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Biological Management of Aspergillus Rot of Pomegranate

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ABSTRACT

Pomegranate (Punica granatum L.) is one of the oldest edible fruit tree species. Many fungal pathogens of pomegranate fruit that infect in the orchard and cause significant losses during storage. In the present study taken up to find out the important postharvest diseases of pomegranate in Dharwad, Aspergillus rot, Penicillium rot and Colletotrichum rot were observed. Among the plant extracts tested in vitro against Aspergillus niger, highest mycelial growth inhibition (72.20 %) was observed in 10 % garlic bulb extract, followed by , 10 % neem leaf extract (71.10 %), 10 % tulsi leaf extract 64.40 %. Garlic bulb extract at 10 % was most effective (78.09 %) in inhibition of spore germination followed by 10 % tulsi leaf extract (71.80 %), 10 % neem leaf extract (71.10 %). Least inhibition (12.51 %) was observed in 5 % parthenium leaf extract. Antagonism of biocontrol agents on A. niger was tested in vitro and Bacillus subtilis (77.00 %), Trichoderma harzianum (76.72 %), T. viride (76.65 %), T. virens (isolate 2) (74.00 %). were found to be effective. Among the postharvest treatments, 10 % neem leaf extract (23.12) and T. viride spore suspension (23.72) were effective in reducing the Per cent Disease Index (PDI) of Aspergillus rot of pomegranate compared to control with PDI of 61.20.

Keywords: *Postharvest diseases of pomegranate, Aspergillus rot, Botanicals and Biocontrol agents*

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruit tree species. Even though it originated in central Asia, it has a broad geographic distribution and adaptation to different climatic conditions. *P. granatum* L. belongs to the family Punicaceae and is the most commercially produced species of the genus. Pomegranate also has many medicinal uses. Pomegranate is rich in antioxidants, polyphenolic substances, fatty acids, vitamin C (which is excellent for the immune system), alkaloids, resinous substances, and flavonoids which act as anti-bacterial, antivirus, anti-carcinogen, and anti-inflammatory substances. The phenolic compound ellagic acid, found in the juice and seed oils of pomegranate, has anti-cancer and anti-oxidant properties (Mortan, 1987).

There are many fungal pathogens of pomegranate fruit that infect in the orchard and cause significant losses during storage. After harvest fungal pathogens that cause significant losses in quantity and quality include: *Botrytis cinerea* Pers.; Fr.; *Aspergillus niger* Tiegh.; *Penicillium spp*.; *Alternaria spp*.; *Colletotrichum gloeosporioides* etc. (Holland *et al.*, 2008; Palou *et al.*, 2009; Snowdon, 1990; Sonawane *et al.* 1986; Srinivasulu, 2001).

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Postharvest diseases of fresh fruits are traditionally being controlled by synthetic chemical fungicides (Eckert and Ogawa, 1985). However, when harvested fruits are treated with fungicides to manage postharvest diseases, there is greater likelihood of direct human exposure to them. Also, the development of resistance in pathogens to fungicides applied for controlling the postharvest diseases (Spotts and Cervantes, 1986; Spalding, 1982) underlines the necessity to develop new and effective methods of controlling postharvest diseases that are perceived as safe by the public and pose negligible risk to human health and environment. Biological control of postharvest diseases is one of the options to overcome these problems in the management of postharvest diseases. Use of antagonistic microorganisms and plant derivatives as postharvest treatments are gaining worldwide interest as an alternative or as supplement for the existing pesticides. These products will help in reducing the cost, environmental hazards and development of resistance by the pathogens.

The present study was taken up to study the postharvest diseases of pomegranate and to evaluate different non chemical management tools like botanicals and biocontrol agents.

MATERIALS AND METHODS

Pomegranate fruits infected by different postharvest pathogens, showing typical symptoms were collected from Dharwad market and from pomegranate orchards. Symptomatology was studied and the fungi associated with disease were isolated by following standard tissue isolation method. Pathogenicity of the organisms was proved by proving Koch's postulates.

A) *In vitro* evaluation of botanicals:

Antagonistic activity of the below mentioned botanicals was tested *in vitro.*

Sl. No.	Scientific name	Vernacular name	Family	Part used
1	*Allium sativum* L.	Garlic	Amaryllidaceae	Bulb
2	*Azardirachta indica* Juss.	Neem	Meliaceae	Leaves
3	*Clerodendron inermii* Gaertn.	Kashmir bouquet	Verbenaceae	Leaves
4	*Chromolaena odoratum* L.	Communist weed	Compositae	Leaves
5	*Lantana camara* L.	Lantana	Verbenaceae	Leaves
6	*Ocimum sanctum* L.	Tulsi	Lamiaceae	Leaves
7	*Parthenium hysterophorous* L.	Congress grass	Compositae	Leaves
8	*Tridax procumbens* L.	Tridax	Compositae	Leaves and flowers

Preparation of stock solution of Botanicals:

Fresh leaves/bulb of each botanicals plant was collected and washed first in tap water and then in distilled water. Then, 100 g of fresh sample was crushed in a mixer grinder by adding 100 ml sterile distilled water (1:1 w/v). The extract was filtered through two layers of muslin cloth. Final filtrate thus obtained was used as stock solution.

i) Mycelial growth inhibition:

Antifungal activity of botanicals was tested using the poisoned food technique as suggested by Nene and Thapliyal (1982). Stock solutions of 5 ml and 10 ml were mixed 95 and 90 ml of sterilized molten PDA medium respectively to get 5 and 10 per cent concentrations. Twenty ml of the poisoned medium was poured into each of the 90 mm sterilized petriplates. Each plate was seeded with 0.5 cm mycelial discs taken from the periphery of eigh day old fungal culture and Per cent inhibition of mycelial growth over control was calculated when the growth of the fungus is full in control plate by using the formula given by Vincent (1927).

ii) Spore germination inhibition:

Effect of botanicals on spore germination of the test fungi was assessed by per cent inhibition of conidial germination. A single drop of the conidial suspension of the test organisms was added to the well of a series of cleaned cavity slides, to which a single drop of different botanicals (double the required concentrations) was also added to get the required concentrations of 5 and 10 per cent. The wells were immediately covered by using coverslips on the cavity slides and the periphery was smeared with Vaseline. Control was maintained with distilled water. The cavity slides were kept in the petriplates lined with moist blotting paper and were incubated at room temperature. Observations were made from ten microscopic fields from each slide. Per cent germination was calculated from the number of total conidia and germinated conidia in each microscopic field. Further, the percent inhibition of spore germination was calculated by using the formula given by Vincent (1927) for each botanical.

B) *In vitro* evaluation of biocontrol agents

From the actively growing cultures of both fungal bioagents and test pathogens, 0.5 cm fungal disc were transferred aseptically to petriplates containing PDA, simultaneously by leaving sufficient space in between two discs. In case of bacterial biocontrol agents, mycelial discs of the test fungus was kept at opposite ends and bacterium was streaked at the center. A pathogen disc alone placed at the center of the petriplate served as control. Colony diameter of both the test fungus and bioagents were measured when control plate is fully covered and per cent inhibition was calculated by using the formula given by Vincent (1927).

C) *In vivo* evaluation of bioagents and botanicals

In vivo studies were carried out against stem end rot of citrus by imposing various bioagents and botanicals by following pre inoculation method given by Bhuvaneswari (1999). Apparently, healthy and uninjured fruits were washed in 1:1000 mercuric chloride for 30 seconds followed by rinsing twice in distilled water and allowed to dry. Small wounds were made by pinching sterile paper pins. Cotton swabs dipped in suspensions of the bioagents and botanicals and swabbed over the wounded surface of the fruit followed by inoculation of the pathogen keeping the cotton swab dipped in spore suspension of the pathogen. The time interval between the postharvest treatments' application and inoculation was 12 h. Fruits were provided with sufficient relative humidity by placing cotton swabs dipped in water along with them. Observations were taken on eighth day after inoculation by following 0-5 scale given by Prasanna Kumar (2001).

Grade	Per cent disease on the fruit surface
0	No disease
1	01 – 5 %
2	5.1 – 10%
3	.10.1 – 25 %
4	25.1 – 50 %
5	50.1 – 5 %

Per cent Disease Index (PDI) was calculated by following the formula given by Wheeler (1969).

$$PDI = \frac{\text{Sum of individual disease rating}}{\text{No. of samples}} \times \frac{100}{\text{Maximum disease grade}}$$

RESULTS AND DISCUSSION

During the present investigations, the postharvest diseases of pomegranate observed are Aspergillus rot, Penicillium rot, and Colletotrichum rot. Pathogenicity was proved and the symptoms observed are described below:

Aspergillus rot: Caused by *Aspergillus niger* v. Teighem

Fruits affected by *A. niger* showed brownish discoloration over the outer rind. Appearance of black spore heads on the affected fruit surface was observed later. Severely affected fruits became depressed, slimy and rotten emitting a fermented odour. (Plate 1).

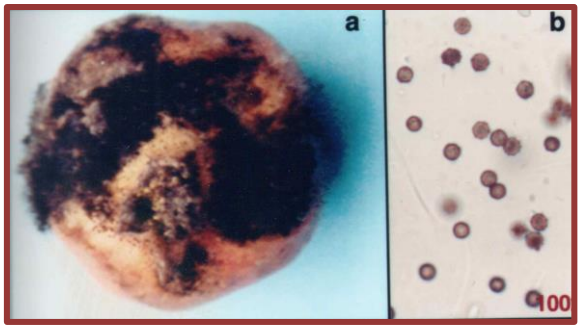

Plate 1: a) Black mold rot of pomegranate
b) Conidia of *Aspergillus nige*

Penicillium rot: Caused by *Penicillium Sp.*

Fruits affected by *Penicillium* sp. showed dark brown irregular patches over the outer rind. As the disease progressed, inner core and the arils became soft. Later, entire rotted fruit surface was covered with bluish growth of the fungus. (Plate 2).

Plate 2: a) Penicillium rot of pomegranate
b) *Penicillium sp*. Phialides with conidia

Colletotrichum rot: Caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

Brown spherical depressed scattered spots occurred on the pericarp of the fruits. In advanced stages, the spots coalesced to form necrotic rotten patches over the surfaces of the fruit. When such diseased fruits were cut open the rotting was observed. Brown to dark brown colored seeds were seen in infected fruits. However, the rotting was not too deep. No smell was emanating from such affected fruits. (Plate 3.)

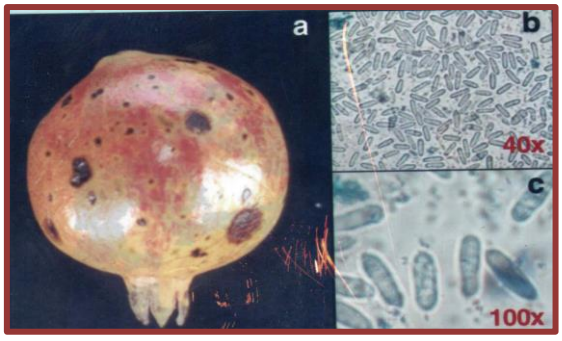

Plate 3: a) Colletortrichum rot of pomegranate
b, c) *Colletotrichum gloeosporioides* spores

A. *In Vitro* evaluation of botanicals: (Table 1)

i) Mycelial growth inhibition:

Of all the plant extracts tested, mean (5 and 10 per cent together) mycelial inhibition is highest inhibition (64.69 %) in neem leaf extract followed by garlic bulb extract (61.65 %), tulsi leaf extract (60.49 %). All the treatments at 10 per cent were found to be significantly superior over 5 percent. Among the individual concentrations, 10 % garlic bulb extract was found to be most effective with 72.20 % inhibition, followed by , 10 % neem leaf extract (71.12 %), 10 % tulsi leaf extract 64.40 %. Least inhibition (11.13 %) was observed in 5 % parthenium leaf extract.

ii) Spore germination inhibition:

Of all the plant extracts that were tested, mean (5 and 10 per cent together) inhibition of spore germination of *Aspergillus niger*, garlic bulb extract (63.89 %), tulsi leaf extract (63.80 %) and neem leaf extract (62.17 %) were found to be most effective. Least inhibition (19.80 %) was observed in parthenium leaf extract. Among the treatments at two different concentrations, 10 % garlic bulb extract was most effective (78.09 %) followed by 10 % tulsi leaf extract (71.80 %), 10 % neem leaf extract (71.10 %). Least inhibition (12.51 %) was observed in 5 % parthenium leaf extract.

Various workers earlier in their studies reported the antifungal activity of plant extracts against *A. niger*. Sobti *et al.* (1995) reported the antifungal activity of neem and tulsi against *A. niger*. Yin *et al.* (1998) and Arun *et al.* (1995) also reported the antifungal of garlic and Thoppil *et al.* (2000) reported the antifungal activity of tulsi against *A. niger*. The present results once again emphasized their effectiveness.

Table 1: *In vitro* evaluation of botanicals against *Aspergillus niger* of pomegranate.

S. No	Plant extract	Percent inhibition of					
		Mycelial growth			Spore germination		
		5 %	10%	Mean	5 %	10 %	Mean
1	Chromolaena leaf extract	22.23 (28.10)*	32.28 (34.56)	27.26 (31.36)	19.65 (26.36)	27.40 (31.56)	23.53 (28.96)
2	Clerodendron leaf extract	17.45 (24.67)	27.80 (31.81)	22.62 (28.24)	34.13 (35.71)	56.12 (48.47)	45.13 (42.09)
3	Garlic bulb extract	51.10 (45.63)	72.20 (58.19)	61.65 (51.91)	49.69 (44.09)	78.09 (62.02)	63.89 (53.06)
4	Lantana leaf extract	30.45 (33.43)	36.32 (37.04)	33.39 (35.24)	29.49 (32.82)	37.34 (37.69)	33.42 (35.25)
5	Neem leaf extract	58.25 (49.67	71.12 (57.49)	64.69 (53.58)	53.24 (46.88)	71.10 (56.41)	62.17 (52.14)
6	Parthenium leaf extract	11.13 (19.44)	20.00 (26.56)	15.57 (23.00)	12.51 (20.72)	27.10 (31.34)	19.80 (26.03)
7	Tridax leaf extract	17.88 (24.94)	25.22 (30.12)	21.55 (27.53)	21.13 (27.27)	29.67 (32.92)	25.40 (30.09)
8	Tulsi leaf extract	56.58 48.77	64.40 (53.31)	60.49 (51.04)	55.80 (48.12)	71.80 (57.92)	63.80 (53.02)
	Mean	31.80 (34.33)	43.29 (41.13)	37.56 (37.73)	33.30 (35.24)	49.82 (44.91)	41.49 (40.08)
	Source	Sem ±		CD at 1 % Level	Sem ±		CD at 1 % Level
	Plant extract (P)	0.53		2.08	0.54		2.13
	Concentration (C)	0.26		1.04	0.27		1.06
	P x C	0.75		2.95	0.77		3.01

*Figures in the parentheses are angular transformed values.

B. *In vitro* evaluation of biocontrol agents against *A. niger*: (Table 2)

Studies on the antagonism of biocontrol agents on *A. niger* revealed that, *Bacillus subtilis* (77.00 %), *Trichoderma harzianum* (76.72 %), *T. viride* (76.65 %), *T. virens* (isolate 2) (74.00 %). were most effective and were statistically on par with each other. *T. virens* (isolate 1) (67.12 %), *T. reesei* (66.91%) were also effective, followed by *Pseudomonas fluorescens* (63.10 %). Lowest inhibition (62.82 %) among those tested was observed in *T. pseudokoningi.*

Antagonistic effect of *Trichodermaa spp.* against *A. niger* has been reported by earlier workers. Bharat Rai *et al*. (1980) reported that *T. viride* was found to be parasitic on Aspergillus. Similar reports of effectiveness of *Trichoderma spp.* against Aspergillus were reported by Prabhu and Urs (1998), Calistru *et al*. (1997). Hence they can be used as one of the components in Integrated Disease Management.

Table 2: *In vitro* evaluation of biocontrol against *Aspergillus niger* of pomegranate.

Sl. No.	Biocontrol agent	Per cent inhibition of mycelial growth
1	*Bacillus subtilis*	77.00 (61.35)*
2	*Pseudomonas fluorescens*	63.10 (52.28)
3	*Trichoderma harzianum*	76.72 (61.12)
4	*T. pseudokoningi*	62.82 (51.41)
5	*T. reesei*	66.91 (54.92)
6	*T. virens* (isolate 1)	67.12 (54.98)
7	*T. virens* (isolate 2)	74.00 (59.32)
8	*T. viride*	76.65 (61.08)
	SEm ±	0.60
	CD at 1% level	2.53

*Figures in the parentheses are angular transformed values.

C) *In vivo* evaluation of bioagents and botanicals against Aspergillus rot of pomegranate: (Table 3)

All the treatments tested significantly reduced the Aspergillus rot compared to control. Lowest PDI among the biocontrol agents and plant extracts tested *in vivo* were recorded in 10 % neem leaf extract (23.12) and *T. viride* spore suspension (23.72). Garlic bulb extract (30.10), *T. viride* culture filtrate (33.78) were also effective compared to control while *B. subtilis* was least effective with PDI of 44.41. However, Benomyl @ 0.1 % produced least PDI of 10.10 and was the most effective of all the treatments compared to control (61.20).

The results of the experiment are in accordance with those of Srinivasulu (2001) who reported that, *T. viride* spore suspension and neem leaf extract were effective in reducing the Aspergillus rot of pomegranate. Similarly, Jitender Singh and Majumdar (2001) also reported the effectiveness of garlic bulb and neem leaf extracts in reducing the fruit rot of pomegranate. Also, chemical check (Benomyl @ 0.1 %) was found to be superior to other biological treatments. Similar results of superiority of chemical control over plant extracts and biocontrol agents in were reported by Shirshikar (2002) and Prasanna Kumar (2001) in case of mango. However, considering the advantages of these treatments which are cheap, safe and ecofriendly in nature, botanicals and biocontrol agents can be used in management of postharvest diseases of pomegranate.

Table 3: Effect of biocontrol agents and botanicals as postharvest treatments on Aspergillus rot of pomegranate

Sl. No.	Postharvest Treatment	Per cent Disease Index (PDI) at 8 DAI
1	*Bacillus subtilis* (10^7 - 10^8 cfu/ml)	44.41 (41.78)*
2	Garlic bulb extract (10%)	30.10 (33.23)
3	Neem leaf extract (10%)	23.12 (28.06)
4	Trichoderma *viride* spore suspension (5 X 10^5 spores/ ml)	23.72 (28.76)
5	*T. viride* culture filtrate	33.78 (35.55)
6	Benomyl (0.1%)	10.10 (18.40)
7	Control	61.20 (51.42)
	SEm	0.32
	CD at 1% level	1.38

DAI –Days after inoculation.

*Figures in the parentheses are angular transformed values.

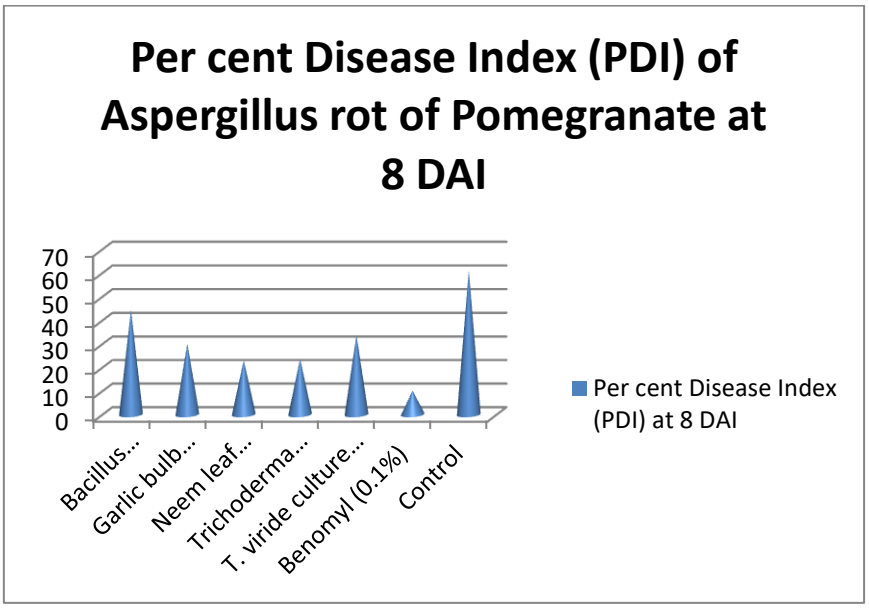

Fig. 1: Effect of various postharvest treatments on Aspergillus rot of Pomegranate

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