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PROMISING ANTIFUNGAL POTENTIAL OF SELECTIVE BOTANICAL EXTRACTS, FUNGICIDES AND *TRICHODERMA* ISOLATES AGAINST *ALTERNARIA SOLANI*

M. SARFRAZ[1], S.A. KHAN[2], A. MOOSA[2,*], A. FARZAND[1], U. ISHAQ[1], I. NAEEM[2], W.A. KHAN[2]

*E-mail: annu_77@live.com

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ABSTRACT. *In vitro* antifungal potential of *Trichoderma* isolates, selective botanical extracts and fungicides against *A. solani* was evaluated. *Trichoderma* isolates, *i.e. T. harzianum*, *T. viride* and *T. hamatum*, were tested for their antifungal effect by dual culture technique at 48, 96, 144 and 172 hrs. *T. hamatum* produced the highest inhibition of *A. solani in vitro*, followed by *T. hazianum* and *T. viride* after 172 hrs. Methanolic leaf extracts of *Elettaria cardamomum, Syzygium aromaticum, Curcuma longa* and root extract of *Parthenium hysterophorus* showed up to 100% inhibition of *A. solani*, compared to control, while methanolic stem and leaf extracts of *P. hysterophorus* produced up to 90% inhibition of the pathogen. *In vitro*, six different systemic fungicides Triger 25% EC (Tebuconazole), Solex (Carbendazim 40% + Triadimefon 10%), Dew (Difenoconazole), Amistor Top SC (Azoxystrobin + Difenoconazole), Corel 25% EC (Difenoconazole), Reflex (Difenoconazole + Propiconazole) were tested against *A. solani* at 5, 10 and 15 ppm concentrations after 48, 96, 144 and 172 hrs. Corel and reflex at all concentrations produced best growth inhibition of *A. solani*. The inhibition was maximum by all fungicides at 15 ppm after 172 hrs. All fungicides had a promising inhibitory effect on *A. solani*, except Solex. It can be concluded from the present investigation that a combination of these strategies can be used in integrated disease management of *A. solani* on potato.

Keywords: *Trichoderma harzianum*, *T. viride*, *T. hamatum*, *Parthenium hysterophorus*, *Elettaria cardamomum, Syzygium aromaticum.*

[1] University of Agriculture, Faisalabad, Sub-Campus Burewala-Vehari, Pakistan
[2] Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan

INTRODUCTION

Early blight of potato, caused by *Alternaria solani*, is one of the major and widely distributed disease in potato or tomato growing areas, but more prevalent in tropical and temperate areas. The disease is a potential threat to potato crops that are cultivated in irrigated areas or in heavy dew conditions (Rotem, 1994). Dark brown to black lesions or spots with concentric rings that look like a 'target board', are characteristic symptoms of the disease (Van der Walls *et al.,* 2001). The disease also reduces market quantity and quality of the produce (Pscheidt, 1985). There are reported evidences of 5-50% yield loss due to early blight of potato (Neergaard, 1945). *A. solani* is an asexually reproducing fungus that produces conidia that are dispersed by wind or rain splashes on lower leaves of the plant and germinate to infect the plants (Rotem, 1994). Early blight of potato can be controlled by different methods that include cultural practices, such as site selection and preparation, crop rotation (3-5 years), nutrition management, sanitation at the end of cropping season and before sowing of next crop, avoiding water stress and use of disease-free planting material (Madden *et al.*, 1978). All above described cultural practices may fail to give effective control of the disease due to the presence of sufficient inoculum and favorable environmental conditions for the pathogen (Van der Walls *et al.,* 2001). Due to devastating nature of *A. solani* it would be more effective to control this pathogen through integrated disease management approach.

Spraying of protectant fungicides is most effective method to control early blight (Teng & Bissonnette, 1985). However, the use of fungicides has potentially threatening effects on human health and environment. The use of fungicides can be optimized to be in a safer range by integrating with other relatively safer alternate approaches, such as use of biological control agents and botanical extracts. Total number of sprays can be reduced by properly timed initial and subsequent application of fungicides. The lower and senescing foliage must receive aerial fungicide treatment to prevent the disease development (Van der Walls *et al.,* 2001).

Trichoderma species has the potential to be used as effective biological control agents due to strong mycoparasitism of fungal pathogens and fast-growth potential (Whipps & Lumsden, 2001). The antagonistic ability of *Trichoderma* is due to production of volatile and non-volatile metabolites, competition for food, space and nutrition, ability to secrete cell wall degrading enzymes and antibiosis (Küçük & Kyvanç, 2008). Plant extracts have antifungal potential due to production of antimicrobial compounds, that are toxic to fungal pathogens (Ngadze, 2014). Plants produce toxic secondary metabolites, such as tannins, flavonols, coumarins, saponins, phenolics and quinones (Cowan, 1990). Plant extracts are a bio-safe

approach to manage fungal pathogens and can be integrated with fungicides to reduce the use of fungicides. To explore the antifungal potential of new biological control agents and botanical extracts is a continuously ongoing research.

The present *in vitro* investigations were carried out with an objective to compare the efficacy of some promising botanical extracts, *Trichoderma* isolates and fungicides *in vitro*, to find the most effective management options, which can be integrated *in vivo* to achieve effective control of the disease.

MATERIALS AND METHODS

Pure cultures of *Trichoderma* isolates and *A. solani*

Purified cultures of *T. hamatum* (Accession # 0769), *T. viride* (Accesion # 0167) and *T. harzianum* (Accession # 0860) were obtained from First Fungal Culture Bank of Punjab University of Lahore, Pakistan, that contains the pertinent information regarding how they were isolated from natural environment. The cultures were maintained on potato dextrose agar medium at 25±1ºC for future use.

A. solani was isolated following standard isolation procedure described by Pathak (1987) from infected potato leaves, collected in sterile polythene bags from Research Farm Area Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Infected leaves were washed with distilled water, surface sterilized with sodium hypochlorite (10%), cut into small bits (5 mm) and transferred to PDA medium aseptically, followed by incubation at 25±1°C for 24 hrs. After 24 hrs, colony growth of the pathogen was observed and it was purified using single spore culture method. *Alternaria solani* was identified microscopically based on spore morphology described in literature. The conidia were dark brown, transversely and horizontally septate with a distinct beak as described by Ellis & Ellis (1987).

Dual culture inhibition of *A. solani* by *Trichoderma* isolates

Antagonistic effect of *Trichoderma* isolates on *A. solani* was evaluated by dual culture method. Freshly, prepared pathogen culture was taken, 5 mm mycelial blocks were cut and transferred at one edge of the Petri plates containing PDA medium, followed by incubation at 25±1°C for 48 hrs. After 48 hrs, 5 mm culture blocks of *Trichoderma* isolates were transferred at opposite edge of the same plate. For all *Trichoderma* isolates the same procedure was repeated. Control was maintained by inoculating the medium with the pathogen only. For each treatment, five replications were maintained. The plates were incubated at 25±1°C in an incubator (Morton & Stroube, 1955). Experiment was repeated once. Radial colony growth of the pathogen was measured and percent inhibition of the pathogen by antagonistic agents was calculated by using the formula of Vincent (1947).

$$I=(C-T)/C\times 100,$$

where, I= Inhibition of the pathogen; C= Growth in control plate and T= Growth in treated plate.

Growth inhibition of *A. solani* by botanical extracts

The antifungal potential of methanolic botanical extracts against the pathogen was evaluated by poisoned food method of Nene & Thapliyal (2000). Fresh samples of different plants, *i.e. Parthenium hysterophorus*, Turmeric

(*Curcuma longa*), Clove (*Syzygium aromaticum*), Cardamom (*Elettaria cardamomum*), well known for their antifungal activity and medicinal properties were collected. Samples were washed with sterilized distilled water, followed by cleaning with sodium hypochlorite (10%) and drying for 48 hrs by keeping in an incubator at 70ºC. The samples were grinded to powder and 10 g of each sample was taken, dissolved in 100 mL methanol and left for 48 hrs. The extract was filtered through a coarse sieve and double layer of filter papers. Final pH of all botanical extracts was maintained at 6.5 by adding acidic (HCL) or basic buffer (NaOH) solutions. With 10 mL of each botanical extract, 100 mL PDA medium was poisoned and poured in sterilized Petri plates. The poisoned medium was inoculated with 5 mm culture blocks of the pathogen, followed by incubation at 25±1°C. For each treatment, five replicates were maintained. Control was maintained by amending the medium with sterilized distilled water only instead of botanical extracts. The experiment was repeated once. After every 24 hrs, interval colony growth was observed and colony diameter was recorded. Percent inhibition of the pathogen, compared to control, was calculated by using the formula of Vincent (1947).

Growth inhibition of *A. solani* by fungicides

In vitro investigation was carried out to evaluate antifungal potential of six different fungicides, *i.e.* Triger 25% EC (Tebuconazole), Amister Top SC (Azoxystrobin + Difenoconazole), Corel 25% EC (Difenoconazole), Solex (Carbendazim 40% + Triadimefon 10%), Reflex 30% EC (Difenoconazole + Propiconazole), Dew (Difenoconazole) against *A. solani*. Stock solution of each fungicide was prepared to maintain 5, 10 and 15 ppm concentrations. PDA 100 mL was poisoned by each fungicide at three different concentrations and poured into sterilized Petri plates. In case of control treatment, PDA was amended with equal concentration of sterilized distilled water. Poisoned medium after solidification was inoculated with culture blocks of the pathogen 5 mm in diameter and incubated at 25±1°C. Each treatment was replicated five times. The experiment was repeated once. Colony growth was recorded at every 48 hrs interval till 192 hrs. Percent inhibition of the pathogen, compared to control, was calculated by using the formula of Vincent (1947).

Statistical analysis

Experiments were conducted in completely randomized design (CRD). Data was subjected to statistical analysis using M-Stat (Ver. 2.3, Faisalabad, Pakistan). Treatment means were separated by Least Significant Difference (LSD) and Tukey's HSD tests.

RESULTS AND DISCUSSION

It was found that *T. hamatum* had the best inhibitory potential on growth inhibition of *A. solani*. After 192 hrs, all species produced maximum inhibition of the pathogen. After 192 hrs, best inhibition was observed by *T. hamatum* (45.05%), followed by *T. harzianum* (43.00%) and *T. viride* (37.29%) (*Table 1*). Strong mycoparasitism was observed by *T. hamatum.* Colony diameter and inhibition were significantly affected by time. *Trichoderma* species can be a safer alternate strategy to be integrated in disease management

program of early blight of potato. Inhibition was significantly increased with the passage of time. The present results match with many previous findings (Jee & Kim, 1987; Sabalpara *et al.,* 2009; Devi *et al.,* 2012; Javaid *et al.,* 2014; Moosa *et al.,* 2017). *Trichoderma* have varying antagonistic potential and this difference might be due to the genetic potential and origin of the isolate (Moosa *et al.,* 2017). *Trichoderma* inhibit the growth of the pathogen through its rapid growth potential and competition for food and space (Devi *et al.,* 2012). *Trichoderma* inhibit the pathogen through mycoparasitism (Doley & Jite, 2012). In present investigation, strongest mycoparasitism was observed by *T. hamatum*. *Trichoderma* also inhibit the pathogen through the production of volatile and non-volatile compounds (Tapwal *et al.,* 2011; Sumana & Devaki, 2012). Trichodermin, dermadin, sesquierpene, harzianum A, harzianolide, and trichodermol are the antibiotics produced by *Trichoderma* (Küçük & Kyvanç 2008; Nakkeeran *et al.,* 2002). *Trichoderma* also inactivate the enzymes produced by the pathogen and induce host resistance (Ozbay & Newman, 2004).

Table 1 - *In vitro* inhibitory effect of *Trichoderma* isolates on *A. solani*

Treatment	Time (hrs)	Mean colony diameter (cm)	Inhibition (%)
T. harzianum	48	2.10g*	7.50j*
T. viride	48	2.17g	4.40k
T. hamatum	48	2.23g	1.76l
Control	48	2.27g	0.00m
T. harzianum	96	2.90ef	12.90h
T. viride	96	3.03de	9.10i
T. hamatum	96	2.80f	15.91g
Control	96	3.33c	0.00m
T. harzianum	144	2.90ef	29.44e
T. viride	144	3.03de	29.10f
T. hamatum	144	2.80f	31.60d
Control	144	4.10b	0.00m
T. harzianum	192	2.90ef	43.00b
T. viride	192	3.20de	37.29c
T. hamatum	192	2.80f	45.05a
Control	192	5.10a	0.00m

*Mean values followed by same alphabets are not significantly different from each other, analyzed by using LSD test at $P \leq 0.05$, values are mean of five replicates.

Table 2 - *In vitro* inhibitory effect of botanical extracts on growth inhibition of *A. solani*

Treatment	Mean colony diameter (cm)	Inhibition (%)
P. hysterophorus stem	0.40b*	90.26b*
P. hysterophorus root	0.00c	100.00a
P. hysterophorus leaf	0.40b	90.26b
Clove	0.00c	100.00a
Cardamom	0.00c	100.00a
Turmeric	0.00c	100.00a
Control	4.10a	0.00c

*Mean values followed by same alphabets are not significantly different from each other, analyzed by using LSD test at $P \leq 0.05$, values are mean of five replicates.

All methanolic botanical extracts showed promising inhibition of *A. solani* at 10% concentration after 8 days. Clove, turmeric, cardamom and *P. hysterophorus* root extracts produced the best inhibition after 8 days. Hence, their biopesticides potential can be further investigated and included in integrated disease management program of early blight of potato caused by *A. solani*, while, *P. hysterophorus* stem and leaf extracts produced 90.26% inhibition of the pathogen (*Table 2*). Plants naturally produce antifungal aromatic secondary metabolites, such as quinones, saponins, flavones, coumarins, flavonols, phenols, and tannins (Cowan, 1999). In the present study, the selected methanolic botanical extracts produced promising inhibition of *A. solani*. The results are in line with the results of Masih *et al.* (2014), who observed that the aqueous extracts of *Curcuma longa* showed inhibitory effect on the growth of *Aspergillus fumigates, Fusarium solani, Alternaria solani* and *Helminthosporium* spp. The results are also supported by the findings of Raza *et al.* (2016), who tested five different plant extracts against *A. solani in vitro*, and found that all botanical extracts, including *P. hysterophorus*, significantly inhibited mycelial growth of the pathogen. Moosa *et al.* (2016) also revealed that *P. hysterophorus* showed 100% inhibition of *P. capsici*. The findings are also supported by several previous reports (Touba *et al.,* 2012; Pawar & Thakar, 2007).

Different fungicides, *i.e.* Corel, Triger, Reflex, Amister Top, Dew and Solex, at 5, 10 and 15 ppm concentrations, were evaluated for their inhibitory action against *A. solani.* It was found that Corel and Reflex had the best inhibitory potential against the pathogen than Amister, Dew, Triger and Solex (*Figs. 1, 2* and *3*). The fungicides with best inhibition are recommended to be integrated in disease management program with botanical extracts and *Trichoderma*. At 15 ppm, maximum inhibition was observed by all fungicides, except Solex (*Fig. 3*).

Time and concentration had a significant effect on inhibition of the pathogen. Hang-Cheng *et al.* (2007) observed that Tebuconazole strongly inhibits the growth of *R. solani*, associated with rice sheath blight, which supports the inhibition potential of Triger (Tebuzonazole) in the present findings. Similar results were also found by Xiu-Rong (2011), Horsfield *et al. (*2010), Koley *et al.* (2016).

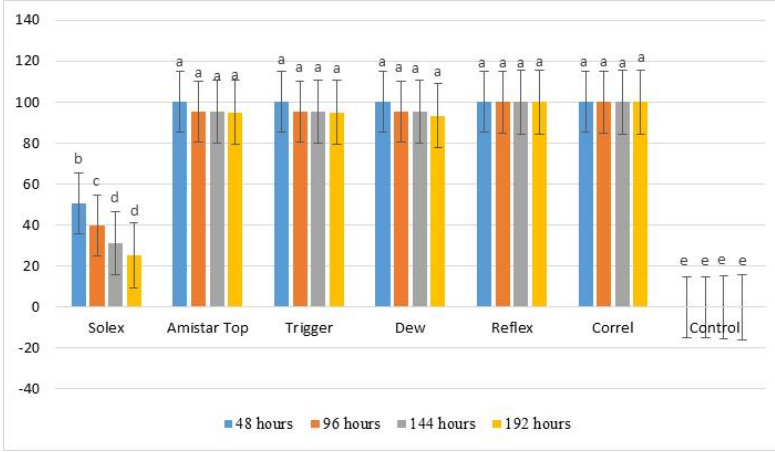

Figure 1 - Effect of fungicides on colony growth inhibition of *A. solani* at 5 ppm. Mean values followed by same letter are not significantly different at *P*≤ 0.05, analyzed by Tukey's HSD test.

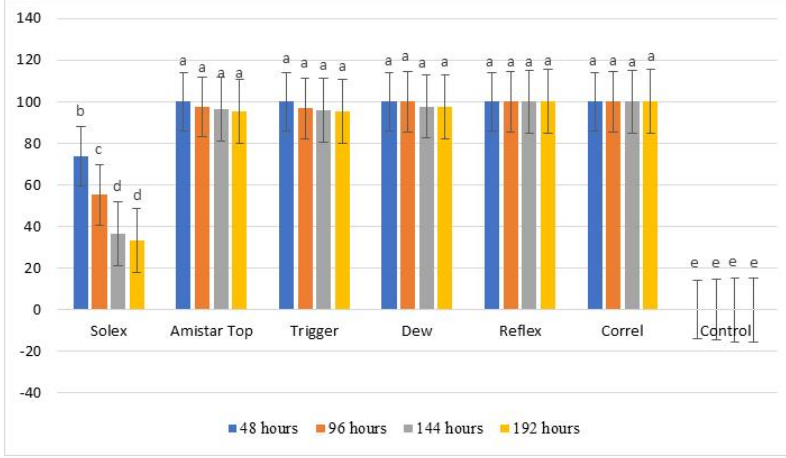

Figure 2 - Effect of fungicides on colony growth inhibition of *A. solani* at 5 ppm. Mean values followed by same letter are not significantly different at *P*≤ 0.05, analyzed by Tukey's HSD test.

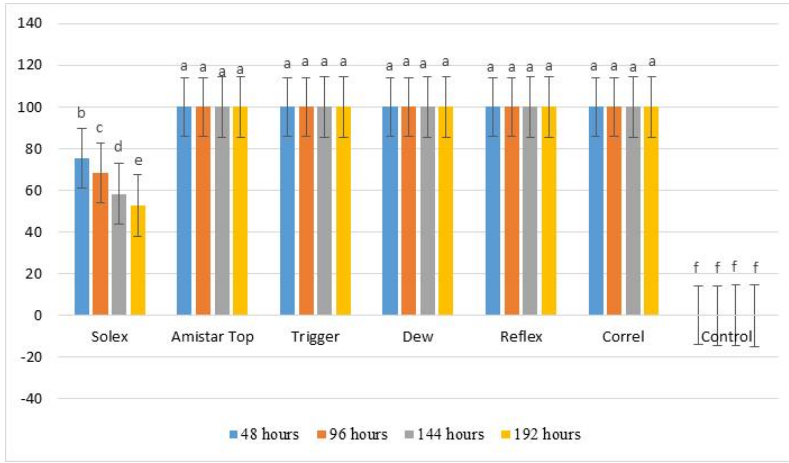

Figure 3 - Effect of fungicides on colony growth inhibition of *A. solani* at 15 ppm. Mean values followed by same letter are not significantly different at *P*≤ 0.05, analyzed by Tukey's HSD test.

The results of carbendazim are opposed by the results of Chourasiya (2013). The fungicides evaluated in the present findings were promising against *A. solani*, hence, they can be used in combination with *Trichoderma* and botanical extracts at low dose, that have least negative impact on human health and environment.

CONCLUSION

Based on the findings of present investigation it can be concluded that the evaluated *Trichoderma* isolates botanical extracts and fungicides can be combined to control early blight of potato. The dose of fungicides can be optimized or reduced to a safer range to be incorporated in integrated disease management of early blight of potato.

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