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Antagonistic Effect of the Phylloplane Yeast against *Fusarium*, *Colletotrichum* and *Penicillium* spp.

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A B S T R A C T

Fusarium, *Colletotrichum* and *Penicillium* spp. are the well-known pathogen that causes post-harvest plant diseases of the crops. Mainly it influences the fruit crop which plays the most important role in the economy of the many fruit-producing countries, so for the management of these post-harvest pathogens, it should need a specific management strategy. So, keeping in view the study was investigated in the laboratory of the Dept. of Mycology and Plant Pathology, Institute of Agricultural Sciences, BHU, Varanasi. These pathogens were tested against thirteen different phylloplane yeast isolates by dual culture technique. Yeast species mainly compete with the pathogen for nutrients and after contact with a pathogen, it leads to cell wall perforation due to producing lytic enzymes as well as acts as the host defense activator in the plant. Herein were observed that isolates Y4 (33.3%) and Y6 (33.3%) showed maximum and Y13 (4.4%) minimum percent inhibition against *Colletotrichum* spp. while maximum and minimum percent inhibition was showed by isolate Y3 (40%) and Y5 (4.4%) against *Fusarium* spp. respectively. Isolate Y4 (51.1%) and Y7 (51.1%) showed maximum inhibition as compared to isolate Y11 (35.5%) that showed minimum inhibition but no per cent inhibition was observed by isolate Y10 (0%) against *Penicillium* spp.

Introduction

Post-harvest losses of fruits and vegetables are 20-50 % in developing countries and one of the causes of these losses include post-harvest diseases. Environment-friendly management of post-harvest diseases is the need of the hour due to the harmful effects of

synthetic fungicides on human and other non-target organisms leaving residual effects as well as the development of resistance in pathogen populations. Due to the increasing concern of the public about safe consumable foods. Yeasts seem to be a very viable option for the management of postharvest pathogens of fruits as well as vegetables. Currently, only a few yeast-based biocontrol products are available in the market. Aspire to contain *Candida oleophila* was registered by U.S Environmental Protection Agency in 1995 for control of postharvest rots of citrus fruit but is no longer available in the market as it was unable to control previously established as well as a latent infection. This was the first launched yeast-based biocontrol product. Schemer containing *Metschnikowia fruticola*, Candifruit containing *Candida sake*, ProYeast-ST and ProYeast-ORG containing heat-tolerant strains of *Metschnikowia fruticola* are some yeast-based biocontrol products available in some countries for commercial use but these are yet to become popular among the formers. *Candida saitonana* based products, Biocure (derivative of chitosan) and Biocoat (derivative of lysozyme) are still awaiting registration. Keeping the above view, the present investigation has been carried out aiming to use potential phylloplane yeast isolates isolated from different fruit phylloplane against post-harvest pathogens.

Materials and Methods

Sample collection

Fresh leaf or fruit samples of banana, citrus, mango, apple, and grapes were collected during different seasons in separate brown paper packets from well-grown established trees of selected fruit from the Banaras Hindu University campus and brought to the Department of Mycology and Plant Pathology, (BHU) and marked the sample properly for a further research experiment.

Preparation of media

Yeast Dextrose Peptone Agar media

YDPA was prepared for isolation, maintenance, and multiplication of yeast by promoting the growth. Yeast Dextrose Peptone Agar medium prepared from the following ingredients (Cold Spring Harbor Protocols, 2010).

After (YDPA) medium get ready it was then gently poured in the conical flask and sterilized at 15 psi at 21°C for 20 minutes in the autoclave. After pouring, the media was kept in room temperature for cooling and then it was used for further plating and slant preparation.

Potato Dextrose Agar media

PDA mainly prepared for the maintenance of fungal pathogen culture. The PDA was used during a dual culture plating process after adding 10 gm of yeast extract per litre for testing the antagonistic potential of yeast isolates against different pathogen

Isolation of yeast from leaf sample following leaf imprint (Schultz *et al.*, 1998) and fruit surfaces by washing (Chalutz and Wilson, 1990)

For isolation of yeasts, the leaf imprint method as described by (Schultz *et al*., 1998) was followed with a little modification. Initially, the Leaf samples were cut into a size of approximately 2x2 cm and then placed on solidified Yeast Dextrose Peptone Agar medium. For each sample, a minimum three replications were taken. Two pieces of leaves were placed in each Petri plate containing Yeast Dextrose Agar medium such that the upper surface of one piece and the lower

surface of other pieces of the leaf sample remain in contact with the medium. The inoculated plates remain undisturbed in laminar airflow for the duration of 4 hours and then the sample pieces were removed with the help of sterilized forceps. Then the plate was incubated for 2 days at 280°C in the BOD incubator. The same procedure was followed for every sample for isolation.

Apart from leaf imprint method (Schultz *et al*., 1998) the Yeast samples were also isolated from fruit surfaces by shaking fruits in sterile distilled water for 5 minutes (Aoudau, 2017) after putting in flask under aseptic conditions followed by serial dilution (Johnson and Curl, 1972) by tenfold and streaking the same on Yeast Dextrose Peptone Agar media maintaining three replications for each sample collected.

Maintenance of yeast isolates

After 2 days of incubation in the BOD chamber, the isolates were collected by streaking yeast colonies (Sherman, F., 2002)on the Yeast Dextrose Peptone Agar medium and then maintained in slants containing Yeast Dextrose Peptone Agar medium. Slants were prepared by pouring melted medium in a test tube then test tubes were properly plugged with cotton. After solidification, the prepared slants were inoculated with yeast cells from a single colony that developed on the plate. Then slants were incubated at 27±1ºC for three days at least and refrigerated for further use. Sub-culturing of the isolates was done as when required.

Isolation of post-harvest pathogen

The direct plating technique described by Pitt and Hocking (1985) was employed. The fruit samples were surface-sterilized for 3 minutes with 1% sodium hypochlorite (NaOCl) and rinsed in four successive changes of sterile distilled water. Four small pieces from the margin of lesion of each sample were directly inoculated on prepared plates of Potato dextrose agar which contain (g/L) of ingredients mentioned in Table. 1. The medium was supplemented with chloramphenicol (250 mg per liter) as a bacteriostatic agent (Smith and Dawson, 1944). The plates were inoculated at 28 ± 1 °C for 5 to 7 days. Three replicates were prepared for each sample. The resulting fungi were isolated, purified and identified according to their macro and micro characteristics. By all the morphological and cultural characters three pathogens viz; *Colletotrichum spp*, *Penicillium* spp, and *Fusarium* spp were purified and preserved in the slant for further experiment.

Identification of fungal genera and species

The pure isolated fungi were identified following the most documented keys in fungal identification (Raper and Fennell, 1965; Barnett and Hunter, 1972; Pitt, 1985, Moubasher, 1993; Alexopoulos and Mims, 1996; Klich, 2002; Agrios, 2005). After that pathogen isolates were maintained in PDA slants and refrigerated for further use. Sub-culturing was done at a regular interval of 1 month.

In vitro screening of yeast isolates for their biocontrol activity against the post-harvest pathogen

In this experiment, all the isolates of yeast were tested for its biocontrol activity against different post-harvest pathogens using dual culture technique (Sabalpara, 2009, Karunanithi and Usman, 1999). Initially, the 5 mm size pathogen bit was cut with the help of cork -borer (5 mm) and the bit was placed in the Petri plate containing Yeast Potato Dextrose Agar media 2 cm away from the

periphery of the Petri plate. Simultaneously, the yeast isolate was streaked gently with the help of a streaking needle (Bio Plas Astral 7020) exactly opposite to the pathogen and kept the same distance from the Petri plate periphery. For control pathogens were placed in a similar manner on the PDA plate with any antagonist. Both theplates were incubated at 28±1ºC in BOD incubator for 6 days. The percentages of reduction of linear mycelial growth of pathogenic fungi were calculated using the formula as given by Vincent (1927)

$$I = \left(\frac{C - T}{C} \right) \times 100$$

Where,

I = Percent inhibition over control.
T = Growth of test pathogen in the presence of antagonist (mm). The per cent inhibition data were analyzed statistically using a completely randomized design (C.R.D)
C= Growth of test pathogen without the antagonist (mm).

Results and Discussion

Isolation of unicellular fungi during from fruits their phylloplane

A total of 13 yeasts were isolated from fruits and phylloplaneby following the imprinting washed or unwashed leaf pieces or by fruit washing methods on the Yeast Dextrose Peptone Agar medium. All isolates of yeast having a different source of isolation. Among 13 isolates of yeast, source of four isolates are banana leaf and two isolates were isolated from banana fruit washing, citrus leaf become source of one isolate while, one isolate of yeast isolated from fruit washing, grapefruit washing leads to becoming source of two isolates and consequently, mango leaf, mango fruit, and apple fruit become source of only one isolate of yeast.

In-vitro screening of yeast isolates against the fungal pathogen

All thirteen yeast isolates were screened *in vitro* against fungal pathogen viz; *Colletotrichum spp*, *Penicillium* spp and *Fusarium* spp by dual culture method presented in Table 1 (fig .1), Table 2(fig. 2) and Table 3 respectively. Y4 and Y6 isolates showed a maximum per cent inhibition upto 33.3 % against *Colletotrichum spp* followed by Y3 that showed inhibition by 28.8 %. Y13 isolates showed a minimum per cent inhibition of 4.4 % only. Y3 isolates showed maximum inhibitory effects by 40 % on *Fusarium spp* followed by Y4 and Y10 that showed inhibition by 33.3%.Y5 showed minimum per cent inhibition by 4.4%. Y4 and Y7 isolates showed maximum inhibitory effect by 51.1 % on *Penicillium spp* followed by Y11 that showed inhibition by 35.5 %. Y10 isolate showed no inhibitory effect.

This study details the isolation of yeast from fruit phylloplane as well as fruit washing and further the antagonistic activity of the isolates were also studied as losses of fruits and vegetables due to post-harvest diseases is high. As the consumption of fruits and vegetables treated with fungicides is harmful, there is a need for an environment-friendly method of managing disease without causing an adverse effect on human health. Nowadays, public concern about healthy eating habits has also increased. Keeping these in mind this experiment was conducted as yeasts remain still unexplored in the field of biocontrol for plant pathogen.

In vitro screening of yeast isolates for biocontrol activity

In vitro screening of all the 13 isolates was done against fungal pathogen *viz.*, *Colletotrichum, Fusarium,* and *Penicillium* using a medium suitable for the growth of both yeast and pathogen. Different isolates

showed a varying amount of percent inhibition. It is generally found that Potato Dextrose Agar Medium (PDA) supports the growth of only fungus pathogen but not yeast isolates. So, in dual culture, a PDA medium having 1 % yeast extract was used that supported the growth of both yeast isolates and pathogen.Y4 and Y6 isolates obtained from banana leaf and mango fruit washing respectively showed a maximum per cent inhibition by 33.3 % on *Colletotrichum* spp.

Table.1 Composition of Yeast Dextrose Peptone Agar

Yeast extract	**:**	**10.00g**
Peptone20.00g	**:**	20.00g
Dextrose	**:**	20.00g
Sodium chloride	**:**	1.00g
Agar agar	**:**	20.00g
Distilled water	**:**	1000ml
Chloramphenicol	**:**	150mg

Table.2 Composition of Potato Dextrose Agar Media

Peeled potato	**:**	**200.0 g**
Dextrose	**:**	20.0g
Agar agar	**:**	20.0g
Distilled water	**:**	1000ml

Table.1 Growth and behavior of *Colletotrichum* spp. against yeast isolates

S.No	Isolate No	Radial growth(cm)	Percent inhibition (%)
1.	**Control**	**4.5**	**-**
2.	Y1	4.2	6.6
3.	Y2	3.5	22.2
4.	Y3	3.1	31.1
5.	Y4	3.0	33.3
6.	Y5	3.2	28.8
7.	Y6	3.0	33.3
8.	Y7	4.1	8.8
9.	Y8	4.0	11.1
10.	Y9	3.7	17.7
11.	Y10	3.3	26.6
12.	Y11	3.9	13.3
13.	Y12	3.6	20
14.	Y13	4.3	4.4

Table.2 Growth and behavior of *Fusarium* spp. against yeast isolates

S.No	Isolate No	Radial growth (cm)	Percent inhibition (%)
1.	**Control**	**4.5**	**-**
2.	Y1	3.7	17.7
3.	Y2	3.5	22.2
4.	Y3	2.7	40
5.	Y4	3.0	33.3
6.	Y5	4.3	4.4
7.	Y6	3.3	26.6
8.	Y7	4.0	11.1
9.	Y8	3.1	31.1
10.	Y9	3.9	13.3
11.	Y10	3.0	33.3
12.	Y11	3.4	24.4
13.	Y12	3.2	28.8
14.	Y13	4.1	8.8

*Mean of three replications.

Table.3 Growth and behavior of *Penicillium* spp against yeast isolates

S.No	Isolate No	Radial growth	Percent inhibition (%)
1.	**Control**	**4.5**	**-**
2.	Y1	3.6	20
3.	Y2	4.1	8.8
4.	Y3	3.5	22.2
5.	Y4	2.2	51.1
6.	Y5	3.4	24.4
7.	Y6	3.0	33.3
8.	Y7	2.2	51.1
9.	Y8	3.9	13.3
10.	Y9	3.2	28.8
11.	Y10	4.5	0
12.	Y11	2.9	35.5
13.	Y12	3.1	31.1
14.	Y13	4.2	6.6

*Mean of three replications.

Fig.1 Growth and behaviour of *Colletotrichum* spp against yeast isolates

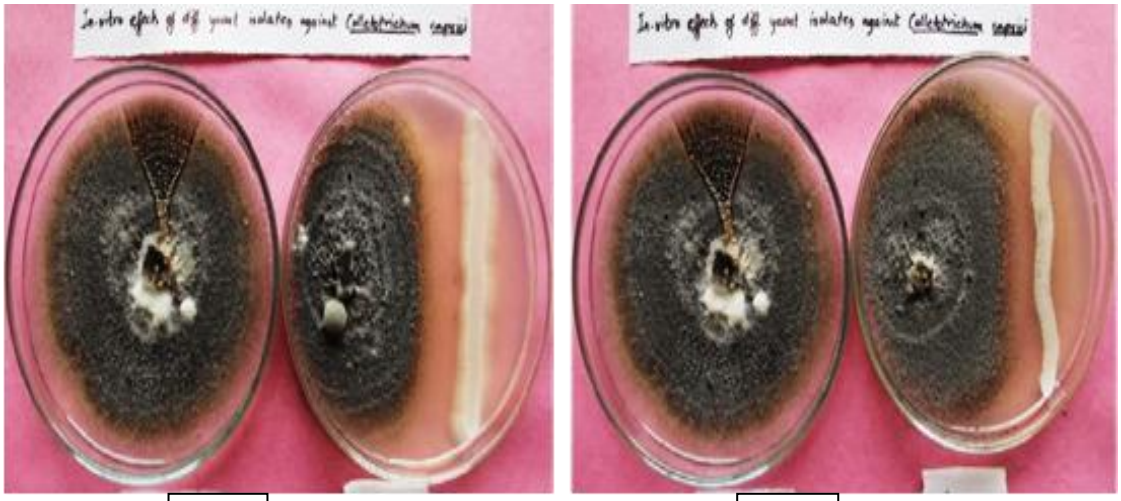
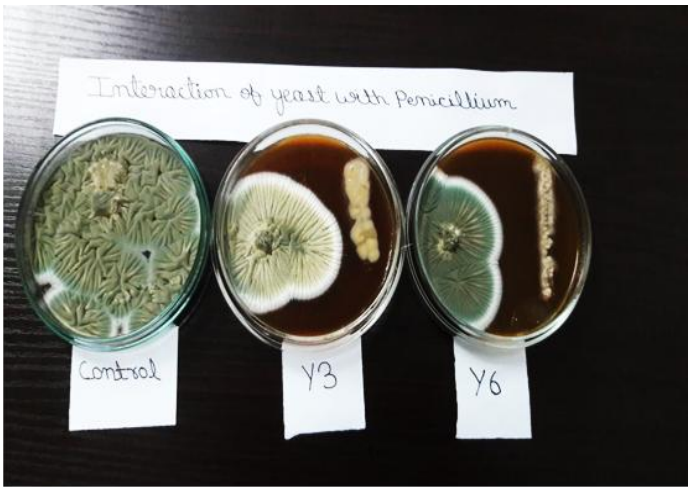


Fig.2 Growth and behaviour of *Penicillium* spp against yeast isolates



De Lima *et al.*, (1997) reported five yeast strains out of 580 strains provided antagonistic activity against *Colletotrichum gloeosporioides* by reducing mycelial growth and conidial germination *in vitro*. *Meyerozyma guilliermondii* showed a reduction in mycelia growth and conidial germination by 60% and 100% respectively.

Y3 isolates obtained from banana leaf provide maximum inhibition by 40% on *Fusarium* spp. Suzzi *et al.*, (1995) reported that few strains of 586 natural wine yeast (*Saccharomyces cerevisiae, Candida, Pichia, Zygosaccharomyces, Saccharomycoides*) from grape berries could provide complete inhibition of pathogen when screened against fungal pathogens like *Alternaria alternate, Aspergillus niger, Colletotrichum acutatum. Fusarium oxysporum* and some sclerotial fungi like *Rhizoctonia solani, Macrophomina,* etc. Y4 and Y7 isolates obtained from banana

leaf and banana fruit washing respectively showed a maximum percent inhibition by 51.1 % on *Penicillium* spp. Scherm *et al.*, (2003) isolated *Candida guillermondii* yeast from fig and (*Ficus carica*) and cactus pear (*Opuntiaficus indica*) grown in untreated orchards in Italy. They observe that yeasts either applied alone or in the presence of various additives on Golden Delicious and Fuji variety of apple reduced apple rot caused by *Penicillium expansum* with upto 100% efficacy. They also reported competition for nitrogen as the mode of biocontrol activity as several nitrates significantly inhibited the antagonistic capability of *C. guillermondii.*

In conclusion, plants are colonized by the number of epiphytic and endophytic microorganisms. The numbers of microorganisms isolated from within plant tissues are lower than the surface of healthy plants. Some of the microorganisms are present at the surface of the plant without causing any harm to the plant. The numbers of epiphytic microorganisms depend on the physical and nutritional conditions of the phylloplane. The leaf surface has a hostile environment as it is exposed to rapidly fluctuating temperature and relative humidity, as well as a repeated alternation between presence and absence of free moisture due to rain and dew.

The isolated yeasts were further tested for antagonistic activity against *Fusarium*, *Colletotrichum*, and *Penicillium* by using dual culture techniques. Different isolates showed varied per cent inhibition of different test pathogens.

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