Evaluation of Antifungal Efficacy of Selected Microorganisms Against Apple Rot Pathogens

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Abstract

Biological control of post harvest pathogens of fruits and vegetables is an effective alternative to chemical based treatments because of its minimal impact on the environment and human health. The antifungal efficiency of antagonistic microorganisms occurring naturally on apple fruit surface (*Trichoderma viride, T. hamatum, T. harzianum, Bacillus subtilis, Pseudomonas fluorescens* and one yeast isolate) against post harvest pathogens of apple (*Alternaria alternata, Monilinia fructigena, Trichothecium roseum, Aspergillus niger, Penicillium expansum)* was evaluated both under *in vitro* and *in vivo* conditions. Overall maximum growth inhibition of all the test fungi (71.97%) was reported with *T. hamatum* followed by the yeast isolate (66.63%), *B. subtilis* (64.98%) and *T. harzianum* (62.43%).

1. Introduction

Fruits and vegetables suffer from a number of fungal diseases after harvest. These losses in fruits and vegetables ranges from 10 to 40 per cent depending on the crop, variety and management practices adopted during crop growth. Among the different fungal pathogens, *Alternaria alternata, Botrytis cinerea, Glomerella cingulata, Monilinia fructigena* and *Penicillium expansum* are the dominant ones causing post harvest losses. *Penicillium expansum* Link causes the post harvest disease known as blue mould and is considered the most important disease of stored apples (Turechek, 2004). In most instances, the post harvest pathogens gain entry to susceptible fruit tissues and cause infection via entry through fruit's surface wounds that are generated through the process of harvesting and post harvest handling.

Traditionally, post harvest diseases are controlled by the application of synthetic fungicides. However, their harmful effects on environment as well as on human health limit their application. It is also reported that some pathogens have become fungicide resistant, thereby limiting the use of synthetic fungicides. Therefore, biological control has emerged as an alternative tool for the management of post harvest rots of fruits and vegetables. Besides having several advantages over synthetic fungicides, the rate of degradation of biopesticides is much faster and they are less toxic to non-target organisms. Several commercial products originated from antagonists are available, such as Kodiak (*B. subtilis* strain

GB03), RhizoPlus (*B. subtilis* FZB24), TrichoVir (*T. viride*) etc. The aim of this study was to test the activity of selected microorganisms as potential antagonists against isolated strains of causal agents of apple fruit rot.

2. Materials and Methods

2.1. Fungal pathogens

Isolates of *Alternaria alternata, Monilinia fructigena, Trichothecium roseum, Aspergillus niger* and *Penicillium expansum* were obtained from apple fruit samples (cv. Starking Delicious) expressing rot symptoms. Apple fruit samples were collected during survey made during 2015 and 2016 from different markets/stores/godowns of Shimla and Solan districts of Himachal Pradesh. The pathogens were isolated using standard phytopathological techniques. The diseased fruits were surface sterilized with absolute alcohol under aseptic conditions. Small bits of 1 to 2 mm size were taken from junction of diseased and healthy part of fruit with the help of sterilized sharp blade and scalpel. These bits were surface sterilized with sodium hypochlorite (1.0%) for 10 to 20 seconds and washed thrice with sterilized distilled water under aseptic conditions. The bits were then placed on the sterilized filter paper to remove the excess moisture and subsequently transferred to sterilized Petri plates containing potato dextrose agar (PDA) medium. The inoculated Petri plates were incubated at 25±1 °C in BOD incubator and examined daily for mycelial growth. The fungal growth developed in Petri

plates was purified by hyphal tip technique and was further cultured on slants containing PDA. These slants of culture were preserved at 5 °C in the refrigerator for further studies. Stock cultures were maintained by regularly sub-culturing after 20 to 25 days.

The causal organisms were identified by studying their morphology and comparisons with standard authentic descriptions from literature (Marie-Jose et al., 2004; Al-Hindi, 2011; Jurick, 2014; Hamid et al., 2014; Vico et al., 2014).

2.2. Antagonists

Six antagonistic microorganisms (*Trichoderma viride, T. hamatum, T. harzianum, Bacillus subtilis, Pseudomonas fluorescens* and one yeast isolate) were isolated from the natural environment i.e. neglected apple orchard with minimal or no pesticide spray history.

Fruit samples irrespective of cultivar types were collected in the month of Aug and Sept from Shimla district of Himachal Pradesh. One gram of sample of fruit peel was taken in 25 ml of sterilized distilled water in a mortar and macerated with the pestle. The extract was then vortexed briefly and 0.1 ml serial dilution (0.10) of the extract was placed onto nutrient yeast extract dextrose agar (NYDA) and potato dextrose agar (PDA) medium in sterilized Petriplates. After 48 h of incubation at 25±0.5 °C, different colonies were picked and transferred to NYDA and PDA slants or media plates. They were further purified by streaking and/or hyphal tip method. The separated, purified colonies were tentatively grouped as fungi, bacteria and yeast.

Fungal isolates were identified on the basis of cultural and morphological characteristics. Bacterial isolates were identified on the basis of Gram staining. Yeast isolates were identified on the basis of microscopic examination and cultural characteristics.

2.3. In vitro antagonistic activity assay

Isolated fungal antagonists were tested for their antagonistic activities against test pathogens by dual culture method (Denis and Webster, 1971). Culture discs (3 mm diameter) of each of antagonists and the test pathogens were taken from margin of their vigorously growing culture and transferred aseptically to solidified PDA (Potato Dextrose Agar) medium contained in Petriplates (90 mm) on the opposite sides facing each other at a distance of 1 cm from the margin of the plate. The Petriplates containing only culture of the test pathogens served as control. The experiment was laid out in CRD and each treatment was replicated thrice and the Petriplates were incubated at 25±1°C in a BOD incubator. The colony diameter of test pathogens was recorded till the control plates achieved full growth of the test fungus and per cent inhibition was calculated by the formula given by Vincent (1947):

$$I = \frac{C-T}{C} \times 100$$

Where:
I=Per cent inhibition

C=Linear growth in control (mm)
T=Linear growth in treatment (mm)

The antagonistic activity of isolated bacteria and yeast against the test pathogens was studied by streak plate method (Utkhede and Rahe, 1983). The Petriplates containing sterilized PDA were streaked at the centre with 48 hours old colonies of bacteria/yeast with the help of inoculation needle. Mycelial bit (3 mm diameter) of the test pathogens were placed on opposite sides of the streak at a distance of 1 cm from the margin of the plate. The Petriplates containing only culture of the test pathogens served as control. Each treatment was replicated thrice under Completely Randomized Design (CRD) and incubated at 25±1 °C in BOD incubator. Per cent inhibition of the radial growth of test pathogens, if any, was calculated with the same formula as described above.

2.4. In vivo antagonistic activity assay

The Starking Delicious apple fruits were selected free of wounds and being homogeneous as much as possible in physiological maturity stage and size. Selected fruits were washed with sterile water and then air dried for 15-20 min. These fruits were then inoculated separately by well method of Granger and Horne (1924) with the test pathogens. Twelve hours after inoculation, fruits were dipped in effective fungal, bacterial and yeast inoculum. The inoculum was prepared and adjusted to the concentration of 10^8 cfu/ml with water. Each treatment was replicated thrice containing five fruits per replicate for a particular test fungus. Untreated inoculated fruits were kept as control for comparison. Observation on per cent rotting of fruits was taken after 6 days of inoculation when the control fruits rotted completely. Per cent fruit rot was recorded by the formula given by Srivastava and Tandon (1968):

$$\text{Per cent fruit rot} = \frac{W-w}{W} \times 100$$

Where,

W=weight of the fruits before inoculation
w= weight of the fruits after removal of the rotten tissue

3. Results and Discussion

3.1. In vitro antagonistic activity assay

From the data presented in Table 1, it is clear that overall maximum growth inhibition (71.97%) of all the test fungi was reported with *T. hamatum* followed by the yeast isolate (66.63%), *B. subtilis* (64.98%), *T. harzianum* (62.43%) and *T. viride* (60.31%). Least growth inhibition was observed with *P. fluorescens* (54.00%). Irrespective of different biocontrol agents, maximum growth inhibition (88.93%) was recorded in *M. fructigena* followed by *T. roseum* (66.14%), *A. niger* (60.22%), *P. expansum* (58.60%) and *A. alternata* (43.05%) (Figure 1).

From the interaction studies it is clear that mycelial growth of *M. fructigena* and *T. roseum* was significantly inhibited by all the biocontrol agents. *T. hamatum* provided maximum

Table 1: *In vitro* evaluation of antagonists against post harvest fungal pathogens of apple

Antagonist	Growth inhibition (%)					
	A. alternata	*T. roseum*	*M. fructigena*	*A.niger*	*P. expansum*	Mean
T. viride	43.27	77.07	91.33	21.95	67.94	60.31 (50.93)
T. hamatum	46.83	81.74	87.23	77.51	66.56	71.97 (58.01)
T. harzianum	25.93	31.61	93.93	72.65	88.06	62.43 (52.18)
P. fluorescens	10.76	46.95	85.88	62.93	63.50	54.00 (47.28)
B. subtilis	64.53	75.60	82.49	61.66	40.59	64.98 (53.70)
Yeast	67.01	83.87	92.70	64.62	24.96	66.63 (54.69)
Mean	43.05 (40.99)	66.14 (4.39)	88.93 (70.54)	60.22 (50.88)	58.60 (49.93)	
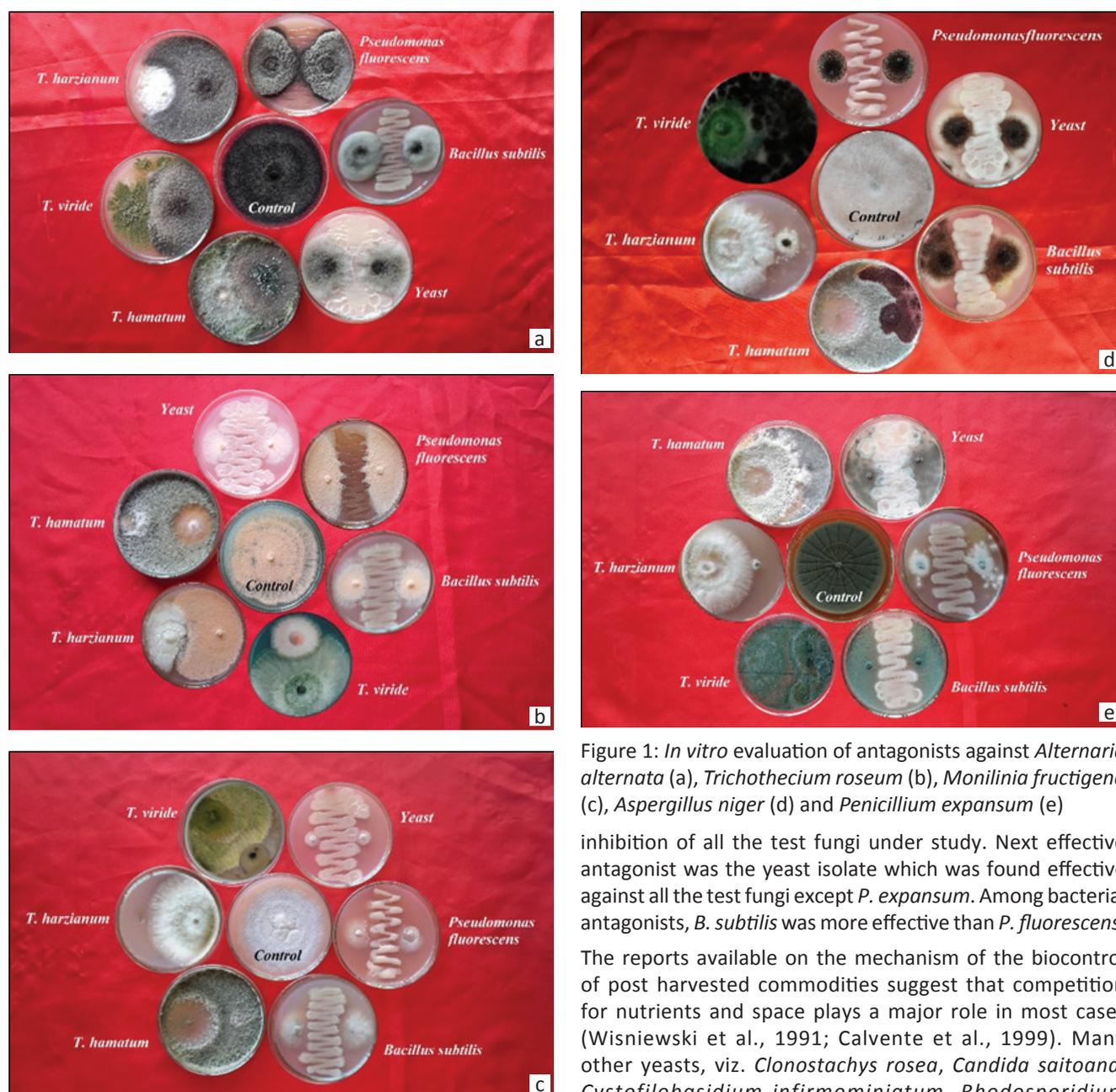
Figure 1: *In vitro* evaluation of antagonists against *Alternaria alternata* (a), *Trichothecium roseum* (b), *Monilinia fructigena* (c), *Aspergillus niger* (d) and *Penicillium expansum* (e)

inhibition of all the test fungi under study. Next effective antagonist was the yeast isolate which was found effective against all the test fungi except *P. expansum*. Among bacterial antagonists, *B. subtilis* was more effective than *P. fluorescens*.

The reports available on the mechanism of the biocontrol of post harvested commodities suggest that competition for nutrients and space plays a major role in most cases (Wisniewski et al., 1991; Calvente et al., 1999). Many other yeasts, viz. *Clonostachys rosea*, *Candida saitoana*, *Cystofilobasidium infirmominiatum*, *Rhodosporidium*

paludigenum, Pichia caribbica, P. fermentans, P. guilliermondii and *P. membranifaciens* have been found effective against various post harvest rot causing pathogens of fruits (El-Ghaouth et al., 2003; Chan et al., 2007; Liu et al., 2011; Fiori et al., 2012; Wang et al., 2010; Xu et al., 2013).

3.2. In vivo antagonistic activity assay

The biocontrol agents screened under *in vitro* conditions for their effectiveness against post harvest pathogens of apple fruits were further evaluated under *in vivo* conditions and data obtained on per cent fruit rot has been presented in Table 2. From the perusal of data it is clear that *T. hamatum* provided maximum control (80.69%) of all the test fungi under study followed by the yeast isolate (76.29%). Other effective biocontrol agents in the order of merit were *B. subtilis* (68.55%), *T. harzianum* (65.02%) and *T. viride* (61.66%). *P. fluorescens* was least effective among all the biocontrol agents.

The cumulative reaction of various test fungi towards differently treated fruits showed maximum rotting due to *P. expansum* (43.43%) followed by *A. alternata* (42.95%), *M. fructigena* (42.85%), *A. niger* (42.05%) and *T. roseum* (41.20%). The interaction studies between biocontrol agents and test fungi revealed that none of the biocontrol agent provided complete control of any of the test fungi *in vivo*. However, significant disease control of all the test fungi in comparison to control was achieved with *T. hamatum* (80.69%) and the yeast isolate (76.29%).

Many recent studies have demonstrated the effect of various *Trichoderma* species on post harvest rot diseases caused by many fungal pathogens (Odebode, 2006; Patale and Mukadam, 2011; Hafez et al., 2013). *T. harzianum* has been used to control the fungal diseases caused by *A. alternata*, *P. expansum* (blue mould on apples), *Botrytis cinerea* (grey mould on apples) and *Rhizoctonia* sp. (Agrios, 1997; Batta,

Table 2: Effect of biocontrol agents against post harvest rotting of apple cv. Starking Delicious

Antagonist	Growth inhibition (%)						
	A. alternata	*T. roseum*	*M. fructigena*	*A.niger*	*P. expansum*	Mean	Per cent control
T. viride	36.99	38.06	40.44	36.00	40.19	38.34 (38.24)	61.66
T. hamatum	23.33	15.67	18.17	20.44	18.92	19.31 (26.06)	80.69
T. harzianum	34.07	35.22	37.78	31.77	36.07	34.98 (36.24)	65.02
P. fluorescens	45.66	47.06	46.88	53.77	55.07	49.69 (44.80)	50.31
B. subtilis	31.66	32.67	34.00	28.82	30.12	31.45 (34.10)	68.55
Yeast	28.93	19.75	22.66	23.57	23.66	23.71 (29.13)	76.29
Control	100.00	100.00	100.00	100.00	100.00	100.00 (89.96)	-
Mean	42.95	41.20	42.85	42.05	43.43	-	-

1999; Batta, 2003; Biswas, 1999; Harman and Kubicek, 1998; Dutta and Das, 1999; Omarjee et al., 2001). Other species of *Trichoderma*, namely *T. pseudokoningii, T. koningii, T. hamatum, T. gamsii, T. atroviride, T. virens* and *T. viride* have also been used as biological control agents to suppress the growth of various pathogenic fungi (Odebode, 2006; Ngullie et al., 2010; Jagtap et al., 2013; Shaikh and Nasreen, 2013).

A saprophytic strain of *Pseudomonas syringae* marketed under the trade name of Biosave™ provided biological control of grey mould, blue mould and Mucor rot on pear and apple fruits (Janisiewicz and Marchi, 1992; Jeffers and Wright, 1994; Mari et al., 2014). Another species of *Pseudomonas* namely *Pseudomonas fluorescens*, has been reported to control grey mould caused by *Botrytis* sp. (Mikani et al., 2008). *Bacillus subtilis* applied to wounded apples reduced fruit rot caused by *B. cinerea, A. alternata, P. expansum* and *P. malicorticis* (Leibinger et al., 1997; Wang et al., 2010). It has been reported that the post harvest brown rot of stone fruits can also be controlled by the application of *Bacillus subtilis* and *Pseudomonas* sp. (Pusey and Wilson, 1984; Smilanick et al., 1993).

4. Conclusion

The results of the present study indicate that isolate *Trichoderma hamatum* has great potential as antagonist of post harvest pathogens of apple and should be included in further investigations as potential microorganism for production of biofungicides intended to be used for protection of apple fruits from rot causing pathogens.

5. References

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