



Full Length Article

Effect of Salicylic Acid, DL- β -amino-n Butyric Acid and Acibenzolar-s-methyl + metalaxyl on Mycelial Growth and Spore Germination of *Alternaria mali* in vitro and on Young Apple Seedlings

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Abstract

In order to determine the effects of chemical elicitors used for inducing resistance in plants against necrotic leaf spot caused by *Alternaria mali*, Salicylic acid (SA) with 0-700 ppm, DL- β -amino-n-butyric acid (BABA) and Acibenzolar-s-methyl + metalaxyl (ASM) with 0-1500 ppm concentrations were tested on PDA. *A. mali* isolated from Red Jim apple variety and having high virulence was used. The spore germination tests were conducted at 24°C for 12 h on water agar medium included different concentrations of chemicals and the effects on hyphal length were determined. SA decreased the mycelial growth of *A. mali* with increasing concentration and it was inhibited at 700 ppm completely. SA had the same effect on spore germination inhibition at 300 ppm concentration. BABA had no significant effect on mycelial growth with increasing concentration. However, the hyphal development was decreased with increasing concentration and hyphal lysis occurred just after spore germination at 800 ppm concentration. Mycelial growth of *A. mali* was decreased by increasing ASM concentration as well, but it was not inhibited completely. However, spore germination decreased by increasing concentration and hyphal lysis occurred at 500 ppm. *In vivo* tests, plant activators were found effective with increasing concentration. SA, BABA and ASM at 100 ppm concentration reduced disease severity by 89.5%, 87.6% and 87.6% on young apple seedlings, respectively. Plant activators that induce host resistance have potential for the control of necrotic leaf spot disease. © 2012 Friends Science Publishers

Keywords: Salicylic acid; DL- β -amino-n-butyric-acid; Acibenzolar-s-methyl; *Alternaria mali*; Apple

Introduction

Necrotic leaf spot caused by *Alternaria mali* Roberts is an important disease after apple scab and shows common symptoms in some cultivars (Ozgonen & Karaca, 2006). The first signs of symptoms of the disease are small, round, brown spots on the leaves. Spots are 2-5 mm in diameter and surrounded by purple margin and sometimes become darker and more irregular showing secondary expansion. Severe infection results in defoliating on apple trees. Chemical control of *A. mali* provided through using of fungicides such as iprodione, mancozeb and captan (Lee & Kim, 1986; Osanai *et al.*, 1987).

Induced resistance to plant diseases has been a method used as an alternative to the fungicides against plant pathogens in recent years. To induce systemic and local resistance against diseases, biotic such as bacterial and fungal cell wall fragment, weakened or dead spore cultures, non-pathogenic strains and abiotic inducers such as UV, heavy metals, herbicides, ethylene and other chemicals are used. A wide range of special compounds such as salicylic acid, butyric acid isomers and acibenzolar-s-methyl were used effectively as abiotic agents and these chemicals

applications resulted in systemic acquired resistance in plants (Kuc, 1995; Yang *et al.*, 1997; Cohen, 2002). Chemical applications activate natural defense mechanisms against pathogens in plants that provide protection from the stress conditions and external factors (Dempsey & Klessig, 1995). Salicylic acid, Beta amino butyric acid and Acibenzolar-s-methyl are important signaling molecule in promoting resistance in plants. Active substances are rapidly taken from leaves and act as a systemic signal in the plant (Jakab *et al.*, 2001). Induced resistance and its mechanisms have been investigated for the control of many plant diseases (Baysal *et al.*, 2005; Alkahtani *et al.*, 2011).

Present study aimed to evaluate the antimicrobial action of inducers including salicylic acid, DL- β -amino-n butyric acid and acibenzolar-s-methyl + metalaxyl on mycelial growth and spore germination of *A. mali* in vitro and on young apple seedlings under controlled conditions.

MATERIALS AND METHODS

Materials

In this study, virulent *A. mali* isolated from Red Jim apple

cultivar was used. Salicylic acid (Merck, S4404331), DL- β -amino-n-butyric acid (Fluka, GA13766), Acibenzolar-s-methyl + metalaxyl (BION, Syngenta) were used as inducers.

Effect of Chemicals on Mycelial Growth and Spore Germination of *A. mali* In Vitro

To determine the chemical effects on *A. mali*, different concentrations of salicylic acid (SA), DL- β -amino-n-butyric acid (BABA) and acibenzolar-s-methyl + Metalaxyl (ASM) were tested. Mycelial growth retardation were tested for SA at the concentration of 0-700 ppm and for BABA and ASM between 0-1500 ppm adjusted from stock solutions; while concentrations were selected as 0.1-300 ppm for SA and 0.1-800 ppm for BABA and 0.1-500 ppm for ASM for spore germination.

Potato dextrose agar (PDA) composed of potato infusion from 200 g, D(+)-glucose (2%) and agar (1.5%) per liter was prepared (pH 5.6). Then, PDA was divided into 10 mL poured into the 15 mL glass test tubes and sterilized at 121°C and 1 kPa. After cooling, different concentrations of chemicals from stock solutions were added into the medium then poured into the petri dishes. Media without addition of chemicals was prepared for comparison. The fungus was cultured on PDA plates at 24°C for 7 days. Chemicals added to petri dishes were inoculated with 6 mm diameter of mycelial disc of *A. mali* using cork borer and incubated at 24°C. After 5 days, colony diameters were measured. Experiment was conducted in a completely randomized design with 5 replicates.

Water agar (WA) was prepared at amount of 10 ml in glass test tubes. After autoclaving and cooling, different concentrations of chemicals were added and poured into the petri dishes. *A. mali* was cultured on PDA for 10 days and sterilized water was added to the cultures and spores scraped with a spatula. Then, mixtures of spores and mycelium were suspended through two layers cheesecloth. Spore suspension concentration was adjusted to 10^6 spore mL⁻¹ using haemocytometer and 100 μ L of spore suspension was spread over the surface of the media using a rod then incubated at 24°C. Hyphal length of germinated spores was measured after 12 h under light microscope with ocular micrometer. Experiment was conducted with 5 replicates. Measurements of hyphal length of germinated spores were performed measuring 10 hyphal length in 3 microscopic field of view totally 30 measurement from each replicates.

Effect of Chemicals on *A. mali* in Apple Seedlings under Controlled Conditions

Experiment was conducted on young seedlings cv Red Jim and plants were maintained under controlled conditions at 24 \pm 2°C, RH 70 \pm 5%, 14 h photoperiod. Stock solutions of SA, BABA and ASM were prepared at 25, 50 and 100 ppm concentration and sprayed to leaves of young apple

seedlings using hand sprayer. After 24 h, spore suspension at 10^6 conidia mL⁻¹ of *A. mali* were sprayed to leaves. Control plants were sprayed with distilled water before inoculation. Disease severity was evaluated using 0-5 scale by Horsfall (1986): where: 0: No signs of symptoms, 1: lesions covered leaf surface by 0-3%, 2: lesions covered leaf surface by 4-6%, 3: lesions covered leaf surface by 7-12%, 4: lesions covered leaf surface by 13-25%, 5: lesions covered leaf surface by 26-50%.

Statistical Analysis

Data were subjected to analysis of variance and the differences were compared by LSD multiple comparison tests. The effects of chemicals on the development of the mycelial growth and spore germination of pathogen (% effect) were calculated by the Abbott formula (Karman, 1971).

RESULTS

Effect of Chemicals on Mycelial Growth of *A. mali*

The effects of different concentrations of SA, BABA and ASM on mycelial growth of *A. mali* were determined. The results of the effect of SA at 0-700 ppm were summarized in Table I. SA decreased the mycelial growth with increasing concentration. The mean colony diameter was 84.7 mm in control while it was decreased to 19.0 mm at 600 ppm concentration. SA inhibited the mycelial growth at 700 ppm completely.

Concentrations of 100-1500 ppm BABA did not affect the mycelial growth of *A. mali* and colony diameter did not differ significantly (Table II). Although BABA reduced the mycelial growth of *A. mali* with increasing concentration up to 1500 ppm, it was not inhibited completely. The colony diameter was found 49.0 mm in control and 44.3 mm at 1500 ppm, respectively. Similarly, ASM did not inhibit the mycelial growth of *A. mali* completely, although it reduced the mycelial growth with concentration up to 1500 ppm. The colony diameter in control petri dishes was found as 62.0 mm but it was reduced to 44.3 mm at 1500 ppm (Table 3).

Effect of Chemicals on Spore Germination of *A. mali* In Vitro

The effects of different concentrations of SA, BABA and ASM on hyphal growth of *A. mali* were determined. SA decreased spore germination of *A. mali* with increasing concentration (Table IV). Hyphal length of germinated spore was 58.1 μ m in control, while it decreased to 3.2 μ m at 200 ppm concentration and completely inhibited at 300 ppm concentration. Similarly, BABA and ASM decreased spore germination of *A. mali* with increasing concentration, also (Table V & VI). Hyphal length of germinated spore was decreased to 2.5 μ m at 800 ppm for BABA and 2.0 μ m

Table 1: The effects of SA on mycelial development of *A. mali*

SA concentrations (ppm)	Means colony diameter (mm)	% Effect**
100	83.0 e*	2.0
200	73.3 d	13.4
300	55.3 d	34.6
400	36.3 c	57.1
500	36.0 c	57.5
600	19.0 b	77.6
700	0.0 a	100.0
Control	84.7 e	-

Table 2: The effects of BABA on mycelial development of *A. mali*

BABA concentrations (ppm)	Means colony diameter (mm)	% Effect
100	49.0 ab*	10.9
200	49.3 ab	10.3
300	49.0 ab	10.9
400	49.0 ab	10.9
500	48.7 ab	11.5
600	46.0 a	16.4
700	46.0 a	16.4
800	46.0 a	16.4
900	46.3 a	15.8
1000	45.3 a	17.6
1100	45.3 a	17.6
1200	45.3 a	17.6
1300	45.3 a	17.6
1400	45.3 a	17.6
1500	44.3 a	19.4
Control	55.0 ab	-

Table 3: The effects of ASM on mycelial development of *A. mali*

ASM concentrations (ppm)	Means colony diameter (mm)	% Effect
100	61.7 cd*	0.5
200	61.3 cd	1.1
300	60.0 c	3.2
400	60.0 c	3.2
500	59.3 c	4.3
600	55.0 c	11.3
700	54.7 c	11.8
800	55.7 c	10.2
900	49.0 ab	21.0
1000	49.0 ab	21.0
1100	49.7 ab	19.9
1200	45.7 a	26.3
1300	46.0 a	25.8
1400	45.7 a	26.3
1500	44.3 a	28.5
Control	62.0 e	-

*Means within the column was following by different letters are significantly different (P=0.05) according to Fisher's LSD test
 **% effect was calculated using Abbott formula

at 500 ppm for ASM, respectively and completely inhibited at these concentrations.

Effect of Chemicals on Disease Severity of *A. mali* In Vivo

Disease severity (%) of *A. mali* on leaves was reduced by all tested chemicals with increasing concentration (Table VII). The disease severity was 70% at control plants, while it was decreased to 7.3% at 100 ppm of SA concentration. BABA

Table 4: The effects of SA on spore germination of *A. mali*

SA concentrations (ppm)	Means hyphal length (µm)	% Effect
0.1	56.9 f	2.2
1	56.7 f	2.5
5	56.3 f	3.2
10	43.2 e	25.6
50	33.1 d	43.1
100	12.4 c	78.7
200	3.2 b	94.5
300	0.0 a	100
Control	58.1 f	-

Table 5: The effects of BABA on spore germination of *A. mali*

BABA concentration(ppm)	Means hyphal length (µm)	% Effect
0.1	57.3 f	1.5
1	57.2 f	1.6
5	55.8 f	4.1
10	34.8 e	40.1
50	24.0 d	58.7
100	23.7 d	59.2
200	19.0 d	67.3
400	12.6 c	78.3
600	7.3 b	87.4
800	2.5 a	100.0
Control	58.1 f	-

Table 6: The effects of ASM on spore germination of *A. mali*

ASM concentration (ppm)	Means hyphal diameter (µm)	% Effect
0.1	56.8 f	2.4
1	56.3 f	3.2
5	52.2 f	10.3
10	41.3 e	29.0
50	17.2 d	70.4
100	13.3 cd	77.1
200	9.3 c	84.0
300	9.1 c	84.3
400	5.5 b	90.5
500	2.0 a	100.0
Control	58.1 f	-

Table 7: The effects of plant activator on *A. mali* in vivo

Treatments	Disease severity (%)	% Effect
Control	70.0 a*	-
SA 25 ppm	25.3 e	63.8
SA 50 ppm	15.3 f	78.1
SA 100 ppm	7.3 g	89.5
BABA 25 ppm	34.0 d	51.4
BABA 50 ppm	14.0 f	80.0
BABA 100 ppm	8.7 fg	87.6
ASM 25 ppm	50.7 b	27.6
ASM 50 ppm	39.3 cd	43.8
ASM 100 ppm	8.7 fg	87.6

*Means within the column was following by different letters are significantly different (P=0.05) according to Fisher's LSD test

and ASM showed similar effect on disease and the disease severity was found 8.7% at 100 ppm concentration for BABA and ASM, respectively.

DISCUSSION

All tested compounds reduced the mycelial development of pathogen and/or hyphal length from germinated spores *in vitro* and provided control by inducing resistance *in vivo*.

In our study, SA, decreased the mycelial growth of *A. mali* with increasing concentration and completely inhibited at 700 ppm. Özgönen *et al.* (2001) reported that SA inhibited the mean colony diameter of *F. oxysporum* f.sp. *lycopersici* at 0.6 mM completely *in vitro*. El-Moughy (2002) revealed that the inhibitory effect of SA and acetyl salicylic acid to the growth and sporulation of some plant pathogenic fungal disease including *Fusarium solani* f.sp. *pisi*, *Rhizoctonia solani*, *Sclerotium rolfsii*. In present study, BABA had no effect on the mean colony diameter of *A. mali* between 100-1500 ppm concentrations. Similarly, ASM did not reduce the mycelial development of *A. mali* *in vitro*. In some studies reported that despite the ineffectiveness on the development of mycelial growth of pathogens *in vitro*, it prevent the growth of pathogen in plants by promoting plant resistance against diseases following applications (Cohen *et al.*, 1994; Sunwoo *et al.*, 1996; Tosi *et al.*, 1998; Agostini *et al.*, 2003; Özgönen, 2004).

According to the results of microscopic examination, SA, BABA and ASM showed a decreasing effect on spore germination of *A. mali* with increasing concentration. SA had a similar effect on the germinated spores and it was inhibited at 300 ppm completely. The hyphal growth from germinated spore reduced compared to control up to 50 ppm of BABA, significantly. ASM reduced the mean hyphal length of germinated spores up to 10 ppm concentration, also. In another study, SA inhibited spore germination of *F. oxysporum* over 5 mM completely (Mahdy *et al.*, 2009). Similarly, Porat *et al.* (2003) tested concentrations of BABA between 1 to 100 mM to *Penicillium digitatum* in grapefruit and revealed that increasing concentration exhibited direct antifungal activity and inhibited spore germination and germ tube elongation *in vitro*.

In the present study, SA, BABA and ASM reduced the disease severity by 89.5%, 87.6% and 87.6% at 100 ppm concentration, respectively. Induced-resistance compounds evaluated for the control of diseases of other agricultural crops and provided significant reduction in disease. BABA applied at 50 mM concentration for 6 h as seed treatment provided the maximum seedling vigor and protected the plants by 75% compared to control. After the second inoculation to plants, disease severity in control and BABA treated plants were 71-76% and 10-12%, respectively (Shailasree *et al.*, 2001). Ishii *et al.* (1999) tested the different concentrations of ASM on *Cladosporium cucumerinum*, *Colletotrichum lagenarium*, *Fusarium oxysporum* f.sp. *cucumerinum* and *Corynespora cassiicola* in cucumber; *Venturia nashicola* in pear, *A. alternata* pathotype Japan pear and *Gymnosporangium asiaticum*;

Botrytis cinerea in grapevine and *Didymella bryoniae* in melon. ASM did not inhibit the mycelial development and conidial germination of all tested fungal pathogens *in vitro*. However, ASM at 100 µg.mL⁻¹, controlled the *C. lagenarium* and *C. cucumerinum* in cucumber; *G. asiaticum* in pear (75.9%) under pot conditions effectively. Brisset *et al.* (2000) reported that ASM induced the systemic acquired resistance in pear against fire blight of pear caused by *Erwinia amylovora*. ASM at 100 and 200 mg/l a.i was applied before artificial inoculation of Golden Delicious seedlings was provided protection against disease. The level of protection against seedling disease in the greenhouse and garden trees were 50% and 69%, respectively. Smith-Becker *et al.* (2003) reported that ASM provided protection via the systemic acquired resistance against fungal pathogen *Colletotrichum lagenarium* and cucumber mosaic virus of melon. ASM at 50 or 100 µg/mL concentrations provided complete protection against fungal pathogens and the spread of cucumber mosaic virus was delayed effectively under greenhouse conditions. In another study, Alkahtani *et al.* (2011) reported that abiotic elicitors including oxalic acid, potassium oxalate, salicylic acid, Bion, Fungastop and Photophor induced resistance against powdery mildew (*Sphaerotheca fuliginea*) disease of cucumber via biochemical change in both pathogenesis-related proteins (PR) and phytoalexin accumulation in treated plants comparing with the control. Pretreatment of cucumber plants with all tested elicitors recorded a decrease in powdery mildew disease severity but Bion recorded the most effective inducers (63.8–72.4%).

This study presents the practical relevance on the use of SA, BABA and ASM by single application of young seedlings before pathogen attack for controlling necrotic leaf disease in apple caused by *A. mali*.

CONCLUSION

SA, BABA and ASM have been widely investigated for the control of disease of agricultural crops. The induced-resistance products evaluated for control of diseases have provided significant reduction. Current results confirmed the effectiveness of these products for control of foliar disease of apple. SA showed maximum inhibitory effect on radial growth of fungus. SA and BABA were highly active on spore germination *in vitro* and *in vivo* as pure active substance. Also, ASM showed similar effect *in vivo* and could be used beside the natural compounds as a potent resistance activator against *A. mali*. In conclusion, it would be better to use abiotic inducers as alternatives to the fungicides against necrotic leaf spot of apple.

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