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BIOCHEMICAL CHARACTERIZATION OF *RALSTONIA SOLANACEARUM* CAUSING BACTERIAL WILT OF CHILLI AND *IN- VITRO* EVALUATION OF BIO AGENTS, ANTIBIOTICS AGAINST PATHOGEN

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ABSTRACT
Bacterial wilt of chilli caused by *Ralstonia solanacearum* (E.F. Smith) Yabuuchi, is one of the most rigorous pathogen on solanaceous crops with a very wide host range. It is very difficult to control because of soil borne nature. The present studies were conducted by collection and isolation *R. solanacearum* from chilli field of Bagalkot. The isolation and the initial characterization of strains were carried out by using 2, 3, 5-triphenyltetrazolium chloride (TTC) which differentiate the virulent and avirulent colonies. Biochemical test was conducted, Positive reaction are obtained in all tests conducted such as gram staining, potassium hydroxide loop test, catalase oxidase test, kovacs oxidase test, levan production from sucrose, Lipase production and utilization of sugars *viz.,* lactose, maltose, sorbitol and mannitol. The bio agents such as *Bacillus spp* and *Pseudomonas spp* were expressed the suppression of pathogen under *in-vitro* and combination of Streptocycline and Copper oxychloride has got maximum inhibition among the entire antibiotic used in present study.

INTRODUCTION

Chilli (*Capsicum annum*) is one of the most popular vegetables in the India and world. It is the second most important vegetable crop. The India production was about 1,687,330 MT green Chillies produced on 1,40,040 ha in 2015 (Anonymous, 2015). Chilli crop has suffering from several biotic and abiotic stresses in its growing season. Among those stresses, bacterial wilt of Chilli caused by *R. solanacearum* (Yabuuchi *et al.,*1995) is one of the most devastating and wide-spread diseases of crops worldwide (Poussier *et al.,* 1999). *R.solanacearum* infects more than 200 species in 50 families (Hayward, 1991), including tomato, potato, eggplant, pepper, tobacco, banana, and peanut (Swanson *et al*. 2005; Ji *et al*. 2006) but causes colossal losses in chilli production (Begum, 2012). The identification of this pathogen was also carried out in Brazil from bell pepper (Garcia *et al.,* 2013) and its presence in hot and sweet pepper was reported by Begum *et al*. (2012). This aforementioned reason therefore makes this soil-borne pathogen difficult to control.

It is a Gram negative, aerobic, motile and rod shaped bacterium belonging to genus Ralstonia, β-subdivision of the class Proteobacteria (Yabuuchi *et al.,* 1995). Distinct geographical distribution of biovars and races of *R. solanacearum* suggest separate evolutionary origins among the strains. Race 1 strains can be found in humid areas throughout the world and attack many solanaceous crops *i.e.* pepper, tomato, and eggplant. It also attacks tobacco and many plants of other families. It requires an optimum temperature as high as 35°C. Strains of Race 2 attack banana and Heliconia and can be found mostly in hot places of South America. Race 3 strains are found at upper altitude in the tropics, subtropical and moderate areas. This race mostly attack potato and, some solanaceous weeds. Race 3 has a lesser optimum temperature as 27°C and appears mainly as biovar 2. Strains of Race 4 are mainly destructive on ginger, while strains of Race 5 (biovar 5) are particular on *Morus spp.* (French *et al.,* 1995).

At present, bacterial wilt caused by *R. solanacearum* is extending its host range, aggressiveness and is posing a serious threat to crop production (Begum, 2005; Begum , 2011). Currently this disease problem is becoming intense and is wreaking havoc to chilli production in many parts of India and Karnataka. The conditions during monsoon seasons succumb chilies to bacterial wilt, but this problem is often concealed and mystified with other disease problems. Based upon this study was planned to study the pathogenic and

virulent behavior of collected *R. solanacearum* sample to characterize and differentiate the most virulent/aggressive colonies or strains of *R. solanaceraum* based on morphological, cultural, pathological, biochemical characters. The paper deals with the efficacy of antibiotics under *In-vitro* condition along with isolation of bio agents from collected soil sample from chilli field.

MATERIALS AND METHODS

Collection of samples

Collection of chilli samples which is infected by *R. solanacearum* cause bacterial wilt in chilli plant and were collected from major chilli growing area of Bagalkot.

Isolation of the bacterial pathogen and maintenance

The isolates of *R. solanacearum* were isolated in Nutrient Agar (NA) plate from the wilted chilli areas by streaking the bacterial ooze streamed out into the water from the infected stem (Figure 1). The plates were then incubated at 28°C for at least 24 h.

Identification of virulent and avirulent isolates by TTC media

TTC media (Hugh and Leifson, 1953) were used for isolation and maintainance of bacterial pathogenic isolates. TTC media (one liter) contained Peptone (10gm), Casein Hydrolysate (1.0gm), Glucose (5.0gm) and Agar (15gm), In TTC media 5 ml of 1% 2, 3, 5- triphenyltetrazolium chloride was added to the sterilized medium before pouring into the plates.

Pathogenicity test (Koch's postulates) of *Raslstonia solanacearum*

Koch's postulates were followed to prove pathogenic nature of *R. solanacearum* isolate given by Rahman, 2015. For proving pathogenicity soil drenching method of inoculation (Artal, 2012) by using 10^8 cfu/ml of inoculum.

Biochemical tests for confirmation of *R. solanacearum*:

Isolates were studied according to specific biochemical tests for *R. solanacearun* i.e., gram staining (Schaad, 1980), potassium hydroxide loop test (Suslow *et al.,* 1982), catalase oxidase test (Schaad 1980), kovacs oxidase test, levan production from sucrose (Schaad, 1980), Lipase production (Sierra 1957), production of fluorescent pigment (King *et al.,* 1954) and utilization of sugars *viz.,* lactose, maltose, sorbitol and mannitol (Hayward 1964).

Collection of rhizosphere soil and isolation of antagonistic bio-agents

Soil sampling

The soil samples were collected randomly from the rhizosphere of chilli growing field. The sterilized polythene bags were used for sampling and the collected soil immediately proceeded for isolation of bio agents through serial dilution technique.

In-vitro assay of bio-agents and antibiotic against *R. solanacearum*

The bio agents and antibiotics are evaluated to find out the effective management procedure. The *In-vitro* antibiotic assay was done by using completely randomized design (CRD) with two replication having seven treatments and filter disk paper technique are used in antibiotic study. The list of antibiotics used to find the efficacy is given in table 3. The streak methods are fallowed to analyze bio agents. A scale was used for grouping the bio-agents based on per cent inhibition of the growth of *R. solanacearum* which is given in Table 1.

RESULTS

Isolation of the pathogen and maintenance of pure culture

Isolation was done by streaking a loopful of flowing ooze containing the bacteria on to sterile nutrient agar medium which yield well separated, typical, yellow, mucoid, colonies of bacterium on nutrient agar medium after 72 hours of incubation at 30°C and pure colonies obtained were further streaked on to the nutrient medium kept for incubation at 30°C for 72 hours (Figure. 2).

Virulent and avirulent colonies Identification

The virulent (colonies with pink colour center or characteristic red center and whitish mucilaginous margin) and avirulent (smaller, dark red colonies) strains colonies of *R. solanacearum* were identified in Triphenyl Tetrazolium Chloride (TTC) medium containing 0.005% TTC (Kelman (1954) in figure 2 and 3. This will help finding the virulent and avirulent strains. Serial dilution of *R. solanaciarum* on TTC medium was carried out and showed in Figure. 4.

Proving pathogenicity (Koch's postulates)

Koch's postulates were followed to prove pathogenic nature of *R. solanacearum* isolate. For proving pathogenicity soil drenching method of inoculation (Artal 2012). Eighteen days old seedlings of chilli were used for inoculation. Before inoculation, the plants were starved for 24 h by avoiding watering. The characteristic symptoms were observed on chilli plants after ten to fifteen days of inoculation. Re-isolations were carried out from these infected plant and comparisons were made with original culture to confirm the identity of the

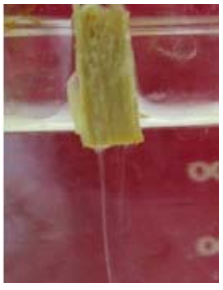
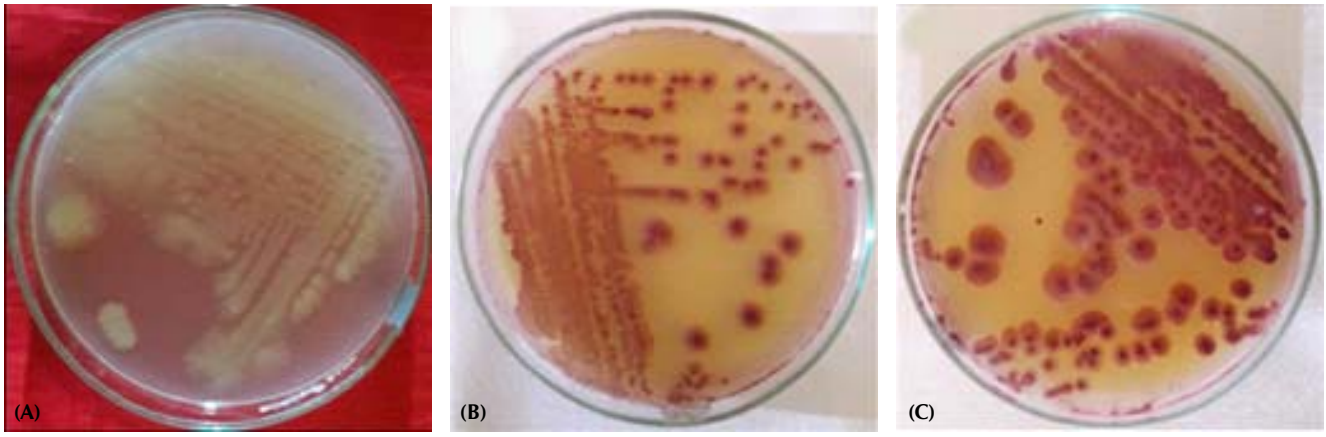


Figure 1: oozing of bacterial cells from *R. solanacearum* infected stem

Table 1: Scale used for grouping of bio-agents based on per cent inhibition of the growth of *R. solanaciarum*.

Sl.No	Per cent inhibition of growth of *R. solanaciarum* by bio-agent	Grade
1	>90	I
2	76-90	II
3	51-75	III
4	26-50	IV
5	1-25	V
6	0	VI



Legend: (A) NA medium and (B& C) TTC medium

Figure 2: Streak method of isolation of *R. solanaciarum* on NA and TTC medium

Table 2: Identification, biochemical characterization of *R. solanacearum*

Biochemical test	Reaction
Gram's stain test	+
Potassium hydroxide solubility test	+
Catalase oxidase test	+
Kovac's oxidse test	+
Lipase production	+
Levan test	+
Sugar fermentation test	
Lactose	+
Maltose	+
Sorbitol	+
Mannitol	+

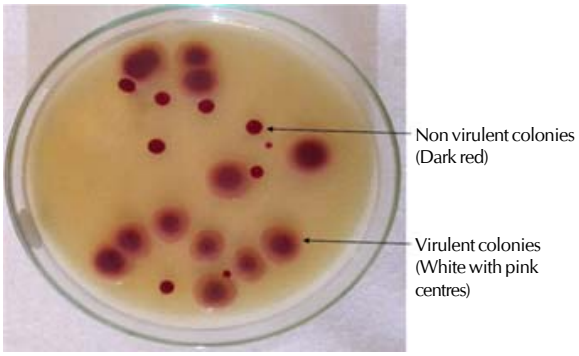


Figure 3: Differentiation of *R. solanaciarum* virulent and non virulent colonies on TTC medium

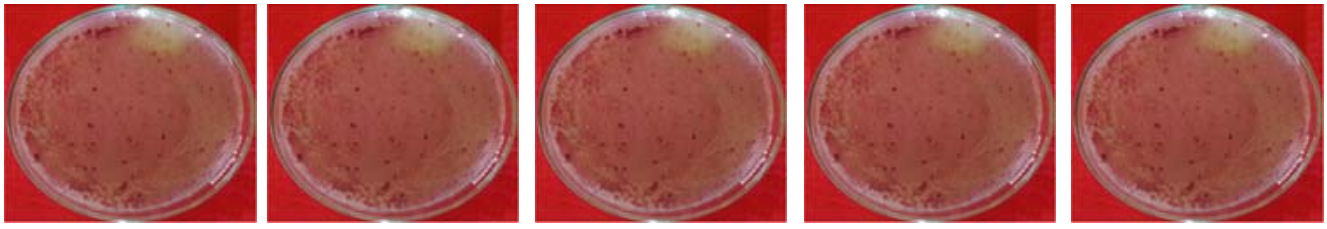


Figure 4: Serial dilution of *R. solanaciarum* on TTC medium

Table 3: Effect of antibiotics against *R. solanaciarum* under *in vitro* condition

	Antibiotics	R1	R3	Total	Mean
T1	2-Bromo-2 Nitro propane 1,3-dial (Bactirinash 200- 500ppm)	17.00	17.92	34.92	17.46
T2	2-Bromo-2 Nitro propane 1,3-dial (Immuno modulater- 500ppm)	17.36	17.92	35.28	17.64
T3	Copper oxy chloride(Blue copper-500ppm)	14.50	14.82	29.32	14.66
T4	Copper oxy chloride(Blitox-500ppm)	14.81	15.02	29.83	14.92
T5	Steptocyclin Sulphate(Caynamisin- 500ppm)	18.20	18.53	36.73	18.37
T6	Steptocyclin Sulphate(K-Cycline 500ppm)	17.90	18.12	36.02	18.01
T7	Streptocycline 500ppm + Copper oxychloride 0.3%)	21.52	21.60	43.12	21.56
Total	121.29	123.93	245.22		
Mean	17.33	17.70	GM	17.52	
S Em ±		0.14	CF	4295.2	
C D 5%		0.49	Treatments	7	
1%		0.74	Replications	2	

Legend: T-Treatments, R- Replications, GM- Grand Mean

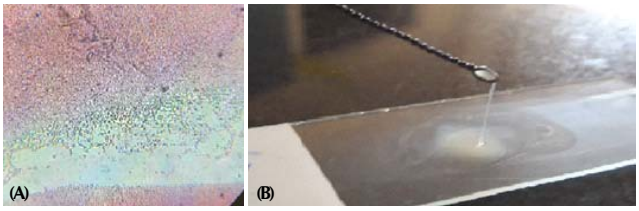


Figure 5: Figure showing Gram staining reaction and KOH of Gram-ve *R. solanacearum*

pathogen. The re-isolated culture resembled the original mother culture and thus pathogenicity test (Koch's postulates) was confirmed.

Biochemical characterization of *Raslstonia solanacearum*

Gram's stain test

The Gram's staining reaction were performed using crystal violate. The microscopic results showed that *R. solanacearum* retained counter stain (pink colour) did not retain violet colour which shows that it is a gram negative bacteria (table 2 and Figure 5).

Potassium hydroxide solubility test

The gram negative test of *R. solanacearum* was also confirmed by KOH test (table 2 and Figure 5). The positive test indicated by an elastic thread or viscious thread observed when loop raised from the bacterial solution by inoculation loop a few centimeters from glass slides. It is an easier and faster technique of distinguish bacteria by Suslow *et al.* (1982).

Catalase oxidase test

Catalase oxidase test found positive by seeing the bubbling which is due to evolution of O_2 gas by break down of hydrogen peroxide (H_2O_2) in to H_2O and O_2 (table 2).

Kovac's oxidse test

The oxidation ability of *R. solanacearum* was tested by using Kovac's oxidse test. The result showed pathogen can able to form deep blue color with oxidase reagent (table 2).

Levan test

Levan was performed in NA medium containing 5% sucrose, levan sucrase, which catalyzes the synthesis of levan form sucrose, is produced by a number of bacteria including *R. solanacearum*. The result showed that *R. solanacearum* able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 2). Ghaly *et al.* (2007) reported the production levan in *Bacillus licheniformis* and observe the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose.

Lipase production

Positive lipase activity observed *R. solanacearum* showed hydrolysis of Tween 80 as an esterase activity, because the substrate is freely soluble in water and precipitated around colonies. Sierra (1957) showed similar type of results (table 2).

Sugar fermentation test

The isolates of *R. solanacearum* is able to oxidize the sugars which are indicated by colour change. The results showed that pathogen can able to oxidize the Dextrose, sucrose, manitol and lactose by producing acid and gas (Table 3). Shahbaz *et al.* (2015) reported The acid production in sugar fermentation test by *R. solanacearum* 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 colour (table 2).

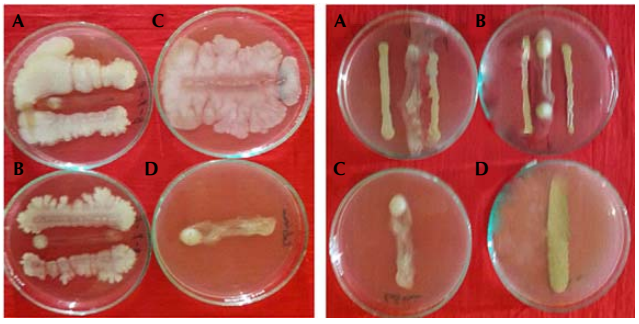


Figure 6: Figure showing the inhibition of bio control against *R. solanaciarum*

Isolation of antagonistic bio-agents from rhizosphere soil

Bio agents were isolated by serial dilution technique. Based on the different morphological characters and response to the UV light, the colonies were purified. Two bio agents were isolated *i.e. Bacillus spp* and *Pseudomonas spp.*

In-vitro* antagonistic efficacy of bio-agents and antibiotic against *R. solanacearum

In-vitro* efficacy of bio agents against *R. solanacearum

The bio agents *i.e. Bacillus spp* and *Pseudomonas spp* were tested against *R. solanacearum* to know the antagonistic efficacy through cross streak method under *in vitro* condition (Figure 6). Both tested bio agents were expressed the suppression of *R. solanacearum* colonies under *in-vitro* condition. Later both tested bio agents were categorized as grade II based on the scale as mentioned in table 1.

In vitro* efficacy of antibiotics against *R. solanacearum

Total seven different commercial available antibiotics are analyzed under *In-vitro* condition against *R. solanacearum*. To find out the effectiveness antibiotics to manage the chilli wilt Figure 7. Among the tested antibiotics copper oxy chloride contain commercial products such as blue copper and blitox showed least inhibition of pathogen *i.e.* 14.66 and 14.92 per cent respectively. Bacteriostasis action containing antibiotics such as 2-Bromo-2 Nitro propane 1,3-diol (Bactirinash 200-500ppm) and 2-Bromo-2 Nitro propane 1,3-dial (Immuno modulater-500ppm) has showed 17.46 and 17.64 per cent respectively. Protein synthesis inhabiting antibiotics such as steptocyclin sulphate (Caynamisin-500ppm) and steptocyclin sulphate (K-Cycline 500ppm). Maximum inhibition of 21.67 mm recorded in streptocycline 0.5g (500ppm) + copper oxychloride 3.0g (3000ppm) per liter of water and which found significantly superior than all the antibiotics (Table 3, Figure. 8).

DISCUSSION

Cultural traits on different media are important tool for their identification. For *R. solanacearum,* these are best studied.

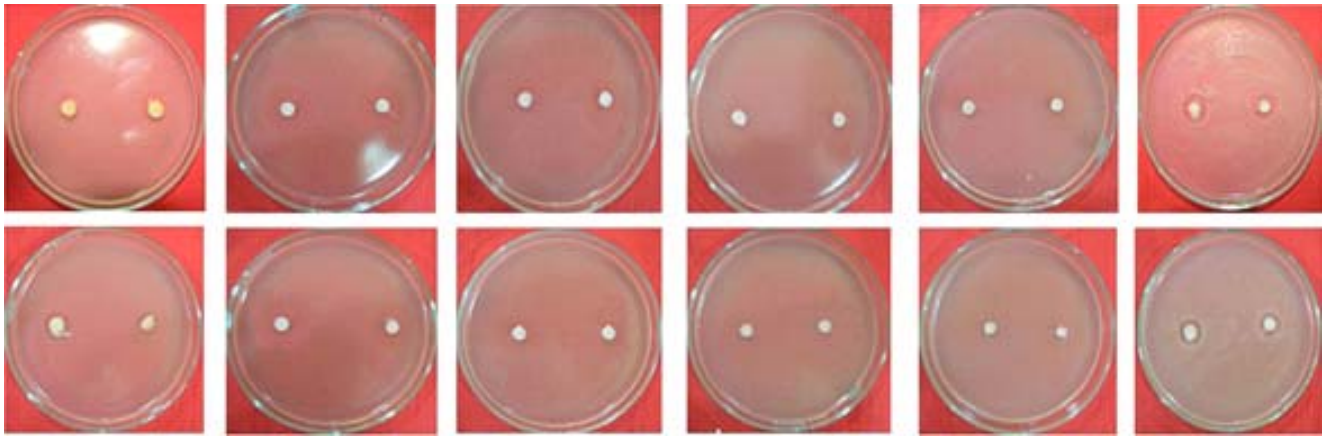


Figure 7: Effect of antibiotics against *R. solanaciarum* under *in vitro* condition

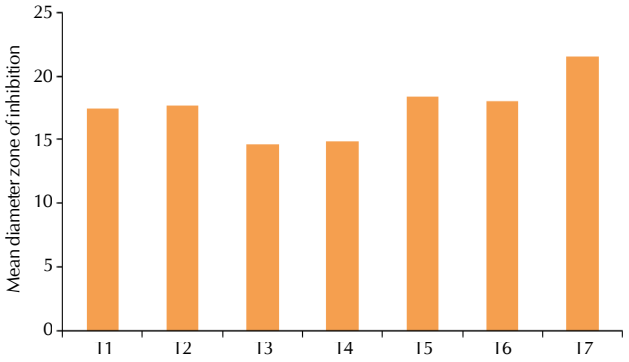


Figure 8: Effect of antibiotics against *R. solanaciarum* under *in vitro* condition

Virulence and avirulence of an isolate can be resolute on the basis of colony traits by using TTC medium. Fluidal colonies with reddish pink center and irregular margins are usually virulent while dark coloured non-fluidal colonies with darker red colours and smooth margins are normally avirulent. These results are in harmony with the findings of Rahman, 2010; Shahbaz, 2015. Gram reaction by staining is a necessary initial step for the identification and classification of bacteria present study found gram negative nature of bacteria and it have fragile cell walls, which are bound by an outer membrane. This membrane is readily disrupted on exposure to 3% KOH releasing the viscous DNA (Suslow *et al.,* 1982, Rahman, 2010; Shahbaz, 2015). The *R. solanacearum* bacteria produced gas bubbles when these were mixed with a drop of H_2O_2 on glass slide, it gives a clue for presence of aerobic bacteria (Schaad, 1980). In Kovacs oxidase test positive isolates produced purple colour when mass of bacterial growth is rubbed on filter paper impregnated with oxidase reagent. Similar results found by (Kovacs, 1956). The *R. solanacearum* colonies of Levan positive cultures produced colonies that were raised convex and mucoid (Schaad, 1980). According to Sierra (1957) hydrolysis of Tween 80 is a sign of lipolytic activity or, more correctly, esterase activity, because the substrate is freely soluble in water and present pathogen showed positive reaction. Efficacy of bio agents *Bacillus spp* and *Pseudomonas spp* shows suppression against tested pathogen. *In-vitro* antibiotic assay, maximum inhibition of 21.67 mm recorded in streptocycline 0.5g (500ppm) + copper oxychloride 3.0g (3000ppm) per liter of water and which found significantly superior than all the antibiotics. Toppo1 and Tiwari, 2015 and Mallesh and Lingaraju, 2015 conducted *In-vitro* studies to find management. Wilt incidence varied due to the species complex of the pathogen at molecular level and various soil factors. The findings of the present study will step forward in determination of the population structures of *R. solanacearum* to design an effective molecular based analysis of *R. solanacearum* with special emphasis on its integrated management.

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