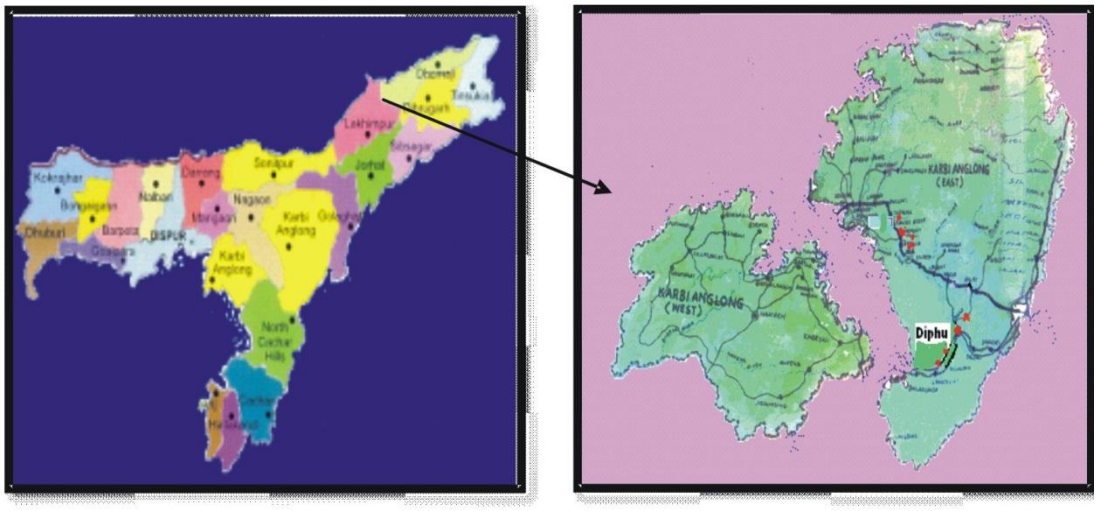

Fig. 1: Map of Assam showing the Hill District Karbi Anglong

Table 1: List of the localities selected for survey along with natural disease incidence

Location ▼ Disease►	**Per cent incidence**
	Bacterial wilt in Brinjal
Taralangso	51.87
Rongnihang	48.67
Manja	44.36
Bokolia	47,33
Patradisha	50.45
Hapjan	26.42
Borlangphar	37.33
Doldoli	33.24
Sub Division average	**36.54**
Deopani	45.22

Japorajan	42.71
Dilaojan	38.22
Santipur	32.12
Sarihajan	29.32
Bokajan	28.82
Balipothar	29.22
Tinglijan	26.48
Sub Division average	**34.01**
Hamren	33.42
Tumpreng	32.66
Kherony	44.90
Uliukunchi	26.24
Donkamukam	26.31
Jenkha	24.57
Baithalangso	26.78
Rongkhoi	24.28
Sub Division average	**29.90**
District average	**33.48**

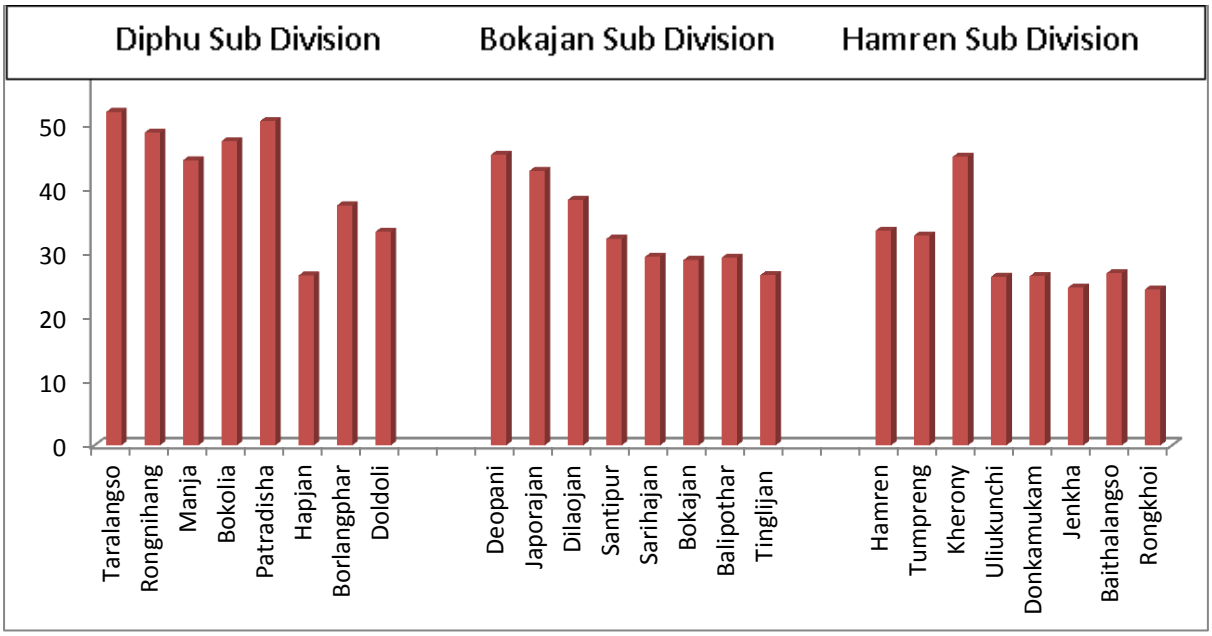

Fig. 2: Incidence (%) of Bacterial Wilt on Brinjal at different localities in Karbi Anglong Distrct

Characteristics disease symptoms of the bacterial wilt infected plants

The characteristic symptoms include wilting of the foliage followed by collapse of the entire plant. The wilting is characterized by dropping and slight yellowing of leaves and vascular discolouration. Drying of plants at the time of flowering and fruiting are also characteristic to the disease condition. The infected cut stems pieces when dipped in water, a white milky

stream of bacterial oozes coming out which is the diagnostic symptom for bacterial wilt. (Plate 1)

Ooze test for preliminary diagnosis of bacterial diseases:

Infected stem of target plants was cut obliquely at the base and placed in test tube containing sterile distill water for bacterial ooze. White colored slimy bacterial cells oozed out from all the suspected diseased samples Infected leaf sections also showed the oozing of bacterial cells , indicating that the disease is caused by bacteria .Samples without characteristics disease symptoms did not produce any ooze indicating healthy samples.

Plate 1: Characteristics disease symptoms of Bacterial wilt of Brinjal

Isolation of the bacteria from the diseased plant parts:

Bacterial isolates obtained from diseased plant samples of both Bhut Jolokia and Brinjal plants were studied on nutrient sucrose agar medium (NSA). Both the colonies were observed as Light pink in colour, opaque, circular, medium surface, entire margin with low convex elevation.

Pathogenicity Test:

Koch postulation of the isolates were performed to prove the pathogenicity by root inoculation technique. Inoculated seedlings reproduced the typical symptoms observed on the naturally infected plant within 7-10 days.

In control plants inoculated with sterile distilled water without bacterial inoculums, no such symptoms were reproduced.

Tobacco Hypersensitive (HR)Test:

Most of the phytopathogenic bacteria produce hypersensitive reaction on tobacco leaves[9]. Approximately 10^7 cfu/ml of freshly cultured bacteria (48hrs old) isolated from Brinjal were injected separately into the interveinal areas of tobacco leaves. Complete collapse of the tissue after 24-48 hrs. recorded as positive result. No such type of collapsed tissues was observed in control plants. This indicated that the pathogenic bacterial isolates were obtained from the diseased plant samples

Identification and characterization of the bacterial isolates:

Isolated bacteria from infected samples of Brinjal and Bhut Jolokia were subjected to various morphological, cultural, physiological/ biochemical tests in order to identify the characteristics of bacterial genera.

Morphological Characters:

Electron microscopy of bacteria:

Electron microscopy of the bacterial isolates were performed using JEOL-100CX-II Transmission Microscope as described in Materials and Methods.Shape, size,flagellation were determined and it was found that the isolated bacteria from brinjal were the same bacterial isolate as rod shaped ,measured as is 0.5-0.7 x 1.5-2.0 µm in size motile with one polar flagella. (Plate2)

The test bacterial isolates were also gram negative, non spore former and capsulated.

Cultural characteristics of the bacterial isolates:

Colony characters of the bacteria on NSA medium in culture plates:

Colony characters of bacteria were determined on NSA medium. The colour, shape, surface, margin, elevation, opacity, size of the colonies and their solubility were accounted for identification and observed that the colonies were light pink in colour, opaque, circular, medium surface, entire margin with low convex elevation and size measuring ranging from 3.0 mm to 4.0 mm in diameter.

Plate 2: Bacterial isolate Showing Monotrichous Flagellation

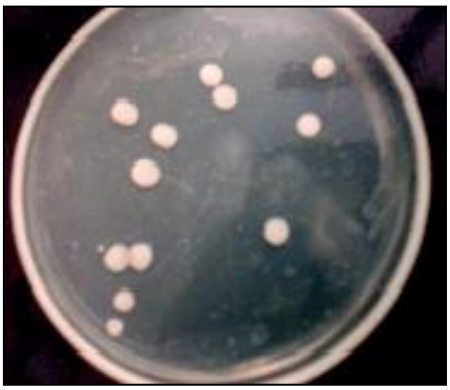

Plate 3: Colonies of Bacterial Isolate

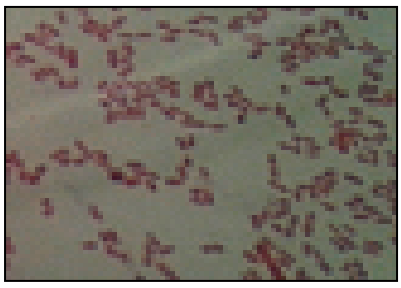

Plate 4.1. Gram negative cells

Plate 4.2 Starch Hydrolysis positive

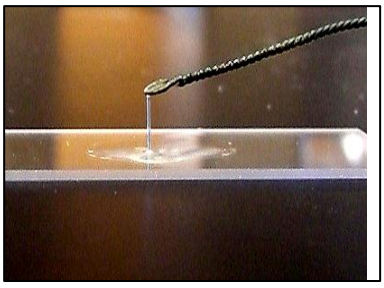

Plate 4.3 KOH Positive

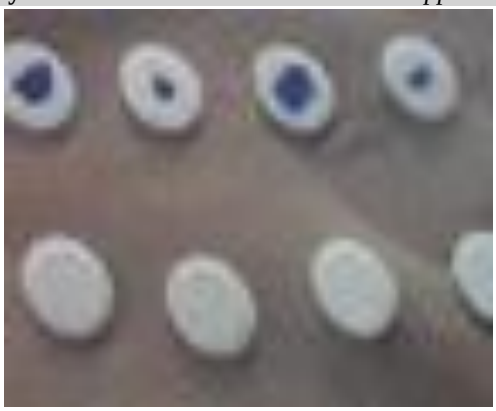

Plate 4.4. Oxidase test Positive

Plate 4.5. Ammonia ProductionNegative

Growth Characters on stab culture:

Growth characters of the bacterial isolates on stab culture were recorded after 48 hr of incubation at 28±1ºC. The results are presented on Table 1

Oxygen requirement:

Oxygen requirement was tested by stab inoculation in quadruplicate tubes of NSA for both the isolates with 0.005 per cent bromocresol purple. The agar surface of one series of tubes was covered with sterile liquid paraffin upto a height of 1cm and the other set of series without paraffin. The tubes were incubated at 28±1ºCand observations was recorded at an interval of 48hr for 2 weeks.

It was observed that the isolates produced growth within 72 hr and changed the violet colour of the medium to yellow in the tubes without paraffin indicating that the isolate are strictly aerobic. Whereas there was no growth of the isolate with paraffin (Table 2)

KOH Test:

One drop of bacterial suspension was mixed thoroughly with 1 drop of 3 per cent KOH and the results indicated that both the isolate were gram negative. The KOH tests further confirmed the results tested with gram staining (Table 2) Plate4.3.)

Biochemical characters of the bacterial isolates:

The various biochemical characters of the isolates were done and presented in Table 2. (. Plate 2.)

Sugar fermentation test

The isolates of *R. solanacearum* is able to oxidize the sugars which are indicated by color change (reddish to yellow). The results of Sugar fermentation test clearly showed that all groups of R. *solanacearum isolates* obtained from the wilted brinjal plants were able to oxidize the four (4) basic sugars (Dextrose, sucrose, manitol and lactose) by producing acid and gas. The acid production in sugar fermentation test by bacterial isolates was indicated by the colour change from reddish to yellow, gas production was noted by the appearance of gas bubbles in the inverted Dhuram's tubes and the oxidation of sugar manitol by the bacterial isolates indicated by the production of yellow to red color. (Table 2).

Biovar differentiation

The biovar of *R. solanacearum* isolates was identified by utiliztion of disaccharides and hexose alcohols. The result of the biovar test showed that all seven groups of *R.solanacearum* isolates oxidized disaccharides (Sucrose, lactose, maltose) and sugar alcohols(manitol, sorbitol and dulcitol) within 3-5days .The oxidation reaction was indicated by the change of color. The results revealed a change of colour blue to yellow colour indicating the oxidization of sugars by bacterial isolates. Therefore, *R. solanacearum* isolates belong to biovar III the other hand all the control plates of different sugars and sugar alcohols remain unchanged. The differentiation of biovars of *R. solanacearum*

based on the utilization of carbohydrates was reported previously by Hayward[5] (1964), He *et al*[6]. (1983). They observed that biovar III oxidizes both disaccharides and hexose alcohols whereas Biovar I oxidize hexose alcohols but not disaccharides, biovar II oxidizes only disaccharides and biovar IV oxidizes only alcohols.

SUMMARY AND CONCLUSION

The morphological, cultural, pathologicaland physiological/biochemical characters of the isolate were summarized as followes. The identification of the isolates of wilt infected Brinjal was done as *Ralstonia solanacearum* Race 1 following the description of various pathogenic genera and their species and race in the 8th Edition, Bergey;s Manual of Determinative Bacteriology, 1974 (Table)

Rod shaped, 0.5-0.7 x 1.5-2.0) µm in size, scattered or in pairs, gram-negative, non spore former, one polar flagella and capsulated.

Colony surface medium, light pink, circular, entire, low convex, opaque, average 3.5mm in dia, filiform growth on NSA slants and on stab culturr, aerobic, produced slime on KOH solution and cause hypersensitive reaction on tobacco.

Catalase production positive, starch hydrolysis positive, liquefied gelatine, H_2S production positive, utilized glucose, oxidatively reduced nitrate. Gas production, ammonia production negative, lack in arginine dihydrolase, 3 keto lactose and potato soft rot test were found negative. The bacteria could not produce the water soluble and water insoluble pigments. Growth on nutrient agar was inhibited by 0.6% NaCl but slight growth occurred on 0.1% Triphenyl tetrazolium chloride.

Based on the characters tested the isolates were identified as *Ralstonia solnacearum* Race 1 biovar III the causal agent of bacterial wiltof Brinjal found virulent in the Hill District Karbi Anglong of, Assam.

Table 2: Characteristics of the local strain of *Ralstonia solanacearum*

Colony Character	Morphological character	Cultural Character	Pathological Test	Biochemical test
Shape(Circular) Surface(Medium) Edge(Entire) Colour(Light Pink) Elevation (Low Convex) Opacity(Opaque) Colony Size(3.0-4.0mm .	Size: 0.5-0.7 x 1.5-2.0)µm Shape:Rod shaped Flagella No. one Capsule:(+) Spore: (-)	Gram Reaction (Gram negative), Oxygen requirement (Aerobic), KOH test: (+) Growth Character:filiform Pigment production on King's B medium (-)	THR: (+) Potato soft rot : (-) Pathogenicity Test : (+)	Catalase production: +ve Starch hydrolysis:+ ve Gelatin liquefication:+ ve H_2Sproduction: + ve Levan Production: - ve Nitrate reduction: +ve Ammonia production: -ve 3-Ketolactose test: -ve Glucose utilization: Oxidative Arginine hydrolase : -ve Gas production:+-ve Sugar fermentation test +ve Growth on 0.6% NaCl: Inhibited Growth on 0.1% TTC: Slight

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