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Pathogenesis-related Protein and Phytoalexin Induction against Cucumber Powdery Mildew by Elicitors

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ABSTRACT

Six different abiotic elicitors (oxalic acid, potassium oxalate, salicylic acid, Bion, Fungastop and Photophor) were used to study their effect on induced resistance of cucumber (*Cucumis sativus* L.) against powdery mildew (*Sphaerotheca fuliginea*) disease. The inducers efficiency was evaluated depending on disease severity calculation and measure the biochemical change in both pathogensis Related Protein (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. Bion recorded the most effective inducers (63.8 and 72.4%) while potassium oxalate recorded the lowest effective one (37.0 and 58.3%) in both single and booster spray. Induced resistance of cucumber against powdery mildew recorded an increase in PR-proteins (peroxidase, polyphenoloxidase, Chitinase and β-1, 3 glucanase) activity as well as an increase accumulation of phytoalexins. Application of abiotic agents in these experiments enhances the induced resistance in cucumber against powdery mildew. It would therefore be the proposal to use abiotic inducers as alternatives to the fungicides and one of a wide range of disease management tools.

Key words: *Cucumis sativum, Sphaerotheca fuliginea*, abiotic inducers, β-1, 3 glucanases, chitinase, polyphenoloxidase, peroxidase

INTRODUCTION

Powdery mildew caused by *Sphaerotheca fuliginea* is a serious disease on cucumber (*Cucumis sativus* L.) grown both in fields and greenhouses. Powdery mildew infects leaves, stems and fruits. The disease is very common in various Geographical regions and under a variety of growing on cultivated cucurbits (Farrag *et al*., 2007; Romero *et al*., 2007). Powdery mildew caused by *Sphaerotheca fuliginea* is one of the most important economic diseases. Several disease control methods were adapted to minimize yield loss in cucumber (Kiss, 2003). These including chemicals, biological breeding for resistance and plant extracts. Several disease control methods were adapted to minimize yield loss in cucumber. These including, chemical, biological breeding for resistance and plant extracts (Liu *et al*., 2010).

Plants have developed a wide range of defense mechanisms to successfully adapt to a changing environment in response to the attacking inducers. The cucumber (cv. *Jinyan* No. 4) was exposed to white and other monochromatic lights inducers and tested the effects on plant response to *Sphaerotheca fuliginea*, defense-related gene expression and metabolic changes. High levels of H_2O_2 and Salicylic Acid (SA) and stronger expression of defense genes such as pathogenesis-related proteins (PR-1) was produced due to treatment with light inducers (Wang *et al*., 2010). A spray inoculation of cucumber plants with different inducers before a challenge inoculation with the pathogen *Sphaerotheca fuliginea* induced systemic resistance to powdery mildew. The induction of systemic resistance against powdery mildew facilitates the development of a wide range of disease management tools (Reuveni and Reuveni, 2000). Plant resistance can be induced locally and systemically to become more resistant to diseases through various biotic or abiotic stresses. It is characterized by protection against a broad range of pathogens, by a set of induced proteins and by its dependence on Salicylic Acid (SA). Various chemicals have been discovered that seem to act at various points in these defense activating networks and mimic all or parts of the biological activation of resistance (Oostendorp *et al*., 2001).

Plant PR proteins are represented by 17 protein families, including β-1, 3-glucanases, chitinases and peroxidases. PR proteins have been shown to be directly involved in plant immunity associated with protective mechanisms. The expression levels of PR proteins can be regulated by various stress-related situations, including wounding, salinity, chemical elicitors, hormones and UV-light. The magnitude of PR accumulation varies among the different PR families and members of families, depending on the type of stimuli (Gorovitsa and Czosnek, 2008).

There is increasing evidence that, in some cases, constitutively expressed genes encoding enzymes associated with normal plant metabolism play critical roles in the induction of plant defences against pathogens (Eckardt, 2004). The enhanced induction of Peroxidase (PO) and Polyphenoloxidase (PPO) might have contributed for the induced systemic resistance triggered by various biotic and abiotic inducers (Tian *et al*., 2006; Imran *et al*., 2007; Barilli *et al*., 2010).

Phytoalexins are antimicrobial, low-molecular-weight secondary metabolites they act as an effective defense mechanism of plants against microbial pathogens. The molecular background of this pathogen-triggered induction of secondary biosynthesis has been rarely investigated in intact plants and tissues, as it is intimately integrated into plant morphology and development. A plethora of examples demonstrate the elicitor-induced biosynthesis of antimicrobial secondary compounds (alkaloids, flavonoids/isoflavonoids, terpenes, etc.) (Angelova *et al*., 2010).

Thus, the objective for the current study was to use six different abiotic elicitors to induce the resistance of cucumber plants against powdery mildew and studying the biochemical changes related to induce resistance as PR-proteins and phytoalexin accumulation.

MATERIALS AND METHODS

All cultivation, inducers treatment, inoculation trails Was conducted in protected green house in Ghat area, Riyadh, Saudi Arabia in January 2010, while the chemical analyses was conducted in same year in Plant Pathology Institute and Faculty of Science, Cairo University, Egypt.

Pathogen inoculum and induction of systemic resistance: Three days after induction, the plants were infected with spore suspension (3×10^5-conidia mL^{-1} water) of *S. fuliginea*. Five seedling of highly susceptible cucumber cultivar (Beta alpha) was used in these experiments; five replicates were used for each treatment. Spraying cucumber seedling at 2nd true leaf growth stage carried

out foliar application with abiotic inducers. Five additional replicates were sprayed with water as a control. Booster application was similar to single spray with all (identical) procedure but differed in additional spray at 5th leaf growth stage and the plant was inoculated with the pathogen only after three days from second spraying.

Six abiotic inducers were used with following dose; Oxalic, Salicylic acid, Potassium oxalate, Fungastop (0.4% Citric acid+0.2% mint oil+1.0% Citrus pulp+12% Fish oil+1.0% glycerol+0.1% ascorbic acid), Bion ([1, 2, 3] Benzo thiadiazole-7-carbothioic acid-5-methyl ester) and Photophor (12.0% Copper sulphate+13.0% methionine and riboflavin+20% sodium dioctyl sulfo succenate) 10, 4, 15, 15 mM, 0.30 g L^{-1} and 4 mL L^{-1}, respectively. Disease severity in each treatment was estimated 10 and 20 days after infection with the pathogen.

Peroxidase and polyphenoloxidase assay: Peroxidase and polyphenoloxidase enzymes were extracted according to Maxwell and Bateman (1967) while the peroxidase and polyphenoloxidase activity was determined according to the method described by Allam and Hollis (1972).

Chitinase and β-1, 3 glucanase enzyme assay: One gram of plant tissue was homogenized with 0.2 M Tris-HCl buffer (pH 7.8) containing 14 mM B-mercaptoethanol at the rate of 1/3 w/v. The homogenate was centrifuged at 3000 rpm for 15 min under cooling. The supernatant was used to determine enzyme activities (Tuzun *et al*., 1989). Chitinase activity determination was carried out according to the methods of Monreal and Reese (1969). β-1, 3 glucanase activity was determined according to the method of Abeles and Forrence (1979).

Phytoalexin contents: Phytoalexin content was estimated as flavone substances of flavonoid which act as the precursor and main source of phytoalexin. One gram of fresh tissue was grinded in 80% methanol for 20 min, then 40°C water bathing for 50 min followed by vacuum filter. The residual was washed with 80% methanol and mixed with the above filtrate. Methyl alcohol was removed from the mixture by using the rotary evaporator. The aqueous residue was de-ester by ether and then hydrolysis by 4 N HCl at 100°C for 90 min. The hydrolysate was cooled and extracted with ethyl acetate. The ethyl acetate fractions obtained were dried at 38°C and then re-suspended in absolute methanol (McNally *et al*., 2002). The extracts was centrifuged and diluted, the supernatant was filtered with organic filter membrane (0.45 μm). Ten microliter of the extractions were analyzed by using High-Performance Liquid Chromatography (HPLC) system (PerkinElmer) on a C-18 reverse phase column (250 mm×4.6 mm) at 272 nm according to the methods of Gertz (1990) and Fofana *et al*. (2002).

Data statistical analysis: The obtained data were statistically analyzed using the Analysis of Variance (ANOVA) one way with the MSTAT-C statistical package The Least Significant Difference procedure (LSD) was used at 0.05 level of probability (Fisher, 1948).

RESULTS

A significant reduction ($p = 0.5$) in *S. fuliginea* disease severity as a result of using any of the tested abiotic inducers compared with the control (Table 1). Nevertheless, variations among the five inducers according to their efficiency were detected. Bion ranked as the most effective abiotic inducer (72.4 and 63.8%) followed by Photophor (68.6 and 58.1%), Fungastop (66.0 and 48.71%), Oxalic acid (65.0 and 47.9%) and Salicylic acid (61.5 and 41.3%), respectively. Whereas, potassium oxalate was the lowest effective inducer.

Table 1: Effect of different concentrations of abiotic inducers (as foliar treatment) on disease severity of powdery mildew (*S. fuliginea*) using Beta alpha cultivar

		The mean of disease severity after inoculation (days)							
		Single spray				Single spray			
Treatments	Dose	10	20	X`	% Efficiency	10	20	X`	% Efficiency
Oxalic acid	10 mM	30.9	35.4	33.1	47.9	19.6	25.4	22.5	65.0
Salicylic acid	4 mM	33.7	40.8	37.3	41.3	22.4	27.2	24.8	61.5
Potassium oxalate	15 mM	36.8	43.2	40.0	37.0	24.9	28.8	26.9	58.3
Fungastop	15 mM	30.3	34.8	32.6	48.7	19.3	24.4	21.9	66.0
Bion	0.3 g L^{-1}	20.1	25.9	23.0	63.8	16.9	18.6	17.8	72.4
Photophor	4 ml L^{-1}	24.2	28.9	26.6	58.1	18.6	21.8	20.2	68.6
Control	-	54.7	72.3	63.5	0.0	55.5	73.2	64.4	0.0

LSD value at 5% for (Treatment×Spray×Days) = 4.401

In both applications, it was found that the efficiency of booster spray in reducing disease severity exceed remarkable than that of single ones. Also, early spray (10 days) was much better than the second spray (20 days) after infection.

Peroxidase activity of cucumber plants after inoculation with *S. fuliginea* and treatment with abiotic inducers are listed in Table 2. A significant increase ($p = 0.05$) in peroxidase activity in the plants treated with any of the tested abiotic inducers compared with the control (healthy or infected ones). However, such increases were obvious in booster spray than single ones in all treatments under study. Level of Peroxidase activity was increased notable 2 and 4 days after the artificial inoculation then decreased 6 days after inoculation in single spray treatment. Whereas, peroxidase activity levels increased with increasing inoculation time (0 to 6 days) in booster spray treatment except Photophor and Fungastop which decreased at 6 day in booster spray.

Fungastop inducer gave the highest peroxidase activity all over the time course of the experiment. Both single (21.4) and booster (33.6) treatments. However, variations in the ranking of the abiotic inducers in their activities were found. Bion ranked as the second in single spray (20.4). While, Potassium oxalate ranked the second (33.4) in booster spray.

On the other hand, the differences in peroxidase activity between the control (healthy or infected) were negligible in both single and booster spray treatments. In general, polyphenol oxidase in cucumber plants treated with the six abiotic inducers was slightly higher than that of the control. This confirmed in both single and booster treatments. Such increases however, were fluctuated over the time course of the experiment (Table 3). Fungastop, Oxalic acid and Bion revealed the highest polyphenol oxidase levels (3.4, 3.3 and 3.2), respectively. Whereas, Photophor was the lowest one (2.3) in single spray treatment. Salicylic acid (3.8), Oxalic acid (3.4) and Bion (3.1) revealed the highest Polyphenoloxidase levels, respectively. While, Potassium oxalate and Fungastop (2.6) revealed the lowest ones in booster spray treatment.

The tested abiotic inducers increased significantly the level of Chitinase activity in cucumber treated plants than untreated ones (control) (Table 4). This increase was slightly higher in single spray than booster. Moreover, the increase reached the highest value 6 days after inoculation in both treatments (single and booster spray). The most effective abiotic inducers in single spray were Photophor (6.7) followed by Bion (5.5), Salicylic acid and Potassium oxalate (4.7), Fungastop (4.1) and Oxalic acid (3.5) in descending order. While, the most effective inducers in booster spray were

Table 2: Peroxidase activity after days of artificial inoculation with powdery mildew (*S. fuliginea*) and inducer treatments on cucumber plant cv. Beta alpha

	Peroxidase activity after inoculation (days)									
	Single spray					Booster spray				
Treatments	0	2	4	6	X`	0	2	4	6	X`
Oxalic acid	15.2	18.9	20.4	16.8	17.8	23.8	29.0	29.8	32.4	28.7
Salicylic acid	14.4	20.0	21.9	9.6	16.5	28.4	32.6	30.1	35.4	31.6
Potassium oxalate	16.2	17.0	20.6	12.6	16.6	32.1	35.5	29.6	36.3	33.4
Bion	20.3	22.3	23.8	15.0	20.4	27.9	28.4	30.8	34.2	30.3
Fungastop	17.3	25.0	29.0	14.3	21.4	32.9	36.4	35.2	29.9	33.6
Photophor	19.9	20.9	20.6	11.4	18.2	30.1	26.0	33.7	30.1	30.0
Control (infected)	13.2	15.6	18.6	10.0	14.4	20.4	24.1	26.6	29.3	25.1
Control (health)	13.2	14.6	16.5	14.5	14.7	20.4	22.5	25.3	27.6	24.0

LSD value at 5% for (Treatment×Spray×Days) = 1.371

Table 3: Polyphenoloxidase activity after days of artificial inoculation with powdery mildew *(S. fuliginea)* and inducer treatments on cucumber plant cv. Beta alpha

	**Polyphenol oxidase activity after inoculation (days)									
	Single spray					Booster spray				
Treatments	0	2	4	6	X`	0	2	4	6	X`
Oxalic acid	2.5	3.5	3.9	3.3	3.3	2.9	3.5	3.3	4.0	3.4
Salicylic acid	2.2	3.3	1.8	2.8	2.5	3.3	4.6	3.4	3.9	3.8
Potassium oxalate	2.2	2.7	3.0	2.5	2.6	2.9	2.8	2.1	2.5	2.6
Bion	2.2	4.2	3.7	2.7	3.2	3.3	3.2	2.9	3.1	3.1
Fungastop	2.0	3.8	5.0	2.7	3.4	3.5	2.6	2.2	2.2	2.6
Photophor	1.6	2.8	2.3	2.4	2.3	2.1	2.6	3.3	3.9	3.0
Control (infected)	1.4	1.9	2.1	2.3	1.9	1.7	2.2	2.5	3.0	2.3
Control (health)	1.4	1.4	1.7	3.2	1.9	1.7	2.2	2.4	2.6	2.2

LSD value at 5% for (Treatment×Spray×Days) = 0.129. **Polyphenol oxidase activity expressed as the change in absorbance at 495 nm

Table 4: Chitinase activity after days of artificial inoculation with powdery mildew *(S. fuliginea)* and inducer treatments on cucumber plant cv. Beta alpha

	Chitinase activity after inoculation (days)*									
	Single spray					Booster spray				
Treatments	0	2	4	6	X`	0	2	4	6	X`
Oxalic acid	2.3	3.2	3.9	4.9	3.5	3.5	4.3	3.5	4.7	4.0
Salicylic acid	2.3	4.2	5.8	6.4	4.7	2.1	3.8	7.7	7.4	5.3
Potassium oxalate	2.9	4.3	5.5	6.3	4.7	2.0	4.4	7.0	7.3	5.2
Bion	2.6	4.7	6.6	8.3	5.5	1.0	2.8	3.2	3.9	2.7
Fungastop	1.4	2.8	4.5	7.9	4.1	1.6	2.5	4.4	3.1	2.9
Photophor	4.0	5.6	7.7	9.7	6.7	1.2	2.8	3.9	6.9	3.7
Control (infected)	1.0	1.2	2.4	4.5	2.3	1.9	2.3	2.7	3.4	2.6
Control (health)	1.0	1.1	2.0	2.5	1.7	1.9	2.1	2.7	2.9	2.4

LSD value at 5% for (Treatment×Spray×Days) = 0.122. *Chitinase activity expressed as Mm N-acetyle glucose amine equivalent released/gram fresh weight/60 mint

Table 5: β-1,3 glucanase activity after days of artificial inoculation with powdery mildew (*S. fuliginea*) and inducer treatments on cucumber plant cv. Beta alpha

	β-1,3 glucanase activity after inoculation (days)*									
	Single spray					Booster spray				
Treatments	0	2	4	6	X`	0	2	4	6	X`
Oxalic acid	2.4	2.6	4.2	5.3	3.6	4.1	5.2	5.8	6.8	5.5
Salicylic acid	2.3	2.8	3.3	3.6	3.0	3.5	5.8	6.2	6.7	5.5
Potassium oxalate	3.3	4.6	4.1	5.5	4.4	3.9	6.2	5.5	7.1	5.7
Bion	2.4	2.5	3.3	4.2	3.1	4.7	5.8	6.1	6.4	5.7
Fungastop	3.6	2.5	4.0	7.5	4.4	3.9	5.7	5.4	5.9	5.2
Photophor	3.1	2.8	4.6	5.7	4.0	4.2	5.8	5.5	6.1	5.4
Control (infected)	2.3	2.5	3.2	3.6	2.9	4.0	5.0	5.3	5.8	5.0
Control (health)	2.3	2.4	2.7	3.3	2.7	4.0	4.6	5.0	5.2	4.7

LSD value at 5% for (Treatment×Spray×Days) = 0.119. *β-1, 3 glucanase activity expressed as mM N-acetyle glucose amine equivalent released/gram fresh

Table 6: Phytoalexin accumulation after days of artificial inoculation with powdery mildew (S. fuliginea) and inducer treatments on cucumber plant cv. Beta alpha

	Phytoalexin accumulation after inoculation (days)*									
	Single spray					Booster spray				
Treatments	0	2	4	6	X`	0	2	4	6	X`
Oxalic acid	1.3	6.8	9.6	8.0	6.4	6.6	8.2	8.2	3.1	6.5
Salicylic acid	1.6	4.5	8.4	7.3	5.5	5.8	6.7	5.2	4.9	5.7
Potassium oxalate	1.2	5.3	9.4	12.1	7.0	3.2	8.5	3.3	2.4	4.4
Bion	0.9	8.1	15.6	14.5	9.8	7.2	13.7	14.1	9.3	11.1
Fungastop	1.1	6.4	12.1	15.3	8.7	14.2	15.2	15.1	12.2	14.2
Photophor	1.2	3.5	9.8	13.8	7.1	14.0	14.9	15.3	14.8	14.8
Control (infected)	1.2	1.1	1.4	2.8	1.6	1.6	0.8	0.1	0.1	0.7
Control (health)	1.3	1.3	1.2	0.8	1.2	1.1	1.4	1.3	0.9	1.2

LSD value at 5% for (Treatment×Spray×Days) = 0.109

Salicylic acid (5.3) followed by Potassium oxalate (5.2), Oxalic acid (4.0), Photophor (3.7), Fungastop (2.9) and Bion (2.7), respectively.

Level of **β-**1, 3 glucanase was increased in cucumber plants treated with the six abiotic inducers than the control (Table 5). Such inducers however, were detected 6 days after inoculation in both single and booster treatments. Potassium oxalate expressed the highest value of **β-**1, 3 glucanase activity either in single (4.4) or booster (5.7) application. Nevertheless, other inducers varied in their efficiency according to the type of application. At single spray, Fungastop gave the same level of Potassium oxalate (4.4) followed by Photophor (4.0) and oxalic acid (3.6), respectively. Whereas, Bion gave the same level of Potassium oxalate (5.7) followed by oxalic acid and Salicylic acid (5.5) and Photophor (5.4) in booster spray. On the other side, the overall mean of **β-**1, 3 glucanase activity in booster spray was higher than in single one.

Accumulation of phytoalexin in cucumber plants pretreated with the abiotic inducers was highly significant than untreated plants (Table 6). Variations in the level of phytoalexin among the examined inducers were noted. Bion and Fungastop induced the highest level of phytoalexi in

single spray (9.8 and 8.7), respectively. However, the level of phytoalexin was almost two fold in booster spray treated with Fungastop and Photophor (14.2 and 14.8), respectively.

In contrary, phytoalexin production induced by potassium oxalate, Fungastop and/or Photophor in single spray increased by increasing inoculation period (0 to 6 days). While, the level of phytoalexin was almost constant over the experimental period in booster spray treatment.

DISCUSSION

Plants can defend themselves against fungal infection by natural means induced by biotic and abiotic elicitors. The treating of cucumber plants with different tested abiotic inducers showed significant reduction in powdery mildew disease severity as well as there was increasing in biochemical changes in treated plants. All these data was in agreement with (Ali *et al*., 2006; Palma *et al*., 2009; Barilli *et al*., 2010). The most effective inducer was bion while the lowest one was potassium oxalate. This reduction in disease severity may be due to an increase in defense-related enzymes such as peroxidase, polyphenoloxidase, Chitinase and β-1, 3 glucanase. (Eckardt, 2004; Cao *et al*., 2006; Tian *et al*., 2006). The present investigation revealed a significant increase in enzymatic activities for (Peroxidase, polyphenoloxidase, Chitinase and β-1, 3 glucanase) as a result of treated cucumber plants with any of the six abiotic inducers compared with the control. These results were confirmed either at single or booster sprays with Bion, Photophor and Fungastop inducers give the highest enzymatic activities and reduced powdery mildew disease severity in cucumber plants. The oxidative enzymes play an important role in induced resistance by the oxidation of phenols to oxidized toxic products (quinone) which limit fungal activity. Peroxidases also, catalyse the final polymerisation step of lignin synthesis, which increases the ability of tissue to lignify which may restrict the fungal penetration (Tian *et al*., 2006; Imran *et al*., 2007; Gorovitsa and Czosnek, 2008; Barilli *et al*., 2010). On the other hand the Chitinase and β-1, 3 glucanase enzymes play roles in plant defence against fungi by hydrolyze their cell wall, this findings was in harmony with those found by Tian *et al*. (2006), Imran *et al*. (2007), Gorovitsa and Czosnek (2008) and Barilli *et al*. (2010).

On the other hand, the abiotic inducers used in the present study showed as a remarkable accumulation of phytoalexin in cucumber treated plants especially at booster spray. The accumulation took place (2-6) days after application. It is well known that phytoalexin play an important role in the defence mechanism against fungal infection in the plants. They indicate that phytoalexin were highly fungi-toxic substances with ability to restrict the growth of the infected hyphae of the invading fungi. This action leads to the reduction of the fungal development on the plants. Present results in the present investigation are in agreement with Veshkurova *et al*. (2006), Okada *et al*. (2007) and Chong *et al*. (2009).

CONCLUSION

Application of abiotic agents in these experiments increased the enzymatic activities and phytoalexin accumulation in cucumber plants. These substances reduced subsequently powdery mildew disease severity. Therefore, it can be suggested using of abiotic inducers as a mean of disease control against powdery mildew in cucumber plants. The induction of systemic resistance against powdery mildew by abiotic agents facilitates the development of a wide range of disease management tools.

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