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Virulence variation in *Alternaria mali* (Roberts) and evaluation of systemic acquired resistance (SAR) activators for the management of Alternaria leaf blotch of apple

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Abstract

Alternaria leaf blotch, caused by *Alternaria mali*, is an economically important disease of apple (*Malus* x *domestica* Borkh.). Twenty one isolates of *Alternaria mali* (Am-1 to Am-21) were obtained during the isolate collection. The virulence was tested on detached leaves of susceptible Red Delicious cultivar and Am-1 showed highest virulence. Two greenhouse trials were conducted using two year old grafted seedlings of Red Delicious apple cultivar to assess the efficacy of seven SAR activators, 2,6-dichloroisonicotinic acid (INA), benzothiadiazole S-methyl ester (BTH), β-aminobutyric acid (BABA), K_2HPO_4, K_3PO_4, $Ca(OH)_2$ and $CaCO_3$ applied 48 hrs before and after spore inoculation. The SAR activators and a conventional synthetic fungicide (penconazole) were evaluated against most virulent isolate Am-1. Distilled sterilized water was sprayed on control plants. All the SAR activators significantly lowered the disease intensity as compared to control. BABA was most effective with least disease intensity before and after pathogenic inoculation. Penconazole proved superior to all the SAR activators, except BABA. The application of SAR activators before pathogen inoculation showed significantly lower disease intensity (12.71%) in comparison to SAR application after pathogen inoculation (14.77%). This induced resistance exploiting natural defense machinery of plants could be proposed as a non-conventional and eco-friendly approach for plant protection.

Key words: Apple, Alternaria leaf blotch, Virulence variation, Detached leaf technique, SAR Activators

Introduction

The apple (*Malus* x *domestica* Borkh.) is the most ubiquitous of temperate fruits cultivated in Europe and Asia from antiquity. India ranks 7th with an annual production of 2163400 MT of apple fruit (FAO, 2012). In India apple is predominantly grown in Himalayan states like Jammu and Kashmir (J&K), H.P. and Uttaranchal which account for about 90% of the total production of country (Anonymous, 2002). Like other horticultural crops apple is attacked by several pathogens which impair the quality and quantity of the fruit. However, huge losses of the crop are incurred mostly by fungal diseases. The major fungal diseases include scab, Alternaria leaf blotch, powdery mildew, collar rot, root rot, sooty blotch and fly speck etc. Amongst these, Alternaria leaf blotch caused by *Alternaria mali* is prevalent in all the apple growing areas of world and is one of the economically important apple diseases. In J&K, the occurrence of disease (*Alternaria mali*) was reported by Shahzad et al. (2002). The disease previously considered to be of minor significance has now attained the status of major disease (Anonymous, 2000) and is prevalent in almost all apple orchards of Kashmir valley with a potential threat to existing apple plantation. One of the significant aspects of the biology of an organism is the morphological and physiological characters of an individual within a species which are not fixed. This holds true with fungi also, thus variability studies are important to document the changes occurring in the population and individuals. The variability is a well-known phenomenon in genus *Alternaria* and such variability may be seen in virulence also. Shahzad (2003) reported prevalence of Alternaria leaf blotch of apple in all the districts of Kashmir with varied degree of incidence and

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intensity. Variation in susceptibility among different apple cultivars was also recorded. This variation may be attributed to many factors including variability in pathogen. Understanding population structure of pathogen is of paramount importance for devising a successful disease management programme. Thus variation in the virulence was studied and the most virulent isolate was evaluated against the SAR activators.

Among the various disease management strategies, chemical application still dominates our approach involving frequent fungi-toxicant interventions throughout the world (Koller et al., 2004). Kashmir valley with temperate environmental conditions is a favourable place for the development of fungal diseases. To combat these diseases a need-based spray schedule comprising 6-8 sprays of fungi-toxicants is in vogue (Anonymous, 2002). This poses serious environmental concerns and selection pressure on pathogen population. The development of fungicide resistance in pathogenic populations is one of the most serious constraints because substantial changes in the populations of several major plant pathogens in their sensitivity to fungicides have been observed. This frequently leads to significant crop damage and forcing either discontinuation or modification in the use of important chemicals. The frequent and successive use of fungi-toxicants is likely to develop resistance among the pathogen (Koller et al., 2004; Thind, 2008) and as such threaten the success of spray programme due to the occurrence of resistance in the pathogen (Koller and Wilcox, 1999; Thind, 2008). Some isolates of *Alternaria mali* have developed tolerance to fungicides like iprodione, mancozeb and captan (Lee and Kim, 1986; Osanai et al., 1987; Asari and Takahashi, 1988). The use of resistant cultivars is an alternative to minimize the disease. However, in apple it is difficult to develop resistant cultivars because of long juvenile phase and self-incompatibility. Hence, use of SAR activators in the disease management seems environmentally safe as compared to current pesticides (Vallad and Goodman, 2004). Unlike traditional pesticides, SAR activators do not exhibit any direct antimicrobial activity but prime the plants for resistance. Thus provide a safe way to control disease without asserting direct selection pressure on pathogen populations. SAR is based on multiple natural defense mechanisms, and this makes it less likely that a pathogen can readily develop resistance to this control measure. Over the last 30 years a number of compounds (SAR activators) have been shown to be instrumental in enhancing disease resistance or at least decreasing the disease symptoms in plants. The use of SAR activators for disease control is safe application because SAR activators neither have any toxic effect on pathogens, plants and animals, nor show any inhibitory effect on plant growth, development and yield. Increased pathogen insensitivity to synthetic fungicides coupled with public demands to reduce pesticide use, stimulated by greater awareness of environmental and health issues has placed more emphasis on alternative pathogen control strategies.

Since the crop and disease are of paramount importance to J & K state (India) and no such studies of virulence variation in *Alternaria mali* isolates and management through eco-friendly approaches (SAR activators) have been conducted in the State, therefore the present study was undertaken with the objectives:

To study virulence variation and ascertain most virulent isolate in *A. mali* and to evaluate SAR activators against the most virulent isolate.

Materials and Methods

Collection of isolates

Apple leaves from susceptible cultivar (Red Delicious) exhibiting typical symptoms of Alternaria leaf blotch were collected from twenty one apple orchards across seven districts of Kashmir valley, *viz*., Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian. For isolation of fungal pathogen, the diseased leaf area along with some healthy portion was cut into small bits with a sharp sterilized blade. These bits were surface disinfected in 0.1 per cent mercuric chloride ($HgCl_2$) for about 30 seconds followed by three washing in sterilized distilled water to remove the last traces of $HgCl_2$. After blotting dry with sterilized filter papers, these bits were transferred to sterilized potato dextrose agar medium (PDA) in sterilized petriplates. Three such bits were placed in each petriplates and incubated for seven days at 24±1°C. The isolates were purified through single spore isolation technique (Johnston and Booth, 1983) by transferring germinating conidia to petriplates containing sterilized PDA medium. On the basis of cultural and morphological characters, establishment of pathogenicity and comparison with authentic description (Roberts, 1924; Shahzad, 2003), the fungus was identified as *Alternaria mali*. The pure cultures were maintained on PDA slants and stored at 4°C in a refrigerator. The isolates thus obtained were designated as Am-1 to Am-21.

Determination of the virulence of Alternaria mali isolates

The virulence of *Alternaria mali* isolates (Am-1 to Am-21) was tested on detached leaves of susceptible Red Delicious cultivar through "Detached leaf technique" as follows:

Preparation of inoculum

The isolates of *A. mali*, were multiplied on PDA medium for 10 days at 24±1°C in BOD (Biological oxygen demand) incubator. After 10 days of incubation, spore suspension was prepared by flooding culture plate with sterilized distilled water. Culture plates were scraped with a sterilized razor blade, strained through a double layer of sterile cheesecloth into a 150 ml flask. Spore concentration was adjusted with haemocytometer to 4 x 10^5 conidia per millilitre. The prepared conidial suspensions were preserved at -80°C until use.

Inoculation method

In order to work with a highly virulent isolate, twenty one isolates of *Alternaria mali* were tested. Healthy leaves were collected, washed thoroughly under running water, followed by three washings in sterilized water. After mild rinsing of leaf surface with sterilized water, the leaves were dried with a laboratory towel and placed in petriplates. Each petriplate was lined with a wet blotter paper on the bottom for maintaining high humidity. A set of three leaves, was placed in each petriplate. Each set was replicated thrice. The leaves were inoculated (outside the petriplates) with a conidial suspension (4×10^5 spores/ml) with an atomizer to the whole surface. Before inoculation celite (0.5%) was applied to the leaves with the help of a brush. Leaves in the petriplates were incubated at 24±1^{0}C in BOD incubator with 12 hour dark and 12 hour light adjustment and monitored for symptom development. Ten days after inoculation, number of lesions and size of lesions were recorded. The size of lesion was recorded by taking two perpendicular measurements and their average was calculated, while as total lesion area was calculated by multiplying number of lesions with size of lesions. Isolate which showed highest virulence (highest total lesion area) was selected for further studies. The experiment was laid in CRD (completely randomized design) with three replications.

Evaluation of SAR chemicals

Plant material

Two year old grafted seedlings of Red Delicious apple cultivar obtained from Division of Pomology, SKUAST-Kashmir were used as host plants for the studies. This cultivar is highly susceptible to Alternaria leaf blotch caused by *Alternaria mali*.

Greenhouse conditions

Seedlings were grown in pots of 30 cm x 30 cm size, filled with clay loam soil in the greenhouse. The greenhouse was maintained on temperatures of 25 ± 5°C, humidity of 68-80%, and 12 hourly light. The plants were used 8 weeks after planting (young shoots were 10-15 cm long with 6-10 leaves per shoot). This environment was maintained during the entire period of the experiment.

Application of SAR chemicals

Seven SAR inducing compounds *viz*: INA, BTH, BABA, K_2HPO_4, K_3PO_4, $Ca(OH)_2$ and $CaCO_3$ were evaluated against the most virulent isolate (Am-1) of the pathogen on potted plants cv. Red Delicious under greenhouse conditions. Test plants were divided in two sets each for chemical sprays 48 hrs before spore inoculation and 48 hrs after spore inoculation. Inoculum was provided to the test plants by spraying with spore suspension of ten days old culture. Spore suspension of 4 x 10^5 conidia / ml was prepared adopting the method as discussed earlier in 'preparation of inoculum'. Tween 20 (0.1%) was added to the inoculum. The plants were inoculated by spraying the spore suspension on both leaf surfaces to the drip point with an atomizer. Before inoculation celite (0.5%) was applied to the leaves of the plants with the help of a brush. SAR chemicals were used at three different concentrations (diluted with distilled sterilized water) as inducing agent by spraying on leaves 48 hours before and after inoculation. INA and BTH were tested at 50, 100 and 200 ppm concentration, BABA at 500, 1000 and 2000 ppm, K_2HPO_4, K_3PO_4, $Ca(OH)_2$, $CaCO_3$ at 25, 50 and 100 mM. Penconazole was tested at 300, 400 and 500 ppm. Distilled sterilized water was sprayed on control plants. Three replications were maintained for each treatment. All the chemicals were sprayed to the runoff with an atomizer. Symptom development was evaluated 15 days after spore inoculation according to a rating system from 0-5 (Filajdic and Sutton, 1991) with slight modification (Table 1). The experiment was laid in Factorial CRD with three replications. The experiment was repeated in the 2nd year.

Per cent disease intensity (PDI) was calculated using Mc Kinney's (1923) formula:

$$\text{Per cent disease intensity} = \frac{\text{Sum of all the numerical ratings}}{\text{Number of leaves examined x maximum disease rating}} \times 100$$

Per cent disease control was calculated using formula as adopted by Burpee and Latin (2008):

Per cent disease control= [(C – T)/C] x 100, where C and T = disease severity in control and treated plots, respectively.

Table 1. Scale (0-5) for disease rating.

Numerical value	Criteria
0	No symptoms
1	0 - 3% leaf area covered with lesions
2	4 - 6% leaf area covered with lesions
3	7 - 12% leaf area covered with lesions
4	13 - 25% leaf area covered with lesions
5	26 - 50% leaf area covered with lesions or chlorotic leaf with petiole infection

Results and Discussion

Virulence variation

In the present study all the isolates were successful in producing the disease lesions with variation in number and size of lesions (Table 2). The number of lesions produced by the isolates varied from 6.3 to 14.3 with the least produced by Am-2 and maximum by Am-16. The minimum lesion size of 2.9 mm was recorded in Am-17 while the maximum of 10.2 mm was observed in Am-1. Our findings are in agreement with Thrall et al. (2005) who found significant differences in the lesion size produced by the *Alternaria brassicicola* isolates on *Cakile maritima*. Kumar (2004) also reported variation in lesion size and lesion number produced by the isolates of *Alternaria triticina*. Among the two isolates of *Alternaria carthami* one produced more severe leaf spots than other when inoculated on safflower cultivars (Rai and Kumari, 2009). However the observations are contradictory to the findings of Quayyum et al. (2005) who did not find any significant variation in the lesions produced by the isolates of *Alternaria panax* on detached leaflets of ginseng. The isolate Am-1 was highly virulent as compared to other isolates with the highest total lesion area of 122.4 mm. Filajdic and Sutton (1992) found all the eight isolates of *A. mali* pathogenic on delicious seedlings with varied virulence. Varma et al. (2006) reported variability in virulence among isolates of *Alternaria solani*. In contrast Van der Waals et al. (2004) found a moderate degree of variation in virulence among different isolates of *Alternaria solani*. It can be argued that variation in the isolates may be inherent since isolates were collected from different sites. Physiological characters are influenced by environmental conditions which may be responsible for such variability. Moreover isolates in these sites may have adapted for many years which may be responsible for this variation. The virulence variation of *A. mali* in Kashmir valley may be because of the mixed cultivars in our orchard eco-system, diversity in sites and selection pressure due to indiscriminate use of fungicides. One more possible explanation for the variation found among isolates of *Alternaria mali* could be natural chance mutations, combined with the fact that the fungus can produce abundant number of spores in a relatively short period of time. Brierley (1920) suggested that variation in fungi imperfecti may be due to mutation, or by splitting of an originally impure genetic constitution or of gametic or somatic segregation from heterozygotes. Variants, considered as due to mutation, were found in single spore cultures of *Alternaria mali* (Roberts, 1924). Thus Am-1 being most virulent isolate was used for the following studies.

Efficacy of SAR activators for the management of disease

All the SAR activators along with fungicide (Penconazole) at three different concentrations before and after pathogen inoculation significantly lowered disease intensity as compared to control (Table 3). None of the treated or control plants died as a result of *Alternaria* attack during the course of the two year study. Likewise none of the SAR agents and fungicide evaluated was phytotoxic to the test seedlings. Pooled data shows that disease intensity ranged from 1.59-18.22 per cent in SAR activators treatments (before inoculation) in comparison to 35.53 per cent in control. Similarly it ranged from 3.94-23.99 per cent after inoculation in comparison to 35.74 per cent in control. Chemically-induced SAR has been found effective against various pathogens (Schneider et al., 1996; Kuc, 2001). The least disease intensity of 3.94 per cent (after inoculation) was recorded in BABA which was at par with penconazole having a disease intensity of 3.76 per cent. Several studies indicated that the SAR compounds are useful in the management of fungal pathogens (Christiansen et al., 1999; Kessmann et al., 1994) with the level of pathogen suppression comparable with synthetic fungicides. Consequently, induced resistance could provide systemic protection against pathogen attack

to substitute for, or supplement control by conventional synthetic fungicides. However penconazole (synthetic fungicides) recorded significantly lower disease intensity than SAR chemicals other than BABA. These findings are in agreement with Agostini et al. (2003) and Percival and Haynes (2008) who reported SAR activators to be generally less effective than standard synthetic fungicides in the control of foliar pathogens. Percival et al. (2009) reported penconazole superior over Messenger (a.i. Harpin protein), *Phoenix* (a.i. Potassium phosphite) and Rigel (a.i. Salicylic acid derivative) in controlling apple and pear scab. The other SAR chemicals in decreasing order of their efficacy were K_3PO_4, BTH, INA, $Ca(OH)_2$, K_2HPO_4 and $CaCO_3$ (Figure 1a). K_3PO_4 and K_2HPO_4 showed 79.85 and 57.17 per cent control in 2009. Gottstein and Kuc (1989) suggests phosphate application for disease control because of the effectiveness of systemic resistance induced by phosphates, their low cost, low animal toxicity, nutrient value and comparative safety for the environment. The BTH which showed 68.82 per cent control has been tested against several other pathogens (Ruess et al., 1995; Oostendrop et al., 1996). In the present findings INA showed 64.97 per cent control which is in agreement with the findings of Kessmann et al. (1994) who reported that the foliar spray of INA reduced fire blight (*Erwinia carotovora*) by 45 per cent compared to non-inoculated control. Calcium based chemicals used in our study were effective in combating the disease. Our results are in agreement with the findings of Yoon et al. (1989) who reported effectiveness of application of calcium compounds against *Alternaria mali* and also found higher calcium content in apple leaves responsible for imparting resistance to *Alternaria mali* than in the leaves of susceptible ones.

Table 2. Virulence variability in *Alternaria mali* isolates.

Isolate	No. of lesions*	Size of lesions (mm)*	Total lesion area(mm)
Am-1	12.0	10.2	122.4
Am-2	6.3	9.1	57.3
Am-3	10.0	4.6	46.0
Am-4	10.0	8.5	85.0
Am-5	8.3	6.1	50.6
Am-6	11.0	3.4	37.4
Am-7	9.6	5.6	53.7
Am-8	11.6	6.2	71.9
Am-9	11.6	5.8	67.2
Am-10	6.6	6.4	42.2
Am-11	12.3	8.3	102.0
Am-12	9.0	4.5	40.5
Am-13	13.0	5.7	74.1
Am-14	12.0	6.2	74.4
Am-15	12.3	3.1	38.1
Am-16	14.3	4.3	61.4
Am-17	6.0	2.9	17.4
Am-18	8.6	5.6	48.1
Am-19	8.3	5.1	42.3
Am-20	11.3	3.3	37.2
Am-21	12.0	4.6	55.2
$CD_{(P = 0.05)}$	2.41	0.74	

*After 10 days of inoculation and mean of three replications

Table 3. Efficacy of SAR activators against *Alternaria* leaf blotch of apple cv. Red Delicious (mean±SD).

Treatment	Conc. (ppm)/ *: mM	Disease intensity (%)					
		2009		2010		Pooled	
		BI	AI	BI	AI	BI	AI
INA	50	14.42±1.60	16.56±0.70	13.95±0.67	15.75±0.63	14.18±0.96	16.15±0.58
(2,6-dichloroisonicotinic acid)	100	11.50±1.16	12.30±1.28	12.05±0.09	12.85±0.29	11.77±0.56	12.57±0.74
	200	9.28±0.72	10.22±0.37	9.30±0.27	9.70±0.35	9.29±0.48	9.96±0.33
BTH	50	11.40±0.95	14.75±1.18	10.93±0.32	13.25±0.52	11.16±0.32	14.00±0.40
(benzothiadiazole-S-methyl ester)	100	9.77±0.43	12.83±0.41	9.20±0.30	10.93±0.43	9.48±0.10	11.88±0.02
	200	7.99±0.42	9.42±0.70	7.50±0.24	10.00±0.74	7.74±0.33	9.71±0.49
BABA	500	9.00±0.42	9.93±0.70	8.55±0.44	9.10±0.11	8.77±0.29	9.51±0.34
(β-aminobutyric acid)	1000	4.65±0.58	6.23±0.54	5.25±0.29	7.05±0.36	4.95±0.28	6.64±0.09
	2000	1.47±1.05	4.03±0.14	1.72±0.41	3.86±0.30	1.59±0.36	3.94±0.21
*K_2HPO_4	25	16.39±1.18	19.37±0.96	17.23±0.28	18.35±0.23	16.81±0.72	18.86±0.38
	50	15.04±1.66	16.24±0.94	14.75±0.31	16.00±0.10	14.89±0.76	16.12±0.44
	100	10.82±1.51	12.97±0.39	10.15±0.34	12.34±0.32	10.48±0.90	12.65±0.34
*K_3PO_4	25	6.90±0.55	10.86±0.10	7.16±0.33	11.05±0.28	7.03±0.39	10.95±0.19
	50	6.21±0.30	8.89±0.25	6.98±0.42	8.95±0.52	6.59±0.14	8.92±0.18
	100	4.39±0.90	5.48±0.27	4.18±0.23	6.00±0.25	4.28±0.55	5.74±0.03
*$Ca(OH)_2$	25	15.34±0.79	21.27±0.69	15.01±0.30	20.10±0.28	15.17±0.36	20.68±0.48
	50	10.72±1.54	16.51±0.72	10.25±0.04	17.00±0.39	10.48±0.78	16.75±0.17
	100	10.03±0.60	10.54±0.49	11.16±0.30	12.08±0.10	10.59±0.16	11.31±0.29
*$CaCO_3$	25	18.46±1.25	24.13±1.15	17.99±0.18	23.85±0.21	18.22±0.70	23.99±0.55
	50	14.68±0.62	21.33±0.52	15.00±0.25	21.75±0.60	14.84±0.23	21.54±0.07
	100	11.65±0.73	13.82±0.58	11.35±0.56	14.09±0.22	11.50±0.37	13.95±0.28
Penconazole	300	8.49±0.78	7.90±0.49	8.11±0.27	6.98±0.36	8.30±0.44	7.44±0.42
	400	5.25±0.96	4.79±0.66	5.76±0.31	4.48±0.47	5.50±0.41	4.63±0.56
	500	3.29±0.79	3.68±1.06	3.00±0.04	3.85±0.25	3.14±0.40	3.76±0.53
Control	-	35.24±0.52	35.47±0.12	35.82±0.25	36.01±0.19	35.53±0.48	35.74±0.25
Mean		12.69	14.83	12.74	14.71	12.71	14.77

BI: Before inoculation; AI: After inoculation

$CD_{(P = 0.05)}$	2009	2010	Pooled		2009	2010	Pooled
BI/AI	0.28	0.11	0.15	Chemical x Concentration x BI/AI	1.50	0.58	0.81

The application of SAR activators before pathogen inoculation showed significantly lower disease intensity (12.69%) in comparison to 14.83% in SAR application after pathogen inoculation (Table 3). The per cent disease control recorded by each SAR activator before inoculation proved superior to after inoculation (Figure 1b). The plants utilize their own defense mechanism for restriction of pathogen development. As markers of resistance, physiological changes always appear in certain intervals after application of the biotic and abiotic inducers against pathogens (Schonbeck et al., 1993). As in application of SAR activators before pathogen inoculation, these physiological changes (markers of resistance) occur before the pathogen inoculation. In SAR activators application after pathogen inoculation these changes occur after pathogen infection. This may be the reason that application of SAR activators before inoculation proved superior to the application after inoculation. Our observations are in accordance with the findings of numerous workers (Schneider et al., 1996; Kuc, 2001; Bokshi et al., 2003; Gozzo, 2003; Soylu et al., 2003). However penconazole was statistically at par in both the cases of application, because of its systemic activity and direct inhibition of the pathogen.

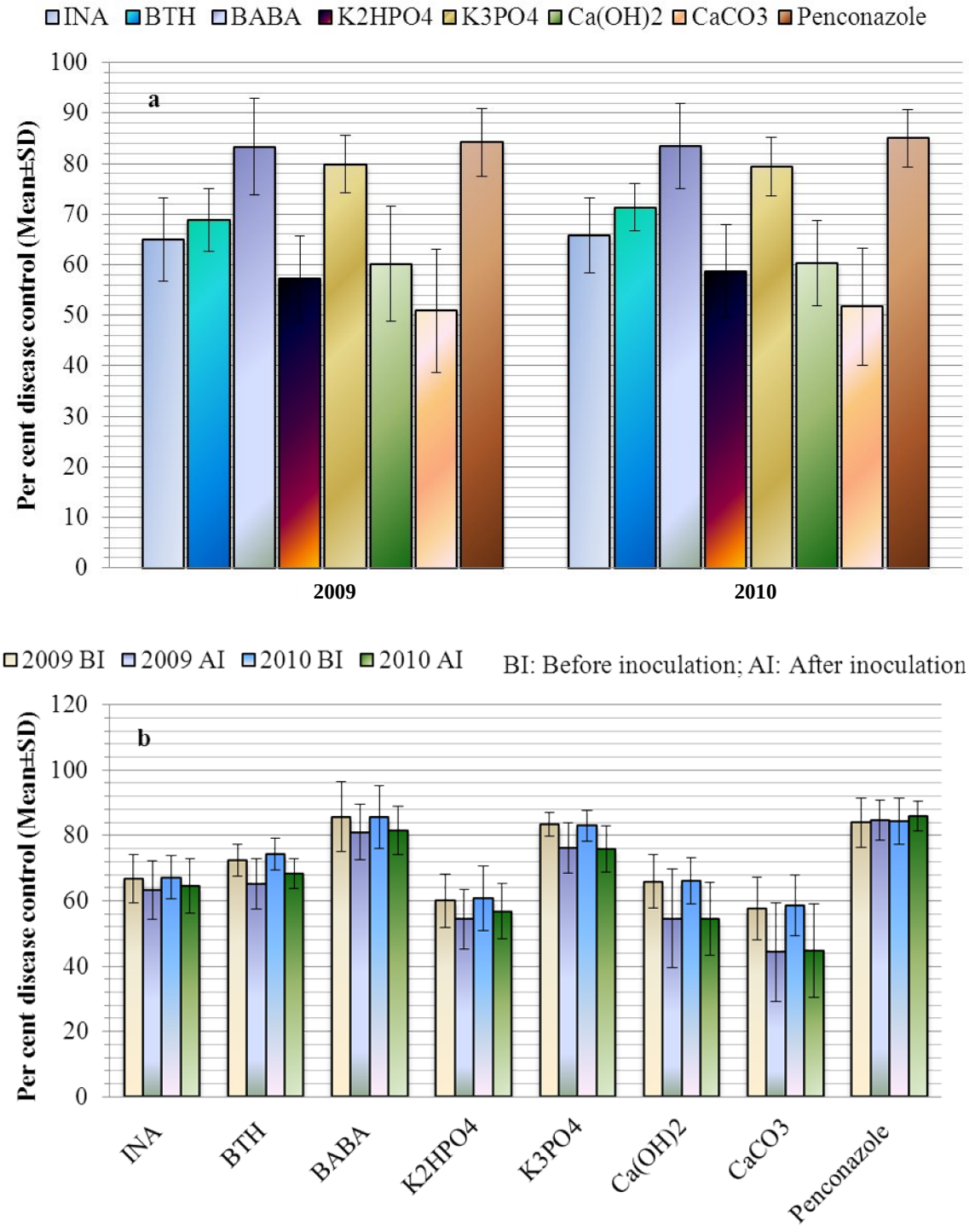

Figure 1. Per cent disease control of Alternaria leaf blotch of apple by SAR activators

In present study application of BABA before and after inoculation of the *Alternaria mali* proved superior to all other SAR activators tested. Similar observations have been recorded by Amzalek and Cohen (2007) who found BABA significantly better than BTH and INA against sunflower rust. The post infection inhibitory effect of BABA better than other SAR activators is because the BABA operates quite rapidly to stop growth of mycelia in the mesophyll as reported by Amzalek and Cohen (2007).

All the three concentrations of each chemical significantly differed from each other with the least disease intensity observed in highest concentration of each chemical.

Conclusion

Alternaria mali isolates exhibited considerable variation in their virulence. The wide variation of isolates indicated that the fungus has a high potential to adapt to resistant cultivars or fungicides. The evidence of random distribution of variation in pathogen population has practical implications for breeding programs as well as in the management of Alternaria leaf blotch. Since the pathogen cannot adapt to SAR chemicals and there is not any such report, so the SAR chemicals were evaluated against the pathogen. All the SAR chemicals except $Ca(OH)_2$ and $CaCO_3$ have been evaluated for the first time against *Alternaria mali*. SAR compounds, especially BABA were effective in managing the disease. This induced resistance exploiting natural defense machinery of plants could be proposed as an alternative, non-conventional and eco-friendly approach for plant protection.

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