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ISOLATION AND CHARACTERIZATION OF *RALSTONIA SOLANACEARUM* FROM INFECTED TOMATO PLANTS OF SOAN SKESAR VALLEY OF PUNJAB

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Abstract

The most devastating disease of tomato is bacterial wilt (BW) caused by *Ralstonia solanacearum* which occur in hot and humid areas of Pakistan. Control of bacterial wilt is difficult, because the pathogen is soil-borne and has a wide host range. There is a need to establish resistance against this pathogen through biotechnological approaches. As a first step, infected tomato leaf samples were collected from various parts of soan skesar valley of Punjab, Pakistan. Isolations were performed on triphenyl tetrazolium salt (TTC) medium. Five samples, each of ten plants, from seven fields were used for isolation. Bacteria with similar characteristics were isolated from all positive samples which produced fluidal colonies that were either entirely white or white with red centre after incubation for 48 hours at 28ºC on TTC medium. They were found positive for gram staining, KOH test, Kovacs oxidase test, Catalase test, Oxidation/fermentation of glucose, Hydrolysis of tween 80 and negative for Arginine dihydrolase, Levan production, Salt tolerance, Lecithinase detection, Gelatin hydrolysis and production of Fluorescent pigment. Wilting and necrosis were observed for pathogenicity test and chlorosis/necrosis was observed for hypersensitivity. Isolates were identified as *Ralstonia Solanacearum*. . Out of 10 diseased plants, 7 were found positive and 3 were negative.

Introduction

Factors contributing low yield of tomato include inappropriate agronomic practices, continuous monocropping, use of non-certified seed and above all, a number of biotic and abiotic stresses. These include bacteria, viruses, fungi and nematodes etc. Four bacterial pathogens of tomato are very important all over the world which are *Ralstonia solanacearum*, *Clavibacter michiganensis*, *Xanthomonas campestris* pv. *vesicatoria* and *Pseuodomonas syringe* (Laterrot, 1998). *Ralstonia solanacearum* is the causal agent of Bacterial Wilt (Fujiwara *et al*., 2008). It represents the major limitation in the production of solanaceous crops all around the world (Hayward, 1991*)* and is a destructive pathogen (Kelman *et al*., 1994) which causes significant damage on many important crops under disease-favorable weather conditions (Doan & Nguyen, 2005).

The youngest leaves of tomato are the first to be affected and have a flaccid appearance, usually at the warmest time of day (Vanitha *et al*., 2009). Wilting of the whole plant may follow rapidly if environmental conditions are favorable for the pathogen. The vascular tissues of the stem show a brown discoloration and, if the stem is cut crosswise, drops of white or yellowish bacterial ooze may be visible. Control is difficult due to high variability of the pathogen, limited possibility for chemical control, high capacity of the pathogen to survive in diverse environments and its extremely wide host range (Nguyen & Ranamukhaarachchi, 2010). The progression of plant disease involves a complex interplay between bacterial virulence and plant defense mechanisms. We are focusing upon characterizing the bacterial pathogen responsible for causing bacterial wilt which will develop a better understanding of the pathogen and will also provide a base line for the improvement of tomato varieties against this very serious pathogen.

Materials and Methods

Samples collection: A total of 10 diseased tomato plants were collected from different areas of Pakistan. From each plant 5 samples were made. Field diagnosis of diseased plant samples were done by critically observing the bacterial wilt symptoms. Simple random sampling technique was adopted for collection of samples.

Isolation of *R. solanacearum*: TTC (2,3,5-triphenyltetrazolium chloride) media (Hugh & Leifson,1953) and 523 media (Kelman, 1954) were used for isolation and maintenance of bacterial pathogenic isolates. Both media were steam sterilized at 121°C and 15-psi pressure for 20 minutes. In TTC media 5ml of 1% 2,3,5-triphenyltetrazolium chloride were added to the sterilize medium before pouring into plates.

Following methods were used for isolation of *Ralstonia solanacearum* from the disease plants.

1. Stem segments (about 10cm in length) from collar region of wilted plants were rinsed with sterilized distilled water containing 1% Clorox. The tubes were stirred on Vortex mixture and turbid bacterial suspensions were obtained. A loop full of turbid suspension was streaked on 2,3,5-triphenyltetrazolium chloride media (TTC) and incubated at 30°C for 2 days, reddish fluidal colonies were again streaked on TTC plates and the process was repeated till purified bacterial cultures were obtained with the homogeneity in colony morphology.

2. Stem sections were kept in sterilized distilled water for few minutes and pressed, bacterial exudates coming out from stem sections were directly streaked on TTC plates.

3. Vascular portion of infected stem sections were cut into small pieces, washed and sterilized by the same procedure as described above and were directly placed on TTC plates, incubated at 28-30°C for 2-3 days. Reddish fluidal colonies of bacteria were purified as described earlier.

4. Dried soil samples (10g) dissolved in 100 ml of sterilized distilled water. The samples were agitated for 20 minutes and dilutions then carried out by adding 1 ml of sample to 9 ml of sterilized distilled water to a dilution of 10^{-2} and 10^{-3}.

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Characterization of the pathogen: Different biochemical tests were performed to characterize the *Ralstonia solanacearum.*

a. Gram staining: A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 30 seconds and then washed with running tape water for one minute. It was then flooded with iodine for one minute, rinsed in tape water and decolorized with 95% ethanol until colorless runoff. After washing the specimen was counter-stained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at 10X, 40X and 100X using oil (Schaad,1980).

b. **Potassium hydroxide test:** Bacteria were aseptically removed from Petri plates with a tooth pick or an inoculating wire loop, placed on glass slide in a drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads (Suslow *et al*., 1982) (Table 2).

c. Catalase oxidase test: Young agar cultures (18-24 hrs) and 3% hydrogen peroxide (H_2O_2) were used to observe production of gas bubbles. A loop full of bacterial culture was mixed with a drop of H_2O_2 on a glass slide and observed for the production of gas bubbles with naked eye and under a dissecting magnification of 25X (Schaad, 1980) (Table 2).

d. Levan production from sucrose: Single colonies of bacterial cultures on 523 media were streaked on nutrient agar plates supplemented with 5% sucrose. Plates were then incubated at 28°C for 48 hrs. Separate, discrete colonies were observed for the Levan production with naked eye (Schaad, 1980) (Table 3).

e. Kovacs oxidase test: Oxidase reagent (1% tetra-methyl-p-phenyl diamine dihydrochloride) solution (100 ml) was prepared and kept in rubber-stopper dark bottle. A drop of reagent was added to a piece of filter paper, placed within a glass Petri dish. Small quantities of the inoculum were rubbed on the filter paper impregnated with 1% (w/v) oxidase reagent solution. Bacteria were then noted for the development of purple colour in 10-60 seconds (Table 2).

f. Production of fluorescent pigment: Plates were inoculated with bacterium and incubated for 48 hrs at 28-30°C. After that, these plates were placed under a UV lamp and observed for the production of fluorescence (Fig. 2).

g. Gas production from nitrate (demystification): The VMS medium contains succinate as major carbon source and the VNG medium contains glycerol. Medium constituents were dissolved and 100 ml quantities were added to 200 ml capacity Pyrex screw-capped bottles each containing 0.3g Difco Bacto agar. Medium was then dispensed in 5ml quantities into each sterile 15ml capacity Pyrex screw-capped test tubes and were sterilized. Tubes were sealed with 3-4 ml of molten sterile 1% Difco Bacto agar cooled to about 45°C and incubated at 28°C for 7 days. These were examined daily for the production of gas under the seal.

h. Lipase activity on Tween 80 agar: The constituents of the medium were dissolved in distilled water and heated at 100°C with periodic swirling to dissolve the agar. The molten medium was dispensed in 100 ml quantities in 200 ml screw-capped bottles. Using a wire loop bacterial inoculums were radially streaked across separate halves of Petri plates and incubated at 28°C for 7 days. The plates were examined daily for the presence of a dense precipitate around the bacterial growth.

i. Arginine dihydrolase reaction: The basal medium was homogenized by heating at 100°C and was dispensed in 5 ml quantities into each 15 ml capacity Pyrex screw-capped test tubes. The medium in each test tube was inoculated with bacterium by stabbing inoculating needle to the base of the test tube along with control. Each inoculated and control tube was sealed with an overlay of 3-4 ml molten 1% Difco Bacto Agar cooled to about 45°C. Tubes were incubated at 28°C for 7 days and observed for the presence of an alkaline pH change under the agar seal (Table 3).

j. Oxidation / fermentation of glucose: A basal medium described by Hayward (1964) was used for the identification of acid production from sugars by aerobic, gram-negative bacteria. Basal medium was prepared with following composition and procedure. Medium constituents were dissolved and pH was adjusted to 7.0-7.1. Color of the medium was olivaceous green. Difco Bacto Agar (0.3g) was mixed in 100 ml quantities of the basal medium. Glucose (0.1g) was added into each Pyrex screw-capped test tubes containing 10 ml sterilized distilled water. These solutions were heated at 100°C for 30 minutes to ensure sterility. Semi-solid basal medium in bottles was melted in microwave oven and cooled to 60-70°C; 10 ml of 1% glucose was mixed in basal media by rotation and 5 ml quantities were carefully dispensed into each sterile screw-capped Pyrex test tube. Medium was allowed to solidify at room temperature.

Straight inoculating wire loop heated to red hot was used to pick up inoculums from the bacterial cultures on 523 media. Tubes were stab inoculated and half of these were sealed with 3-4 ml molten, sterile 1 5 Difco Bacto Agar cooled to 45°C. Both sealed and unsealed tubes were incubated at 28°C. Tubes were examined after one day, 2 days and 7 days for gradual pH change at the surface of the open tube.

k. Grouping into biovars: Ten ml of each 10% lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol was added in sterilized screw-capped test tubes and then heated at 100°C for 30 min to sterilize these solutions. Bottles of semi-solid basal medium were melted in water bath and cooled to70°C. Carbohydrate solution (10 ml) was added and mixed in basal media. Five ml solution was poured into labeled tubes. Media was then allowed to solidify at room temperature. Control was kept by adding 10ml sterilized distilled water instead of sugar solution into the basal medium. Bacterial suspensions from individuals isolates (with concentration of 10^8 cfu/ml) were prepared from 48 hrs old cultures on 523 plates. Inoculation of the medium was carried out by adding 1-2 drops of bacterial suspension to each tube containing

sugar solutions, three replicates of sugar solution and control were maintained for each isolate. Test tubes were incubated at 30°C and examined after 2,7 and 14 days for the presence of indicator change from olivaceous green to orange colour on the surface of medium.

Statistical analysis: Data obtained were analyzed by applying Analysis of Variance technique using Completely Randomized Design (CRD) at 5% probability level (Steel & Torrie, 1980).

Results and Discussion

Isolation: When fragments were cut from plant samples in which symptoms of disease were evident, fluidal pinkish red centered colonies, typical of *Ralstonia solanacearum* were observed on TTC medium. Typical isolated colonies were picked and purified for confirmation of bacterial wilt causing pathogen *Ralstonia solanacearum*. Bacterial cultures were multiplied on 523 media and stored in sterilize distilled water for pathogenicity and confirmatory tests (Table 1; Fig. 1).

Virulence of an isolate can be determined on the basis of colony colour on this particular media. Virulent wild type colonies are usually large, elevated, fluidal and either entirely white or with a pale red center; avirulent mutant colonies were butyrous, deep-red often with a bluish border (French *et al*., 1995).

Table 1. Isolation of *Ralstonia solanacearum* from different sample types of diseased tomato.

Location	Sample type	No	Response	Color
Field-1	STB	3	-	Pink
	STEB	1	-	Pink
	VPB	1	-	Pink
	RB	2	-	Pink
	SDB	1	-	Pink
	SB	2	-	Pink
Field-2	STD	1	+ + + +	Pink
	STED	1	+ + +	Pink
	VPD	2	+ + + +	Pink
	RD	1	+ + +	Pink
	SDD	2	+ + + +	Pink
	SD	1	+ +	Pink
Field-3	STM	2	+ + + + +	Pink
	STEM	1	+ + + +	Pink
	VPM	1	+ + +	Pink
	RM	1	+ + + +	Pink
	SDM	2	+ + + +	Pink
	SM	3	+ + +	Pink

STB= Stem (Field-1); STD= Stem (Field-2); STM= Stem (Field-3); STED= Stem Exudates (Field-1); STED= Stem exudates (Field-2); STEM= Stem exudates (Field-3); VPB= Vascular portion (Field-1); VPD= Vascular portion (Field-2); VPM= Vascular portion (Field-3); RB= Root (Field-1); RD= Root (Field-2); SDM= Seed (Field-3); SDB= Seed (Field-1); SDD= Seed (Field-2); RM= Root (Field-3); SB= Soil (Field-1); SD= Soil (Field-2); SM= Soil (Field-3)

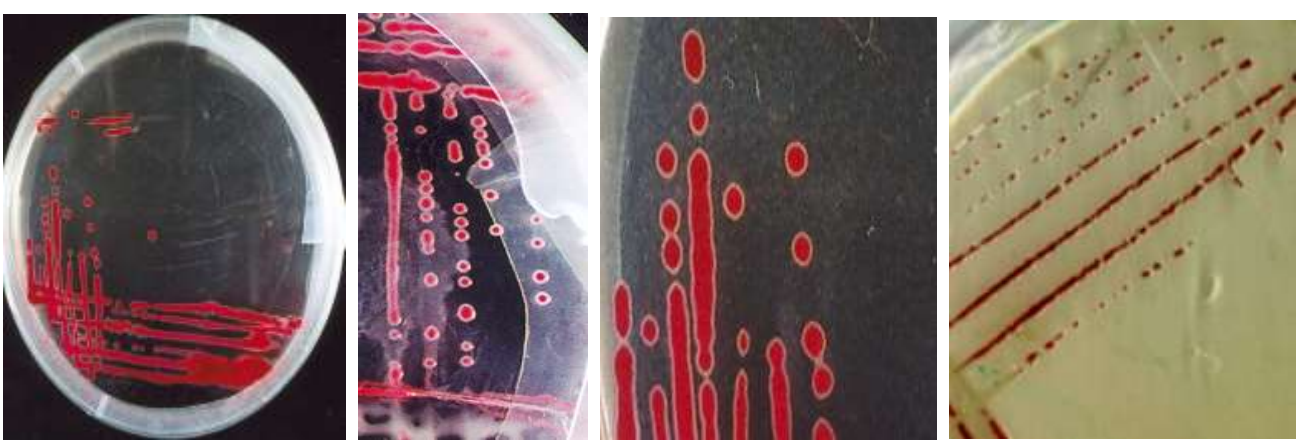

Fig. 1. Isolation and confirmation of *Ralstonia solanacearum* from infected tomato plants.

Characterization: Bacteria mainly Pseudomonads and Xanthomonads and pathogens belonging to other genera, induce HR in the incompatible hosts (Klement 1982). It has been shown that many but not all pathogenic bacteria can induce hypersensitivity necrosis in leaves of tobacco or other non-host plants (Klement, 1963). Since only the phytopathogenic but not the saprophytic bacteria have this property, the tobacco test is ideal for quick detection of the pathogenecity of the bacterium in question (Klement, 1963; Klement *et al*., 1964). The advantage of this method is that it is simple, quick and exact and does not require sterile conditions. This test reduces the number of further physiological, biochemical and pathological investigations, because only the HR positive strain will be investigated.

Hypersensitivity reaction is used for race determination in *Ralstonia solanacearum*. Race 1 showed chlorosis after 2 days followed by wilting after 7-8 days. Hypersensitive reaction after 12-24 hours (Race 2). Chlorosis only after 2-8 days confirmed Race 3 (Lelliott & Stead 1987).

The hypersensitive response occurs only in specific host-pathogen combinations in which the host and the pathogen are incompatible, that is the pathogen fails to infect the host. It is thought that this happens because of the presence in the plant of a resistance gene (R) which recognizes and is triggered into action by the elicitor molecule released by the pathogen.

Isolates of pathogen were tested for hypersensitive response. Slight localized chlorosis followed by necrosis and collapse of whole tissue was evident in HR positive isolates. In isolates that gave a very strong hypersensitivity response, symptoms were evident even after 24 hrs of inoculation. These were considered as strongly pathogenic. Isolates that developed symptoms after 72 hrs were weakly pathogenic, while isolates that showed symptoms after 7 days were considered as slightly pathogenic (Table 2). These results are inline with the findings of Lelliott & Stead (1987).

Table 2. Potassium hydroxide test, catalase oxidase test and kovacs oxidase test for confirmation of *Ralstonia Solanacearum*.

S. No	Isolates	KOH test (Loop formation)	Catalase oxidase test	Kovacs oxidase (purple color)	G-Reaction
1.	RS-STM1	+	+	+	-
2.	RS-STM2	+	+	+	-
3.	RS-STM3	+	+	+	-
4.	RS-STM4	+	+	+	-
5.	RS-STM5	+	+	+	-
6.	RS-STEM1	+	+	+	-
7.	RS-STEM2	+	+	+	-
8.	RS-STEM3	+	+	+	-
9.	RS-STEM4	+	+	+	-
10.	RS-STEM5	+	+	+	-
11.	RS-VPM1	+	+	+	-
12.	RS-VPM2	+	+	+	-
13.	RS-VPM3	+	+	+	-
14.	RS-VPM4	+	+	+	-
15.	RS-VPM5	+	+	+	-
16.	RS-RM1	+	+	+	
17.	RS-RM2	+	+	+	
18.	RS-RM3	+	+	+	
19.	RS-RM4	+	+	+	
20.	RS-RM5	+	+	+	
21.	RS-SDM1	+	+	+	
22.	RS-SDM2	+	+	+	
23.	RS-SDM3	+	+	+	
24.	RS-SDM4	+	+	+	
25.	RS-SDM5	+	+	+	
26.	RS-SM1	+	+	+	
27.	RS-SM2	+	+	+	
28.	RS-SM3	+	+	+	

+ Positive
- Negative

Biochemical tests: Many diagnostic and identification tests are based upon structural and chemical properties of bacteria (Lelliott & Stead 1987). The chemical compositions of certain substances in bacterial cells can be detected with specific staining techniques. Information about the presence or absence of such substances is used for identification of bacteria (Agrios, 1997). This reflects fundamental biochemical and biophysical differences in the bacterial cell wall.

Gram staining: All 15 isolates which gave positive hypersensitive response were tested for gram reaction as described by Schaad (1980). Bacteria retaining reddish pink colony color showed that these are gram -negative (G-ve), while gram-positive (G+ive) bacteria stained violet blue. All 15 isolates exhibiting hypersensitive response were found to be gram- negative. These results are in coincidence with the findings of Suslow *et al*., (1982).

KOH test: All isolates found gram-negative by gram staining gave a positive KOH loop test (Table 2). Slime threads were formed when fresh bacterial cultures (24-48 hrs old) were mixed with 3% KOH solution.

Formation of slime threads or loop is positive test and is indication of being gram-negative because gram-negative bacteria have relatively fragile cell walls which are bounded by an outer membrane. This outer membrane is readily disrupted by exposure to 3 % KOH releasing the viscous DNA. So those slime threads are actually DNA so

this test is lytic release of DNA. But gram- positive bacteria by contrast possess a thicker, more rigid cell wall which resists the disruptive effect of KOH (Table 2).

Catalase oxidase test: All gram negative bacteria produce gas bubbles when these are mixed with a drop of H_2O_2 on glass slide. Production of gas bubbles gives a clue for presence of aerobic and facultative anaerobic bacteria (Schaad, 1980). In my studies all the isolates tested produced gas bubbles during these tests (Plate), indicating that these might be *Ralstonia solanacearum* (Table 2).

Catalase is a hemi enzyme capable of decomposing hydrogen peroxide to water and oxygen gas (Klement, *et al*., 1964).

Levan production from sucrose: The tested isolates were negative for levan production, and Levan formation results from the enzymatic activity of levan sucrase on sucrose. The glucose is metabolized and the fructose is polymerized. Alginates may also be formed on sucrose agar (Fett *et al*., 1986), (Gross & Rudolph, 1984, 1987). Levan formation is responsible for the production of mucoid colonies by some *Pseudomonas.* Production of levan as an extra cellular capsule or slime layer results in colonies which are characteristically raised, convex and dome shaped in appearance (Schaad, 1980). This test helps in eliminating fluorescent *Pseudomonas* from non-fluorescent *Pseudomonas* because levan is produced from sucrose by some fluorescent *Pseudomonas* spp., like pathovar of *Pseudomonas syringe* (Table 3).

Table 3. Lipase activity on Tween 80 Agar (precipitate), arginine dihydrolase reaction and levan production from sucrose test for confirmation of *Ralstonia solanacearum*.

S. No	Inoculums	Lipase activity on Tween 80 Agar (precipitate)	Arginine dihydrolase reaction	Levan production from sucrose
1.	RS-STM1	+	-	-
2.	RS-STM2	+	-	-
3.	RS-STM3	+	-	-
4.	RS-STD1	+	-	-
5.	RS-STD2	+	-	-
6.	RS-STEM1	+	-	-
7.	RS-STEM2	+	-	-
8.	RS-STED1	+	-	-
9.	RS-STED2	+	-	-
10.	RS-STED3	+	-	-
11.	RS-VPM1	+	-	-
12.	RS-VPM2	+	-	-
13.	RS-VPD1	+	-	-
14.	RS-VPD2	+	-	-
15.	RS-VPD3	+	-	-
16.	RS-RM1	+	-	-
17.	RS-RM2	+	-	-
18.	RS-RM3	+	-	-
19.	RS-RD1	+	-	-
20.	RS-RD2	+	-	-
21.	RS-SDM1	+	-	-
22.	RS-SDM2	+	-	-
23.	RS-SDM3	+	-	-
24.	RS-SDD1	+	-	-
25.	RS-SDD2	+	-	-
26.	RS-SM1	+	-	-
27.	RS-SM2	+	-	-
28.	RS-SD1	+	-	-

+ Positive
- Negative

Kovac's Oxidase Test

In Kovacs oxidase test positive isolates produce purple color when mass of bacterial growth is rubbed on filter paper impregnated with oxidase reagent. This test is used for differentiation between aerobic and anaerobic bacteria (Kovacs, 1956). In our studies, the tested isolates showed variable response in development of color. Isolates RS- STM1, RS-STM2, RS-STM4, RS-STEM3, RS-STEM4, RS-VPM1, RS- VPM2, RS- VPM3 and RS-VPM5 developed purple color within 10 sec and were categorized as positive for this reaction, while isolates RS-STM3, RS- STEM1 and RS- VPM4 developed color in 60 sec and were categorized as delayed positive. Isolates RS-STM5, RS- STEM2 and RS- STEM5 were oxidase negative as they did not develop purple color (Table 2).

Kovac`s oxidase test is particularly important for differentiation of the Gram-negative bacteria. *Ralstonia solanacearum* gives a positive reaction. This test is used to detect the presence of cytochrome C oxidase (oxidase positive) (Lelliott & Stead, 1987).

Arginine dihydrolase test (Thornley, 1960): In this test color change from purple to red is considered as positive, while isolates in which color change is not evident are considered as negative for the reaction. All non-

fluorescent isolates were found negative for the reaction except isolates. This test is mainly of importance for the differentiation of the *Pseudomonas*, both fluorescent and non-fluorescent.

Bacteria differ in their ability to produce ammonia from arginine under anaerobic conditions. Color change is an evidence of alkaline pH change under gas seal. Among *P. seudomonadacae* there is a clear distinction between those species which give a positive reaction such as *P. aeruginosa* and *P. fuorescens* and those which are unable to produce ammonia from arginine under anaerobic conditions such as *P. syringe*, *P. anthropogonis* and *Ralstonia solanacearum* (Thornley, 1960).

This is a test for the presence of two enzymes that permit certain *Pseudomonas* to grow under anaerobic conditions. These enzymes generate ATP by the conversion of arginine to ornithine with the generation of CO_2 and NH_3. It is the alkaline reaction of NH3 production that is detected by the test.

Production of fluorescent pigment: All tested isolates were found fluorescent while remaining behaved as non-fluorescent. Presence of green, diffusible fluorescent pigment was evident in fluorescent strains, whereas non-fluorescent cultures produced brown diffusible pigments which are the characteristic of *Ralstonia solanacearum* strains (Fig. 2).

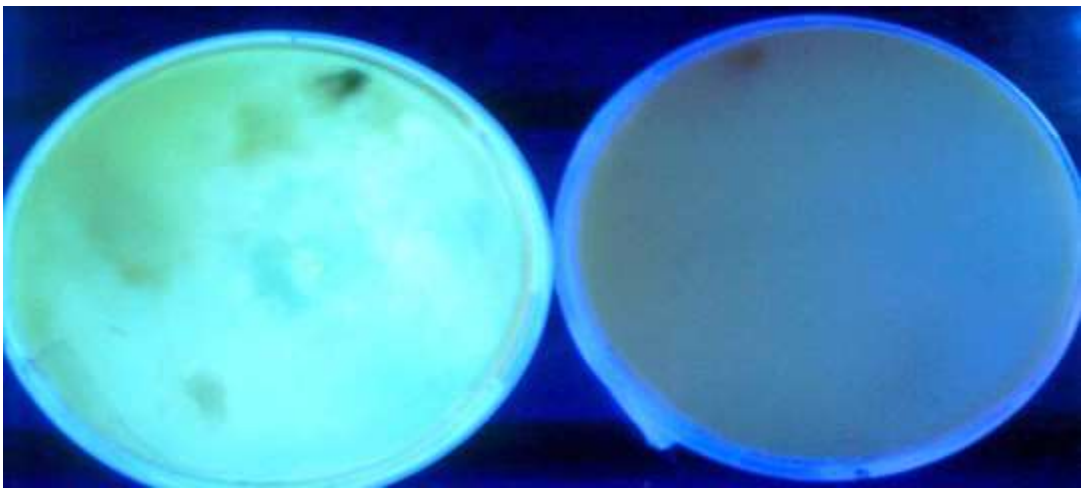

Fig. 2. Production of Fluorescent Pigment under a UV in the Plates inoculated with bacterium for 48 hrs at 28-30°C.

Siderophores are synthesized according to demand (iron starvation) act as high –affinity chelators for ferric ions (Neilands, 1974). They are part of a complex iron acquisition system which also comprises membrane receptors, reductases and digestive enzymes, and which is tightly regulated (Neilands, 1982; Neilands, 1984; Ecker *et al*., 1986).

In aerobic environments the concentration of free ferric ions usually is too low to support growth (Maximal 10-17 M at pH 7, Neilands 1984), due to the precipitation of iron in form of oxy-hydroxy.

The production of specific siderophores may enable a plant pathogenic bacterium to grow within its host plant. Excretion of siderophores with a very high affinity to iron may thus be a pre-condition for the development of any bacterial disease, or in other words, a virulence factor.

In *Pseudomonas*, several different kinds of pigments are found, the most important diagnostically being the green, water-soluble, fluorescent pigments (pyoverdines) produced on iron-deficient media. These pigments fluoresce most strongly under ultra-violet radiation of long wave length.

Oxidation-fermentation of glucose: All tested isolates were found positive for oxidation of glucose .There was a gradual pH change at the surface of the open tubes resulting in color change from green to yellow, no pH change was observed in sealed tubes.

Most bacterial plant pathogens are oxidative (respiratory) however, members of Pseudomonaceae such as genus *Pseudomonas* and *Ralstonia* are oxidative in nature as compared to members of family Enterobacteriaceae such as Erwinia which are fermentative bacteria (Hugh & Leifson, 1953; Hayward, 1964).

Bacteria utilize glucose and other carbohydrates using certain metabolic pathways. Some are oxidative routes but others involve a fermentative reaction. The oxidation-fermentation test is also known as the "oxferm" test is used to determine which route is used. This test is used to differentiate between species, especially Gram-negative rods.

Oxidative organisms can only metabolize glucose or other carbohydrates under aerobic conditions i.e. oxygen is the ultimate hydrogen acceptor. Other organisms ferment glucose and the hydrogen acceptor is then another substance e.g., sulphur. This fermentative process is independent of oxygen and cultures of organism may be aerobic or anaerobic.

In aerobic respiration pyruvate is oxidized to CO_2 and H_2O however, under anaerobic conditions, pyruvate cannot be oxidized; instead it undergoes fermentation and yields lactic acid or alcohol. Because the main process of energy is cut off (Agrios, 1997).

Several selective media for isolation from latently infected material or difficult substrates like soil, waste, or surface water have been described. SMSA medium as modified by Elphinstone *et al*., (1996) has been used successfully in Europe (e.g. Elphinstone *et al*., 1998; Wenneker *et al*., 1999). Isolates of *R. solanacearum* rapidly loose virulence when maintained on laboratory media; however, the organism can easily be maintained for years in sterile distilled water or on agar slants covered

wit h sterile mineral oil and stored at room temperature (Shew & Lucas, 1991). Isolation from symptomatic material can easily be performed by using YPGA medium non-selective medium or Kelman's tetrazolium medium. In some cases when secondary infections are present, isolation on selective media is necessary. A presumptive test in the field can be the water-streaming test as described under disease symptoms or a serological agglutination test using a field kit in the form of a lateral flow device (Danks & Barker, 2000).

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