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# CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF PANTOEAANANATIS CAUSING FRUITLET BLIGHT OF NAGPUR MANDARIN

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Nagpur mandarin (Citrus reticulata Blanco) is a nutritionally rich and bioactive compound-laden fruit of significant economic importance, particularly as an export commodity of India. However, its production is adversely affected by various biotic and abiotic stresses. One of the most destructive diseases affecting Nagpur mandarin is fruitlet blight, caused by the bacterium Pantoea ananatis, which affects the fruit at the berry size stage. In the present study, the causal bacterium was isolated and characterized using a range of cultural, physiological, and biochemical methods. On nutrient agar medium, the bacterial colonies appeared pale yellow, convex, shiny, mucilaginous, and smooth-edged, but non-flat. Microscopically, the bacterium was observed as small, rod-shaped, Gram-negative cells, occurring singly or in chains, with a characteristic pinkish colour after Gram staining. The bacterium's tolerance to various concentrations of sodium chloride (NaCl) and potassium chloride (KCl) was assessed. P. ananatis exhibited moderate growth at 2% concentrations of both salts, while higher concentrations (4% to 10%) inhibited its growth. The optimum temperature for bacterial growth was found to be between 25°C and 30°C, which yielded the highest colony counts. In terms of pH response, optimal growth was recorded within the pH range of 6.5 to 7.0, with moderate growth observed at pH 6.0 and 7.5. Biochemical characterization of P. ananatis revealed positive reactions for potassium hydroxide (KOH) solubility, hydrogen sulfide (H, S) production, catalase activity, indole acetic acid production, starch hydrolysis, and gelatin liquefaction. However, the bacterium tested negative for the Methyl Red (MR) test.

Key words: Biochemical, Cultural, Fruitlet blight, Nagpur mandarin, Pantoea ananatis, Physiological characteristics

# Introduction

Citrus is a significant global fruit crop belonging to the Rutaceae family and Aurantioedae sub-family. Its origins can be traced back to Southeast Asia, with the Northeast region of India serving as the home for various citrus species. In India, citrus cultivation gained immense significance due to its medicinal and nutritional values, contributing to an industry of considerable importance (Dinesh Kumar *et al.*, 2023). India stands as the sixth-largest citrus fruit producer worldwide, with citrus fruits ranking third in production after mangoes and bananas. In India, the most commercially important citrus species

are Mandarin (*Citrus reticulata*), Sweet Orange (*Citrus sinensis*), and Acid Lime (*Citrus aurantifolia*). Among these, mandarin (*Citrus reticulata* Blanco) is the most common citrus fruits grown in India and occupies nearly 40 percent of the total citrus cultivation area in India. Amongst all the cultivars, Nagpur mandarins are produced in the highest quantity in India. Nagpur mandarin are extensively grown in the Vidarbha region, encompassing Nagpur and Amravati divisions in Maharashtra, as well as some parts of Madhya Pradesh. In Nagpur and Amravati districts of Maharashtra, theycontribute significantly, with a production of 9.65 lakh MT on 1.15

**ABSTRACT** 

lakh ha area (Anonymous 2022). Citrus production faces various challenges leading to low productivity, including problems such as plant pathogens, insect pests, nutritional imbalances, and physiological disorders (Zhang *et al.*, 2012). However, citrus production has been affected by the damage of diseases, resulting in substantial economic losses (Gohel *et al.*, 2022).

Citrus diseases are mainly caused by fungi, viruses and bacteria. Among these, bacteria pose a significant threat to citrus cultivation, causing diseases like citrus canker, citrus greening, and the emerging concern of fruitlet blight, particularly affecting mandarins. Fruitlet blight, once considered a minor and non-listed disease among the major diseases in India, has now emerged as a significant bottleneck. It adversely affects the quality of fruits and results in premature fruit drop during the berry's growth stage. Fruitlet blight in Nagpur mandarin was first reported by Das et al., (2020) and confirmed bacteria Pantoea ananatis caused this disease. This disease has impacted nearly 30% of trees with over 20% of fruitlets affected in the Vidarbha region (Das et al., 2020). In Vidarbha, the prevalence of fruitlet blight has been reported to range from 4.64% to 19.12% in Nagpur mandarin (Anonymous, 2023). Over the past three years, fruitlet blight has emerged as a major problem, especially during the Ambia bahar flush when the fruits are in the berry size in the Vidarbha region of Maharashtra. Affected tissue initially exhibits an irregular water-soaked appearance that quickly darkens to brown or black and becomes sunken. The affected fruitlets eventually dry up, wrinkle, and drop off, causing significant damage.

This bacterium has also been reported to be pathogenic on several economically important horticultural crops including muskmelon (Kido *et al.*, 2008); peach (Liao *et al.*, 2016); and onion (Carr *et al.*, 2010) and also in rice (Azizi *et al.*, 2019).

Fruitlet blight, which is most active during warm weather and on large succulent fruits, is spread by various sucking pest insect injuries, wind injury, and dashing rain. This disease may threaten the Nagpur Mandarin



**Fig. 1:** Collected of naturally infected fruitlet blight Nagpur mandarin berry size fruits.

production system if effective management strategies are not implemented. Though the research has been conducted on its management of this disease but scarcity of literature is available on the biochemical characterization of this bacterium. Therefore, studies on cultural, physiological and biochemical analysis of *P. ananatis* were carried out. The present investigation strengthens the information on the cause of fruitlet blight, cultural, physiological and biochemical characterization of a pathogen asthe cultural, morphological and biochemical features of *P. ananatis* can be used for identification isolate.

#### **Material and Methods**

# Collection of disease specimen and Isolation

Typical symptoms of fruitlet blight were observed on mandarin fruits at the berry stage and were collected from the AICRP on Fruits experimental field (Lat. 20.7029170, Long. 77.066170) during the *Ambia bahar* season, specifically in February–March 2023. The collected specimens were processed for pathogen isolation following the protocol outlined by Das *et al.*, (2020). The purified isolate was maintained on nutrient agar (NA) medium for further studies.

# Cultural and morphological characterization

The pure isolate was streaked on nutrient agar medium to allow colony development. Individual colonies were observed for cultural and morphological traits, including colony shape, pigmentation, elevation, margin characteristics, and Gram staining reaction, as per the method described by Carr *et al.*, (2010).

#### Physiological characterization

a) **Temperature effect:** An experiment was conducted to know the optimum temperature requirement

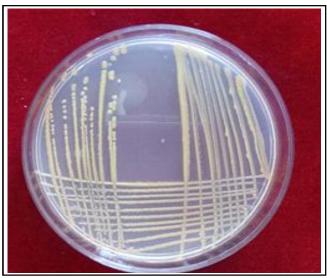


Fig. 2: Pure growth of isolated fruitlet bacterium on NA media.

for the growth of *P. ananatis* causing fruitlet blight of Nagpur Mandarinusing NA medium as a basal medium. A loopful of 48 hours old bacterial culture was in oculated to solidified Petriplates. The inoculated Petriplates were incubated at different temperatures *viz.*, 10, 15, 25, 30 and 35°C for 72 hours. Observations were recorded for the growth of bacterial colonies in the inoculated Petriplates at specific temperature levels. Colony growth is categorized using the method that Patil *et al.*, (2017) outlined (colony growth: '-': no growth, '+': reduced growth, '++': moderate growth, '+++': maximum growth).

b) pH effect: The effect of hydrogen ion concentration on the growth of the bacterial isolate was studied by adjusting the pH of the nutrient agar as per the procedure given by Bhure *et al.*, (2019). The pH was adjusted to 5, 5.5, 6, 6.5, 7 and 7.5 using appropriate buffers. A loopful of 72 hours old bacterial culture was separately streak on to the surface of the medium having varied pH levels. Inoculated plates were incubated at 25°C temperature for 72 hours. After the incubation period, observations were recorded for the development of colonies on the media having different pH. Colonies growth was recorded and theapproach described by Patil *et al.*, (2017) is used to categorize colony growth (colony growth: '-': no growth, '+': reduced growth, '++': moderate growth, '+++': maximum growth).

c) Tolerance to NaCl and KCl: Adding salt to a culture medium increases the ionic concentration of the medium and affects the osmotic pressure and membrane permeability across the bacterial cell membrane. Thus, salt acts as a selective agent and allows only the growth of salt-tolerant or halophilic organisms. The concentration of NaCl *i.e.* 2% (2g/l), 4% (4g/l), 6% (6g/l), 8% (8g/l), and 10% (10g/l) were made in nutrient agar medium and 20 ml of medium of each salt concentration was poured in sterilized Petri plates. The Petri plates inoculated with *P.ananatis*. Inoculated Petri plates incubate for 24 hours at  $25 \pm 2^{\circ}$ C and observe for visible growth or colour change. If no growth is seen, re-incubate the Petri plates and observe for growth for up to 72 hours and check for

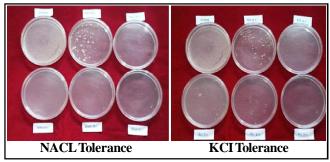


Fig. 3: Tolerance to NaCl and KCl.

turbidity or colour change at 48 and 72 hours. A positive test is indicated by bacteria growth and colour change and a negative test is indicated by no bacterial growth and no colour change of the medium. Growth of isolates in 2% medium was considered as salt tolerance.

KCl test was performed by using the concentration of KCl i.e. 2% (2g/l), 4% (4g/l), 6% (6g/l), 8% (8g/l), and 10% (10g/l) in glucose nutrient medium (glucose 6 g/l, 3 g/l beef extract, 5 g/l peptone, and marinating pH 7.2) and 20 ml of medium of each salt concentration was poured in sterilized Petri plates as per protocol of Gerges et al., (2007). The Petri plates inoculated with P. ananatis. Inoculated Petri plates incubate for 24 hours at 25± 2°C and observe for visible growth or colour change. If no growth is seen, re-incubate the Petri plates and observe for growth for up to 72 hours and check for turbidity or colour change at 48 and 72 hours. A positive test is indicated by bacteria growth and colour change and a negative test is indicated by no bacterial growth and no colour change of the medium.

#### **Results and Discussion**

# Collection of disease specimen and Isolation

Specimens displaying prominent symptoms of fruitlet blight were carefully collected in paper envelopes and subsequently brought to the laboratory for further examination. A specific portion of the infected fruits was selected for isolation. Infected portion of fruit were cut into small pieces and sterilized with 1% aqueous sodium hypochlorite solution for 1 min, then washed by giving three changes of sterile distilled water to remove traces of sodium hypochlorite. The bits were macerated in sterile distilled water and allowed for diffusion of bacteria from cut ends for 30 minutes. A loopful of crushed leachate (oozed bacterial cell) was streaked on nutrient agar plates, aseptically and incubated at temperature  $27\pm2^{\circ}\text{C}$  for two



Fig. 4: Growth rate at pH 5.0.

Table 1:	Cultural and	morphological	characteristics	of <i>P</i> .
	ananatis.			

S.N.	Name of test	Appearance/Reaction
1	Colony shape	Circular
2	Pigmentation	Pale yellow
3	Elevation	Convex
4	Margin	Smooth
5	Texture	Slightly mucoid
6	Gram staining	Small, rod shaped, pink in
		colour, gram negative reaction

days. The single pale yellow coloured, convex and smooth margin, non- flat, mucous shiny colonies observed on nutrient agar medium. Then these bacterial colonies were transferred on to fresh nutrient agar plates and slants having NA mediumby strake plate method.

The isolation procedure employed in the present study follows the methodology outlined by Das *et al.*, (2020), and the cultural characteristics of the bacterium observed are akin to those reported earlier by Carr *et al.*, (2010), Leonila *et al.*, (2017), and Das *et al.*, (2020). The pathogenicity test indicated that the bacteria induced typical black, water soaked; sunken symptoms on mandarin fruitlets and after isolation gave the same colonial colour texture as the originally inoculated. Isolated bacterium, suggesting their pathogenic nature.

#### Cultural and morphological characteristics

Cultural traits, including colony shape, pigmentation, elevation, margin, texture, and Gram staining of isolated pathogenic bacterium were studied using NA medium as basal culture medium.

The visual examination revealed that the *Pantoea* ananatis isolate exhibited circular colonies with a pale yellow pigment, a convex elevation, a smooth margin encompassing the entire colony, and a slightly mucoid appearance on the NA medium (Table 1). Gram-stained

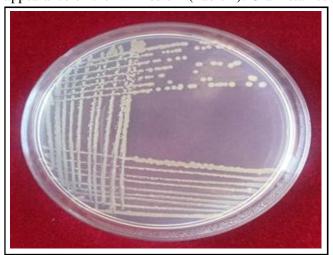


Fig. 5: Growth rate at pH 6.0.

**Table 2:** Effect of temperature level on the growth of *Pantoea ananatis*.

S.	Temperature	Colony Growth	
NO.	level <sup>o</sup> C	24 hour	72 hour
1	10	_	+
2	15	+	++
3	20	+	++
4	25	++	+++
5	30	++	+++
6	35	+	++
(Colony growth: '-': no growth, '+': less growth, '++': moderate growth, '+++': maximum growth)			

bacterial mounts were examined under a light microscope at 100X magnification with oil immersion. The *P. ananatis* isolate displayed a gram-negative characteristic, appearing as small rod-shaped structures either singly or in chains. The application of the counter stain Safranin revealed a pinkish colourationconfirming the gramnegative nature of the bacterium. Thus, based on the Gram staining results, it was convincingly determined that the isolated *P. ananatis* belonged to the category of gramnegative bacteria.

According to the findings of Das et al., (2020), bacterial colonies of Pantoea ananatis isolated from Nagpur mandarin fruitlet blight were characterized by a shiny, yellow, convex, and circular appearance with entire margins. The bacterium was identified as Gram-negative and facultatively anaerobic. These observations are consistent with those of Kido et al., (2008), who reported P. ananatis as a Gram-negative, facultative anaerobe isolated from internal fruit rot of muskmelon. Carr et al., (2010) noted colonies with yellow pigmentation and dark centers. Likewise, Achbani et al., (2016) observed that P. ananatis colonies on Yeast Peptone Glucose Agar medium were consistently yellow and opaque, and described the bacterium as rod-shaped, occurring singly or in chains, reaffirming its Gram-negative nature. Our findings are further supported by Leonila et al., (2017), who described colonies with a yellow pigment, smooth mucoid texture, and convex to heterogeneous margins on Nutrient agar medium.

# Physiological characterization

a) **Temperature effect:** The effect of varied temperature levels (10, 15, 20, 25, 30 and 35°C) on the growth of *Pantoea ananatis* was investigated over a 72-hour period on NA agar medium. The results of this study are detailed in Table 2.

The data revealed that the optimal temperatures for the growth of the pathogen were 25°C and 30°C, as these temperatures exhibited a considerably higher number of

**Table 3:** Effect of pH level on the growth of *Pantoea* ananatis.

S.	рH	Colony	y Growth
NO.	level	24 hour	72 hour
1	5.0	_	_
2	5.5	_	_
3	6.0	+	++
4	6.5	++	+++
5	7.0	++	+++
6	7.5	+	++
(Colony growth: '-': no growth, '+': less growth,			
'++': moderate growth, '+++': maximum growth)			

colonies. Growth was modest and slow at 24 hours, but over time (72 hours), the maximum growth was observed at 25°C and 30°C. At 35°C, moderate growth was observed up to 72 hours, while at 10°C, less growth was observed after 72 hours.

Leonila et al., (2017) conducted a similar investigation into temperature requirements and found that the Pantoea genus thrives well at a cordial temperature range of 25-30°C, categorizing the bacterium as a mesophile. In general classification, mesophiles are bacteria that prefer temperature between 20 to 45°C. Likewise, Son et al., (2006) observed vigorous growth of Pantoeaagglomerans within a temperature range of 5 to 45 °C, with optimal growth occurring between 25 and 35 °C. Gour et al., (2000) also noted in their studies that X. axonopodispy. vignicola, the causal agent of cowpea leaf blight, exhibited maximum growth at a temperature of 30°C. The identified optimal temperature range for P. ananatis bacterial growth was 25-30°C, with a minimum temperature of 10°C and a maximum of 35°C.

**b) pH effect:** The effect of different pH levels (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) on the growth of *Pantoea ananatis* was observed and the data presented in Table 3.

The data clearly indicated that the highest growth of *P. ananatis* occurred within the pH range of 6.5 to 7. Additionally, moderate growth was observed at pH 6.0 and 7.5. However, no growth was observed at pH 5.0 and 5.5.

The findings from the currentstudy correlates with previous research, particularly with Gour *et al.*, (2000), who obtained similar results while investigating *X. axonopodis* pv. *vignicola*. They observed maximum pathogen growth at a pH of 7.0, with a significant (number of colonies) declinein growth at pH values both higher and lower than 7.0, reaching a minimum at pH 5.0. Likewise, Leonila *et al.*, (2017) recorded the highest number of *Pantoeagenuscolonies* colonies at pH of 7

**Table 4:** Salt sensitivity of *P. ananatis* at different concentration level.

S. No.	Tolerance to NaCl	Colony Growth
1	2% concentration	++
2	4% concentration	_
3	6% concentration	_
4	8% concentration	_
5	10% concentration _	
(Colony growth: '-' no growth, '+' less growth, '++' moderate growth, '+++' maximum growth)		

and 8.0, with optimum growth observed at pH 7.0. These results are consistent with findings from studies on *Pantoeaagglomerans* CPA-2 and *P. agglomerans* IMA2 by Son *et al.*, (2006) and Silini-Cherif *et al.*, (2012), respectively. Other research has also highlighted that *Pantoea* tends to thrive best in culture medium with pH values ranging from 6.0 to 7.0 (Costa *et al.*, 2002). As the bacterium exhibit optimal growth in a pH range from 5.5 to 7.5, it can be classified as neutrophilic bacterium.

c) Tolerance to NaCl and KCl: Evaluating NaCl tolerance is a significant factor that can offer valuable insights into the ecological preferences and adaptability of a bacterium. In this particular test, media were formulated with NaCl concentrations of 2%, 4%, 6%, 8%, and 10%. The plates were inoculated with the bacteria and then incubated for 72 hours at  $25 \pm 2^{\circ}$ C. The impact of different salt concentrations on the growth of *P. ananatis* was observed, and the resulting data is presented in Table 4.

The growth of the bacterium *P. ananatis* was examined across various salt concentration levels. *P. ananatis* exhibited moderate growth at a 2% salt concentration, while its growth was inhibited from 4% to 10% salt concentration. Following incubation, bacterial growth was observed only in the 2% NaCl medium, indicating a moderate level of growth for *P. ananatis* (Table 4). When a bacterium is noted to have a 2% NaCl tolerance, it signifies its ability to flourish in a medium containing 2% NaCl. Therefore, describing a bacterium as halophilic and capable of thriving in a 2% salt

**Table 5:** KCl sensitivity of *P. ananatis* at different concentration level.

S. No.	Tolerance to KCI	Colony Growth
1	2% concentration	++
2	4% concentration	_
3	6% concentration	_
4	8% concentration	_
5	10% concentration	_
(C-1		

(Colony growth: '-' no growth, '+' less growth, '++' moderate growth, '+++' maximum growth)

concentration implies its adaptation to and capacity for growth in a moderately saline environment.

Comparable findings were reported by Leonila et al., (2017) concerning Pantoeaagglomerans strain. They observed that P. agglomerans exhibited a notable tolerance to high salt concentrations, with optimal growth at 100 mM. Additionally, the bacterium demonstrated favourable outcomes at concentrations of 400, 800, and 1000 mM NaCl. It was noted that as the salt concentration increased, the growth of the bacterium decreased. Silini-Cherif et al., (2012) also reported similar results when studying a strain of *Pantoeaagglomerans*. Bacteria that grow ambiently in a salt concentration of <1.7% (<0.2 to 0.3 M) are considered non-halophiles. Bacteria that require NaCl concentration of 1.7 to 4.8% (0.3 to 0.7 M), 4.8% to 20% (0.7 to 3.4 M), and 20% to 30% (3.4 to 5.9 M) salt for their ambient growth are called slight halophiles, moderate halophiles, and extreme halophiles respectively. If the non-halophiles tolerate salt concentrations of up to 2.5M, they are called halo-tolerant bacteria (Edbeib et al., 2016).

#### Tolerance to KCl

The test is performed to verify the potassium chloride tolerance of *Pantoea ananatis*. In this test, media is prepared with 2%, 4%, 6%, 8%, and 10% KCl concentration. The plate was inoculated with bacteria and incubated for 72 hours at  $25 \pm 2^{\circ}$ C. The effect of different KCl concentrations on the growth of *P. ananatis* was observed, and the resulting data is presented in Table 5.

Pantoea ananatis demonstrated a moderate level of growth at a 2% KCl concentration, but its growth was impeded when exposed to salt concentrations ranging from 4% to 10%. After the incubation period, bacterial growth was evident solely in the medium containing 2% potassium chloride, signifying a moderate growth level for *P. ananatis*.

In practical terms, if a bacterium is described as having a 2% KCl tolerance, it means that the bacterium can withstand and proliferate in a medium containing 2% potassium chloride. The tolerance of a bacterium to a 2% concentration of potassium chloride implies the ability of the bacterium to survive and grow in an environment where the concentration of KCl is 2%. This characteristic provides insights into the bacterium's adaptability to conditions with elevated levels of potassium chloride (Edbeib *et al.*, 2016).

Fruitlet blight is a major and destructive bacterial disease of Nagpur mandarin (*Citrus reticulata*), caused by *Pantoea ananatis*. In the present study, the pathogen

was isolated and subjected to cultural, morphological, and physiological characterization. The successful isolation and identification of the causal organism provide a foundation for future diagnosis and management strategies aimed at controlling this economically damaging disease.

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