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Efficacy of antagonistic bacteria against postharvest fungal rot pathogens of *Citrus reticulata* Blanco

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Mandarin (*Citrus reticulata* Blanco) is very good source of vitamin C, B, minerals and lots of antioxidants. Due to higher moisture, sugar, nutrient content, softness and low pH value orange is vulnerable to pathogen attack. So orange production faces tremendous loss specially due to post harvest fungal attack. In the present study, survey and collection of rotten orange from different market areas of Alipurduar have been carried out and symptoms were noted. *Fusarium solani, Penicillium polonicum* and *Aspergillus* sp. have been successfully isolated and identified from the rotten orange following completion of Koch's postulate. Isolated fungi are reported to produce strong mycotoxin which can cause dangerous health hazards. In order to delay the postharvest rot of orange, fungicides are used which have some negative effect on health. Therefore, using some potential biocontrol agents like *Pseudomonas putida, P. aeruginosa* and *Bacillus subtilis*, following *in vitro* interaction experiments with fungal pathogens, attempts were made for *in vivo* experiments to achieve post harvest management practices. It has been observed that among the biocontrol agents tested, *P. aeruginosa* was found to be most effective against all pathogens.

Keywords : *Citrus reticulata*, Biocontrol agents, *Fusarium solani, Penicillium polonicum,* postharvest fungal disease

INTRODUCTION

Citrus fruits form the third largest fruit industry next to the banana and mango and hold an important place in the economy of the country (NHB database 2016). It is very good source of bioactive compounds such as vitamin C, vitamin B, carotenoids, flavonoids, alkaloids, phenols, tannins and hydroxycinnamic acids (Yasmeen and Mazhar 2019) which has different health benefits and support immune system. But orange is vulnerable to pathogen attack due to higher moisture, sugar, nutrient content, softness. On the other hand, packaging, storage, transportation, improper handling may result in decay and growth of microorganisms which ultimately results spoilage because of the changing physiological state of the fruits (Oviasogie *et al*. 2015).

So, orange production faces tremendous loss specially due to post harvest fungal attack (Abdullah *et al*. 2018) limiting extended storage. It was observed that in India, the extent of damage varied from 29.9 to 43.8% in sweet orange (Sharma and Verma, 2013).

Many pathogens are reported to be associated for postharvest losses of citrus fruit like *Penicillium digitatum* (Sharma and Raj, 2018) *Aspergillus niger, A fumigatus, A. flavus, Rhizopus stolonifer, Alternaria, Fusarium, Mucor* (Sharma and Verma 2013). Molecular phylogenetic analysis of *Alternaria alternata,* a new pathogen associated with post bloom fruit drop of *Citrus reticulata* have been reported (Ray *et al.* 2015).

Chemical treatment of fruits to minimize post-harvest rot is a common practice which causes many health hazards. Biological control is one of the safest sustainable ways to combat the postharvest deterioration (Moraes Bazioli, 2019). So, microbial antagonists are no doubt less toxic alternatives to synthetic fungicides. Different antagonists like many species of *Trichoderma,* Saccharomyces and non-saccharomyces yeast, *Pseudomonas, Bacillus* are very common which are used as effective biocontrol agent against

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many postharvest rot pathogens including citrus rot (Vu *et al.* 2020). So, the present investigation designed to assess the efficacy of antagonistic bacteria like *Pseudomonas aeruginosa*, *P. putida* and *Bacillus subtilis* against postharvest fungal rot pathogens of orange (*Citrus reticulata*).

MATERIAL AND METHODS

Material

Fresh orange and orange (*Citrus reticulata* Blanco) which were found with symptoms of fungal infection were purchased and collected from different local markets of Alipurduar town. Biocontrol agents- *Pseudomonas aeruginosa, P. putida* and *Bacillus subtilis* for this study are provided by Laboratory of Molecular Plant Pathology, Dept. of Botany, NBU.

Isolation of pathogens

Pathogens were isolated in PDA following the method of Ray et al 2022). Infected fruit samples were collected in plastic bags from the markets and quickly transferred to the laboratory. Collected fruits were washed and dried. Infected region was cut into small pieces and sterilized with 0.1% $HgCl_2$ for 30-50 seconds. Pieces were removed and rinsed in several changes of sterile distilled water soaked three times in sterile distilled water for 40-60 seconds, orange pieces were then placed on sterile paper towels to dry for 5 minutes. The orange sections were placed on Potato Dextrose Agar medium. The inoculated plates were incubated at 27º C for 7 days and observations were made daily for possible microbial growth. After 5-7 days of growth, sub-culturing was done to obtain pure cultures of the isolates. Microscopic observations and identifications were made for the isolated fungal pathogens on the basis of mycelium behavior, sporulation, spore structure, conidial size.

Biocontrol agents *Pseudomonas aeruginosa, P. putida* and *Bacillus subtilis* were maintained by subculturing in nutrient agar media.

Pathogenicity test

Pathogenicity test was carried out following the method of Khuna et al (2022) with slight modification. Fresh orange from markets were collected, surfaces were disinfected by rubbing with 1.5% (v/v) sterile sodium hypochlorite solution. They were then subsequently washed three times with sterile distilled water. The surface disinfected fruits were then air-dried at room temperature (25 ± 2 ^{0}C) for 10 min. After being air-dried, a uniform wound (5 pores, 1 cm in depth and 1 mm in width) was made at the equator of each fruit using aseptic needles. Conidial suspensions of all fungal isolates were prepared from each fungal culture grown on PDA at 25 ^{0}C for two weeks and suspended in sterile distilled water. Hundred microliters of the conidial suspension were dropped onto the wounded fruits. Accordingly, control fruits were also wounded and treated with sterile distilled water. Each fruit was then placed in a separate sterile plastic box and stored in BOD chamber at 27 ^{0}C. The experiments were independently repeated twice.

In vitro test of biocontrol agents against pathogens

Dual culture method has been carried out following the method of Lal *et al*. (2022) with little modification. Bacterial culture of *Pseudomonas aeruginosa, P. putida* and *Bacillus subtilis* were multiplied in nutrient broth for 24 hours in BOD at 27 ^{0}C. 5mm block of fungal culture from growing region of 7 days old cultures of each pathogen were used. Plates of nutrient agar were prepared and pairing of pathogens and antagonistic bacteria were done making control of each case i.e., growing pathogen only in two plates for each set without biocontrol agents to make comparison with treated plates. The per cent inhibition in mycelial growth of the pathogens compared to the control was calculated following the standard method (Sharma *et al*. 2020).

In vivo application of biocontrol agents

Healthy fresh orange of uniform size purchased from the market of Alipuduar Bada Bazar. Fruits were surface sterilized as before and inoculated through pin pricked method following standard technique (Sharma *et. al*. 2020). *In vivo* evaluation was made making the combination (i) Orange inoculated (dipping 10^5 spores/ml for) with pathogen only, (ii) pretreated with bacteria and

after required interval inoculated with pathogens, (iii) pretreated with pathogens followed by antagonist, (iv) control set prepared with dipping the healthy orange in water only.

RESULTS AND DISCUSSION

Post-harvest rot infected oranges (Fig.1A, E) were collected from different market of Alipurduar. Pathogens were isolated and identified the genus on the basis of characters of cultures, sporulation on media and microscopic study. Three pathogens are *Fusarium solani* (SUB14560653 apdu_bot_fs PP946876)*, Penicillium polonicum* (SUB14582196 apdu_bot_pp PP976622) and *Aspergillus* (Fig.1 B, F and I respectively) isolated from rotten sweet orange of which *Penicillium* is most abundant like other studies (Sharma and Raj, 2018) and *Aspergillus* is least but reverse results revealed previously (Akintobi *et al*. 2011, Oviasogie *et al.* 2015) may be due to different environmental factors. Pathogenicity test of each pathogen with fresh orange establish the same symptoms in fruits and appearance of the same fungi in cultures.

Fusarium has two types of conidia found in media macroconidia and microconidia (Fig.1 C&D). Size of macroconidia is 3 to 4 µm× 3 to 7µm and microconidia is 7 to 12 µm× 3 to 4 µm. Diameter of spores of *Penicillium* and *Aspergillus* are 2.5 to 5 µm and 5 to 10 µm respectively.

In vitro interaction of pathogen with bio-control agents

Study of *in vitro* control of pathogens with bio-control agents like *Pseudomonas aeruginosa, P putida and Bacillus subtilis* were carried out (Fig 2). Three replicas of each combinations were made. After pairing with bacterial biocontrol agents with *Fusarium* it is observed that initially in presence of *P. aeruginosa* pathogen is unable to grow up to 5 days but submerged growth of mycelia was noticed in 7 days which gradually occupied half of the plate when pathogen attends full growth in control plate. When paired with *Bacillus subtilis* fluffy growth of fungus was observed in the vicinity and attends only 1.5 cm in diameter even after 20 days as its further growth has been ceased. Pairing with *P. putida* reveals almost normal growth of pathogen in submerged condition.

Growth of *Penicillium* in presence of *P. aeruginosa* was absent even the control plates attend maximum growth. *Penicillium* form 3.6 cm inhibition ring beyond that it cannot grow further as *Penicillium* forms antibiotics which works against this *Bacillus subtilis.* So, *B. subtilis* has a very little effect on it though growth of pathogen in this inhibition zone was also not observed. Almost same results were obtained when *Penicillium* paired with *P. putida*.

Table 1: *In vivo* evaluation of *Pseudomonas aeruginosa* (Pa) against postharvest rot of orange caused *Fusarium* and *Penicillium*

Treatment of fruit	% of rot after different days of interval							
	3 days	6 days	9 days	12 days	15 days	18 days	21 days	33 days
Healthy without pin prick	0	0	0	0	0	0	0	51.2
Healthy with pin prick	0	0	0	0	0	0	3.72	70.51
Pseudomonas aeruginosa (Pa)	0	0	0	0	0	0	0	21.27
Fusarium solani	0.41	7.57	27.66	47.03	81.54	100		
Pre-treated with Pa	0	0	0	21	70	100		
Post-treated with Pa	0	0	2.95	37.17	71.34	100		
Penicillium polonicum (Pp)	1.62	17.50	43.4	73.00	100			
Pretreated with Pa	0	0.55	3.48	6.82	20.04	86	100	
Post treated with Pa	0	5.23	34.51	74.12	100			

Five oranges were inoculated for each treatment combinations.

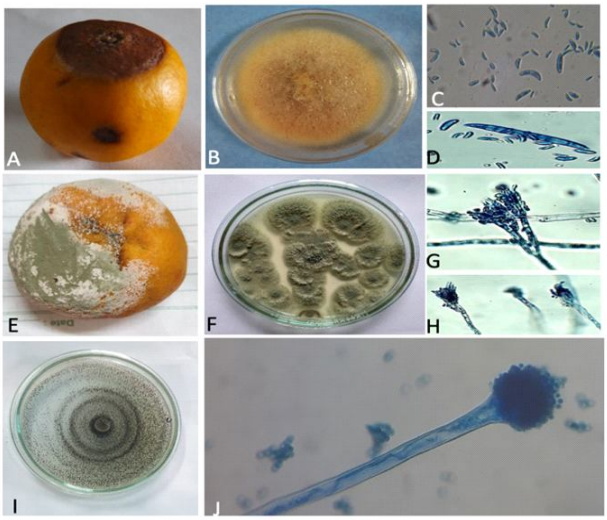

Fig. 1: Postharvest rot of Orange A-*Fusarium* rot, B-culture of *Fusarium solani,* C&D-microspore and macrospore of *F.solani*; E-*Penicillium* rot, F-culture of *Penicillium polonicum*, G&H-conidiophores of P.*polonicum*; I-*Aspergillus* culture, J-conidia and conidiophore of *Aspergillus*

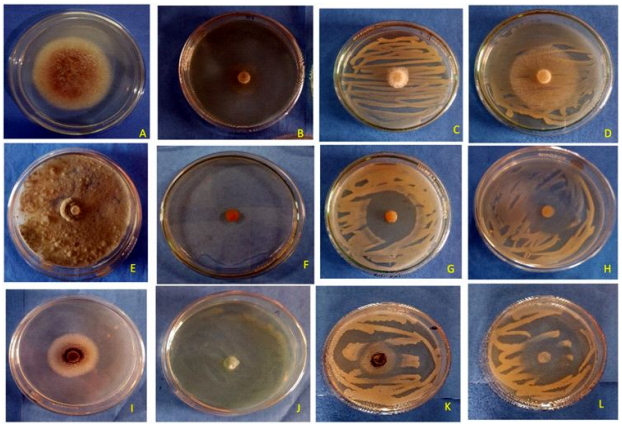

Fig. 2: (A-L) : *In vitro* pairing of *Fusarium* (A), *Penicillium* (E) and *Aspergillius* (I) - post harvest rot pathogens of orange with antagonistic bacteria (*P.aeruginosa, B. subtilis* and *P. putida*). B-D: *Fusarium* with *P. aeruginosa* (B), *B. subtilis* (C) and *P.putida* (D) F-H: *Penicillium* with *P. aeruginosa* (F), *B. subtilis* (G) and *P. putida* (H) J-L: *Aspergillus* with *P. aeruginosa* (J), *B. subtilis* (K) and *P. putida* (L)

In vitro antagonistic tests against *Aspergillus* was found to be significant in presence of *P. aeruginosa* and *P. putida* and minimum inhibition was noticed by *B. subtilis* (Fig 3)

In vivo evaluation of biocontrol agents against pathogens

Efficacy of *Pseudomonas aeruginosa* (Pa) carried out *in vivo* condition in different combination (Table 1) mentioned in methodology as the antagonist *P. aeruginosa* shows best

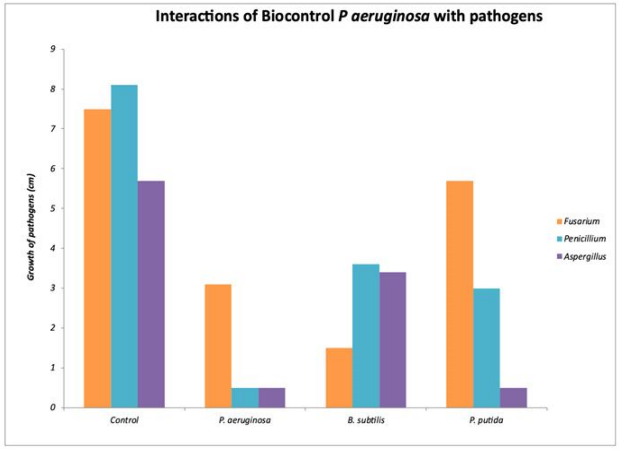

Fig. 3: *In vitro* interaction of rot pathogens of orange with *Pseudomonas aeruginosa*

results in *in vitro* interactions. *Penicillium* and *Fusarium* were used as pathogens for the *in vivo* experiment. In case of *Fusarium*, pre-treatment with bio-control agent shows good control of rot. Rot started in pathogen inoculated oranges after 3 days whereas in *P. aeruginosa* pretreated oranges, rot started after 9-12 days, rot of post-treated oranges started after 7 days. Only bacterial treated oranges did not show rot symptoms even after 4 weeks, delayed rot was observed after 30 days. It is very significant that when one of the bacterial pre-treated oranges peeled off in 30 days none of the flakes of orange shows any symptoms of rot though size of flakes has been reduced little bit. Healthy orange shows natural rot after 3 weeks. *Penicillium* treated oranges showed initiation of infection within 3 days, 70% rot observed within 12 days and fully spoiled by *Penicillium* within 15 days. Bacterial (*P. aeruginosa*) pretreated orange resist *Penicillium* infection almost up to 6 days. Whereas bacterial post treated (initially inoculated with pathogen) oranges showed infection just after three days. It is clear from the experiment that *Penicillium* rot of orange is very fast. So, it may be recommended that self-life of orange may be extended when healthy fruits will be treated with antagonist *P. aeruginosa.*

In vitro test of ten bacterial isolates were done against green mold causing *Penicillium digitatum* where promising control of pathogen had been shown by *B. subtilis* and *Agrobacterium radiobacter* (Mohammadi et al 2017). Biological antagonist *Pseudomonas protegens* an endophytic bacterium suppresses mycelial

growth of *Botryosphaeria dothidea* causes ring rot of apple (Huang *et. al.* 2022). Wasim et al (2024) confirmed that the maintenance of shelf life is better when apples were treated with *P. aeruginosa* in contrast to untreated apples during postharvest period. So, the present investigation is very significant in context to the contemporary research.

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DECLARATIONS

Conflict of interest. Authors declare no conflict of interest.

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