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Bio Efficacy of Bio Agents and Botanicals against *Alternaria alternata* (Fr.) Keissler Causing Leaf Spot of Pomegranate

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A B S T R A C T

Pomegranate (*Punica granatum* L) is one of the important fruit crops grown in India. Leaf spot of pomegranate caused by *Alternaria alternata* (Fr.) Keissler has becoming a major limiting factor in cultivation of pomegranate in some regions of India. The present investigation was carried out to test the efficacy of bio-agents and botanicals *in vitro*. All the seven fungal and two bacterial antagonists tested, exhibited significant mycelial growth inhibition of *A. alternata*. However, *T. viride* recorded significantly highest mycelial growth inhibition (86.85 %) of the test pathogen, followed by *T. hamatum* (82.04 %), *A. niger* (81.11 %). Among eleven botanicals tested, significantly highest average mycelial growth inhibition was recorded with *A. sativum* (75.56 %), followed by *Z. officinale* (73.64 %), *A. indica* (71.17 %).

Introduction

Pomegranate (*Punica granatum* L.) is an ancient, delicious fruits consumed worldwide, gaining lot of attention of the world over, because of its high economic value and nutritional values. It is one of the important fruit crops in arid and semi-arid regions commercially important in both tropical and subtropical countries known for its drought tolerance which thrives well in dry tropical conditions with marginal soils of low fertility. Being the most adaptable subtropical fruit crop, its cultivation has increased rapidly creating its image as an important cash crop in global market. Globally India is ranked first in area and production. During 2015-16, pomegranate was cultivated over 2.09 lakh ha with an annual production of 24.42 lakh MT and productivity of 12.00 MT/ha in India (Anonymous, 2016). Maharashtra considered as pomegranate basket of India contributes more than 70 per cent of the total area under pomegranate followed by Andhra Pradesh, Uttar Pradesh, Rajasthan, Gujarat and Karnataka which are the leading states; cultivating pomegranate commercially on a large scale.

However, the crop is under threat due to number of serious diseases such as bacterial blight (*Xanthomonas axonopodis* pv. *punicae*), wilt due to *Ceratocystis fimbriata*, anthracnose (*Colletotrichum gloeosporioides*) and leaf spot and severe fruit rotting due to *Alternaria alternata*, *Cercospora* sp., *Pseudocercospora* sp., *Drechslera* sp. and *Sphaceloma* sp.etc., are more or less equally important and harmful in some orchards and also take a heavy toll on the crop (Khosla and Bhardwaj, 2013). Among these; severe spotting and fruit rotting due to *Alternaria alternata*(Fr.) Keissler; remains hitherto unexplored but potentially dangerous pathogen on pomegranate and considered to be an emerging disease.

In recent years, there has been a major thrust on pesticide residue free organic pomegranate production. Taking the task into consideration, efficient botanicals and bioagents need to be explored to fit into the management schedule. Use of bioagents for the management of various diseases of crop plants is eco-friendly and environmentally safe. Therefore, present investigation aimed to evaluate bioagents and botanicals (*in vitro*) against *Alternaria alternata* (Fr.) Keissler causing leaf spot of Pomegranate.

Materials and Methods

Invitro evaluation of bioagents

Seven fungal and two bacterial bioagents were evaluated *in vitro* against *A. alternata*, applying Dual Culture Technique (Dennis and Webster, 1971). Seven days old cultures of the test bioagents and test pathogen (*A. alternata*) grown on PDA were used for the study. Two 5 mm culture discs, one each of the test pathogen and test bioagent were cut out with sterilized cork borer and placed at equidistance, exactly opposite to each other on autoclaved and solidified PDA medium in Petri plates and three plates were incubated at 27±2 ^{0}C. PDA plates inoculated alone with pure culture disc (5 mm) of the test pathogen were maintained as untreated control. Pure cultures and talc based formulations of biocontrol agents *viz., Trichoderma viride, T. hamatum, T. harzianum, T.* (*Gliocladium*) *virens, T. koningii, T. longibrachiatum, Aspergillus niger, Pseudomonas fluorescens* and *Bacillus subtilis* were obtained from the Spawn Production-cum-Biocontrol Laboratory, Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani; maintained and multiplied on appropriate culture media and used for present studies.

Observations on linear mycelial growth of the test pathogen and test bioagent were recorded at an interval of 24 hours and continued till untreated control plates were fully covered with mycelial growth of the test pathogen. Per cent inhibition of the test pathogen with the test bioagent, over untreated control was calculated by applying following formula (Arora and Upaddhyay, 1978).

Per cent Growth Inhibition =

$$\frac{\text{Colony growth in Control plate} - \text{Colony growth in intersecting plate}}{\text{Colony growth in control plate}} \times 100$$

In vitro evaluation of plant extracts

Aqueous extracts of 11 botanicals (as detailed under treatments) were evaluated *in vitro* against *A. alternata*. Leaf / bulb / rhizome extract of the test botanicals were prepared by grinding with mixture-cum grinder. Washed 100 g each leaves / Turmeric rhizome / Onion bulb / Garlic cloves were macerated separately in 100 ml distilled water (w/v) and the macerates obtained were filtered separately through double layered muslin cloth. Each of the filtrate obtained was further filtered through Whatman No. I filter paper using funnel and volumetric flasks (100 ml cap.).

The final clear extracts obtained formed the standard plant extracts of 100 per cent concentration. These were evaluated (each @ 10 %, 15 % and 20 %) *in vitro* against *A. alternata*, applying Poisoned Food Technique (Nene and Thapliyal, 1993) and using Potato dextrose agar (PDA) as basal culture medium. An appropriate quantity of each test aqueous extract (100 %) was separately mixed thoroughly with autoclaved and cooled (40 0C) PDA medium in conical flasks (250 ml cap.) to obtain desired concentrations of 10, 15 and 20 per cent. The PDA medium amended separately with the test aqueous extract was then poured (20 ml / plate) into sterile glass Petri plates (90 mm dia.) and allowed to solidify at room temperature. For each test botanical extract and their respective concentrations, three plates / treatment / replication were maintained and all the treatments were replicated thrice. Upon solidification of the amended PDA medium, all the treatment plates were aseptically inoculated by placing in the centre a 5 mm mycelial disc obtained from a week old actively growing pure culture of *A. alternata*. Plates containing plain PDA without any botanical extract and inoculated with mycelial disc of the test pathogen served as untreated control. All these plates were then incubated at 27± 2 ^{0}C temperatures for a week or till the untreated control plates were fully covered with mycelial growth of the test pathogen.

Observations on radial mycelial growth / colony diameter of the test pathogen were recorded treatment-wise at 24 hours interval and continued till mycelial growth of the test pathogen was fully covered in the untreated control plates. Per cent inhibition of mycelial growth over untreated control was calculated by applying the formula given by Vincent (1927)

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where, C= growth of the test fungus in untreated control plates

T= growth of the test fungus in treated plates

Results and Discussion

In vitro evaluation of bioagents

Results (Plate 1, Fig. 1 and Table 1) revealed that all the bioagents evaluated exhibited fungistatic / antifungal activity against *A. alternata* and significantly inhibited its growth over untreated control. Of the seven fungal antagonists tested, *T. viride* was found most effective and test pathogen recorded least linear mycelial growth (11.83 mm) with highest mycelial inhibition (86.85 %) of the test pathogen. The second and third best antagonists found were *T. hamatum* and *A. niger*, which recorded mycelial growth of 16.17 mm and 17.00 mm, of the test pathogen respectively and inhibition of 82.04 and 81.11 per cent, respectively.

This was followed by *T. harzianum* (col. dia.: 20.33 mm and inhibition: 77.41 %), *T. (Gliocladium) virens* (col. dia.: 23.67 mm and inhibition: 73.23%) and *T. longibrachiatum* (col. dia.: 32.67 mm and inhibition: 63.70 %). The antagonists *Pseudomonas fluorescens* and *Bacillus subtilis* were found least effective with 47.17 mm and 37.83 mm linear mycelial growth and 47.59 and 57.96 per cent mycelial inhibition.

These results are in conformity with the earlier findings of those workers who reported bioagents *viz*., *T. viride*, *T. harzianum*, *T. koningii* and*T. hamatum* had significantly inhibited mycelial growth of *A. alternata* infecting different crops (Gohel *et al.,* 2011; Akbari and Parakhia, 2007; Hudge *et al.,* 2009; Waghunde *et al.,* 2009; Balai and Ahir, 2011, Rajput *et al.,* 2011; Apet *et al.,* 2014).

Table.1 *In vitro* evaluation of bio control agents against mycelial growth of *A. alternate*

Tr. No.	Treatments	Colony Dia. of test pathogen * (mm)	% Inhibition
Fungal antagonists			
T_1	*Trichoderma viride*	11.83	86.85 (68.71)
T_2	*T. hamatum*	16.17	82.04 (64.90)
T_3	*T. harzianum*	20.33	77.41 (61.60)
T_4	*G. virens*	23.67	73.70 (59.13)
T_5	*T. konigii*	29.50	67.22 (55.05)
T_6	*T. longi brachiatum*	32.67	63.70 (52.93)
T_7	*Aspergillus niger*	17.00	81.11 (64.22)
Bacterial antagonist			
T_8	*Bacillus subtilis*	37.83	57.96 (49.56)
T_9	*Pseudomonas fluorescens*	47.17	47.59 (43.60)
T_{10}	Control (Untreated)	90.00	0.00 (0.00)
	S.E. ±	**0.45**	**0.33**
	C.D. (P = 0.01)	**1.338**	**0.99**
		2.391	**1.11**

*: Mean of three replications, Dia.: Diameter,
Figures in parentheses are arcsine transformed values

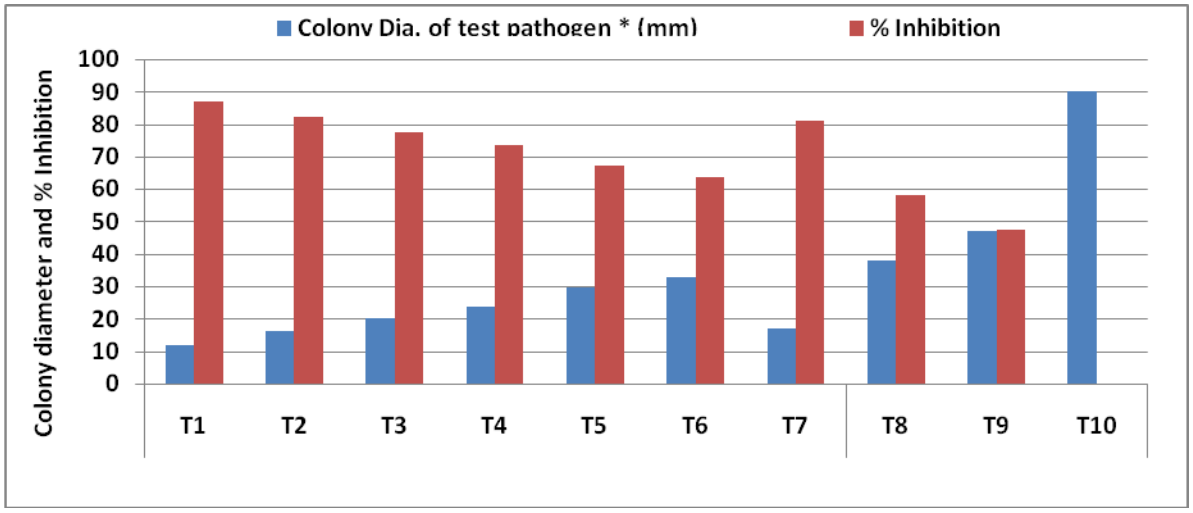
Table.2 *In vitro* evaluation of plant extracts against mycelial growth of*A. Alternate*

Tr. No.	Treatment	Col. Dia.(mm) *at Conc.			Av. Col. Dia.(mm)	% Inhibition*			Av. % Inhibition
		10%	15%	20%		10%	15%	20%	
T_1	Neergudi (*V. negundo*)	44.33	41.00	38.33	41.22	50.74 (45.41)	54.44 (47.53)	57.41 (49.24)	54.20 (47.39)
T_2	Bougainvillea (*Bougainveilliea* spp.)	77.17	72.67	66.67	72.17	14.26 (22.17)	19.26 (26.02)	25.93 (30.60)	19.81 (26.26)
T_3	Dhotra / Dhatura (*D. metal*)	43.67	39.83	34.33	39.28	51.48 (45.83)	55.74 (48.28)	61.85 (51.84)	56.36 (48.65)
T_4	Nilgiri/Eucalyptus (*E. globulus*)	35.67	30.67	24.50	30.28	60.37 (50.97)	65.93 (54.27)	72.78 (58.53)	66.36 (54.59)
T_5	Ghaneri (*L. camera*)	48.50	45.00	39.67	44.39	46.11 (42.75)	50.00 (44.98)	55.93 (48.39)	50.68 (45.37)
T_6	Ginger (*Z. officinale*)	26.67	24.17	20.33	23.72	70.37 (57.00)	73.15 (58.77)	77.41 (61.60)	73.64 (59.12)
T_7	Karanj (*P.pinnata*)	60.67	52.50	47.83	53.67	32.59 (34.80)	41.67 (40.19)	46.85 (43.18)	40.37 (39.39)
T_8	Neem (*A. indica*)	30.00	25.17	22.67	25.94	66.67 (54.72)	72.04 (58.06)	74.81 (59.85)	71.17 (57.54)
T_9	Parthenium (*P.hysterophorus*)	67.33	59.33	53.33	60.00	25.19 (30.10)	34.07 (35.70)	40.74 (39.65)	33.33 (35.15)
T_{10}	Turmeric (*C.longa*)	42.33	35.33	29.83	35.83	52.96 (46.68)	60.74 (51.18)	66.85 (54.83)	60.19 (50.90)
T_{11}	Garlic (*A. sativum*)	24.83	22.67	18.50	22.00	72.41 (58.29)	74.81 (59.85)	79.44 (63.01)	75.56 (60.39)
T_{12}	Control	90.00	90.00	90.00	90.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	S.E. ±	**0.51**	**0.48**	**0.44**		**0.36**	**0.32**	**0.33**	
	C.D.(P=0.01)	**1.49**	**1.40**	**1.30**		**1.05**	**0.95**	**0.96**	
		1.78	**1.84**	**1.89**		**1.52**	**1.28**	**1.30**	

***** Mean of three replications, Figures in parentheses are arcsine transformed value, Dia: Diameter Conc.: Concentration, Av.: Average, Col.: Colony

Plate.1 *In vitro* effect of bioagents on growth and inhibition of *A. alternate*

Fig.1 *In vitro* bioefficacy of bioagents against *A. alternate*



Plate,2 *In vitro* effect of phytoextracts at various concentrations on growth and inhibition of *A. alternata*

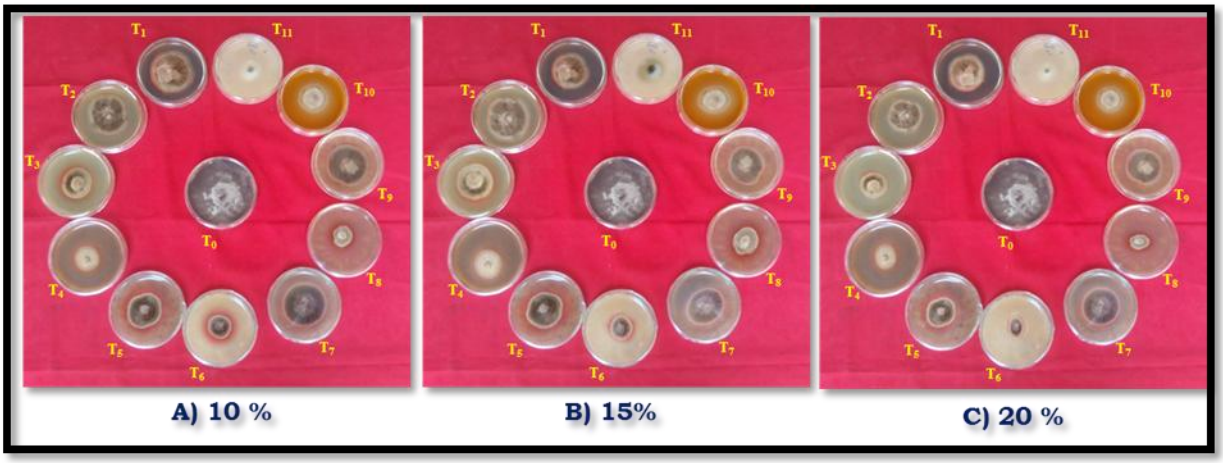
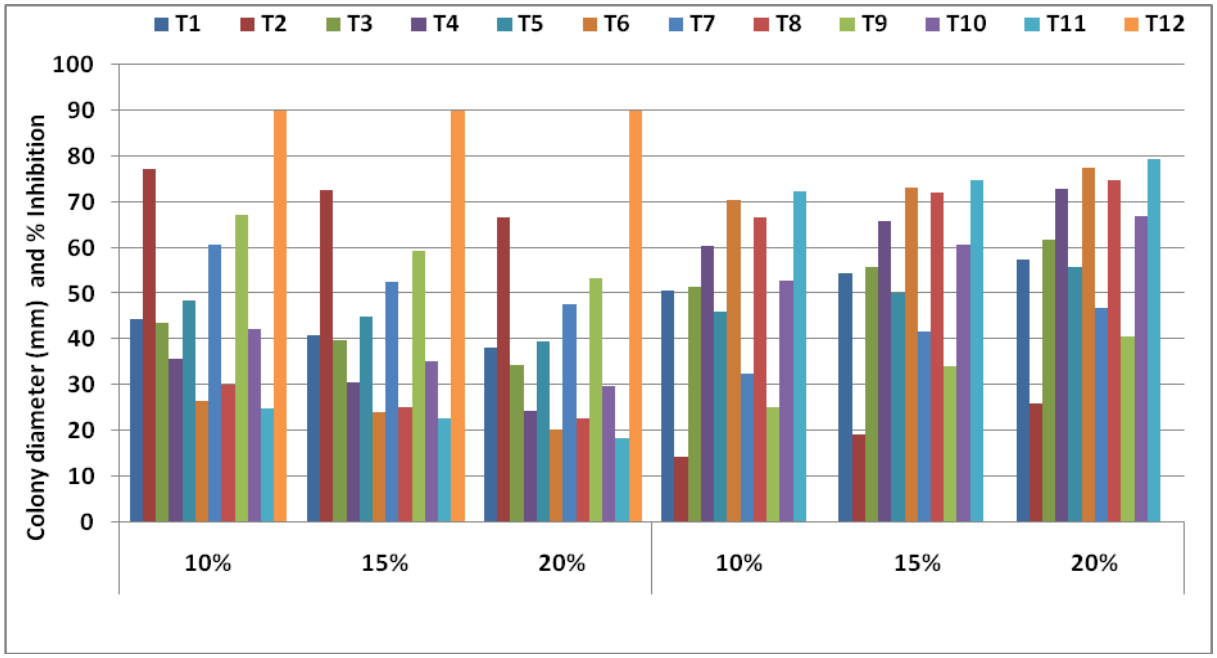


Fig.2 *In vitro* bioefficacy of botanicals against *A. alternate*



The fungistatic / antifungal action exerted by the species of *Trichoderma* and *A. alternata* and other species of *Alternaria* were reported by several workers, *A. macrospora* infecting cotton (Gholve *et al.*, 2014), *A. lini* infecting linseed (Charpe *et al.*, 2014), *A. solani* infecting tomato (Kharbhari *et al.*, 2008) and *A. burnsii* infecting cumin (Vihol *et al.*, 2009).

The fungistatic / antifungal action exerted by the species of *Trichoderma* and *A. niger* against *A. alternata* and other species of *Alternaria* may be attributed to their production of volatile and non-volatile substances, cell wall degrading enzymes (glucanases, B1, 3 glucanase), the phenomenon of competition, lysis and antibiosis.

In vitro evaluation of plant extracts / botanicals

Results (Table 2) revealed that all the 11 botanicals tested (each @ 10, 15 and 20 %) exhibited a wide range of radial mycelial growth of *A. alternate* (Plate 2 and Fig. 2) and it was decreased drastically with increase in concentrations of the test botanicals from 10 to 20 per cent.

Mycelial growth

Average radial mycelial growth of the test pathogen was ranged from 22.00 mm (*A. sativum*) to 72.17 mm (*Bougain veilliea* spp.). However, it was significantly least with *A. sativum* (22.00 mm), followed by *Z. officinale* (23.72 mm), *A. indica* (25.94 mm), *E. globulus* (30.28 mm), *C. longa* (35.83 mm),*D. metal* (39.28 mm), *V. negundo* (41.22 mm) and *L. camera* (44.39 mm) whereas *P. pinnata*, *P. hysterophorus* and *Bougainveilliea* spp. recorded comparatively maximum mycelial growth of 53.67, 60.00 and 72.17 mm, respectively. Results revealed that all the 11 botanicals tested exhibited a wide range of mycelial growth inhibition of *A. alternata* and it was decreased drastically with increase in concentrations of the test botanicals from 10 to 20 per cent

Mycelial inhibition

Average mycelial growth inhibition of the test pathogen was ranged from 19.81 % (*Bougain veilliea* spp.) to 75.56 % (*A. sativum*). However, it was significantly least with *A. sativum* (75.56 %), followed by *Z. officinale* (73.64 %), *A. indica* (71.17 %), *E. globulus* (66.36 %), *C. longa* (60.19 %), *D. metal* (56.36 %), *V. negundo* (54.20 %) and *L. camera* (50.68 %) whereas *P. pinnata*, *P.hysterophorus* and *Bougainveilliea* spp.recorded comparatively maximum mycelial growth of 40.37, 33.33 and 19.81 %, respectively.

Thus, on the basis of antifungal activity, the botanicals found most effective in the order of merit were *A. sativum, Z. officinale, A. indica, E. globules, C. longa, D. metal, V. negundo, L. camera, P. pinnata, P.hysterophorus, P.hysterophorus* and *Bougain veilliea* spp which after their further confirmation could be exploited for control of leaf spot of pomegranate (*A. alternata*).

In the present study, of the 11 phytoextracts *A. sativum* extract was found most effective which caused substantial inhibition (> 88%) of *A. alternata.* The antifungal activity of *A. sativum* has been attributed to the presence of diallyl sulphide and other compounds like alllisatin I, II and garli phytocide (Sharma and Prasad, 1980). A number of phytoextracts / botanicals have been studied and reported with potential inhibitory action against many phytopathogenic fungi, bacteria and viruses. The presence of various secondary metabolites *viz.*, alkaloids, quaternary alkaloids, cumarins, flavanoids, steroids / terpenoids, phenolics etc with potential antifungal activity were reported in various plant extracts (Abraham *et al.*,1986; Chopra *et al.*, 1992). The antifungal properties of *A. sativum* against *A. alternate* have been reported earlier by many workers (Mandhare and Suryawanshi, 2009; Balai and Ahir, 2011; Apet *et al.*, 2014; Barman *et al.*, 2016).

Similar effect of the test botanicals / phytoextracts against *A. alternata,* andother *Alternaria* spp. were reported earlier by several workers. Botanicals *viz*. *A. sativum, Z. officinale, A. indica, E. globules, C. longa, D. metal, V. negundo, L. camera, P. pinnata, P. hysterophorus, P. hysterophorus* and *Bougainveilliea* spp etc. were reported to cause significant mycelial growth inhibition of *Alternaria* spp., earlier by several workers (Anamika and Shobita, 2011; Chethana *et al.*, 2012; Waghmare, 2012; Ganie *et al.*, 2013).

In conclusion, all the, bio agents and botanicals evaluated *in vitro* were found fungistatic / antifungal against *A. alternata.* However, bioagents *viz., T. viride, T. hamatum* and *A. niger*; botanicals *viz., A. sativum, Z. officinale, A. indica* were most efficient with significantly highest inhibition of mycelial growth of the *A. alternata* causing leaf spot in pomegranate.

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