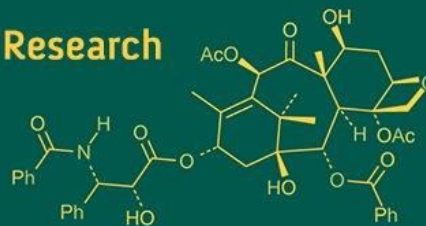
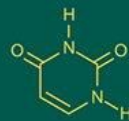
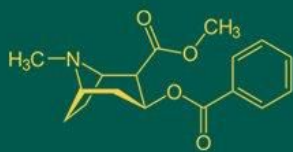


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Biochemical diversity profiling of *Ralstonia solanacearum* (L.) isolates causing bacterial rhizome rot of ginger (*Zingiber officinale* Rosc.) under *in vitro* conditions

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Abstract

Ginger (*Zingiber officinale* Rosc.) is a commercially important spice and medicinal crop and it is affected by serious bacterial rhizome rot disease caused by *Ralstonia solanacearum* (L.). The present investigation was undertaken for biochemical characterization of ten isolates of *R. solanacearum* inducing bacterial rhizome rot of Ginger which collected from different locations under *in-vitro* experiments. Isolates were selected based on morphological, cultural, and biochemical properties and pathogenicity. For these evaluations, all isolates were grown on TZC medium and maintained at 28 ± 2 °C for a period of 48-72 hr. Various biochemical tests such as acid production from various sources of carbohydrates, starch hydrolysis, Gelatin liquefaction, catalase production, urease activity, hydrogen sulphide production, gas formation, KOH solubility and Grams staining tests were conducted to evaluate the biochemical diversity and metabolic activity of *R. solanacearum*. In acid production test, all isolates showed gradual increase over time by change in color of medium. Dextrose and sucrose found suitable acid production than maltose. In starch hydrolysis test out of ten isolates, eight isolates are able to hydrolyse the starch by showing zone formation. Hydrogen sulphide test revealed that, as the time increases few isolates are able to produce strong positive reaction. In gelatin liquefaction test, at early stage all isolate unable to show the gelatinase activity and after 78 hr few isolates showed strong enzymatic activity. In gas production test, none of the isolates showed positive reaction in 24hr, however after 72 hr all isolates demonstrated gas production except control. In urease production test, some isolates form the pink colour in broth media after 48hr and all isolates showed positive reaction after 78hr by producing ammonia. During grams staining reaction all the isolates showed negative reaction due to thin peptidoglycan layer unable to retain the stain. The formation of slime threads occurs due to the release of cellular DNA upon lysis of Gram-negative bacterial cells in the presence of 3% KOH solution. Catalase test was positive for all the isolates except RS-4. The study aids to contribute baseline data for accurate identification, comparison with known strains, and to support future molecular and epidemiological studies for effective management of bacterial wilt in Ginger.

Keywords: Ginger, biochemical diversity, rhizome rot, variability and *R. solanacearum*

1. Introduction

As a high value commercial crop, Ginger (*Zingiber officinale* Rosc.), belongs to the family Zingiberaceae and is primarily cultivated for its aromatic rhizomes, which are used both as a spice and in medicinal applications (Sharma *et al.*, 2010) [30]. The rhizome is versatile in its uses; it is consumed fresh, as a dried powder, in preserved forms like syrups and candies (crystallized ginger), and as a flavoring in beverages. In India, a leading global producer responsible for approximately one third of the world's supply, ginger is widely grown across tropical and subtropical regions (Tarafdar and Saha, 2007) [32]. Its global cultivation history is long, with records indicating its introduction to Ethiopia as early as the 13th century (Jansen, 1981) [13]. Apart from its culinary value, Ginger holds a significant value in traditional medicine, where it has been used to treat many ailments such as influenza, cold, cough and stomach pain (Jansen, 1981; Girma and Digafie, 2004) [13, 8].

Ginger crop is pretentious by many biotic and abiotic stresses that badly influence its growth, quality and yield. Among the biotic stresses, several diseases caused by bacteria, fungi, nematodes, and viruses are of considerable economic importance.

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These pathogens not only affect the crop during the growing period but also cause substantial post harvest losses during storage (Dohroo, 2005; Nirmal Babu *et al.*, 2013) [6, 20]. Major diseases reported in Ginger include soft rot, bacterial wilt, leaf spot, yellows, and nematode infestations, all of which significantly limit production and degrade rhizome quality (Anandaraj *et al.*, 2008) [2].

Among these, bacterial wilt or rhizome rot caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is considered as most destructive disease in Ginger worldwide. The pathogen is a soil borne, Gram-negative bacterium with a wide host range, infecting several solanaceous and zingiberaceous crops (Kelman, 1953; Hayward, 1991) [14, 10]. The epidemiology, the disease is more severe under warm and humid conditions, which are congenial for rapid multiplication and survival of the bacterial pathogen in soil and planting materials (Hayward, 1994) [11].

The bacterial rot of ginger is symptomatically characterized by yellowing of leaves initially followed by wilting of whole plant. Decaying and rhizome softening is the typical symptom leads to gradual death. Foul smell coming out from the infected rhizome is peculiar symptom. The pathogen enters through physical or mechanical wounds or natural openings in the roots and colonizes the vascular bundle, which leads to blocking of water and nutrient translocation and wilting symptoms (Sarma *et al.*, 2015; Gopalakrishnan *et al.*, 2017) [27, 9].

Ralstonia solanacearum shows high genetic and physiological variability, resulting in wide range adaptability and persistence in different environmental conditions (Fegan and Prior, 2005) [7]. Hence, biochemical characterization of the *R. solanacearum* is crucial to understand its physiological behavior, which aids in proper identification, and further supports in molecular and pathogenicity studies for developing effective disease management practices (Saddler, 2005; Kumar *et al.*, 2019) [26, 17].

Biochemical characterization of a microorganism is the preliminary approach and serves as a reliable to accurate identification and to differentiate bacterial strains based on their physiological metabolism and biochemical profile (Denny & ayward, 2001) [5]. The main biochemical experiments such as Acid production by carbohydrates, Starch hydrolysis, Hydrogen sulphide and gas production, Gelatin liquefaction, KOH solubility test, Urease and Catalase test and gram staining provide valuable insights into the pathogenic potential and adaptability of the bacterium to a specific ecological condition (Hayward, 1991; Schaad *et al.*, 2001) [10, 28].

In this regard, the present investigation was conducted with an aim to isolate and characterize *Ralstonia solanacearum* from ginger rhizomes affected by bacterial rot disease, with a specific focus on biochemical profiling. This work helps in developing essential baseline data for the precise and accurate identification of the pathogen and its comparison with existing strains.

2. Material and Methods

This present investigation on *Ralstonia solanacearum* causing bacterial rhizome rot of Ginger with respect to isolation and biochemical variability of was carried out in laboratory of Plant Pathology, College of Agriculture, University of Agricultural and Horticultural Sciences, Shivamogga, Karnataka. The materials and methodology applied during this research were described here.

2.1 Collection of Rhizome Samples

A systemic roving field survey was implemented in major Ginger growing areas of Karnataka and infected rhizome samples which exhibiting typical symptoms of bacterial rot including yellowing leaves, wilting of plant, and rhizome softening or rot were collected for isolation of pathogen. *Ralstonia solanacearum*, causal agent, was isolated by following the protocol of Kelman (1954) [15] using Tetrazolium Chloride (TZC) agar medium.

The base medium was standardized using the ingredients of peptone (10 g), dextrose (10 g), casein hydrolysate (1 g), agar (18 g), and distilled water (1000 ml). Medium was sterilized by autoclaving at 121 °C for 15 minutes after preparation. Immediately after sterilization and prior to pouring the medium into Petri plates, a filter-sterilized 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (TTC) was incorporated at a volume of 500 µl. Incorporation of TTC is very crucial, as it acts as a differencing agent that distinguishes virulent and avirulent colonies of *R. solanacearum* through distinct morphological and pigmentation characteristics.

2.2 Identification of Bacterial Isolates

The isolate was confirmed as *R. solanacearum* through a polyphasic approach, integrating morphological, cultural, and biochemical analyses as described in previous literature (Kelman, 1954; Buddenhagen *et al.*, 1962; Schaad, 1992; Chaudhry and Rashid, 2011) [15, 3, 29, 4].

2.3 Coded names of the Isolates of *R. Solanacearum*

The isolates of *R. solanacearum* collected from different locations were coded as given below. During the survey, ten isolates of *Ralstonia solanacearum* were collected from different major Ginger growing regions of Karnataka. The isolates were designated based on their place of collection as follows: RS-1 from Shivamogga, RS-2 from Sirsi, RS-3 from Hassan, RS-4 from Chikmagalur, RS-5 from Bidar, RS-6 from Shikaripura, RS-7 from Sagara, RS-8 from Hosnagara, RS-9 from Siddapura, and RS-10 from Sakaleshpura.

2.4 Maintenance of Isolates on Kelman's TZC Agar Medium

The isolated colonies of *Ralstonia solanacearum* grown on Kelman's Tetrazolium Chloride (TZC) medium exhibited characteristic morphological features that aided in their preliminary identification. After incubation at 28±2 °C for 48-72 hours, typical virulent colonies appeared as fluidal, irregular, and white with a distinct pink to light red center due to the reduction of 2,3,5-triphenyl tetrazolium chloride. In contrast, avirulent or less virulent colonies were small, dry, and uniformly red without fluidal margins. All ten isolates showed the typical virulent colony morphology, confirming their identity as *R. solanacearum*. These purified cultures were sub-cultured and maintained on TZC slants at 4 °C for further biochemical characterization.

2.5 Biochemical Characters

The biochemical tests such as Acid production, Starch hydrolysis, Production of hydrogen sulphide, Gelatin liquefaction, Gas production, KOH solubility test, Urease test and Catalase test were conducted to know the variability of different isolates. The methodology followed for the

biochemical experiments on biochemical variability as per the method described by (Schaad, 1992)^[29].

2.6 Acid Production from Different Sources of Carbohydrates

The ability of *Ralstonia solanacearum* isolates to produce acid from different carbohydrates was tested using Dye's medium C. Ten millilitres of medium C were dispensed into each test tube and sterilized by autoclaving at 121 °C for 15 minutes. After cooling, filter sterilized carbohydrate solutions maltose, sucrose, and dextrose were added aseptically to the medium at a final concentration of 0.14%. Each tube was inoculated with a loopful of a 24-hour old bacterial culture and incubated at room temperature (28±2 °C) for three days. Acid production was confirmed by a change in the colour of the medium from purple to yellow, indicating carbohydrate fermentation.

The composition of Dye's medium C used for the test was as follows: ammonium dihydrogen phosphate (NH₄H₂PO₄) 0.5 g, dipotassium hydrogen phosphate (K₂HPO₄) 0.5 g, magnesium sulphate heptahydrate (MgSO₄·7H₂O) 0.2 g, sodium chloride (NaCl) 5.0 g, yeast extract 1.0 g, bromocresol purple 0.7 ml of 1.5% alcoholic solution, and distilled water 1000 ml.

2.7 Starch Hydrolysis

Starch hydrolysis test was carried out to check the amylolytic activity of *Ralstonia solanacearum* isolates. Starch agar medium was prepared by dissolving peptone (10 g), beef extract (5 g), soluble starch (2 g), and agar (20 g) in 1000 ml of sterile distilled water, and the pH was set to 7.0. The medium was sterilized by autoclaving and poured into sterile Petri plates. After solidification, the plates were inoculated with a loopful of 24-hour-old bacterial culture and incubated at 30 °C for seven days. Following incubation, the plates were flooded with Lugol's iodine solution and allowed to react for a few minutes. The appearance of a clear zone around the bacterial colony indicated positive starch hydrolysis, whereas the absence of such a zone denoted a negative reaction. The diameter of the hydrolyzed zone was measured in millimeters for each isolate. Lugol's iodine solution was prepared by dissolving iodine (5.0 g) and potassium iodide (10.0 g) in 10 ml of distilled water, followed by dilution to a 1:5 ratio using additional distilled water before use.

2.8 Production of Hydrogen Sulphide

The hydrogen sulfide (H₂S) production test was carried out to determine the ability of *Ralstonia solanacearum* isolates to produce H₂S through the dissimilation of sulfur-containing amino acids such as cystine and methionine. Since ordinary bacteriological peptone contains cystine in sufficient concentration, it served as the substrate for this test. The peptone water medium was prepared using peptone (10.0 g) and sodium chloride (5.0 g) dissolved in 1000 ml of distilled water, with the pH adjusted to 7.0. The medium was dispensed in 5 ml quantities into test tubes and sterilized by autoclaving.

To detect H₂S production, lead acetate test strips were prepared by cutting Whatman No. 1 filter paper into strips measuring 5 × 50 mm. These strips were soaked in a warm, saturated solution of lead acetate, dried, autoclaved, and again dried at 60 °C before use. Each sterilized tube containing the peptone water medium was inoculated with a

loopful of a 48-hour-old bacterial culture. After inoculation, one lead acetate strip was inserted between the cotton plug and the inner wall of the test tube so that it hung just above the broth surface without touching it. The tubes were incubated at 27 °C, and observations were recorded at regular intervals for up to 14 days. The development of black coloration on the lead acetate strip indicated the production of hydrogen sulfide by the bacterial isolate.

2.9 Gelatin Liquefaction

Gelatin liquefaction was tested to determine the ability of *Ralstonia solanacearum* isolates to produce the extracellular enzyme gelatinase, which hydrolyzes gelatin. Gelatin, a protein derived from animal tissues such as skin, ligaments, and bones, is insoluble in cold water but dissolves in hot water (around 37 °C) to form a transparent gel upon cooling. When decomposed by gelatinase-producing bacteria, gelatin loses its gel-forming property, indicating proteolytic activity.

Gelatin liquefaction testing medium was prepared by addition of peptone (10.0 g), beef extract (5.0 g), and gelatin (20.0 g) dissolved in 1000 ml of distilled water and pH was set to 7.0. The prepared medium was sterilized in autoclave followed by pouring into sterile Petri plates and allowed to solidification. Each plate were spot inoculated with a 48-h old bacterial culture and incubated at 30 °C for 48 hours. After incubation, plates were flooded with 0.2% mercuric chloride solution and left to reaction for a few minutes. The appearance of white precipitates around the bacterial growth indicated positive gelatin hydrolysis by confirming the production of gelatinase enzyme by the bacterial isolates.

2.10 Gas Production

The gas production test was carried out check the ability of *Ralstonia solanacearum* isolates to produce gaseous byproducts during carbohydrate fermentation. This test aids in understanding the metabolic activity of the bacterium and its ability to ferment various substrates with the evolution of gas, which is directly associated with pathogenic activity in plant tissues.

For this purpose, nutrient broth was prepared and dispensed in 5 ml quantities into clean test tubes. A small inverted Durham's tube was placed inside each test tube to trap any gas evolved during bacterial growth. The Durham's tubes were completely filled with broth and contained no air bubbles before sterilization. After autoclaving, the broth was allowed to cool and each tube was inoculated with a loopful of the bacterial culture under aseptic conditions. The inoculated tubes were then incubated at 28±2 °C for 3-4 days. The formation of visible bubbles in inverted Durham's tube indicated the gas production while tubes without bubbles confirmed negative means the bacterial isolates did not produce any gas in the nutrient broth.

2.11 KOH Solubility Test

A loopful of a 48-hour old bacterial culture of *Ralstonia solanacearum* from a slant was transferred to a glass slide. Two to three drops of a 3% potassium hydroxide (KOH) solution were added to the sample. The culture was mixed into the KOH solution using a sterile loop for 5-10 seconds. The test was recorded as positive (KOH soluble) if the mixture became viscous and formed a slimy thread that adhered to the loop when it was lifted.

The potassium hydroxide (KOH) solubility test helped determine the Gram reaction of the *Ralstonia solanacearum* isolates. This test supports on the differential susceptibility of bacterial cell walls to alkaline lysis. Gram-negative bacteria, with their characteristic thin peptidoglycan layer is very susceptible to lysis upon vulnerable to a 3% KOH solution. This upheaval releases viscous chromosomal DNA, creating a sticky, stringy consistency when the loop is lifted. On other hand, Gram positive bacteria, prevented by a robust peptidoglycan layer, prevail unaffected and not forming slimy thread.

2.12 Urease Test

The urease activity of *Ralstonia solanacearum* isolates was estimated using urea broth as the test medium. Overnight grown bacterial cultures were inoculated into sterilized test tubes containing urea broth and incubated for 24-72 hours at 28 ± 2 °C. Formation of a pink color in the medium, which indicate positive urease reaction, confirming the ability of the bacterium to hydrolyze urea with the release of ammonia, which increases the pH of the medium. No colour formation indicates negative reaction.

The composition of the urea broth used for the test was as follows: yeast extract (0.10 g), potassium dihydrogen phosphate (KH_2PO_4 , 9.10 g), disodium hydrogen phosphate (Na_2HPO_4 , 9.50 g), urea (20.00 g), and phenol red (0.010 g) dissolved in 1000 ml of distilled water.

2.13 Gram Staining

Place a small drop of sterile water on a clean microscope slide. Remove part of young colony, with a cold, sterile loop, from the agar medium. Smear the bacteria on the slide. The smear should be just discernible. Air-dry and heat-fix the bacteria on the slide by passing the slide four times though a Bunsen flame, but do not overheat it. Flood the slide with crystal violet and set aside for 60 seconds. Rinse under running water. Drain off excess water. Flood with Logo's iodine and set aside for 60 seconds. Wash with 95% ethanol for 30 seconds. Rinse with water. Blot dry. Counter stain with Safranin for 10 seconds. Rinse with water and dry. Examine at 100 x magnification using oil immersion. Gram - negative indicated by appearance of red colour.

2.14 Catalase Test

The catalase test was performed to determine the ability of *Ralstonia solanacearum* isolates to produce the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) into water and oxygen. This enzyme plays a crucial role in protecting bacterial cells from oxidative damage caused by reactive oxygen species generated during aerobic metabolism.

A loopful of 24-48-hour old bacterial culture from a nutrient agar slant was transferred onto a clean glass slide for catalase test. A few drops (20 μl) of readily prepared hydrogen peroxide (3%) solution was added to the bacterial smear. The immediate formation of gas bubbles will indicate the release of oxygen, confirming a positive reaction. While absence of bubble shows as negative result in catalase test resulted that, the isolate didn't have catalase enzyme activity.

3. Results and Discussion

The present study on biochemical profiling of ten isolates of *Ralstonia solanacearum* causing bacterial rhizome rot in

Ginger with a series of biochemical assays were performed. These assays include ability of acid production from various carbohydrates, starch hydrolysis, hydrogen sulfide production, gelatin liquefaction, gas formation, KOH solubility, urease and catalase activity, and Gram staining. The results we observed for each test along with scientific discussion were detailed here.

3.1 Acid Production

The acid production test by different isolates of *Ralstonia solanacearum* in media containing various carbon sources was presented in Table 1, Figure 1 and Plates 1,2,3. The acid production profiles from maltose, sucrose, and dextrose divulge distinct metabolic diversity among the ten isolates over 72 hours. Few isolates, including RS-1, RS-6, RS-7, and RS-10, consistent in showing strong (+++) to very strong (+++++) acid production across all carbon sources, several demonstrated delayed or weaker activity (+). Strains RS-3, RS-5 and RS-8 were slow in metabolizing maltose, showing no primary activity and only medium production by 72 hours. Similarly, RS-2 and RS-4 showed a delayed but increasing reaction to sucrose. For dextrose, isolates RS-4 and RS-9 were initially negative but progressed to medium and strong production. A few isolates, such as RS-8 on maltose and RS-9 on dextrose, were particularly slow to initiate acid production.

Overall, dextrose and sucrose were found to be more effective substrates for acid production compared to maltose, and longer incubation favored increased acidification, confirming the metabolic versatility of *R. solanacearum* isolates.

Similar patterns of variability in acid production among *Ralstonia solanacearum* isolates have been previously reported, reflecting metabolic diversity within the species (Kelman, 1954; He *et al.*, 1983; Hayward, 1991) [15, 12, 10]. These biochemical variations form the basis for biovar differentiation and are widely used as diagnostic tools in the characterization of *R. solanacearum* populations (Priou *et al.*, 2006; Kumar *et al.*, 2015; Saddler, 2005) [23, 16, 26].

The *R. solanacearum* is able to oxidize the sugars which are indicated by colour change (blue to yellow). The results of fermentation test clearly showed that all isolates of *R. solanacearum* was obtained from the wilted Ginger rhizomes samples were able to oxidize the basic sugars viz., Maltose, Sucrose and Dextrose within three days but however, utilization time varied among all the different isolates for these sugars, the oxidation reaction was indicated by the change of colour. The results revealed that, change of colour blue to yellow colour indicating the oxidation of sugars by bacterial isolates. All these results are in conformity with the findings of Rahman *et al.* (2010) [24] and Anand (2014) [1].

3.2 Starch Hydrolysis

The results of starch hydrolysis by ten isolates of *Ralstonia solanacearum* are presented in Table 2 and Plate 4. None of the isolates showed a positive reaction after three days of incubation, indicating the absence of early starch degradation. However, after seven days of incubation, eight isolates (RS-1, RS-3, RS-4, RS-5, RS-6, RS-7, RS-8, and RS-9) exhibited a positive reaction, as evidenced by clear zones around the bacterial growth, with hydrolysis zones ranging from 6 mm to 24 mm in diameter. Among these, isolate RS-6 recorded the highest zone of starch hydrolysis

(24 mm), followed by RS-1 (22 mm) and RS-9 (21 mm). Interestingly the isolates RS-2 and RS-10 remained negative even after seven days of incubation, indicating their inability to hydrolyze starch.

Starch hydrolysis test results showed that, out of ten isolates, eight isolates were able to hydrolyse starch and these showed significant variation compared to other isolates by distinct zone formation. This starch hydrolysis may be due to the ability to produce higher quantity of extra cellular enzyme amylase, which is mainly involved in the hydrolysis of starch (Lammert, 2007) [18]. The results obtained are in accordance with the previous findings of (Prsanna kumar, 2012) and Anand (2014) [22, 1]. The differences observed in starch hydrolysis across the *Ralstonia solanacearum* isolates indicates clear variability in production of amylolytic enzymes. Those isolates tested positive, this trait signifies an adaptive advantage for breakdown of complex starch into simple sugars, as they can utilize additional energy sources in nutrient scarce conditions.

3.3 Hydrogen Sulphide Production

The production of hydrogen sulfide (H₂S) by the *Ralstonia solanacearum* isolates was monitored over a 72 hour period, the results were presented in Table 3 and Plate 5. Beginning, none of the isolates were produced detectable H₂S within the first 24 hours. After 48 hours, a distinct pattern appeared, isolates RS-1, RS-4, RS-5, RS-6, RS-7, and RS-9 showing medium production. For 72-hour mark, these same six isolates exhibited a strong positive reaction, while isolates RS-2 and RS-8 reached only a medium level of production. This pattern of delayed but increasing H₂S production among certain strains aligns with similar findings reported by Anand (2014) [1].

Hydrogen sulfide production increases gradually over time indicating the active metabolic dissimilation of Sulphur containing amino acids such as cysteine and methionine by *R. solanacearum*. The variability among the isolates in hydrogen sulphide production may be due to differences in enzymatic composition and efficacy of metabolism. The results are in accordance with Kelman (1954) [15], Anand (2014) [1] and Hayward (1991) [10], those reported that, variability in H₂S production among *R. solanacearum* isolates has emphasizing the biochemical diversity of the species.

3.4 Gelatin Liquefaction Utilization

In gelatin liquefaction utilization study, after 24 hours of incubation one of the isolates showed the positive reaction for gelatin liquefaction while after 48 hours eight isolates were able to utilize gelatin liquefaction viz., RS-1, RS-2, RS-4, RS-5, RS-6, RS-7, RS-9 and RS-10. whereas, only two isolates RS-3 and RS-8 were failed to utilize gelatin liquefaction even at 48 h after inoculation (Table 3 and Plate 6). These results are in coincidence with the similar of Chaudhry and Rashid (2011) [4] Jooja and Ingle, (2023) [21] and Anand (2014) [1].

The gelatin liquefaction by some isolates confirms their ability to secrete the extracellular protease gelatinase enzyme. This breaks down gelatin into soluble peptides and amino acids, which explains the loss of gel structure observed in the positive tests. However, the failure of other isolates to cause liquefaction highlights a clear variability in either the secretion or the functional activity of this enzyme

across the different strains. The results of present findings in gelatin liquefaction is a variable trait across the *R. solanacearum* isolates substantiates with the findings of Chaudhry and Rashid (2011) [4] and Anand (2014) [1]. Their experiments also entrenched this characteristic as a useful criterion for differentiating strains within the species.

3.5 Gas Production

A relative study of ten *R. solanacearum* isolates to produce gas was carried out, the results were presented in Table 5 and Plate 7. None of the isolates showed positive sign in gas production after 24 h of inoculation. Meanwhile, at 48 h, seven isolates namely RS-1, RS-2, RS-3, RS-6, RS-7, RS-8, and RS-9 showed positive reactions, depicting onset of fermentation and gas evolution. Finally, after 72 h of incubation, all isolates, except the control, demonstrated gas production and intensity of the reaction varied between the isolates.

The formation of gas bubbles in the Durham tubes confirms the fermentative metabolism of *R. solanacearum*, emanate from carbohydrate breakdown under aerobic and facultative anaerobic conditions. In some isolates (RS-4, RS-5 and RS-10), the delayed gas production suggests possible variations in metabolic efficiency and enzyme activity among the strains. The current research outcomes were agree with the observations made by Rahman (2010) [24], who observed that, gas production is a variable and characteristic feature of *R. solanacearum*, attributable to its oxidative and fermentative metabolism during growth on carbohydrate substrates.

3.6 Urease Production

The results of urease production by ten different isolates of *R. solanacearum* isolates were presented in Table 5 and Plate 8. None of the isolates showed urease activity within the first 24 h of inoculation. However, at 48 h, most of the isolates showed a positive reaction (except RS-5 and RS-7) characterized by the development of a pink color in the urea broth, depicting ammonia release and a subsequent pH increase due to urea hydrolysis. After 72 h of incubation, all isolates, except the control, demonstrated urease activity, even though RS-5 and RS-7 showed delayed reactions and turned positive after 72 h.

Urease production is a common biochemical character among *R. solanacearum* isolates, although the time required for enzyme production was varies among strains. This variation in urease activity may be due to the differences in the enzyme production, synthesis rate or regulation mechanisms between isolates. Our findings were matches Anand (2014) [1], who recorded that urease production serves as a important diagnostic tool for distinguishing *R. solanacearum* from other soil and plant associated bacteria, focusing its metabolic adaptability across diverse environments.

3.7 Gram's Staining

The main purpose of Gram staining was to determine the cell wall characteristics of *Ralstonia solanacearum* isolates utilizing crystal violet dye, Gram's iodine, alcohol, and safranin. Microscopic observation discloses reddish pink cells, depicting all isolates were gram-negative in reaction which is a typical character of this bacterium (Plate 9).

Negative reaction occurs due to thin peptidoglycan layer and an outer membrane rich in lipopolysaccharides, which fails to retain the primary stain. The research findings are accurate with Suslow *et al.* (1982) ^[31], who observed *R. solanacearum* as a gram-negative, non-spore forming, rod-shaped, motile bacterium grouped under the family Burkholderiaceae.

3.8 Potassium Hydroxide (KOH) Solubility Test

When a loop or toothpick was gently lifted from a thick bacterial suspension prepared on a glass slide, a few centimeters of viscous slime threads were observed for all isolates, indicating a positive KOH solubility reaction. The formation of these slime threads occurs due to the release of cellular DNA upon lysis of Gram-negative bacterial cells in the presence of 3% KOH solution. This characteristic reaction confirms that all *Ralstonia solanacearum* isolates tested were Gram-negative in nature (Plate 10). These observations are in close agreement with the findings of Suslow *et al.* (1982) ^[31] and Rudrappa *et al.* (2016) ^[25], they also reported similar reactions for Gram-negative bacteria.

3.9 Catalase Test

Catalase test was positive (Plate 11) for all the isolates except RS-4. The overall biochemical tests are associated with the findings of Narasimha and Srinivas, (2012) ^[19] who showed the biochemical characteristics of *R. solanacearum* isolated from wilted tomato plants in Karnataka and other parts of India were compared with authentic strain *R. solanacearum*. Results revealed that, the isolates show positive reaction for oxidase, nitrate reduction, catalase, levan production, citrate utilisation, motility test, H₂S production, salt tolerance at one percent and KOH solubility test. Negative for starch hydrolysis, gelatine hydrolysis, indole production, chromogenesis, esculin hydrolysis and urease test. Anand (2014) ^[1] and (Tripathy *et al.*, 2022) ^[33] conducted experiment on bacterial wilt of Ginger which revealed that different isolates produced strong to weak acids from sucrose, maltose and dextrose. And positive for Hydrogen sulphide production, starch hydrolysis and gelatine liquefaction at different time intervals indicating the existence of variability.

Table1: Acid production by different isolates of *R. solanacearum* in media containing different carbon sources

Isolates	Maltose			Sucrose			Dextrose		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
RS-1	+++	++++	++++	++++	++++	++++	++++	++++	++++
RS-2	+++	++++	++++	-	++	+++	++++	++++	+++
RS-3	-	+	++	++++	++++	++++	++++	++++	++++
RS-4	+++	++++	++++	-	++	+++	-	++	+++
RS-5	-	+	++	++++	++++	++++	++++	++++	++++
RS-6	+++	++++	++++	++++	++++	++++	++++	++++	++++
RS-7	+++	++++	++++	++++	++++	++++	++++	++++	++++
RS-8	-	-	++	++++	++++	++++	+++	++++	++++
RS-9	+++	++++	++++	++++	++++	++++	-	++	+++
RS-10	+++	++++	++++	++++	++++	++++	++++	++++	++++
Control	-	-	-	-	-	-	-	-	-

Note: '-' no acid production, '+' weak acid production, '++' medium acid production, '+++ strong acid production, '++++' very strong acid production.

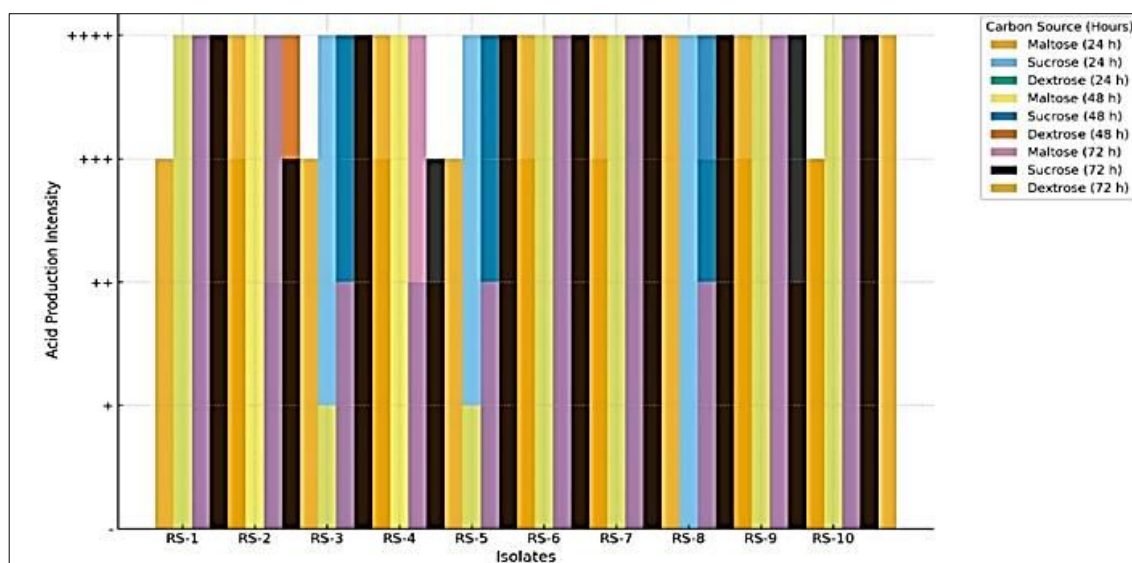


Fig 1: Acid production by different isolates of *R. solanacearum* in various carbon sources and time intervals

Table 2: Starch hydrolysis in different isolates of *R. solanacearum*

Isolates	3 Days of Inoculation		7 Days of Inoculation	
	Reaction	Zone of Starch Hydrolysis (mm)	Reaction	Zone of starch hydrolysis (mm)
RS-1	-	0.00	+	22
RS-2	-	0.00	-	0.00
RS-3	-	0.00	+	9
RS-4	-	0.00	+	6
RS-5	-	0.00	+	10
RS-6	-	0.00	+	24
RS-7	-	0.00	+	17
RS-8	-	0.00	+	15
RS-9	-	0.00	+	21
RS-10	-	0.00	-	0.00

Note: '-' 'Negative' '+' Positive

Table 3: Hydrogen Sulphide production and Gelatin liquefaction from different isolates of *R. solanacearum*

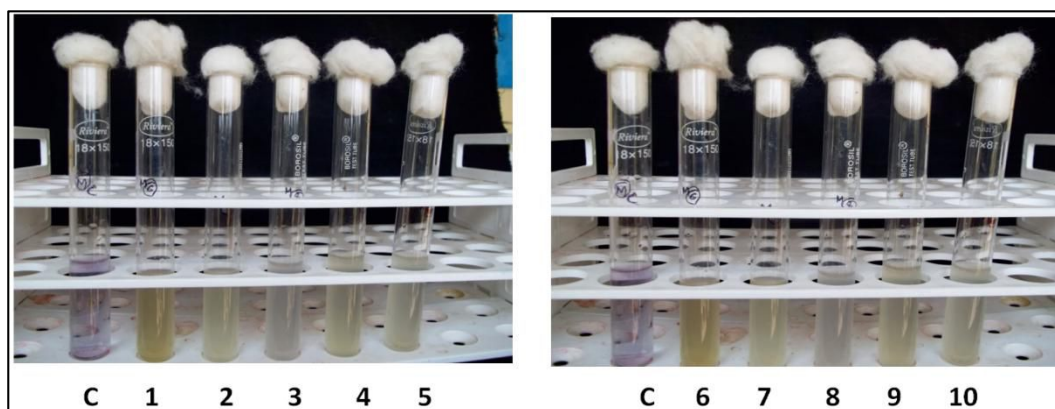
Isolates	Hydrogen Sulphide production			Gelatin liquefaction	
	Duration after Inoculation			Duration after Inoculation	
	24 h	48 h	72 h	24 h	48 h
RS-1	-	++	+++	-	+
RS-2	-	+	++	-	+
RS-3	-	-	+	-	-
RS-4	-	++	+++	-	+
RS-5	-	++	+++	-	+
RS-6	-	++	+++	-	+
RS-7	-	++	+++	-	+
RS-8	-	+	++	-	-
RS-9	-	++	+++	-	+
RS-10	-	-	+	-	-
Control	-	-	-	-	-

Note: '-' 'absent', '+' weak, '++' medium, '+++ strong

Table 4: Gas and Urease production in different isolates of *R. solanacearum*

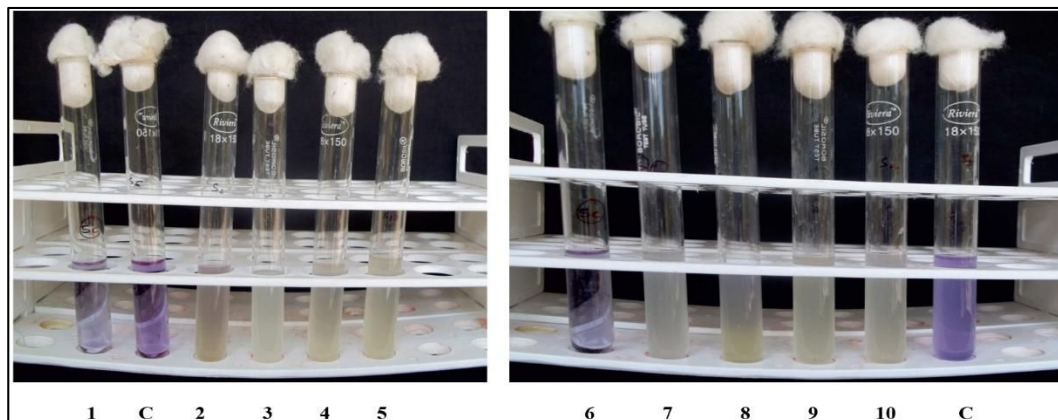
Isolates	Gas Production			Urease Production		
	Duration after Inoculation			Duration after Inoculation		
	24 h	48 h	72 h	24 h	48 h	72 h
RS-1	-	+	+	-	+	+
RS-2	-	+	+	-	+	+
RS-3	-	+	+	-	+	+
RS-4	-	-	+	-	+	+
RS-5	-	-	+	-	-	+
RS-6	-	+	+	-	+	+
RS-7	-	+	+	-	-	+
RS-8	-	+	+	-	+	+
RS-9	-	+	+	-	+	+
RS-10	-	-	+	-	+	+
Control	-	-	-	-	-	-

Note: '-' 'Negative' '+' Positive



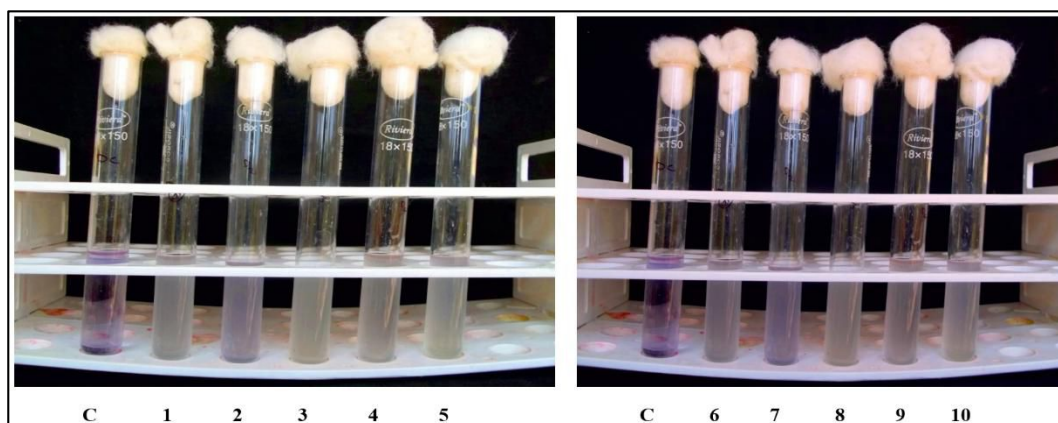
1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara) 8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 1: Acid production by maltose



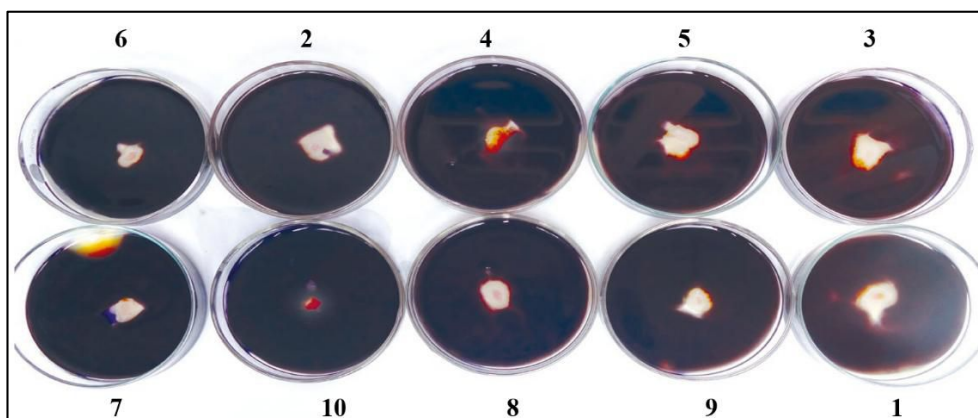
1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara)
8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 2: Acid production by Sucrose



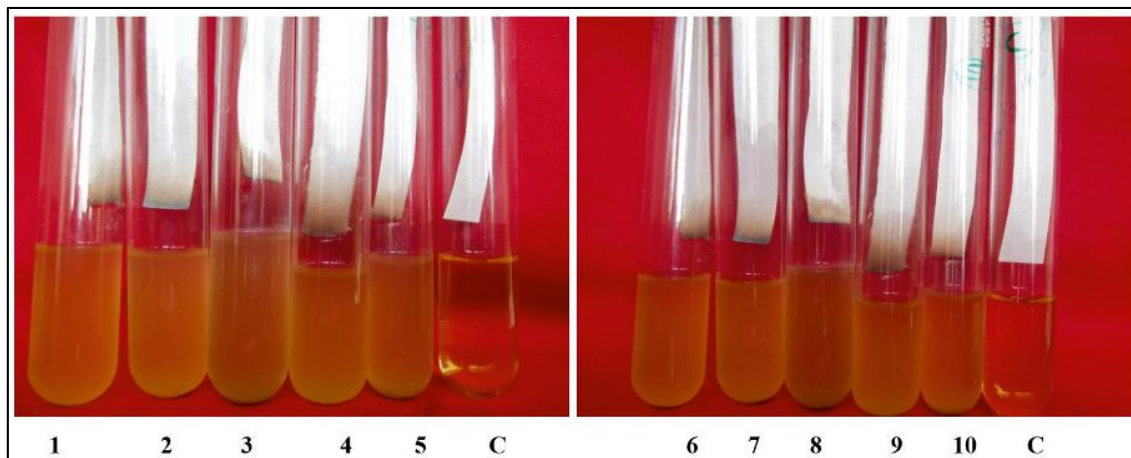
1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara)
8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 3: Acid production by Dextrose.



1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara)
8. RS- 3 (Hosnagar) 9. RS-9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 4: Starch hydrolysis in different isolates of *R. solanacearum*



1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara)
8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 5: Hydrogen Sulphide production

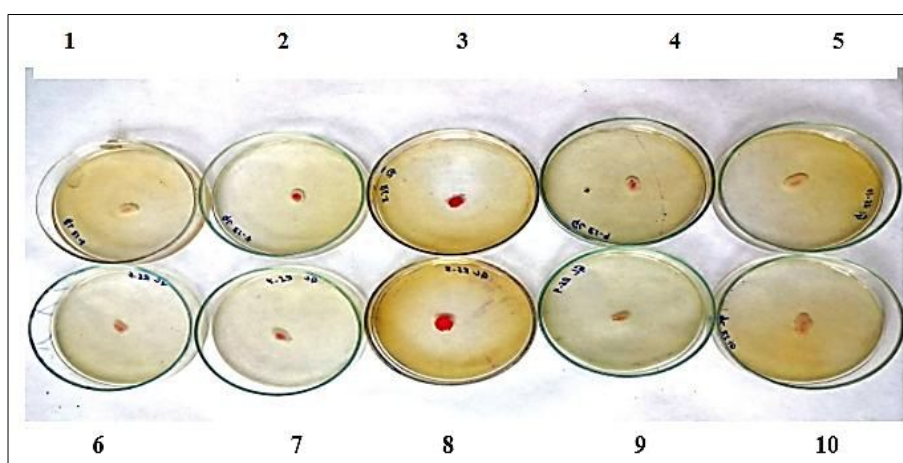
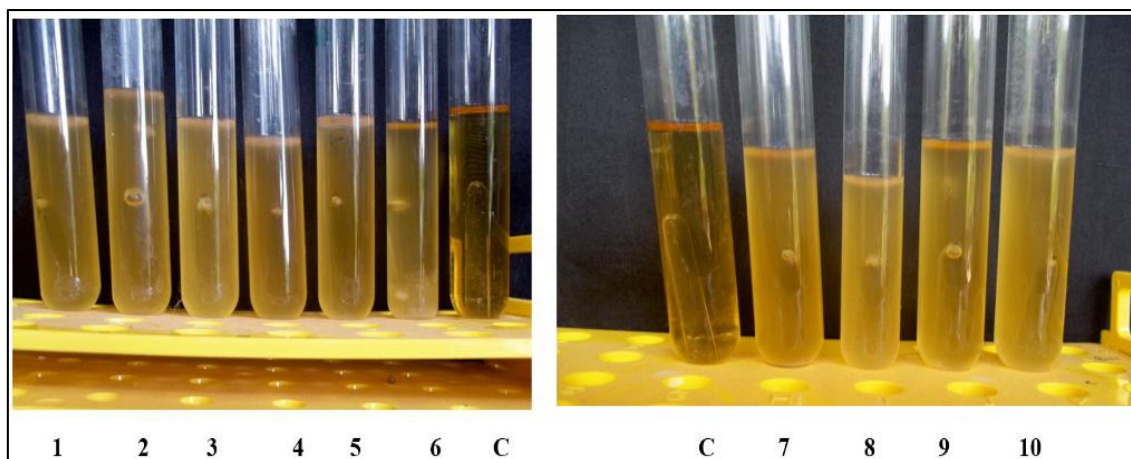
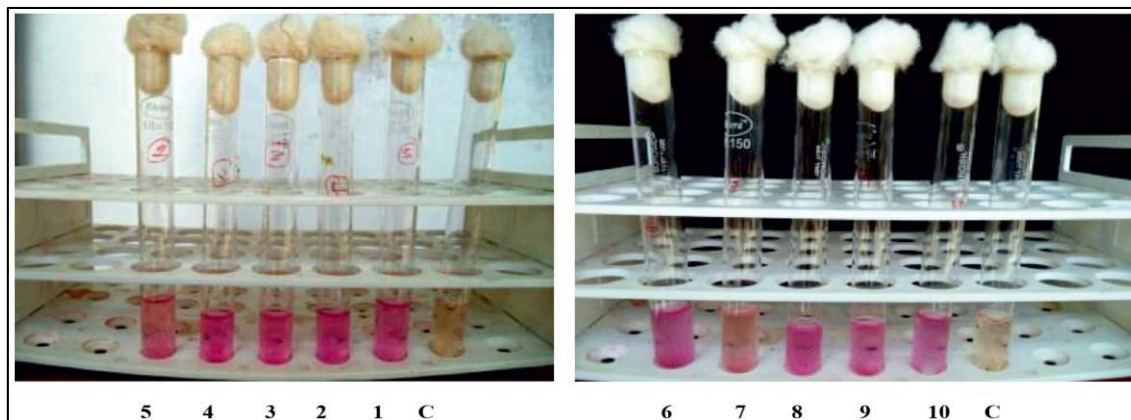


Plate 6: Gelatin liquefactions in different isolates of *R. solanacearum*



1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara)
8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 7: Gas production in different isolates of *R. solanacearum*.



1. RS- 1 (Shivamogga), 2. RS- 2 (Sirsi), 3. RS- 3 (Hassan), 4. RS- 4 (Chikmagalur), 5. RS- (Bidar) 6. RS- 6 (Shikaripura) 7. RS- 7 (Sagara) 8. RS- 3 (Hosnagar) 9. RS- 9 (Siddapura) 10. RS- 10 (Sakaleshpura) and c. control

Plate 8: Urease production in different isolates of *R. solanacearum*

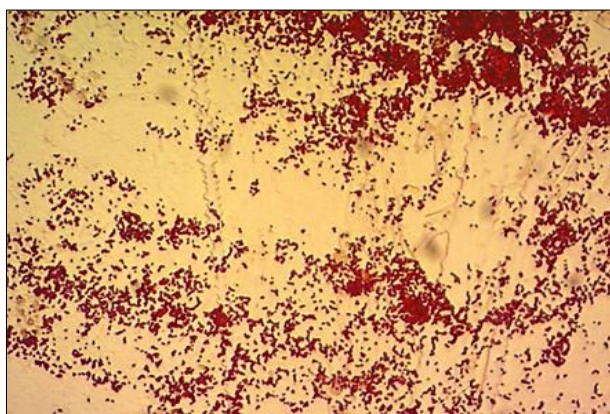


Plate 9: Gram staining colonies of *R. solanacearum*

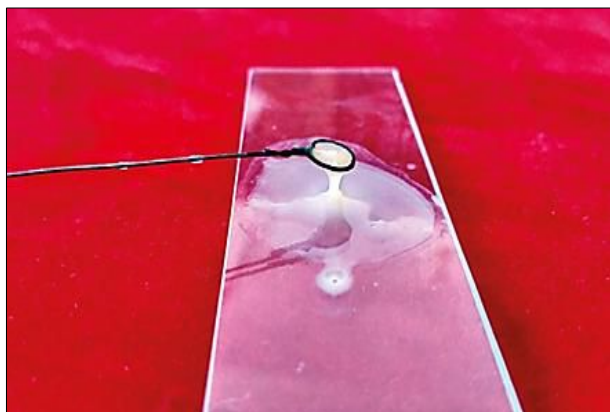


Plate 10: Potassium hydroxide (KOH) solubility test



Plate 11: Catalase test C- Control and P- Positive

Conclusion and Future Line of Work

The present investigation on the biochemical characterization of *Ralstonia solanacearum* isolates causing bacterial rhizome rot in ginger unveil significant variability in their physiological and metabolic properties. All isolates were exhibited diverse biochemical reactions, including differences in acid production, starch hydrolysis, and enzyme activities such as gelatinase and urease. These variations suggested possible physiological diversity and pathogenic adaptability among isolates from different regions. Understanding of these biochemical features gives valuable insights into the mechanisms of virulence and survival of pathogens under changing climatic conditions. Further, molecular characterization using PCR-based detection, sequencing, and phylogenetic analysis can be applied to supplement biochemical outcomes. Screening of different Ginger cultivars and expedition of resistant varieties aids in development of effective management practices for bacterial rhizome rot.

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Competing Interests

All the authors declare that, there is no conflicts of interest exist.

References

1. Anand. Investigations on bacterial wilt of ginger caused by *Ralstonia solanacearum* (E.F. Smith) Yabuuchi *et al.* [M.Sc. thesis]. Dharwad: University of Agricultural Sciences; 2014.
2. Anandaraj M, Dinesh R, Eapen SJ. Diseases of ginger and their management. *Indian Phytopathol.* 2008;61(2):131-139.
3. Buddenhagen I, Sequeira L, Kelman K. Designation of races of *Pseudomonas solanacearum*. *Phytopathology.* 1962;52:726-729.

4. Chaudhry Z, Rashid H. Isolation and characterization of *Ralstonia solanacearum* from infected tomato plants of Soan Skesar Valley of Punjab. Pak J Bot. 2011;43(6):2979-2985.
5. Denny TP, Hayward AC. *Ralstonia solanacearum*. In: Schaad NW, Jones JB, Chun W, editors. Laboratory Guide for Identification of Plant Pathogenic Bacteria. St Paul (MN): APS Press; 2001. p. 151-174.
6. Dohroo NP. Diseases of ginger. In: Diseases of Spices and Plantation Crops. Kalyani Publishers; 2005. p. 85-107.
7. Fegan M, Prior P. How complex is the *Ralstonia solanacearum* species complex? In: Allen C, Prior P, Hayward AC, editors. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. APS Press; 2005. p. 449-461.
8. Hailemichael G, Tilahun D. Annual report on the current status of spices research. Jimma: IAR, Teppi Agricultural Research Sub-Centre; 2004. p. 35-48.
9. Gopalakrishnan C, Karthikeyan M, Nakkeeran S, Kalaiselvi P. Characterization of *Ralstonia solanacearum* causing bacterial wilt in ginger (*Zingiber officinale* Rosc.) in Tamil Nadu, India. J Plant Pathol. 2017;99(2):373-380.
10. Hayward AC. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu Rev Phytopathol. 1991;29:65-87.
11. Hayward AC. The hosts of *Pseudomonas solanacearum*. In: Hayward AC, Hartman GL, editors. Bacterial Wilt: The Disease and Its Causative Agent *Pseudomonas solanacearum*. Wallingford (UK): CAB International; 1994. p. 9-24.
12. He LY, Sequeira L, Kelman A. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 1983;67(12):1357-1361.
13. Jansen PCM. Spices, Condiments and Medicinal Plants in Ethiopia: Their Taxonomy and Agricultural Significance. Ethiopia: Centre for Agricultural Publishing; 1981.
14. Kelman A. The bacterial wilt caused by *Pseudomonas solanacearum*: a literature review and bibliography. North Carolina Agric Exp Stn Tech Bull. 1953;99:1-194.
15. Kelman A. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology. 1954;44:693-695.
16. Kumar A, Bhattacharya R, Ghosh S. Biochemical and molecular characterization of *Ralstonia solanacearum* causing bacterial wilt of solanaceous crops in Eastern India. J Plant Pathol. 2015;97(1):115-123.
17. Kumar S, Devika R, Dinesh R. Biochemical and molecular characterization of *Ralstonia solanacearum* causing bacterial wilt of ginger in Kerala, India. J Pharmacogn Phytochem. 2019;8(5):1390-1396.
18. Lammert JM. Techniques in Microbiology: A Student Handbook. San Francisco: Pearson Education; 2007.
19. Narasimha Murthy K, Srinivas C. *In-vitro* screening of bio-antagonistic agents and plant extracts to control bacterial wilt (*Ralstonia solanacearum*) of tomato (*Lycopersicon esculentum*). J Agric Technol. 2012;8(3):999-1015.
20. Nirmal Babu K, Ravindran PN, Shiva KN. Ginger: The Genus *Zingiber*. CRC Press; 2013.
21. Pooja R, Ingle RW. Isolation and biochemical characterization of *Ralstonia solanacearum* of chilli. Pharma Innov J. 2023;12(10):27-31.
22. Prasannakumar MK, Chandrashekara KN, Vani M, Deepa, Khan ANA. Fingerprinting of *Ralstonia solanacearum* isolates by Rep-PCR and RAPD. Pest Manag Horticult Ecosyst. 2012;18(2):179-187.
23. Priou S, Gutarra L, Alep P, Elphinstone J. *Ralstonia solanacearum* race 2, biovar 2, causing brown rot of potato: detection, diagnosis and management. CIP Training Manual. Lima (Peru): International Potato Center; 2006.
24. Rahman MF, Islam MR, Rahman, Meah MB. Biochemical characterization of *Ralstonia solanacearum* causing bacterial wilt of brinjal in Bangladesh. Prog Agric. 2010;21(1-2):9-19.
25. Rudrappa KB, Suryawanshi AP, Punitkumar ND, Ganesh JK, Lambani K, Singh R. Cultural and biochemical characterization of *Ralstonia solanacearum* causing bacterial wilt in tomato. J Pure Appl Microbiol. 2016;10(4):3111-3115.
26. Saddler GS. Management of bacterial wilt disease. In: Allen C, Prior P, Hayward AC, editors. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. APS Press; 2005. p. 121-132.
27. Sarma YR, Anandaraj M, Kabeerathumma S. Bacterial wilt of ginger: etiology and management. Indian Phytopathol. 2015;68(1):1-9.
28. Schaad NW, Jones JB, Chun W. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd ed. St Paul (MN): APS Press; 2001.
29. Schaad NW. *Xanthomonas*. In: Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. Lucknow: International Book Distributing Co.; 1992. p. 165.
30. Sharma BR, Dutta S, Roy S, Debnath A, Roy MD. Effect of soil physico-chemical properties on rhizome rot and wilt disease complex incidence of ginger under hill agro-climatic region of West Bengal. Plant Pathol J. 2010;26:198-202.
31. Suslow TV, Schoth MN, Isaka M. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology. 1982;72:917-918.
32. Tarafdar J, Saha N. Correlation study on population dynamics of ginger soft rot inciting pathogens under different organic amendments, disease incidence and survival in Darjeeling hill soils. Proc 13th ISTRC Symp. 2007; p. 169.
33. Tripathy A, Senapati AK, Satapathy SN. Biochemical characterization of *Ralstonia solanacearum* causing chilli bacterial wilt. Biol Forum. 2022;14(4A):781-785.