Research Article

Effect of Aspergillus awamori isolates against Alternaria leaf blight of Asalio (*Lepidium sativum L*.) caused by *Alternaria alternata*

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Abstract

Asalio an important medicinal plant with significant pharmacological properties has been observed to be generally affected by many fungal pathogens in India. Phosphate-solublizing Fungi (PSF) functions in soil phosphorus cycle by increasing the bioavailability of soil phosphorus for plants and root associated fungi have been known to benefit plants and are hence referred to as plant growth promoting fungi (PGPF). Present investigation Five isolates of *Aspergillus awamori* namely Zinger-Z, Parthenium-P, Red gram-G, Rice leaf sheath-R, Field bean-B isolated from different rhizosphere and phylloplane were tested In-vitro and In-vivo conditions. The results showed that *Aspergillus awamori* isolates were IAA, Phosphorus solublizing activity, Ammonia producing activity, solublizing index, Biomass determination and efficacy tests were performed against *Alternaria alternata*.The IAA activity of all *Aspergillus awamori* isolates were minimum.However, the phosphorous solublizing and Ammonia producing activity *of A. awamori*-R, *A. awamori*-B and *A. awamori*-Z was higher and medium among the tested isolates.The solublizing index and Phosphate-solublizing activity of all *Aspergillus awamori* isolates was found significant but *A. awamori*-R showed highest Phosphate-solublizing activity (halo zone formation). However, the highest inhibition was recorded in *A. awamori*-G isolate reduction in mycelia growth when growth medium was amended with and without $ZnSO_4$. *A.awamori* culture filtrates of *Aspergillus awamori* amended with $ZnSO_4$ amended culture, exhibited comparatively lower mycelial growth inhibition in comparison to $ZnSO_4$ non amended culture filtrate. The highest biomass production of *A.awamori*-R was recorded in both $ZnSO_4$ amended medium and without $ZnSO_4$ amended medium.

Keywords:*Aspergillus awamori* isolates, *Alternaria alternata*, $ZnSO_4$

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Introduction

Garden cress (*Lepidium sativum* Linn; Family: Brassicaceae) commonly known as Asalio is a fast growing annual herb native to Egypt and West Asia [5]. It's seeds, leaves and roots posses medicinal properties due to presence of imidazole, lepidine, semilepidinoside A and B [2] carotenes, ascorbic acid, linoleic acid, oleic acid, palmitic acid, stearic acid [7] sinapic acid and sinapin[12]. Hence its commercial demand has increased several times in different parts of India viz., states of Madhya Pradesh, Uttar Pradesh, Rajasthan, Gujarat, Maharashtra and Rajasthan more than 8000 ha are under its cultivation [8]. Asalio leaf blight caused by *A. alternata* is major problem for hampering medicinal property crop as well as its yield loss of the crop as it not only produces melanin but also a large variety of secondary metabolites such as carcinogenic alternariol [10], [14]. Therefore, organic solution for such menace is indispensible for retaining the therapeutic value of the crop along with higher yield. Phosphorus-solubilizing microorganisms (PSMs) are abundantly available in the rhizosphere of plants [18] where they compete with other organisms for nutrient and space and in-turn provide nutrient for growth and protection from deleterious micro-organism. Many studies have shown an increase in growth and P-uptake by plants through the inoculation of PSMs (one of the component of PGPF) in pot experiments [17] and as well as in field conditions [16]. The antagonistic fungi *Aspergillus* are a common genus in most agricultural fields of India and are ubiquitous fungus with no specific moisture and pH requirements. Therefore, the present investigation was under taken overcome the menace of this pathogen, the Plant Growth Promoting Fungi (PGPF) with special reference to *Aspergillus awamori isolates* have been used to manage the disease.

Material and Methods

Collection of diseased specimens and purification of the pathogen

Diseased Asalio plants exhibiting typical symptoms of *Alternaria alternata* infection were collected from the experimental field of AICRP on Medicinal Aromatic Plants and Betelvine of Jawaharlal Nehru Krishi Vishwa Vidyalaya ($22^0$49'- 220 80'N; $78^0$21'- $80^0$58'E), Jabalpur in the Central India.

Treatment details of mycoflora used under in-vitro studies

Different PGPF were isolated from the rhizosphere and phylloplane of different crops. The five isolates of *Aspergillus awamori* have been screened against *Alternaria alternata* under *in-vitro* and *in- vivo* conditions.*Aspergillus awamori* isolates: *A. awamori*- Z,(*A. awamori*- Zinger isolate), *A. awamori* –P(*A. awamori*- Parthenium isolate), *A. awamori*-G(*A. awamori*-Red gram isolate), *A. awamori* –R(*A. awamori*-Rice leaf sheath isolate) and *A. awamori* –B(*A. awamori*- Field bean isolate).

IAA producing activity

The presence of IAA-like substances was detected by following the method of [11] in L-tryptophan agar. The fungi were grown on L-tryptophan agar medium in triplicate and incubated at 28 ± 2^0C for seven days in the dark. After seven days of incubation, the fungus grown on L-tryptophan agar medium was added with freshly prepared Salkowsky reagent in triplicate, for each bioagent grown on Petri dish and incubated in the dark for 30 min for development of pink colour. The amount of IAA production was expressed by + and – sign. The - indicates no IAA production;+ faint pink colour and small amount of IAA production; ++ pink colour and medium amount of IAA production; +++ dark pink colour and high amount of IAA production.

Phosphorus solublizing activity

Phosphate solubilizing fungi were isolated by the dilution plate methods on PVK Pikovskaya medium with tri-calcium phosphate as insoluble inorganic phosphate source. Rose Bengal as bacteriostatic agent was added (1 0 ML/l) at concentration 1 /15000 [13].

Pikovskaya's medium with rose Bengal addition was prepared. Sterilized PVK media was poured into sterilized plates, after solidification of the media, fungal strains were placed on the center of plates under aseptic conditions. They were incubated at 28 ± 2^0C for 5 days with continuous observation for colony diameter. The P. solubilizing fungi were detected by the formation of clear halo around their colonies. The performance of each fungus was marked by assigning them + and – sign. The - indicates no phosphorus solublization,+ small amount of phosphorus was dissolved, ++ medium amount of phosphorus was dissolved and +++ high amount of phosphorus was dissolved. The halo formation was observed after 5 and 6 days colonies forming a clear halo around them indicating P solubilisation were counted and further used to determine the P solubilization index. Solubilization index was measured using following [9].

$$SI= \frac{\text{colony diameter+ Halozone diameter}}{\text{Colony diameter}}$$

Ammonia producing activity

For the detection of ammonia production, all the isolates were grown in Petri-dishes containing peptone water agar (peptone: 10.0 g; NaCl: 5.0 g; distilled water: 1000 ml; 7.0 pH). The Petri-dishes were inoculated with seven days old culture of bioagents and incubated at 30 ± 1^0C for 5 days. The accumulation of ammonia was detected by adding Nessler's reagent (0.5 ml per plate). A faint yellow colour indicated a small amount of ammonia, and deep yellow to brownish colour indicated medium to maximum production of ammonia.

Assessment of culture filtrates of beneficial mycoflora added with or without $ZnSo_4$ by poison food technique

Effect of culture filtrate of Aspergillus awamori isolates combined with and without $ZnSo_4$ was assessed against mycelial growth of *Alternaria alternata* bymethod[1]. Filtrate of antagonist(s) culture in PDA broth grown for 10 days with or without addition of $ZnSo_4$ (@ 200ppm) was collected after passing it twice through Whatman filter paper

No. 1. These filtrates were used to amend Petriplates containing PDA at 5 per cent concentration while $ZnSo_4$ at 200ppm and incubated at 25+2°C in a BOD incubator and observations were recorded at regular intervals; an un-amended Petri-plate served as check (control). The observation of radial growth of test pathogen was taken after 48, 72, 96, and 120 hours, respectively. Each treatment was replicated thrice and the experiment was repeated twice.

Per cent inhibition of growth of the pathogens was calculated by using the following formula.

$$\text{Inhibition} = \frac{\text{Radial growth in control(C)}-\text{Radial growth in the treatment (T)}}{\text{Radial growth in control(C)}}$$

Determination of biomass production

The testing of biomass production by the beneficial fungi was done by growing them on potato dextrose broth and amended with and without $ZnSo_4$@200ppm) prepared in 100 ml Erlenmeyer flask and final pH was adjusted to 6.5 to 7.0. They were later inoculated aseptically with 5mm actively grown culture disc of the fungus. Three replications were maintained. The entire set up was incubated for 7days at 25^0C to attain maximum growth and sporulation. Mycelial mat was obtained by filtering on pre-weighed filter paper (Whatman filter paper no.1) (as fresh weight) and dried in hot air oven at 60 ^{0}C until a constant weight (dry weight) was obtained [6].

Results and Discussion

All the isolates of *A.awamori* were screened for IAA production, tri-calcium phosphate solublization and ammonia production under in-vitro conditions All the *A.awamori* isolates produced minimum quantity of IAA but *A.awamori* – G isolate did not produce the indole acetic acid. The maximum solublization of tri-calcium phosphate took place with all the studied isolates of *A.awamori* except Parthenium isolate. The medium ammonifying property of four *A.awamori* isolates (such as Parthenium, rice-sheath, field bean & zinger) were recorded with development of deep yellow colour while lower was recorded with gram isolate of *A.awamori*(**Table 1**). [15] reported that Indole acetic acid production from tryptophan using broth cultures showed the greatest production in case of *A. niger* 710 ug mL^{-1}) followed by *A. terreus* (235 ug mL^{-1}) isolated from the rhizosphere of pepper (The other isolates of *Aspergillus species* tested produced IAA in negligible quantities – *A. clavatus* (100 ug mL^{-1}), *A. flavus* str 1 (98 ug mL^{-1}), and *A. flavus* str2 (120 ug mL^{-1}).

Table 1 Qualitative characterization of beneficial attributes of plant growth promoting rhizosphere and phylloplane fungi

Aspergillus awamori isolates different Rhizosphre soils	IAA Producing activity	Phosphorus solublizing activity	Ammonia Producing activity
*A.awamori (*R)	+(pink) minimum	+++(maximum)	++(deep yellow) medium
A.awamori(P)	+(pink) minimum	++(medium)	+(faint yellow) minimum
A.awamori (B)	+(pink) minimum	+++(maximum)	++(deep yellow) medium
A.awamori (Z)	+(pink) minimum	+++(maximum)	++(deep yellow) medium
A.awamori(G)	-(absent)	+++(maximum)	+(faint yellow) minimum

In vitro solubilization of phosphate was determined on Pikovskaya agar media clear halo zone was recorded on pikovaskaya agar media after 5 and 6 days interval incubation in BOD at 28 c. In vitro Solubilization index was recorded higher significant of Aspergillus awamori isolates. The highest phosphorous solubilization activity was recorded isolate of *A.awamori* –R at incubation 5 and 6 days. The lowest solubilizing activity was recorded *A.awamori*-G at incubation of 5 and 6 days(**Table 2**). [3]reported P-solublizing filamentous fungi (like *A. awamori*) are also well-known producers of lytic enzymes and cell-wall-degrading enzymes, such as β-1,3-glucanases, cellulases, proteases, and chitinases are known to be involved in the activity of some microorganisms against phytopathogenic fungi.

The inhibition of mycelia growth of *A. alternata* by culture filtrate of different *Aspergillus awamori* isolates under poison food technique was found significant (**Table 3**). The highest (28.37mm) inhibition was recorded in T_5 that corresponds to 23.24 percent reduction in mycelia growth. Similar growth inhibition was recorded in culture filtrate of T_2 (29.86mm) & T_1 (29.82mm). The least inhibition was recorded with T_3 (30.32mm) and T_4(30.25mm), respectively among the tested isolates. The growth of the pathogen had increased with every time interval from 48 hours to 96hours. The growth of test pathogen in culture filtrates of T_5 (23.96mm, 27.74mm &33.41mm) was slower

throughout the studied time to rest *A. awamori* isolates. The range of percent mycelia growth inhibition was very narrow among the *A. awamori* isolates and it varied from 23.24 to 17.96 percent.

Table 2 Phosphorus solublizing index for different rhizosphere and phylloplaneof *Aspergillus awamori* isolates

Fungal bioagents	5 days	6 days	Mean
T_1(*A.awamori*-R)	1.23	1.26	1.25
T_2 (*A.awamori*-P)	1.18	1.23	1.21
T_3(*A.awamori*-B)	1.15	1.20	1.18
T_4(*A.awamori* -Z)	1.11	1.14	1.12
T_5(*A.awamori* -G)	1.03	1.10	1.07
Mean	1.14	1.19	
Coefficient of Variation	1.10		
Fungus CD(P<0.05) X Hours	0.02		

Table 3 Evaluation of bioefficacy of culture filtrates of *Aspergillus awamori* isolates against *Alternaria alternata*

Aspergillus awamori isolates	Growth in (mm)				
	48hours	72hours	96 hours	mean	Percent Inhibition
T_1 (A.awamori-R)	25.72(18.83)	29.10 (23.66)	34.65 (32.33)	29.82	19.31
T_2 (A.awamori-P)	24.84(17.66)	29.88 (24.73)	34.85 (32.66)	29.86	19.20
T_3 (A.awamori-B)	26.55(20.00)	29.77 (24.66)	34.64 (32.33)	30.32	17.96
T_4(A.awamori-Z)	25.96(19.16)	29.53 (24.33)	35.26 (33.33)	30.25	18.15
T_5 (A.awamori-G)	23.96(16.50)	27.74 (21.66)	33.41 (30.33)	28.37	23.24
Control	30.64(26.00)	37.26 (36.66)	42.99 (46.50)	36.96	
Mean	26.28	30.55	35.97		
CV	2.80				
FungusCD(P≤0.05)	0.83				
Hours CD(P≤0.05)	0.58				
Fungus x Hours	1.44				
The values in the parenthesis are original value					

Table 4 Evaluation of bioefficacy of culture filtrate amended with $znso_4$ of *Aspergillus awamori* isolates against *Alternaria alternata*

Aspergillus awamori isolates	Growth in (mm)				
	48hours	72hours	96 hours	Mean	Percent Inhibition
T_1 (A.awamori-R)	25.10 (18.00)	28.98 (23.50)	35.05 (33.00)	29.71	18.22
T_2(A.awamori-P)	24.72 (17.50)	30.22 (25.33)	35.36 (33.50)	30.10	17.14
T_3 (A.awamori-B)	25.84 (19.00)	29.10 (23.66)	34.64 (32.33)	29.86	17.80
T_4 (A.awamori-Z)	25.47 (18.60)	30.32 (25.50)	35.46(33.66)	30.42	16.26
T_5 (A.awamori-G)	25.95 (19.16)	27.74 (21.66)	33.41 (30.33)	29.03	20.09
Control	30.64 (26.00)	35.66 (34.00)	42.70 (46.00)	36.33	
Mean	26.29	30.34	36.11		
CV	2.45				
Fungus CD(P≤0.05)	0.72				
Hours CD(P≤0.05)	0.51				
Fungus x Hours CD(P≤0.05)	1.25				
The values in the parenthesis are original value					

The culture filtrate of different *A. awamori* isolates amended with $znso_4$ were tested against mycelia growth of *Alternaria alternata* through poison food technique and were found highly effective against the test pathogen at different time intervals (**Table 4**).The $znso_4$ amended culture filtrate of *A.awamori*-G (29.03mm), *A.awamori*-R(29.71mm) and *A.awamori*-B (29.86mm) were highly and equally inhibitory towards the test pathogen followed by T_2 (30.10mm) and T_1. (30.42mm). The significant increase in growth of test pathogen was recorded from 48 hours (26.29mm) to 96 hours(36.11mm). The percent mycelia growth inhibition by *A. awamori* isolates was in order of T_5(20.09%)> T_1(18.22%)>T_3(17.80%)>T_2(17.14%)> T_4(16.26%), respectively).

Table 5 Effect of micro-nutrient on biomass production of beneficial fungi

With $znso_4$				
Fungal bioagents	Fresh weight	Dry weight	Biomass (%)	pH
T1A.awamori(R)	3.25	0.53	83.69	1.7
T2 A.awamori(P)	3.50	0.71	79.71	1.6
T3 A.awamori(B)	4.47	0.82	81.65	3.9
T_4A.awamori(Z)	2.75	0.46	83.27	1.7
T_5 A.awamori(G)	2.94	0.49	83.33	1.9
Without $znso_4$				
Fungal bioagents	Fresh weight	Dry weight	Biomass (%)	pH
T1A.awamori(R)	3.18	0.49	84.59	1.5
T2 A.awamori(P)	3.39	0.63	81.41	1.3
T3 A.awamori(B)	5.07	0.96	81.06	1.8
T_4A.awamori (Z)	2.03	0.50	75.36	1.4
T_5A.awamori(G)	3.23	0.53	83.59	1.6

To evaluate the effect of micro-nutrient on biomass production, the growth medium of beneficial fungus was amended with and without znso4.The growth medium of beneficial fungus was amended with $znso_4$.The highest fresh weight varied from 2.75 to 3.50 while dry weight 0.46 to 0.71 and pH from 1.6 to 3.9.The highest 83.69 bio mass production recorded with AW-(R) is followed by AW-(G), AW-(Z), AW-(B) and bio mass production least AW-(P). The growth medium of beneficial fungus was amended without $znso_4$.The highest fresh weight varied from 2.03 to 5.07 while dry weight 0.49 to 0.96 and pH from 1.3 to 1.8. The highest 84.59 bio mass production recorded with AW-(R) and lowest bio mass production AW-Z. Copper salts, zinc salts, calcium hydroxide, potassium hydroxide and selected nitrogen sulfur and molybdenum compounds have been found to be highly toxic to pathogenic fungi. Metal ions applied at certain concentrations under laboratory conditions may lead to the death of the tested microorganisms, where in a natural environment they can stimulate microbial growth. The effects exerted by metal ions are determined by their chemical form availability and environmental factors[4].Applications of *A. awamori* cause decline in the pathogen populations and result in enhanced yield was reported by [16].

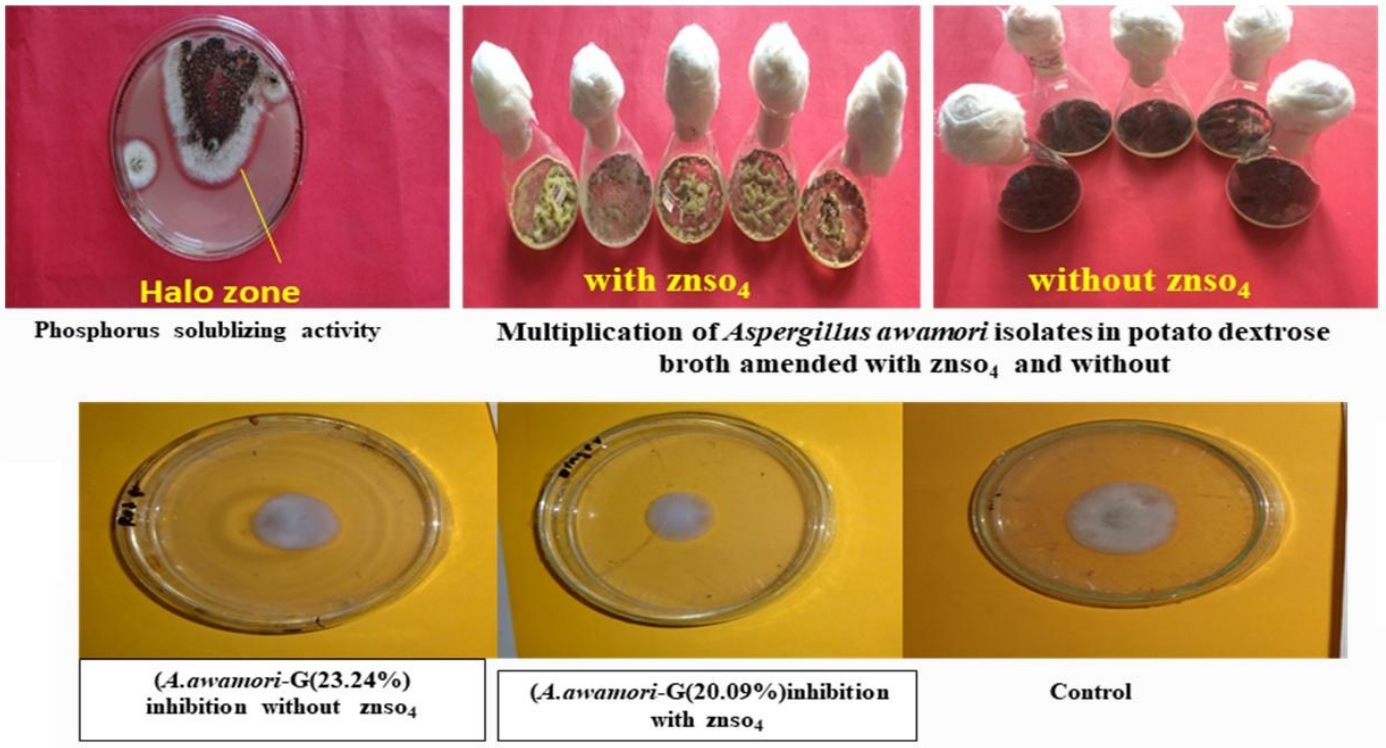

Fig-Table:1.Phosphorus solublizing activity),(Fig-Tab.5 Multiplication of Aspergillus awamori isolates in potato dextrose broth amended with $znso_4$ and without) and (figTab:3,4 A.awamori-G23.24, 20.09 without and with.

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