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ASSESSMENT OF FUNGAL PATHOGENS FROM INFECTED RICE AND ORANGES AND THEIR GROWTH IN ACIDIFIED MEDIA

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ABSTRACT

An attempt was made to isolate the fungal pathogens from rice and infected oranges. Further, the pathogens were identified based on their morphological characteristics and biochemical tests. Biochemical tests like Catalase test, Urease test, Gelatin Hydrolysis test and Carbohydrate Fermentation tests using Glucose (monosaccharide), Sucrose (disaccharide) and Lactose (disaccharide) were performed to identify the pathogens. All the test organisms were able to split the hydrogen peroxide to evolve oxygen. Hence, Catalase positive. Again, Penicillium possessed the enzyme Urease, producing ammonium carbonate, which raised the pH turning the urea agar into pinkish colour with phenol red indicator. The other two test organisms answered negative with Urease Test. Again, all the test organisms responded positively to Gelatin Liquefaction test, turning the gelatin to polypeptides and polypeptides were further hydrolysed to amino acids, marked by liquefaction of the gelatin agar medium even after refrigeration. All the test organisms responded positively with Carbohydrate Fermentation test, using glucose, producing acid and gas as a result of fermentation. Phenol Red used as indicator for Carbohydrate Fermentation tests showed that there was no acid and gas formation for lactose, except *Cladosporium*. Again, for sucrose fermentation reaction was observed for *Alternaria* and *Cladosporium*, but not *Penicillium*. Thus, the three test organisms found were *Alternaria, Penicillium* and *Cladosporium*. An interesting fact was that Acetic acid, an inhibitor of fungi, was used to culture *Alternaria* fungi.

KEYWORDS: *Alternaria*, *Penicillium*, *Cladosporium*, Catalase Test, Urease Test, Gelatin Hydrolysis Test, Acetic Acid, pH Variation.

INTRODUCTION

In a research it was found that, fungus is responsible for plant disease. Depending upon the environmental and atmospheric condition the fungal growth occurs. Mostly the growth of different species of *Penicillium* has been found.[1] *Penicillium* species are opportunistic pathogens basically grows in citrus fruit. The decay of the citrus fruits occurs due to the infection of *Penicillium*. It was seen that, due to repeated use of toxic chemicals can induce Fungicide resistant strain and human health risk factors.[2] The main cause behind the deterioration of fruits is post harvesting techniques like proper transport, marketing, dehydration, weight loss, colour change, microbial spoilage etc. The most important post harvest disease causing fungi includes *Penicillium* species, *Aspergillus* species, *Alteranaria* species and many more. Oranges are basically eaten in raw condition and spoilage fungus typically produces more diverse and greater amounts of extracellular depolymerases.[3] *It has also found that, Alternaria brown spot is highly destructive disease of tangerines and tangerine hybrids of worldwide importance. The disease is conventional in citrus production areas with a Mediterranean climate, characterized by cool, humid winters, hot and dry summers respectively.*[4] The growth of *Alternaria* species is very much influenced by interacting abiotic factors, especially water activity, temperature and pH.The competitiveness of *Alternaria* species is related to their water stress tolerance, production of hydrolytic enzymes and ability to produce mycotoxins. Since *Alternaria* species are common components of the airspora and components of the phyllosphere of many plants. Therefore, they inevitably come in contact with a diverse fungal and bacterial community. Heavy pigmentation of their spores presented over there is able to provide good UV resistance and plays a crucial role to their effective colonization of the phyllosphere/phylloplane of many plant surfaces. They are also having effective tolerance to abiotic factors and are able to produce the necessary hydrolytic enzymes to facilitate effective competitiveness in the ecological niches.[5] Among the fungus, *Cladosporium* species are the most abundant fungi in outdoor and indoor air. As the composition of indoor species reflects the composition of outdoor species, It can be expected to find *Cladosporium* as dominant indoors too.[6] Among the various different citrus fruits grown in India, mandarin orange (*Citrus reticulata*), sweet orange (*C. sinensis*) and acid lime (*C. aurantifolia*) basically occupy the first three positions

producing 41%, 23 % and 23%, respectively, of the total production of citrus fruits. India is the ninth largest mandarin orange producing country in the world. It occupies around 40% of the total area for citrus fruit cultivation in the country. Fungal infection in oranges is very common. Green mould, blue mould and stem-end rot are some major post harvested diseases of mandarin orange.[7] The occurrence of diseases in rice, mainly rice blast (*Magnaporthe oryzae*), is one of the most important cause of yield reduction, and it has been controlled with the calamitous use of fungicides in the conventional production system.[8] Rice (*Oryza sativa*) is a primary grain crop that is cultivated worldwide. *Penicillium spp*. are common contaminants of stored food products, particularly cereal grains, and able to deteriorate grain quality. Most importantly, these fungi can produce mycotoxins, the hazardous secondary metabolites. Mycotoxins, including aflatoxins B1, B2, G1, and G2 are mainly produced by *A. flavus* and *A. parasiticus*. Aflatoxin B1 is one of the most carcinogenic compounds, which is having several adverse effects on human and animal health by direct or indirect consumption of contaminated food.[9]

MATERIALS AND METHODS

Sample Collection

The infected oranges were collected from the markets of Kolkata, West Bengal and the rice seeds were separated from infected rice plants. Those infected plants were again collected from the rice fields of Medinipur and Kolkata, West Bengal, India.

Isolation of Pathogens

A very small quantity of the black infected portions from oranges, were picked up with the help of sterilized forceps and placed on fresh, sterilized PDA (Potato Dextrose Agar) plates within the laminar air flow hood. In case of the rice, the infected seeds and plant parts were directly placed on the sterilized Potato Dextrose Agar plates.

The pathogens on the PDA plates were then incubated at 25^{o}c for 3 days.

Again, for rice, the pathogens were sub cultured onto fresh PDA plates and incubated at 25^{o}c for 3 days.

Identification method of pathogens

Pathogens were identified based on their morphology and biochemical tests.

For morphology study, on a clean slide a drop of lactophenol cotton blue was taken and then a minute amount of the fungal mycelia, were placed on the drop. Teasing of the fungal

mycelia was done using a pair of needles. Then, the coverslip was slowly placed on the mycelia and observed under microscope. The whole process of staining was carried out in the laminar air flow, under aseptic conditions.

Several biochemical tests were also performed to identify the fungi at the genus level. Biochemical tests like Catalase test, Gelatin test, Urease test and Carbohydrate Fermentation test using glucose, sucrose and lactose.

Isolation of fungal pathogen on acid media

The fungi from oranges were also cultured on Nutrient Agar, acidified with glacial acetic acid. For 200ml of Nutrient Agar, 20µl of glacial acetic acid was used to turn the pH to 6.2.

RESULTS AND DISCUSSION

Fungi from infected oranges were identified as *Alternaria* whereas the fungi from rice were identified to be *Penicillium* and *Cladosporium*, based on morphology study and biochemical tests.

Table 1: Plate-culture characteristics of fungi.

Types of Fungi	Media Used	Pigmentation
Alternaria	Potato Dextrose Agar (PDA)	Whitish-green velvet
Penicillium	Potato Dextrose Agar (PDA)	Dark - green
Cladosporium	Potato Dextrose Agar (PDA)	Small blackish-green colonies

Table 2: Morphological characteristics of fungi (Micrograph).

Types of Fungi	Morphological characteristics of fungi
Alternaria	Large segmented conidia with tapering ends
Penicillium	Brush-like formations
Cladosporium	Fragmented branches with small conidia

Fig 1: PDA Plate culture- *Alternaria*;

Fig 2: Micrograph of *Alternaria* conidia

Fig 3: Conidiophore.

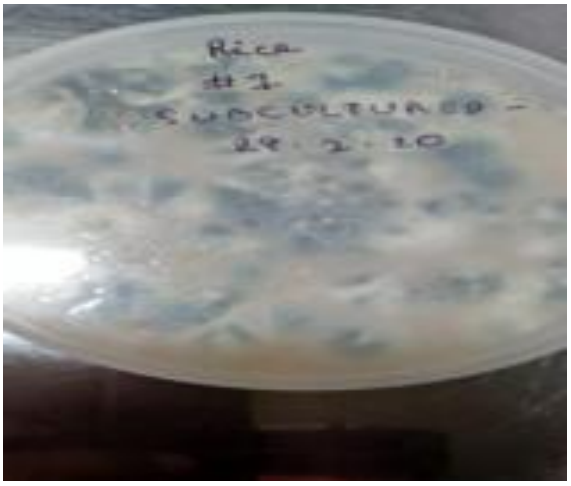

Fig 5: PDA Plate culture- *Penicillium*

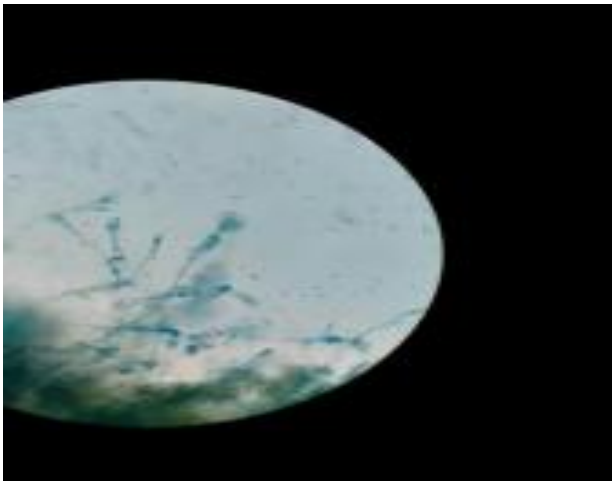

Fig 6: Micrograph of *Penicillium*.

Fig 7: PDA Plate culture- *Cladosporium*.

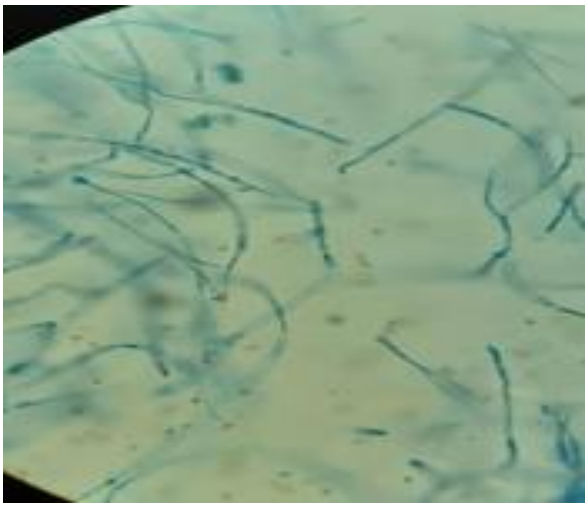

Fig 8: Micrograph of *Cladosporium*.

Biochemical tests of fungi

The cultured fungi from oranges and rice were subjected to several biochemical tests like

Catalase test, Gelatin Hydrolysis test, Urease test and Carbohydrate Fermentation test using glucose, sucrose and lactose.

Table 3: Response of different fungi to biochemical tests.

Types of Fungi	Catalase Test	Urease Test	Gelatin Hydrolysis Test	Carbohydrate Fermentation Tests		
				Glucose	Sucrose	Lactose
Alternaria	(+)	(-)	(+)	(+)	(+)	(-)
Penicillium	(+)	(+)	(+)	(+)	(-)	(-)
Cladosporium	(+)	(-)	(+)	(+)	(+)	(+)

(-) = Negative; (+) = Positive

pH Variation - *Alternaria* growth in Acidified Nutrient Agar

Alternaria was found to have good growth in acidified Nutrient Agar with pH 6.2, with glacial acetic acid.

Response of *Alternaria* fungi to acid medium

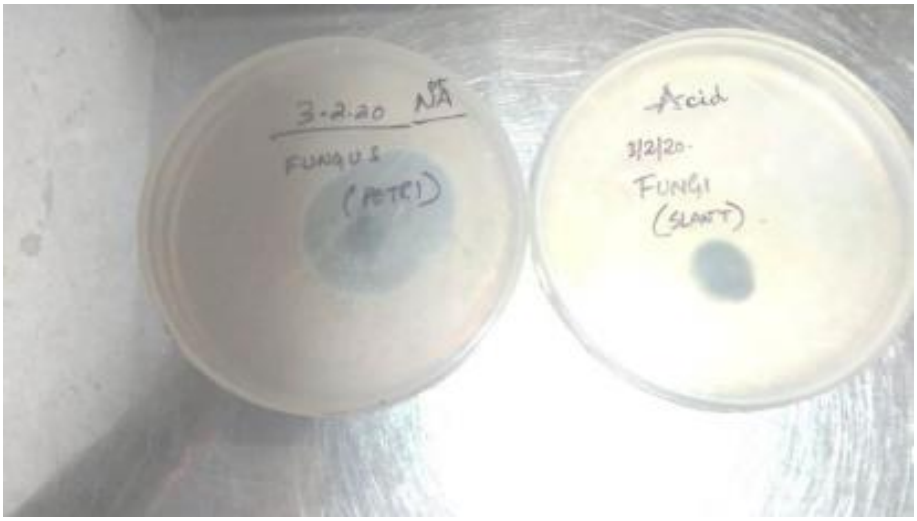

Fig 9: *Alternaria* growth in Nutrient Agar without acid (Left side) and Nutrient Agar with acid (Right side)

After culturing the fungal pathogens onto separate sterile PDA plates and PDA slants, morphological studies under microscope, had helped in identifying the fungi. For *Alternaria*, the whitish-green velvety mat on PDA plates and the positive results of Catalase test , Gelatin Hydrolysis test and Carbohydrate Fermentation tests using glucose and sucrose , had further proved that the fungi was *Alternaria.* Hence, the *Alternaria possesses* the Catalase enzyme by means of which it was able to split the added hydrogen peroxide to water and oxygen. The same was true for *Penicillium* and *Cladosporium* as they were also found to be positive.

Again, *Alternaria* and *Cladosporium* were found to be negative, while *Penicillium* was found to possess the enzyme urease, which hydrolysed urea to form ammonium carbonate, which raised the pH turning phenol red indicator to pink colour. For Gelatin Hydrolysis test, all the test organisms like *Alternaria, Penicillium* and *Cladosporium* had answered positive. Hence, all those organisms had hydrolysed gelatin to polypeptides and polypeptides were further hydrolysed to amino acids, exhibited by liquefaction of the inoculated gelatin agar media, even after refrigeration. Again, for carbohydrate fermentation test, all the test organisms were able to metabolize glucose to produce acid, marked by yellow colouration with phenol red indicator and gas, marked by a gap in the Durham's tube inserted in the carbohydrate broth. But *Penicillium* was not able to metabolize sucrose (disaccharide), whereas *Alternaria* and *Cladosporium* were able to produce acid and gas from the sugar. Lactose fermentation was positive only for *Cladosporium*, whereas negative for *Alternaria and Penicillium*. *Alternaria* was able to grow in low acid concentration media, also, though it was established that acetic acid was an inhibitor of fungi.[10]

CONCLUSION

Thus, the test organisms found were *Alternaria, Penicillium* and *Cladosporium*, identified based on morphological studies and biochemical tests. Also, the interesting fact that on pH variation, *Alternaria* was found to grow profusely. Hence, further research will be carried out in this regard.

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