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Emerging Incidence of *Burkholderia* spp. in Rice Seed Samples of Odisha

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ABSTRACT: A total of 188 samples of paddy seed were collected from different districts of Odisha in 2018-19 and 2019-20 and were used for testing the seed health status. Bacterial pathogens viz. *Xanthomonas* spp., *Pseudomonas* spp. and *Burkholderia* spp. were found associated with seeds. *Burkholderia* spp. was found to be associated with rice seed samples saved by farmers in Odisha, which is a new emerging pathogen causing panicle blight disease. Along with the bacterial pathogens some common fungal pathogens *viz. Helminthosporium oryzae, Curvularia* spp., *Fusarium* spp., *Sarocladium oryzae, Alternaria* spp., *Trichoconis sp., Cladosporium* sp. and *Aspergilus* spp. were found to be associated with the paddy seed samples.

Keywords: Rice Seed, Seed health test, *Burkholderia* spp., *Pseudomonas* spp. , *Xanthomonas* spp.

INTRODUCTION

Rice is the main staple food of most of the states of India and Odisha is one among them. The significance of rice within the agricultural economy of Odisha is of great magnitude, as it has a prominent position in the dietary patterns, economic activities, cultural practices and historical context of the state. The production of rice has a crucial role in determining the level of food security within the state, exerting a substantial impact. But there are many factors which are responsible for low yield in rice crop and the main cause is the infectious diseases that include twenty one fungal, six bacterial, seven viral and four nematode diseases (Hollier *et al.*, 1993; Sharma and Bambawale 2008). Most of these infectious fungal, bacterial and viral diseases are seed-borne in nature. These seed-borne pathogens cause damage at different stages like seed germination, seedling establishment, vegetative growth, reproductive phase as well as at storage period and play an important role in transmitting the disease from seed to seedling and to growing plants (Ashura *et al.*, 1999; Zhou-qi *et al.*, 2016; Shekhar *et al.*, 2020). Due to global warming and fluctuation in climate many minor diseases are converted to major pathogens in rice cultivation (Nandakumar *et al.*, 2007; Mondal *et al.*, 2015). So seed health testing is the convenient method to detect the presence of seed-borne pathogens (Habib *et al.*, 2012; Archana and Prakash 2013). Seed health test reveals the information about the organism carried by the seed and level of infection that will be introduced to another region through the seeds. Such information comes from survey under field condition and seed samples collected from farmers saved seed. The present study was conducted by Seed Technology Research, AICRP on NSP (Crops), O.U.A.T., Bhubaneswar and for this purpose farmers saved rice seed samples were collected from different districts of Odisha and seed health test was conducted following the ISTA method.

MATERIALS AND METHODS

Collection of seed sample: A total of 188 farmers saved rice seed samples were collected from 9 different districts of Odisha namely Baragarh, Baleswar, Cuttack, Ganjam, Khurda, Mayurbhanj, Puri, Rayagada and Sambalpur in 2018-19 and 2019-20.

Seed health testing: Four hundred seeds per seed sample were taken and surface disinfected with 1% freshly prepared sodium hypochlorite solution for 1 minute and 2-3 times rinsed with sterilized distilled water. Three layers of Whatman No.1 filter papers were soaked with sterilized distilled water and fitted in Petri dish. Then on each Petri dish 25 numbers of the seeds were plated on the filter paper in 2-3 concentric rings. The plates were incubated at 25°C for 7 days under alternate light and dark condition (12hr nuv/ 12hr dark) in an incubator to test the fungal association with seeds. Another one set of plates were incubated at 28°C for 48 hours under alternate light and dark condition (12hr nuv/ 12hr dark) in an incubator to test the presence of bacteria in the seed sample. After 7days of incubation pathogenic and saprophytic fungi were counted and the fungi associated with seeds were isolated and identified under the microscope by their colony growth, colour and sporulation. The percent incidence was recorded by the formula

Incidence = Number of infected seeds/ Number of plated seeds × 100

To know the bacterial association with rice seeds, the plates incubated at 28°C for 48 hours, were thoroughly examines under the stereoscopic microscope to see the bacterial ooze or any bacterial growth over the seed surface. The percent incidence was also calculated using the above formula. The seed having bacterial growth over it was aseptically transferred to the Nutrient Agar plates and pure cultures were obtained by repeated streaking of bacteria. The Gram's reaction test and the different biochemical test for sugar utilization, nitrate utilization etc. were conducted to identify the bacterial isolates.

Gram's reaction of the bacterial isolates: Uniform smears of the isolates were made on a clean glass slide, by the help of a sterilized loop using sterile distilled water. The smear was allowed to air dry followed by heat fixing. The smears were stained using crystal violet and safranine as primary and secondary stains respectively following the method of Christian Gram (1884) with modification (Cruickshank, 1968).

Biochemical characterization: Different biochemical tests viz. catalase test, oxidase test, Voges Prausker's test, methyl red test and gelatin liquefaction were conducted using standard protocols (Biyyani *et al.*, 2018).

Indol test: A sterilized test tube containing tryptophan broth was inoculated with 48 hours old bacterial culture and incubated at 37°C for 24hrs. The test was performed by adding 4-5 drops of Kovac's reagent to the broth. A red colour at the surface of the broth indicates a positive result, while a yellow colour indicates a negative result.

Catalase test: Catalase test was performed by taking a drop of 3% hydrogen peroxide and adding to 48hrs old bacterial colony on a clean glass slide. The effervescence indicates catalase activity.

Oxidase test: The bacterial isolates were grown in nutrient agar slants. Oxidase paper discs were kept on fully grown cultures for 48hrs. A colour change to purple indicates positive result.

Voges Prausker's test: The test was performed by adding alpha-naphthol and potassium hydroxide to the Voges Prausker's broth. A cherry red colour indicates a positive result, while a yellow –brown colour indicates a negative result.

Methyl red test: Sterilized glucose phosphate broth tubes were inoculated with the test culture and incubated at 28°C for 48 hrs. After incubation, five drops of methyl red indicator was added to each tube and gently shaken. Red colour production was taken as negative for the test.

Gelatin liquefaction: The overnight cultures of the test isolates were inoculated in sterilized nutrient gelatin deep tubes and incubated for 24hrs at 28°C. Then the tubes were kept in the refrigerator for 30mins at 4°C. The isolates showing liquefied gelatin was taken as positive and those which resulted in solidified of gelatin on refrigeration were recorded as negative.

Growth at 5% NaCl: The nutrient agar medium was prepared with 5% NaCl, poured onto Petri plates and allowed for solidification. A 24hrs old bacterial culture was streaked on the media and incubated for 48hrs at 28°C. The growth on the medium indicated tolerance to high salt concentration.

Growth at 45°C and 65°C: The 24 hrs old bacterial culture was streaked on Petri plates containing NA media and incubated at 45°C for 48 hrs. Another set of Petri plates with bacterial culture were incubated at 65°C for 48hrs. The growth of culture on media indicated positive for survival under high temperature.

H_2S production: The 24hrs bacterial cultures were stab inoculated into the tubes containing Triple Sugar Iron media and incubated at 37°C for 24hrs. Formation of black precipitation in the medium indicating the positive reaction.

Caseinase test: The plates with milk agar media were inoculated with individual bacterial isolates aseptically and incubated at 35°C for 24hrs. The appearance of cleared zones around the colonies indicating the positive result of casein hydrolysis.

Starch hydrolysis test: For starch hydrolysis test, 2g NA was added to 80ml of water and dissolved by successive heating and stirring similarly 2g starch was then thoroughly dissolved in 10ml distilled water separately and added to hot molten agar with through stirring. The plates were then inoculated with individual isolates aseptically and incubated at 28°C for 7days. After scrapping bacterial growth to each plate Lugol's iodine was added. The appearance of blue cleared zones around the colonies indicating the positive result of starch hydrolysis.

Urease test: Test for the presence of the enzyme urease in the isolates which split urea into ammonia and carbon dioxide was studied using Christensen's urea agar medium. Colour change of the slant from yellow to pink was considered as positive result while no change in colour was taken as negative, for indole production by the isolates.

Citrate utilization test: The capability of the isolates to utilise citrate as the sole source of carbon and energy was studied on Simmons citrate agar medium. Colour change of the slant from green to royal blue was considered as positive result while no change in colour was taken as negative.

Nitrate test: Sterilized nitrate broth tubes were inoculated with the test culture and incubated at 37°C for 48 hrs. After incubation, check the presence of gas bubbles in broth then 6-8 drops of nitrite reagent A and B was added to each tube one after another. Red colour production within seconds to a minute was taken as positive for the test.

Carbohydrate utilization test: The organism use carbohydrate differently depending upon their enzyme complement. The pattern of fermentation is

characteristics of certain species, genera or groups of organisms and this property has been extensively used as a method for biochemical differentiation of microbes. To carry out this test various carbon sources were used. First, a litre of nutrient broth was prepared and a pinch of phenol red indicator was added which is responsible for the colour change during the growth of bacteria in the nutrient broth. The nutrient broth of about 10ml was poured into each test tube and durham tubes were placed in each test tube and autoclaved. After autoclaving the test tubes were added with different carbon sources like maltose, fructose, galactose, mannose, sorbitol, mannitol, adonitol, cellobiose and arabinose. To these test tubes 1ml of 48hrs old culture is added and observed for colour change in the test tube. When the bacteria ferment carbohydrate an acid or acid with gas are produced. Depending upon the organisms involved and the substrate being fermented, the end products may vary. Common end products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide and hydrogen. The production of the acid, lower the pH indicator. Colour of the medium changes from red colour to yellow colour if the test positive, while medium remains red in colour if the test is negative. The biochemical test results were used in ABIS online software to identify the bacterial isolates.

RESULT AND DISCUSSION

The seed samples collected from different districts of Odisha were used for seed health testing and the result are furnished in table 1. From the present investigation it was found that seed samples collected from different districts of Odisha were having associated with some fungal and bacterial pathogens. Fungal pathogen association was recorded in all the 188 seed samples, however bacterial association was found only with 48 seed samples. The maximum fungal infection (34.5%) was recorded in the seed samples collected from Baleswar district followed by the seed samples collected from sambalpur district (32.5%). The lowest fungal association (7.5%) was found in the seed samples of Khurda district. The maximum number of seed samples (45.8%) collected from Ganjam district were found to have the bacterial association whereas the minimum number of seed samples to have bacterial association was Cuttack *i.e.* 12%. The maximum bacterial infection in each sample (26%) was recorded in the seed samples collected from Ganjam district followed by the seed samples collected from Sambalpur district (21%). The lowest bacterial association (2.5%) was found in the seed samples of Rayagada district.

Table 1: List of rice seed samples collected from different districts of Odisha.

Sr. No.	District	Number of Sample collected	Variety	Range of fungal association with each seed sample (%)	Number of seed samples with bacterial association (%)	Range of bacterial association with each seed sample (%)
1.	Baragarh	22	Swarna, Pooja, Pratikhya, Kalachampa, Bina, Naveen, Barsha	18.5-27	27.2	4.5-18
2.	Baleswar	18	Prativa, Acharmati, Prachi Sidhanta, Uphar, Mahanadi, Padmini	11-34.5	22.2	5-12.5
3.	Cuttack	25	Kranti, Parijat, Swarna, Ranidhan, Prachi, Indrabati, Pratikhya	9-25	12.0	4.5-8.5
4.	Ganjam	24	Swarna, Lalat, Barsha, Uphar, Kalachampa, Kalajeera, Sidhanta	12-25.5	45.8	10.5-26
5	Khurda	18	Jagannath, Moti, Swarna, Konark, Jagabandhu, Lalat, Krushnabhog	7.5-29	16.6	7.5-20
6.	Mayurbhanj	20	Mayurakantha, Lalat, Surendra, Anjali, Khandagiri	12.5-31	20.0	8-12.5
7.	Puri	24	Asutosh, Mrunalini, Upahar, Gajapati, Acharmati, Padmini, Udaygiri, Sidhanta	10.5-28	25.0	7.5-10.5
8.	Rayagada	15	Mrunalini, Ranidhan Baidyaraj, Pooja, Sarala, Prachi, Lalat, Ranjit, Sindhu,	10-24.5	13.3	2.5-4.5
9.	Sambalpur	22	Swarna, Padmini, Jajati, Kalajeera, Masoori, Manaswini, Mahanadi, Barsha	14-32.5	40.9	11.5-21

Fourteen different bacterial isolates were isolated from 48 seed samples having bacterial association. The bacterial isolates were further purified and tested for different biochemical characterization. The Gram's test confirms that 6 isolates were gram positive and rest 8 isolates were gram negative. The biochemical test results (Table 2 & 3) were used in ABIS online software to identify the bacterial isolate. The gram positive bacterial isolates were found to be *Bacillus* spp. whereas gram negative bacterial isolates belong to the three different genera viz. *Xanthomonas*, *Pseudomonas* and *Burkholderia*. In the seed samples, 20.21% seed samples had the infection of *Xanthomonas* spp. followed by 9.57% infection of *Burkholderia* spp. and 6.38% of *Pseudomonas* spp. in them (Table 4). However most of the seed samples having bacterial association were found to have more than one species of bacterial infection in them.

The fungal mycoflora associated with seed samples were purified and identified by microscopic examination of morphological character of mycelia and spore. A total 8 genera of fungi *viz. Helminthosporium oryzae*, *Curvularia* spp., *Fusarium* spp., *Sarocladium oryzae*, *Alternaria* spp., *Trichoconis sp.*, *Cladosporium sp.* and *Aspergillus* spp. comprising fourteen species were found associated with the seed samples. Among them the most prominent fungal pathogen was *Curvularia* spp. which was associated with 86.17% of seed samples, including *Curvularia lunata*, *Curvularia geniculata* and *Curvularia oryzae*. The second highly infecting fungal pathogen was found to be *Helminthosporium oryzae* with 80.85%. The pathogen *Fusarium* spp. was found in 48.93% of seeds comprising three different species namely *Fusarium moniliforme*, *Fusarium oxysporum* and *Fusarium solani*. The other fungal pathogens found associated with seed samples were *Sarocladium oryzae* (17.02%), *Cladosporium sp.* (6.38%), *Trichoconis sp.*(4.78%), *Alternaria* spp. (6.38%) including *Alternaria* alternata and *Alternaria* padwickii. A common storage fungi *Aspergilus* spp. was also found associated with 67.55% of seeds comprising two different species *i.e. Aspergillus flavus* and *Aspergillus niger*.

Table 2: Biochemical test of bacterial isolates.

Tests	*Bacillus* spp.	*Pseudomonas* spp.	*Xanthomonas* spp.	*Burkholderia* spp.
Gram's test	+ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve
MR	+ve	-ve	+ve	-ve
VP	-ve	-ve	-ve	-ve
Citrate	-ve	+ve	+ve	-ve
Growth at 45°C	+ve	+ve	-ve	-ve
Growth at 65°C	-ve	-ve	-ve	-ve
Growth at 5% NaCl	+ve	+ve	-ve	-ve
Caseinase	+ve	+ve	-ve	+ve
H2S production	+ve	-ve	-ve	-ve
Gelatinase	+ve	+ve	-ve	+ve
Starch hydrolysis	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	-ve	+ve
Nitrate	+ve	+ve	-ve	-ve
Ureaase	+ve	-ve	-ve	-ve

Table 3: Sugar utilization test of bacterial isolates.

Sugars	*Bacillus* spp.	*Pseudomonas* spp.	*Xanthomonas* spp.	*Burkholderia* spp.
Arabinose	-ve	-ve	+ve	+ve
Cellibiose	+ve	-ve	+ve	+ve
Fructose	+ve	-ve	+ve	+ve
Glucose	+ve	-ve	+ve	-ve
Glycerol	+ve	+ve	-ve	+ve
Starch	+ve	-ve	-ve	-ve
Inositol	-ve	-ve	+ve	+ve
Lactose	+ve	-ve	+ve	-ve
Manitol	+ve	+ve	-ve	-ve
Mannose	-ve	-ve	+ve	-ve
Maltose	-ve	+ve	+ve	-ve
Melizitose	-ve	-ve	-ve	-ve
Melibiose	-ve	-ve	-ve	-ve
Rhamnose	+ve	-ve	+ve	+ve
Ribose	-ve	-ve	-ve	-ve
Salicin	+ve	-ve	-ve	+ve
Sorbitol	+ve	+ve	-ve	+ve
Sucrose	+ve	-ve	+ve	+ve
Xylose	+ve	-ve	+ve	+ve

Table 4: Occurrence of seed-borne pathogens in rice samples collected from different districts of Odisha.

Sr. No.	Pathogen	Seed lot infection (%)	Range of infection in samples									
			1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
1.	*Burkholderia* spp.	9.57	5	9	4	-	-	-	-	-	-	-
2.	*Pseudomonas* spp.	6.38	4	5	3	-	-	-	-	-	-	-
3.	*Xanthomonas* spp.	20.21	12	18	4	2	1	1	-	-	-	-
4.	*Alternaria* spp.	6.38	4	3	4	1	-	-	-	-	-	-
5.	*Aspergillus* spp.	67.55	27	21	12	14	18	16	19	-	-	-
6.	*Cladosporium sp.*	6.38	5	7	-	-	-	-	-	-	-	-
7.	*Curvularia* spp.	86.17	47	32	27	25	18	7	6	-	-	-
8.	*Fusarium* spp.	48.93	21	25	18	12	11	5	-	-	-	-
9.	*Helminthosporium oryzae*	80.85	25	19	32	48	12	5	7	4	-	-
10.	*Sarocladium oryzae*	17.02	12	6	5	9	-	-	-	-	-	-
11.	*Trichoconis sp.*	4.78	3	2	3	1	-	-	-	-	-	-

In the present study , the incidence of bacteria reflect the economic importance of various bacterial diseases of rice as the maximum yield loss can be possible by the bacterial diseases as reported by different workers like Trung *et al.* (1993), Nandakumar *et al.* (2007); Rajarajeswari *et al.* (2008); Shekhar *et al.* (2020). Many worker had tested different rice seed samples for seed health test and reported the presence of different fungal pathogens and bacterial pathogens in them. Ashura *et al.* (1999) had tested different seed samples of rice and reported the presence of *Xanthomaonas* oryzae pv. *oryzae, Burkholderia glumae, Pseudomonas putida, Pantoea agglomerans, Acedovorax avenae subsp. avenae* in them. Mondal *et al.* (2015) had also reported the incidence of bacterial panicle blight caused by *Burkholderia glumae* in norther parts of India mostly Uttar Pradesh, Haryana and Delhi. Detection of *Burkholderia* sp. in rice seed samples of Odisha is a threat to rice production in the state because the symptom on rice plants were visible at panicle stage and at that stage, control of that particular disease is very difficult. Similar statements were also given by different workers like Nandakumar *et al.* (2007); Mondal *et al.* (2015); Zhou-qi *et al.* (2016). In favourable conditions these bacteria can cause huge economic loss by reducing rice yield. Under favourable condition *Burkholderia* sp. can cause 73% yield loss (Trung *et al.*, 1993).

CONCLUSIONS

The present study reveals the presence of *Burkholderi*a sp. in rice seeds from different locations of Odisha. Current global climate change may be the cause of increase in bacterial panicle blight, as prolonged hot and humid conditions during the rice-growing season favour the development of serious epidemics of bacteria *Burkholderia* sp. This disease occur more frequently in tropical and semi-tropical regions during growing seasons with higher than normal temperatures and can survive in seeds. In a developing country where farmers have to save their own seed for planting, knowledge of seed health can be very important for crop and disease pest management. Since rice holds the immense cultural, economic significance, seed health management should be a pre requisite for successful rice cultivation and to protect the crop from such emerging pathogens.

FUTURE SCOPE

The bacterial panicle blight disease caused by *Burkholderia glumae* and *Burkholderia gladioli* has sporadic occurrence in the rice growing states of India and can be spread to other parts of the country and may cause the major economic loss to the farmers. As these bacteria can be identified by biochemical tests in any microbiological laboratory, the precautions like seed health testing can be done to check the spread of such new emerging pathogens.

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Conflict of Interest. None.

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