

Original Research Article

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Antibacterial Activity of Antagonists, Botanical and Chemical against *Pantoea stewartii* sub sp. *Indolegenes* Causing Bacterial Leaf Blight of Rice

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ABSTRACT

Keywords

Bacterial leaf blight, *Xanthomonas oryzae* pv. *oryzae*, *Pantoea stewartii* subsp. *Indolegenes*, Biochemical and molecular characterization

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Rice is one of the important cereal crop and known to be affected by many diseases. Bacterial leaf blight of rice is a destructive disease of rice, known to be caused by *Xanthomonas oryzae* pv. *oryzae* and it also be reported to cause by species of *Pantoea*. BLB diseased samples were collected from southern districts of Tamil Nadu and upon isolation, the pathogen was thought to be *Xanthomonas oryzae* pv. *oryzae*. But, the results of biochemical and molecular characterization revealed that the causal agent was not *Xanthomonas*, but a new species of bacterium *Pantoea stewartii* subsp. *Indolegenes* (Accession No. SUB2733370: MF163273; MF163274; MF16327). The present study was conducted to investigate the antibacterial activity of different botanicals, antagonists, chemicals and antibiotics on the growth of *Pantoea*. *In vitro* studies revealed, the extract of *Prosopis* (10.33mm) was proved to having anti-bacterial activity at 10 per cent and *Bacillus subtilis* (8.66mm) was the potent antagonist. The evaluation of different fungicides and antibiotics revealed that copper oxy chloride (5.56mm) and streptomycin (7.13mm) having maximum inhibitory effect on *Pantoea*.

Introduction

Rice is one of the major cereal crops in India and also primary staple food for huge population in Asia, Africa and Latin America. Global rice utilization is projected over around 501.2 million tonnes (milled basis) in 2016-17. In India, rice is being grown in 44.10 Mha area with production of 106.5 million tonnes and productivity of 3.52 MT/ha respectively (USDA, 2016). In Tamil Nadu, rice is grown in an area of 20.16 lakh hectares with the production of 62.53 lakh million tonnes with the average productivity of 3,102 kg/ha

(INDIASTAT, 2015). Around 40% of world's rice crop lost annually due to various biotic stresses like pathogens, insects and weeds (Hossain, 1996). The damage results of rice diseases are enough to feed 60 million peoples in the world (Asghar *et al.*, 2007). The highly valuable crop is affected by diverse fungal and bacterial attacks (Khan, 2009). Bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* is the most important and oldest known bacterial disease of rice in Asia. It considered as a serious bacterial diseases in many of the rice growing regions of the world (Xu *et al.*, 2010). The disease known to be

caused by *Xanthomonas oryzae pv. oryzae* and also reported to be caused by *Pantoea* (Lee *et al.*, 2010; Mondal *et al.*, 2011). *Pantoea* spp are opportunistic pathogens documented to cause different diseases in economically important crop plants including grain discoloration in China (Yan *et al.*, 2010) and leaf blight of rice reported in Korea (Lee *et al.*, 2010), India (Mondal *et al.*, 2011), Venezuela (Gonzalez *et al.*, 2015), Benin and Togo (Kini *et al.*, 2017). *Pantoea ananatis* is the causal agent of the newly emerged rice leaf blight disease reported in northern India (Mondal *et al.*, 2011). The screening of phyto extracts, bio control agents, fungicides and antibiotics under *in vitro* provides preliminary information in short time about their efficacy against the pathogens. It is economical and time saving in planning of control measures for plant diseases in the field condition.

Materials and Methods

Isolation of pathogen

The diseased leaves of rice showing typical bacterial blight (BLB) symptoms were collected in brown paper bags from Tirunelveli, Tuticorin, Madurai, Virudhunagar and Kanyakumari districts of Tamil Nadu, India. Small portion of BLB infected leaf bits sterilized with 1 per cent sodium hypochlorite solution, placed on petridish containing nutrient agar medium and incubated at $27 \pm 2^\circ\text{C}$ for 48 hours. The bacterial colonies were transferred to the nutrient agar slant and pathogenicity test has been performed subsequently.

Characterization of pathogen

To identify the bacterium isolated from blighted rice leaves, different biochemical test and molecular analysis have been performed. The standard bacteriological and biochemical methods were employed *viz.*, Gram staining

(Jonit *et al.*, 2016), KOH test (Jonit *et al.*, 2016), Catalase test (Jonit *et al.*, 2016), Citrate utilization test, Production of yellow pigment on YDC medium (HaliaturRahma *et al.*, 2014), Anaerobic growth test (Jonit *et al.*, 2016), Starch hydrolysis test and tween 80 hydrolysis test (Lelliot and Stead, 1987). In molecular characterization, the isolates which showed higher level of virulence have been selected for characterization based on the earlier symptom expression and percent leaf area blighted.

The genomic DNA has been extracted from the isolated bacterial culture and used for PCR analysis. The purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Inhibitory effect of botanicals against *Pantoea*

The phyto extracts of commonly available plant species like Pungam (*Pongamia pinnata*), Onion (*Allium cepa*), Garlic (*Allium sativum*), Neem (*Azadirachta indica*), Mehandi (*Lawsonia inermis*), Adathoda (*Adathodav asica*), Tulsi (*Ocimum sanctum*), Chilli (*Capsicum annum*), *Prosopis* (*Prosopis juliflora*), *Calatrophis* (*Calatrophis gigantea*), Nochi (*Vitex negundo*), Nithyakalyani (*Vincarosea*), *Lantana* (*Lantana camera*) and Ginger (*Zingiber officinale*) were screened *in vitro*.

Ten per cent of aqueous extract prepared (1:1) and the medium was added with 0.1ml of bacterial suspension (10^9 cell/ml). The

sterilized 5 mm diameter filter paper discs were dipped in phyto extract and placed on petridish which is analysed by measuring the zone of inhibition radius after 48 hrs of incubation to know their anti-bacterial effect on the growth of the *Pantoea*.

Antagonistic effect of different microorganisms to *Pantoea*

Antagonistic effect of *Trichoderma viride*, *Penicillium digitatum*, *Aspergillus niger*, *Trichoderma harzianum*, *Bacillus amyloliquifaciens*, *Pseudomonas fluorescens* and *Bacillus subtilis* have been tested against *Pantoea* under paper disc method.

The medium was added with 0.1ml of bacterial suspension (10^9 cell/ml) and filter paper discs were dipped in previously prepared suspension (10^8 cells or spore/ml) of different microorganisms then placed on petridish. The zone of inhibition radius was measured after 48 hrs of incubation.

Inhibitory effect of fungicides and antibiotics against *Pantoea*

Six fungicides belonging to different chemical groups like, Mancozeb, Copper oxy chloride, Carbendazim, Hexaconazole, Propiconazole and copper hydroxide have been tested at three different concentrations (0.1%, 0.2% & 0.3%) to know their inhibitory effect against *Pantoea* under paper disc method by measuring the zone of inhibition radius after 48 hrs of incubation.

Antibacterial activity of five antibiotics viz., Streptomycin sulphate, Rifamycin, Kanamycin, Ampicillin and Chloromphenical at 250 ppm, 500 ppm and 750 ppm were tested by dipping the paper disc in different antibiotics at three concentrations and inhibition radius measured after 48 hrs of incubation to assess the antibacterial activity of antibiotics on the growth of the *Pantoea*.

Results and Discussion

Isolation and characterization of pathogen

The diseased leaves of rice showing typical bacterial blight (BB) symptoms were visually observed, collected and the disease symptom was characterized by yellowing and orange to brown stripes on the leaf blade. The symptoms enlarged to the entire leaf and showed brown stripes below the leaf tip used for isolation of pathogen. Upon plating on nutrient agar medium, yellow pigmented straw to yellow colored, raised and translucent with smooth margin colonies were obtained after incubation at 28°C for 2 days. The bacteria are gram-negative, facultative anaerobes with small rods either arranged singly or in chains. The isolated bacteria proved to be pathogenic to rice beyond doubt satisfying Koch's postulate.

All the isolates showed positive results in gram staining, KOH test, Catalase test, Citrate utilization test, Production of yellow pigment on YDC medium, Anaerobic growth test, Starch hydrolysis test, Tween 80 hydrolysis medium except some isolates. Some of the biochemical tests gave overlapping results regarding the identification of the causal organism of bacterial blight.

Based on 16S rRNA sequence analysis the causal agent was identified as *Pantoea stewartii* sub sp. *indolegenes*.

The sequencing data obtained has been deposited in NCBI gene bank with accession no. SUB2733370: MF163273 (ASD 16), MF163274 (TN 1), MF163275 (CO 43). The alignment showed maximum (99%) homology with the related sequence in the data bank. The biochemical and molecular analysis revealed that the causal agent was not *Xanthomonas oryzae* pv *oryzae*, but it was *Pantoea stewartii* sub sp. *indolegenes*.

In vitro* evaluation of inhibitory effect of botanicals and antagonistic effect of microorganisms against *Pantoea

The results revealed that among fourteen phyto extracts, the extract of *Prosopis* (10.33mm) followed by Chilli (10.00mm) and Nochi (9.33mm) recorded maximum inhibition radius, significantly better in checking the growth of *Pantoea* under paper disc method. The phyto extract of *Aadathoda* (0.00), Onion (0.00) and *Pongam* (0.00) failed to inhibit the growth of *Pantoea* as the inhibition radius of these treatments were all most zero, same as control. The observations of inhibition radius were recorded in mm, which is statistically analysed and presented in Table 1.

The inhibition radius was recorded significantly more in case of *Bacillus subtilis* (8.66mm) followed by *Bacillus amyloliquefaciens* (7.64mm) than the rest of

antagonists and control treatment, during all the three period of observation. The antagonist *Trichoderma* failed to inhibit the growth of *Pantoeas* in case of control. The inhibitory effect of *Bacillus subtilis* was reduced significantly from first observation to second observation and second observation to third observation *i.e.* 96 hours.

The observations of inhibition radius were recorded in mm, which is statistically analysed and presented in Table 2.

In vitro* screening of antibacterial properties of fungicides and antibiotics against *Pantoea

It is evident from the result, Copper oxychloride (5.56mm) followed by Copper hydroxide (4.87mm) and Mancozeb (3.64mm) recorded the highest inhibition radius significantly at all the three concentrations under paper disc method.

Table.1 Inhibition radius exhibited by botanicals against *Pantoea*

Trt. No	Botanicals	Mean of inhibition radius in mm
1	<i>Prosopis</i>	3.29 ^a * (10.33)**
2	Chiili	3.24 ^a (10.00)
3	Nochi	3.13 ^{ab} (9.33)
4	Tulsi	3.08 ^{abc} (9.00)
5	Ginger	2.91 ^{bcd} (8.00)
6	Garlic	2.85 ^{bcd} (7.67)
7	Neem	2.82 ^{bcd} (7.50)
8	Mehandi	2.79 ^{cd} (7.33)
9	<i>Lantana</i>	2.76 ^{cd} (7.17)
10	<i>Vinca</i>	2.75 ^d (7.10)
11	<i>Calatrophis</i>	2.73 ^d (7.00)
12	<i>Aadathoda</i>	0.71 ^e (0.00)
13	Onion	0.71 ^e (0.00)
14	<i>Pongum</i>	0.71 ^e (0.00)
15	Control	0.71 ^e (0.00)

The treatment means are compared using Duncan Multiple Range Test (DMRT).

In a column, means followed by a common letter (s) are not significantly different (P = 0.05).

* Indicate square root +0.5 transformed values

**Figures in the parenthesis are original values.

Table.2 Inhibition radius due to various microorganisms

T. No	Antagonists	Inhibition radius in mm			
		24hrs	48 hrs	96 hrs	Mean
1	<i>Bacillus subtilis</i>	3.16 (9.50)	2.99 (8.46)	2.92 (8.03)	3.02 ^{a*} (8.66)
2	<i>Bacillus amyloliquefaciens</i>	2.99 (8.43)	2.82 (7.47)	2.74 (7.02)	2.80 ^b (7.64)
3	<i>Pseudomonas fluorescens</i>	2.41 (5.32)	2.20 (4.33)	2.09 (3.87)	2.23 ^c (4.50)
4	<i>Trichoderma viridae</i>	0.71 (0.00)	0.71 (0.00)	0.71 (0.00)	0.71 ^d (0.00)
5	<i>Trichoderma harzianum</i> ,	0.71 (0.00)	0.71 (0.00)	0.71 (0.00)	0.71 ^d (0.00)
6	Control	0.71 (0.00)	0.71 (0.00)	0.71 (0.00)	0.71 ^d (0.00)

The treatment means are compared using Duncan Multiple Range Test (DMRT).

In a column, means followed by a common letter (s) are not significantly different (P = 0.05).

* Indicate square root transformed values

Figures in the parenthesis are original values.

Table.3 Inhibition radius due to various fungicides at different concentrations

Trt. No.	Name of chemicals	Inhibition radius at 0.1% (mm)	Inhibition radius at 0.2% (mm)	Inhibition radius at 0.3% (mm)	Mean of inhibition radius (mm)
1	Copper oxy chloride	2.21(4.39)	2.38(5.18)	2.76(7.12)	2.45 ^{a*} (5.56)**
2	Copper hydroxide	1.96(3.36)	2.27(4.68)	2.65(6.57)	2.20 ^b (4.87)
3	Mancozeb	1.71(2.45)	1.88(3.04)	2.43(5.42)	2.01 ^c (3.64)
4	Propiconazole	1.39(1.45)	1.73(2.50)	2.28(4.70)	1.80 ^d (2.82)
5	Hexaconazole	1.01(0.53)	1.46(1.91)	1.84(2.91)	1.44 ^e (1.78)
6	Carbendazim	0.71(0.00)	0.71(0.00)	0.71(0.00)	0.71 ^f (0.00)
7	Control	0.71(0.00)	0.71(0.00)	0.71(0.00)	0.71 ^f (0.00)

The treatment means are compared using Duncan Multiple Range Test (DMRT).

* Indicate square root transformed values. ** Figures in the parenthesis are original values.

Table.4 Inhibition radius due to various antibiotics at different concentrations

Trt. No	Name of antibiotics	Inhibition radius at 250 ppm (mm)	Inhibition radius at 500ppm (mm)	Inhibition radius at 750 ppm (mm)	Mean of inhibition radius in mm
1	Streptomycin sulphate	2.39(5.22)	2.66(6.61)	3.17(9.56)	2.74 ^{a*} (7.13)**
2	Rifamycin	2.26(4.64)	2.50(5.77)	2.81(7.45)	2.52 ^b (5.95)
3	Kanamycin	0.97(0.45)	1.43(1.55)	1.70(2.39)	1.37 ^c (1.46)
4	Carbenicillin	0.71(0.00)	0.71(0.00)	0.71(0.00)	0.71 ^d (0.00)
5	Ampicillin	0.71(0.00)	0.71(0.00)	0.71(0.00)	0.71 ^d (0.00)
6	Control	0.71(0.00)	0.71(0.00)	0.71(0.00)	0.71 ^d (0.00)

The treatment means are compared using Duncan Multiple Range Test (DMRT).

* Indicate square root transformed values. ** Figures in the parenthesis are original values.

It is clearly evident, in case of most effective and better treatments, higher concentration is positively correlated with maximum inhibition radius. But, Carbendazim (0.00) does not show any effect on the control of *Pantoea*. The observations of inhibition radius were recorded in mm, which is statistically analysed and presented in Table 3.

In accordance with the results of antibacterial effect of antibiotics, the maximum inhibition radius were recorded in Streptomycin sulphate (7.13mm) followed by Rifamycin (5.95mm) under paper disc method. Carbenicillin (0.00) and Ampicillin (0.00) does not show any effect on the control of *Pantoea*. The observations of inhibition radius were recorded in mm, which is statistically analysed and presented in Table 4.

The BLB symptom was observed as yellowing of leaves and orange to brown stripes on the leaf blade. Later, symptom enlarged to the entire leaf showed brown to orange water soaked lesions at margin and below the leaf tip. Similarly, Mondal *et al.*, (2011) revealed that the symptom exhibited as water soaked lesions at the tip of rice leaves and turned light brown, exhibiting a blighted appearance. Morphological and biochemical characterization revealed the pathogen is gram-negative, facultative anaerobes with small rods either arranged singly or in chains and yellow pigmented. The results were in accordance with Mondal *et al.*, (2011) and Gonzalez *et al.*, (2015). Anaerobic growth test is a key test for the identification of the bacterial genera *Erwinia* and *Pantoea*. Most of the isolates exhibit positive result on anaerobic growth test indicated clearly that the organism belongs to Enterobacteriaceae (i.e. facultative anaerobes) not belong to Xanthomonadaceae family (i.e. True aerobes). The production of yellow pigmentation on

YDC medium indicates the isolate belongs to *Pantoea* species. Similar results were observed by Pérez-y-Terrón *et al.*, (2009) and Haliatur Rahma *et al.*, (2014).

Our finding of new bacterial leaf blight of rice caused by *Pantoea stewartii* sub sp. *Indolegenes* coincidental with Mondal *et al.*, (2011) who gave a first report on 'New leaf blight of rice caused by *Pantoea ananatis* in India.

The hazardous effect of chemicals used in plant disease management has been diverted plant pathologists to find out the alternative method with little or no adverse effect on environment. From our experiment, it is clear that leaf extract of *Prosopis juliflora* L.), Chilli (*Capsicum annum* L.) and Nochi (*Vitex negundo*) may have antibacterial activity, which directly affects the growth of *Pantoea* causing BLB of rice. They may kill or inhibit the *Pantoea* bacteria present in radius of poison zone near the paper disc.

Rajeswari (1991) reported that the antibacterial activity of *Prosopis* against bacterial pathogen might be explained to have high level of glycoprotein and tannin. Therefore, in our experiment the extract of *Prosopis* was found most effective in inhibiting the growth of *Pantoea*. In this study, significantly more inhibition radius was recorded in case of *Bacillus subtilis* compared with other treatments.

Our findings were in agreement with Ahmed and Thind (1992) and Manmeet and Thind (2002) who reported the effectiveness of *Bacillus subtilis* and *P. fluorescens* against BLB causing bacteria *in vitro*. Velusamy *et al.*, (2006) who reported that *P. fluorescens* strain produced the antimicrobial antibiotic namely 2, 4-diacetylphloroglucinol (24-DAPG) which plays major role in mechanism of bio control. They also reported that the

compound inhibited the growth of the devastating rice BLB pathogen.

The results indicated that copper oxychloride and copper hydroxide at three different concentrations was found to having the inhibitory effect to *Pantoea*. But, carbendazim (0.00) does not show any effect on the control of *Pantoea*. Chauhan and Vaishnav (1980), Horti (1973) and Tagami and Mizukami (1962) reported the spraying of copper oxy chloride completely inhibits the growth of bacterium. Antibacterial activity exhibited by streptomycin sulphate (34.82%), rifamycin (28.28%) and kanamycin (21.27%) even at higher concentration and those were found highly effective in checking the growth of *Pantoea*. Carbenicillin and ampicillin does not show any effect on the control of *Pantoea*. Rahul singh *et al.*, (2015) reported that streptomycin and streptomycin + copper oxychloride reduced the bacterial disease intensity. Erasmus *et al.*, (2014) reported that streptomycin was found effective in inhibiting the growth of pathogen at higher concentrations significantly. Pereyra *et al.*, (2009), Nayak *et al.*, (2008) and Nithya *et al.*, (2007) also proved that higher concentrations of the antibiotics responded significantly for the control of BLB of rice.

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