Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 1049-1052
Received: 01-06-2020
Accepted: 05-07-2020

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Optimization of media and appraisement of fungicides and bioagents on leaf spot of ashwagandha, *Withania somnifera* (L.) Dunal caused by *Alternaria alternata*

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Abstract
Alternaria leaf spot is a major disease in ashwagandha caused by *Alternaria alternata* which reduces in withaferin-A and withanolides contents and exhibited significant antitumour activity. Effect of five media *viz.*, PDA, CMA, OMA, CA and MEA were optimized at different temperature i.e. (15, 20, 25, 30 and 35^{0}C). It was found CMA at temperature 30^0 C was most favoured for the growth of *A. alternata* (28.00 mm) while lowest growth occurred in media MEA at temperature 15^{0}C i.e. (5.60 mm). Nine fungicides, in which formulation of carbendazim, mancozeb, captan, copper oxy chloride, chlorothalonil, ridomil-MZ, thiophanate methyl, propiconazole and difenoconazole at different concentrations i.e. (100, 500, 1000, 1500 and 2000 ppm) by using poisoned food technique. Propiconazole and difenoconazole showed maximum percentage inhibition of *A. alternata* at all concentrations while thiophanate methyl was least effective. Four bioagents namely (*T. harzianum, T. viride, P. fluorescens* and *B. cereus*) were also evaluated for their antagonistic effect against *A. alternata* by using dual culture technique. The bioassay showed that *T. viride* was most effective with (55.66%) inhibition of mycelial growth while *P. fluorescens* (34.91%) was least effective.

Keywords: Ashwagandha, *Alternaria alternata*, fungicide, bioagent, media, temperature, *Trichoderma viride* and *Withania somnifera*

Introduction
Ashwagandha, *Withania somnifera* (L.) Dunal - belongs to family Solanaceae, is an important medicinal plant that is used in the Indian traditional system of medicine ayurveda and unani. Ashwagandha roots are compared with ginseng roots for their restorative properties and have been popularly known as Indian ginseng. The plant roots, leaves, fruits and seeds contain a number of alkaloids including withanin and somniferine (Asthana and Raina, 1989) [2]. Ashwagandha is affected by various diseases like leaf spot, seedling blight and wilt. The leaves of the plant (Indian chemotype) are reported to contain 12 withanolides, 5 unidentified alkaloids (yield, 0.09%), many free amino acids, chlorogenic acid, glycosides, glucose, condensed tannins, and flavonoids (Khare, 2007) [14]. Among these leaf spot disease caused by *Alternaria alternata* is the most important. This disease causes reduction in withaferin- A and withanolides contents by 15.4% and 76.3% respectively (Pati *et al.*, 2008) [20].
The initial symptoms are leaves having brown to black spot of 2 to 9 mm in diameter surrounded by a yellow halo appear on both dorsal and ventral surfaces of the infected leaves (Inoue and Nasu, 2000) [10]. Different synthetic and non-synthetic media were tested and proved that the synthetic media Leonion's agar, Glucose- peptone agar and Sabourand's agar and non- synthetic media, OMA and PDA were excellent for the mycelial growth and conidial production of *A. alternata* by Nagrale *et al.* (2013) [17]. Kalieswari *et al.* (2016) [11] were tested seven fungicides against *A. alternata* among the fungicides, the minimum diameter of mycelia growth occurred (0.75 cm) and maximum percentage inhibition (91.34%) recorded in mancozeb (0.2%). Kamalalakshmi (1996) [12] found both poisoned food technique and spore germination assay, mancozeb (0.2%) was the most effective fungicide against *A. alternata.* Ganie *et al.* (2013) [6] found the higher inhibition of mycelial growth of *A. solani* was recorded in application of *T. harzianum.* (Ghosh *et al.*, 2016) [8] observed among the fungicides, the commercially available mancozeb was effective at concentration (100 µg/ml) to control the pathogen whereas it can tolerate 1000 µg/ml or more concentration of bavistin. Two plant growth promoting rhizobacterial strains, *Burkholderia cenocepacia* VBC7 and *Pesudomonas poae* VBK1 were able to produce prominent zone of inhibition against leaf spot of Aloe vera (*A. alternata*).

The present investigation was undertaken to evaluation of different media, fungicides and bioagents on growth of leaf spot ashwagandha, *Withania somnifera* (L.) caused by *Alternaria alternata*.

Materials and Methods

Isolation, purification and identification of causal fungus

The initial symptoms of *A. alternata* on ashwagandha leaves were brown to black spot of 2 to 9 mm in diameter surrounded by yellow halo. The infected plants samples, showed characteristic symptoms of spot on ashwagandha local variety, leaves at nursery stage in Poly house of Fruit Nursery Block and at Medicinal and Aromatic Plants Block, were collected during 2017-18 growing seasons from College of Horticulture, VCSG UUHF Bharsar, Pauri Garhwal (Uttarakhand).

infected leaf samples were cut into small pieces (4 mm) by a sharp sterilized blade along with some healthy portions, surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) solution for about 20-30 seconds washed thrice with sterilized distilled water and dried on blotter paper then transferred aseptically on PDA medium containing Petri plates. The plates were incubated at 25±1^0 C for 96 hrs. The fungus was later transferred to PDA slants for future use. The fungal morphological features such as colour, shape and branching habit of the developing mycelia were observed. Mycelium of isolated fungus from host it was first white, rapidly become grayish black. Seven days old fungal hyphae were transferred to a glass slide and teased gently, stained with lactophenol cotton blue and examined under microscope to study the microscopic morphological characters of the fungus. Measurement of the conidia was done with the help of micrometer.

Effect of media at different temperature on growth of *A. alternata* at 75±5% (RH)

In order to find out the optimum media and temperature for the development of the *A. alternata*, growth of fungus on five media i.e. Potato Dextrose Agar (PDA), Corn Meal Aga(CMA), Oat Meal Agar(OMA), Carrot Agar (CA) and Malt Extract Agar (MEA) at five different temperatures i.e. (15, 20, 25, 30, and 35 ^{0}C) were observed. Twenty ml of each media was poured into sterilized Petri plates and fungal disc of (5 mm) was inoculated and incubated at different temperature for five days, observed average radial mycelia growth in (mm), (r) is radius of diameter; Kalieswari (2016) [11] plates were also incubated at room temperature (28 ± 2^{0}C) for five days and observed for the growth of the fungus.

In vitro efficacy of fungicides

Nine fungicides namely carbendazim, mancozeb, captan, copper oxy chloride, chlorothalonil, ridomil-MZ, thiophanate methyl, propiconazole and difenoconazole at different concentrations i.e. (100, 500, 1000, 1500 and 2000 ppm) were evaluated for their efficacy in inhibition of *A. alternata* using the poisoned food technique by (Nene and Thapliyal, 1993) [18]. The plates were incubated at temperature 25±1^0 C for 4 days. Average radial mycelia growth in (mm) was calculated, (r) is radius of diameter, per cent mycelium growth inhibition was calculated by formula and mentioned in below.

In vitro efficacy of bio control agents

Two fungus namely (*Trichoderma harzianum* and *T. viride*), two bacterial bioagents (*Pseudomonas fluorescens* and *Bacillus cereus*) were evaluated for their efficacy against *A. alternata* using dual culture technique described by (Faheem *et al*., 2010) [5]. A set of sterilized Petri plates were taken and 20 ml of sterilized PDA was poured into each of the Petri plate under aseptic condition. After the media got solidified in the plates, the test fungus was inoculated at one end of each Petri plate and the bio-control agents on the opposite end. Each treatment was replicated four times and these Petri plates were incubated at temperature of 25±1^{0}C for 4 days. Per cent mycelium inhibition was calculated using the following formula given by Vincent (1947) [21].

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

Where

C= Colony radial in control

T= Colony radial in treatment

The data had obtained and analyzed by using standard statistical procedure in the simple completely randomized design (CRD) with the help of OPSTAT and Graph Pad (3.05).

Results and Discussion

Effect of media at different temperature on (*A. alternata)*

Among all the media and temperature combinations, maximum radial growth of *A. alternata* presented in (Table 1) in which at temperature 15 ^{0}C was found in OMA i.e. (9.00 mm) and statistically at par with media CMA (7.90 mm) and PDA (7.75 mm). On the other hand the minimum growth was observed in MEA i.e. 5.60 mm. At the temperature of 20^{0}C the maximum radial growth was observed in PDA i.e. 23.00 mm and the minimum observation was recorded in MEA i.e. 17.00 mm and statistically at par with CMA (17.75 mm). At the temperature of 25 ^{0}C the maximum growth of the pathogen in PDA i.e. 26.25 mm which was statistically at par with OMA (24.75 mm) and the minimum was recorded in MEA i.e. 23.11 mm which was statistically at par with CMA (24.25 mm) and CA (24.38 mm). At the temperature 30^{0}C the maximum radial growth was observed in CMA i.e. 28.00 mm which was statistically at par with PDA (27.00mm) and OMA (26.50 mm), the minimum radial growth was observed in MEA i.e. 22.90 mm. At the temperature 35 ^{0}C the maximum radial growth was found in PDA i.e. 11.38 mm which was statistically at par with OMA (10.50 mm) and CMA (10.20 mm) and minimum radial growth was observed in MEA i.e. 8.19 mm. Nagrale *et al*. (2013) [17] were similar observations recorded that OMA and PDA were excellent for the mycelial growth and conidial production of *A. alternata*. Koley and Mahapatra (2015) [15] also recorded that (PDA) and (OMA) among solid media and Richard's broth and Sabouraud's broth among liquid media appeared to be better than other media for growth of tomato early blight causing fungi. (Kaul and Saxena, 1988) [13] reported that fungus sporulation is best in (OMA) media the maximum growth of five isolates of *A. solani* was at 25 ^{0}C followed by 20 ^{0}C, 15 ^{0}C, 10 ^{0}C and 5 ^{0}C with least growth at 35 ^{0}C. Hubballi *et al.* (2010) [9] found among the different medium, host leaf extract medium supported significantly the maximum growth of all the fifteen isolates of *A. alternata* with mean mycelial growth of (89.80 mm) followed by PDA (89.34 mm) and OMA (83.72 mm) and Walksman's medium (80.23 mm). Least mean mycelial growth was observed in water agar (9.84 mm).

Table 1: Effect of media at different temperature on radial growth of (*A. alternata)* after five days of inoculation

Media	Average radial mycelia growth (mm) at different temperature				
	15 ^{0}C	20 ^{0}C	25 ^{0}C	30 ^{0}C	35 ^{0}C
PDA	7.75	23.00	26.25	27.00	11.38
CMA	7.90	17.75	24.25	28.00	10.20
OMA	9.00	19.25	24.75	26.50	10.50
CA	7.03	18.13	24.38	25.75	9.75
MEA	5.60	17.00	23.11	22.90	8.19
Mean	7.46	19.03	24.55	26.03	10.00
S.E.(d)	0.59	0.51	0.82	0.82	0.72
C.D.$_{(p=0.05)}$	1.27	1.11	1.76	1.77	1.55

Efficacy of fungicides

The significant variation among all the fungicides with control at different concentrations (100, 500, 1000, 1500 and 2000 ppm) were observed for mycelium growth inhibition of *A. alternata* and observations presented in (Table 2). Two fungicides namely propiconazole and difenoconazole were most effective at all concentrations which were significantly inhibition the percentage mycelium growth (100%), but at the concentration (1000, 1500 and 2000 ppm) ridomil- MZ found effective which inhibition (100%) the mycelium growth of *A. alternata*. Mancozeb and thiophanate methyl also found effective which inhibition the mycelium growth at 1000,1500 and 2000 ppm i.e. (76.47, 100 and 100%) and (67.70,100 and 100%). Copper oxy chloride also maximum percentage of inhibition mycelia 1000,1500 and 2000 ppm i.e. (66.90,79.27 and 100%). Whereas thiophanate methyl and chlorothalonil were found least effective at concentration 100 ppm which showed minimum mycelium inhibition (24.27 and 24.92%) and captan was found least effective at 500, 1000 and 1500 ppm which showed minimum percentage of mycelium inhibition i.e. (37.80, 54.14 and 61.12%) and chlorothalonil was found least effective at (2000 ppm) which showed (71.09%) minimum mycelium inhibition. Kalieswari *et al*. (2016) [11] tested against *A. alternata* in ashwagandha and found mancozeb maximum percentage of inhibition (91.34%) of mycelial growth, copper oxychloride found effective with (78.87%) mycelial inhibition and less effective in (mancozeb+metalaxyl) i.e. (16.86%), carbendazim (20.90%) and chlorothalonil (25.87%). Bhat *et al.* (2017) tested eight fungicides against *A. alternata* in gerbera among these chlorothalonil 75 WP, mancozeb 75 WP, flusilazole 40 EC and fenarimol 12 EC proved significantly superior in inhibiting the mycelial growth of test fungi (*in vitro)*. Panwar *et al.* (2013) [19] tested seven fungicides i.e. mancozeb, tebuconazole, myclobutanil, tricyclazole, metalaxyl + mancozeb, carbendazim and hexaconazole at (0.05, 0.10 and 0.20%) concentrations, among these three fungicides *viz*., tebuconazole, myclobutanil and hexaconazole completely inhibited growth of test pathogen at all concentrations.

Table 2: Effect of fungicides on per cent mycelium growth inhibition on (*A. alternata*)

Treatments	Per cent radial mycelia growth inhibition at different concentration									
	100 (ppm)		500(ppm)		1000(ppm)		1500(ppm)		2000(ppm)	
	G	I	G	I	G	I	G	I	G	I
Control	25.08	00.00	25.08	00.00	25.08	00.00	25.08	00.00	25.08	00.00
Carbendazim	16.50	34.21	12.90	48.56	9.20	63.31	7.50	70.10	6.25	75.08
Mancozeb	11.39	54.58	9.20	63.31	5.90	76.47	0.00	100	0.00	100
Captan	16.90	32.61	15.60	37.80	11.50	54.14	9.75	61.12	7.00	72.08
Copper oxy chloride	14.20	43.38	11.60	53.75	8.30	66.90	5.20	79.27	0.00	100
Chlorothalonil	18.83	24.92	14.90	40.58	10.00	59.46	8.00	68.10	7.25	71.09
Ridomil- MZ	10.50	58.13	7.75	69.10	0.00	100	0.00	100	0.00	100
Thiophanate methyl	18.99	24.27	13.29	47.01	8.10	67.70	0.00	100	0.00	100
Propiconazole	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
Difenoconazole	0.00	100	0.00	100	0.00	100	0.00	100	0.00	100
S.E.(d)	0.63	-	0.54	-	0.62	-	0.35	-	0.42	-
C.D. $_{(p=0.05)}$	1.32	-	1.13	-	1.29	-	0.74	-	0.88	-

G=Average radial mycelia growth in (mm); I= Average radial mycelia growth inhibition in (%)

Efficacy of bioagents

The significant variation among all the bio control agents with control were observed for mycelium inhibition of *A. alternata* presented in (Table 3). *Trichoderma viride* was found most effective bio control agent which significantly inhibition mycelium growth (55.66%) of the fungus. It was statistically at par with *T. harzianum* (52.79%). Whereas *B. cereus* was found minimum mycelium inhibition (37.30%) and *P. fluorescens* which was found least effective (34.91%).

Cardoza *et al*. (2005) [4] found that *Trichoderma* spp produce the extracellular lytic enzymes and the production of many secondary metabolites. Three kinds of compounds are mainly produced by different species of *Trichoderma*: Peptaibols, polyketides and terpenes, some of them with antifungal activity. Similar results were also reported by Ghosh *et al.* (2002) [7] observed that the bio agent's *viz. T. viride*, *Aspergillus awamori* and *T. hamatum* checked the growth of by *A. alternata* on gerbera. Kumar *et al*. (2005) [16] observed that three antagonist *viz. T. virens*, *T. harzianum* and *T. viride* over grew colony of *A. alternata* but *T. viride* over grew gave best results because it could parasitize pathogen fungus faster than other antagonists. Abdul *et al.* (2001) [1] proved that *Verticillium* sp. was the most effective antagonist in reducing *A. solani* mycellial growth followed by *Beauveria bassiana*, *Bacillus subtilis*, *T. harzianum* and *Arachniotus* species, *Paecilomyces lilacinus* and *Metarhizium anisopliae* were effective against *A. solani*. (Ghosh *et al*., 2016) [8] observed two plant growth promoting rhizobacterial strains, *Burkholderia cenocepacia* VBC7 and *Pesudomonas poae*

VBK1 were able to produce prominent zone of inhibition against leaf spot disease of Aloe vera (*A. alternata).*

Table 3: Effect of bioagents against (*A. alternata*)

Bio control agents	Mycelial growth (mm)±SE(m)	Per cent mycelia growth inhibition (%)
Control	25.08±0.05	0.00
Trichoderma harzianum	11.84±0.34	52.79
Trichoderma viride	11.12±0.48	55.66
Pseudomonas fluorescens	16.45±0.41	34.91
Bacillus cereus	15.80±0.70	37.30
S.E.(d)	0.63	-
C.D. (p=0.05)	1.36	-

Conclusion

Tested five media at five different temperatures for the growth of *A. alternata* among these, CMA at 30^0 C was most favoured for the growth of pathogen. Nine fungicides at five different concentrations in which propiconazole and difenoconazole found complete inhibition of the mycelial growth at 100 ppm, it was concluded that systemic fungicides above mentioned found most effective and when we increased the doses of some fungicides then it inhibited maximum mycelial growth inhibition. Four bioagents among these *T. viride* observed most effective and significantly inhibited the mycelial growth of test pathogen.

Acknowledgement

The Authors would like to acknowledge, Dean and OIC, MAP Block and Fruit Nursery Block, College of Horticulture, VCSG UUHF Bharsar, Pauri Garhwal (Uttarakhand) for extending support.

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