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Trilogy, a Product of Neem (*Azadirachta indica*) Induces Resistance in Cucumber Against *Podosphaera xanthii*

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Abstract: The efficacy of Trilogy, a natural product from neem (*Azadirachta indica* Fuss.), against cucumber (*Cucumis sativus* L.) powdery mildew (*Podosphaera xanthii*) was studied in detached leaf and intact plant experiments. Trilogy significantly retarded several growth parameters of the pathogen, viz. multiple germ tube formation, number of germ tubes, haustoria and colony size on cucumber leaves. Trilogy induced hypersensitive reaction (HR), as evidence by browning of host cell associated with appressoria. Furthermore, increase in protein concentration of intercellular fluid followed treatment with Trilogy. The elicited leaves exhibited significantly high activity of enzymes: Phenylalanine Ammonia Lyase (PAL) and Tyrosine Ammonia Lyase (TAL) along with rapid and distinct accumulation of fungitoxic phenolic compounds (phytoalexin). Based on analysis of foliar tissues, at least five separate phenolic compounds were identified as intrinsic components of cucumber plants. The pathogen *P. xanthii* alone induced the production of other seven identifiable phenolic compounds within the leaves of the cucumber plants. These compounds, displayed a significant increase in concentration as a result of elicitation with Trilogy when the plant was stressed by the pathogen especially cucumerin A and B. The combined amount of these antifungal compounds in treated plants was nearly five times the level found in control plants. Phytoalexin production was triggered by the combination of an eliciting/inoculation treatment. These results provide direct evidence that Trilogy induced resistance in cucumber associated with increased extractable enzymatic activity. Accumulation of flavonoid compounds response to an eliciting treatment after infection creates incompatible interactions with powdery mildew.

Key words: Trilogy, *Azadirachta indica*, cucumber, enzymatic activity, powdery mildew

INTRODUCTION

Cucumber (*Cucumis sativus* L.) and powdery mildew (*Podosphaera xanthii*, syn *Sphaerotheca fuliginea* Castagne; ([Schlecht.] Fr.) Poll. is a highly valuable pathosystem providing insight into the interactions between plants and biotrophic pathogens (McNally *et al.*, 2003). *S. fuliginea* has been subsequently renamed to *Podosphaera xanthii* (Castagne), the organism used in this work (Braun and Takamora, 2000).

Several effective synthetic fungicides are available to control this disease (Das, 1987). However, due to increasing awareness of the illeffects of synthetic pesticides on human and animal health and also to the agroecosystem, research efforts on alternative and more environmentally friendly methods of controlling pests and diseases have proliferated (Lyon *et al.*, 1995). Beside biocontrol agents, the use of plant products in plant disease control seems to be a logical approach (Prithiviraj and Singh, 1995).

Many workers have screened extracts/products from higher plants for antifungal activity (Singh *et al.*, 1990). For instance, leaf extract of *Reynoutria sachalinensis* induced resistance in long English cucumber against *Sphaerotheca fuliginea* through biochemical changes in the host plant

(Daayf *et al.*, 1995) and leaf extract of *Azadirachata indica* induced resistance in barely against leaf stripe pathogen caused by *Drechslera graminea* (Paul and Sharma, 2002).

During the past 40 years, several research groups have studied the defense reactions of cucumber plants attempting to unveil the underlying mechanisms to induced resistance (McNally *et al.*, 2003; Remoero *et al.*, 2004).

Biochemical and molecular-based techniques have revealed putative roles for pathogenesis-related and hypersensitive response proteins such as chitinase (Avdiushko *et al.*, 1993), α and β -glucosidase (Avdiushko *et al.*, 1993), lipoxygenase (Avdiushko *et al.*, 1993), peroxidase (Avdiushko *et al.*, 1993; Chen *et al.*, 2000) and polyphenoloxidase (Avdiushko *et al.*, 1993; Chen *et al.*, 2000) in cucumber.

Biochemical studies examining disease-resistant plants have revealed the increased activities for key biosynthetic enzymes involved in the production of phenolic compounds including: phenylalanine ammonia lyase (Chen *et al.*, 2000),

More recently, phytochemical investigations of powdery mildew-resistant cucumber plants have led to identification of C-glycosyl flavonoids acting as phytoalexins, including the discovery of cucumerin A and B, two novel and structurally complex C-glycosyl flavonoids (McNally *et al.*, 2003).

Three criteria were advanced for establishing the role of phytoalexins in disease resistance: (1) Phytoalexins must be associated with restriction of pathogen development and be conditioned by host-resistance genes; (2) Phytoalexins must accumulate to antimicrobial levels at infection site in resistant plants at the time pathogen development is stopped and (3) there must be evidence that phytoalexins are directly involved in defense and that this defensive role has a measurable benefit for the plant (Hammerschmidt, 1999).

In addition to modernizing phytoalexin theory by including data generated by more recent molecular-based techniques, these criteria introduced the concept of proximity of phytoalexins to the invading pathogen *in planta* as necessary evidence of their intervention within plant-pathogen interaction. Such reactions have been reported to occur as components of systemic acquired resistance in cucumber (Siegrist *et al.*, 1994). As mentioned by Kowalewski (1993), Phenylalanine Ammonia Lyase (PAL) is the key enzyme of the phenylpropanoid pathway which converts phenylalanine to cinnamic acid, which is, in turn, modified by other enzymes resulting in highly antifungal phenolic including phytoalexins (PAL activity is induced by several factors such as pathogen ingress, insect damage and stress).

This study was carried out to explore the potential of Trilogy, for its effect against cucumber powdery mildew. However, *in vitro* experiments, carried out herein, on the development of *P. xanthii* on cucumber leaves and its control by Trilogy indicate that the latter induces resistance in cucumber against powdery mildew and stimulates the production of fungitoxic phenolic compounds in cucumber. The bulk of the fungitoxic activity was found in phenolics in their aglycone form. The presence of these aglycones appeared to correlate with the prophylactic properties of the products. Accordingly, the present work was expanded to determine whether these compounds were phytoalexins and could play a role in the resistance induced by Trilogy in cucumber.

MATERIALS AND METHODS

Plants

Seeds of cucumber (*Cucumis sativus* L., c.v. Corona) were sown in plastic pots (9 cm diameter) containing unsterilized garden soil and maintained in a growth chamber (12 h of light/12 h of darkness, 24°C day/20°C night).

Source of Inoculum

Inoculum for the trails was obtained from three to four week old cucumber plants artificially inoculated with *Podosphaera xanthii* (Syn. *Sphaerotheca fuliginea*, Castagne, ([Schlecht.] Fr.) Poll.

grown in a green chamber at 20-24°C in a 12 h photo period. Inoculum from fresh pustules was collected by removing the conidia with a brush and water. Conidial suspensions were adjusted to 3000 conidia mL⁻¹ and sprayed immediately mainly on the upper leaf surface of the tested plants with hand sprayer.

Trilogy

Trilogy was provided by CERTIS USA, LIC. (CERTIS Crop, Protection). It, a commercial product, contains 70% hydrophobic extract of Neem oil and the remaining 30% is other inert ingredients, including 2% by weight total limonoids, which includes azadiractin and 6 or 7 additional compounds.

Assessment of Powdery Mildew

The prepared spore suspension was sprayed on the leaves of 20 days old cucumber plants and the development of powdery mildew was assessed at 1 week after treatment. Disease severity was estimated by calculating percentage of infected leaf area for all leaves on each plant and the mean for each treatment. A visual estimate for % cover of the whole plant was recorded. Careful attention was given to maintaining consistency throughout the work. This method was used by Paloukidou (2005). Plants were treated with 4 Trilogy concentrations, i.e., 0.5, 1, 1.5 and 2%, one day before inoculation (protective doses) and six days after inoculation (curative doses). Four replicates were used for each treatment giving 16 plants in total and 4 plants for control sprayed with sterile distilled water.

Effect of Trilogy on Conidium Germination of *P. xanthii* on Cucumber Leaves

Cucumber seeds were sown in plastic pots (9 cm diameter) containing unsterilized garden soil maintained a growth chamber. Twenty-day old plants were used for the experiment. Second nodal leaves from the bottom were excised with sharp scissors. The leaves were then arranged on filter paper towels, with the upper surfaces facing up and inoculated with conidia of *P. xanthii* by tapping heavily infested leaves over them. The leaves were then transferred to Petri dishes containing Trilogy solution (0.5, 1, 1.5 or 2%) prepared in 2% sucrose in distilled water (Yarwood, 1964). The control treatment contained 2% aqueous sucrose solution only. Nine replicates for each treatment giving 36 leaves in total, each leaf put separately in a Petri dish. The Petri dishes with the leaves were incubated in growth chamber at 20°C with a 12/12 h photoperiod. The incubation started at the onset of dark cycle. Three leaves representing each treatment were removed at 24 h intervals for three days and fixed immediately in ethyl alcohol acetic acid (3: 1). The fixation was done as described by Carver and Adaigbe (1990). Observations on germination and appressorium formation were made 24 h after inoculation on 100 conidia on each replicate leaf. Observations on percentage of conidia with multiple germ tubes were indicated. The establishment of a functional primary haustorium, were made 24 and 48 h post treatment, on at least 100 conidia per replicate. Hypersensitive host reactions were observed in the cells just below well developed appressoria which had failed to produce further germ tubes, 72 h after inoculation. At least 100 observations were made per treatment and the percentage of the attacked host cells showing a hypersensitive response was calculated. For calculation of the frequency and distribution of germings with multiple germ tubes, 250 germinated conidia were measured.

Estimation of Protein Concentration in Intercellular Fluid

Cucumber leaves were floated on aqueous Trilogy (1, 1.5 and 2%) and distilled water (control) in 9 cm glass *Petri* dishes with adaxial surface in contact with the extract or distilled water. The leaves were incubated for 24, 48, 72 and 96 h; five replicates were taken for each treatment and control. After each incubation period, leaves were removed and blotted dry. Approximately 1 g of these leaves was used for collecting the intercellular fluid using the method described by DeWit *et al.* (1986) and protein concentration in the collected fluid was estimated by Bradfords (1976) method.

Treatment of Plants for Estimation of Enzymes

Twenty-day old cucumber plants were sprayed to run off with different concentrations of Trilogy, i.e., 1, 1.5 and 2%. The plants were then placed in a growth chamber at 20-24°C in 12 h photoperiod. Leaves from control and treatment plants were sampled after 24, 48 and 72 and 96 h of treatment for estimation the activity of phenylalanine ammonia lyase (PAL) and Tyrosine Ammonia Lyase (TAL) enzymes as well as total phenol content. PAL and TAL activity was assayed by the method described by Havir and Hanson (1970).

Estimation of Total Phenol Contents

Total phenols in the ethanol extract were estimated by the method described by Bray and Thrope (1954).

Statistical Analysis

The effect of treatments on intercellular protein contents, PAL and TAL activity and total phenol contents of cucumber leaves were analyzed by analysis of variance (ANOVA).

Elicitation of Induced Resistance

To determine the purpose of this experiment, 3 treatments were created: plants elicited with Trilogy (1%) and inoculated with *P. xanthii* (E₊ I₊), non elicited inoculated plants (E₋ I₊) and control plants, i.e., non inoculated and non-elicited (E₋ I₋). Treatments, E₋ I₊ and E₊ I₊ were inoculated with *P. xanthii* after the emergence of the fourth real leaf. Inoculation was achieved by shaking the leaves of heavy diseased cucumber plants, kept in the nursery, over E₊ I₊ and E₋ I₊ plants. When the infection covered 2% of the leaf surface (10 days after inoculation), the elicited treatment (E₊ I₊) plants were sprayed with Trilogy (1% V/V sprayed until run-off) once a week all over the duration of the experiment (3 weeks). Non-elicited treatments, E₋ I₊ and E₋ I₋, received an equal volume of water applied at the same frequency.

Extraction and Identification of Phytoalexins

Approximately 100 g (fresh wt.) of leaf tissue were harvested from the areas of leaves harboring powdery mildew colonies for E₊ I₊ and E₋ I₊ plants using number 13 cork borer. The modified extraction method of Dercks and Buchenauer (1986) was adopted, as described by Daayf *et al.* (1995), allowing for the determination of free and glycoside-linked phenolics. The frozen dried foliar material was homogenized with 80% methanol at 10 mL g⁻¹, protected from oxidation by replacing oxygen with nitrogen and eliminating light and extracted for 48 h on rotatory shaker. After extraction, the methanolic homogenate was filtered and the residue was washed with 20 mL of 80% methanol. According to McNally *et al.* (2002), chlorophyll, carotenoids, lipids and waxes were removed by partitioning against light petroleum ether three times (Fraction, 1). The methanolic fraction containing the phenolic constituents was evaporated at 38°C and the aqueous residue was partitioned three times with 30 mL of anhydrous ethyl ether. Free phenolic compounds were found in the ether fraction (Fraction, 11). The aqueous function was diluted with an equal volume of 4NHCl and acid hydrolysis was performed for 90 min in a water bath at 100°C. After cooling, the hydrolysate was partitioned against anhydrous ethyl ether (FIII) to recover aglycones and the aglycone-containing ether fraction was evaporated, resuspended in methanol and finely subjected to HPLC analysis. At least 15 HPLC runs from different samples were carried out to properly assess the presence of compounds. All compounds were visualized with photodiode array (PDA) detector (Water Co. Ltd., Montréal, Quc Canada) at 272 nm wavelength known to discriminate flavonoid compound in cucumber (Fawe *et al.*, 1998).

RESULTS

Effect of Trilogy on the Incidence of Powdery Mildew

Plants were treated with Trilogy one day before inoculation or six days after inoculation (Table 1). In both cases the pustule number on all treated plants was significantly reduced as compared with the untreated check. The protective treatment was generally more effective than the curative treatment. Even at the lowest Trilogy concentration (0.5%), the pustule number was reduced by more than 83.5% following pre-inoculation treatment, while the efficacy was 56.2% after post-inoculation treatment.

At the highest concentration of Trilogy, i.e., 2% pre-inoculation treatment showed 94.7% efficacy, while after the protective treatment, it recorded 85.9%.

After protective treatments (one day before) the pustules were 7.0-7.5 mm in diameter which was comparable with the untreated check (15-20 mm), the shape of the pustules was circular. After curative treatments (6 days after) the pustule size was 2-3 mm, some pustules were not circular. Trilogy elicited plants inoculated with *P. xanthii* (E. I.) maintained a low level of infection that never exceeded 5% for the duration of the experiment (3 weeks). By contrast, the level of infection for non elicited inoculated plants (E. I.) progressed rapidly and covered approximately 80% of the leaf surfaces.

Effect of Trilogy on Conidial Germination of *P. xanthii* on Cucumber Leaves

Trilogy was not able statistically to affect the conidial germination or appressorium formation of *P. xanthii* on leaves fixed 24 h after treatment and inoculation (Table 2). However, a significant reduction in the number of germings producing multiple germ tubes was observed at 24 and 48 h post treatment. In the control (2% sucrose solution), 87.9% of conidia formed multiple germ tubes 48 h post inoculation, while only 3.7% did so in the treatment with 2% of Trilogy (Table 3).

There was a significant ($p < 0.05$) effect of Trilogy on colony growth-number of germ tubes and number of haustoria per colony (Table 4). A maximum colony length of 600 μm was recorded in the control, while colony length was only 120 μm following treatment with 1.5% of Trilogy. The effect of Trilogy in reducing the number of haustoria appeared to be dose dependent.

Trilogy greatly affected the host cell response to *P. xanthii* ingress, as indicated by the hypersensitive browning of cell associated with cytoplasmic granulation below appressoria and failure of the fungus to produce multiple germ tubes. The maximum response was observed at 1%, in which more than 3 times as many hypersensitive cells were found compared to the control (Fig. 1). Trilogy also, affected the development of *P. xanthii*, as indicated by the number of germ tubes compared with the control. Most conidia had four germ tubes in control, while in all the treatments conidia with a single germ tube predominated, although a few had multiple germ tubes. The frequency of multiple germ tubes was decreased with increasing Trilogy concentration up to 2% (Table 4).

Table 1: Effect of pre- and post-inoculation treatments with Trilogy against *P. xanthii* on cucumber at one week after inoculation

Application	Concentrations of trilogy (%)	Number of Pustules/ leaf	Efficacy(%)
1 day Pre-inoculation	0.5	14.9bc*	83.5
	1.0	13.8bcd	84.7
	1.5	9.1d	89.9
	2.0	4.8dc	94.7
6 days Post-inoculation	0.5	39.6bc	56.2
	1.0	32.1cd	64.5
	1.5	24.5f	78.2
	2.0	6.5g	85.9
Untreated check	0.0	90.4a	

*Same letter(s) means no significant difference in the students t-test ($p = 0.05$)

Table 2: Effect of Trilogy on conidial germination and appressorium formation by *P. xanthii* on cucumber leaves 24 h after treatment and inoculation

Treatments	Percentage germination	Appressorium formation (%)
Untreated plants (control)	79.4	90.0
Treated plants		
0.5% Trilogy	82.3	93.1
1.0% Trilogy	73.6	94.0
1.5% Trilogy	75.3	87.0
2.0% Trilogy	77.1	85.0

*Differences in percentage germination and appressorium formation were not statistically significant ($p>0.05$) by student t-test

Table 3: Effect of Trilogy on multiple germ tube formation by *P. xanthii* conidia 24 and 48 h after treatment and inoculation

Treatments	Percentage of conidia with multiple germ tubes	
	24 h	48 h
Untreated plants (control)	15.10 ^a	87.90 ^a
Treated plants with		
0.5% Trilogy	4.30 ^b	37.50 ^b
1.0% Trilogy	1.60 ^c	12.40 ^c
1.5% Trilogy	1.64 ^c	3.50 ^d
2.0% Trilogy	2.53 ^b	3.70 ^d

In columns, values with the same superscript letter do not differ significantly $p<0.05$ by student's t-test

Table 4: Effect of Trilogy on gemling development of *P. xanthii* on cucumber leaves 72 h after treatment

Treatments	Length of colony (μ m)	No. of germ tubes/colony	No. of haustoria/colony
Untreated plants (Control)	600 $\pm$ 71.5	4.0 $\pm$ 0.6	3.5 $\pm$ 0.8
Treated plants with			
Trilogy 0.5%	320 $\pm$ 100	3.6 $\pm$ 0.6	1.6 $\pm$ 3.0
Trilogy 1.0%	330 $\pm$ 70.1	3.9 $\pm$ 0.9	1.2 $\pm$ 0.2
Trilogy 1.5%	120 $\pm$ 65.0	2.8 $\pm$ 0.5	1.1 $\pm$ 0.1
Trilogy 2.0%	160 $\pm$ 110	2.5 $\pm$ 0.8	1.0 $\pm$ 0.1

*Based on at least 100 independent observations ($\pm$ standard deviation)

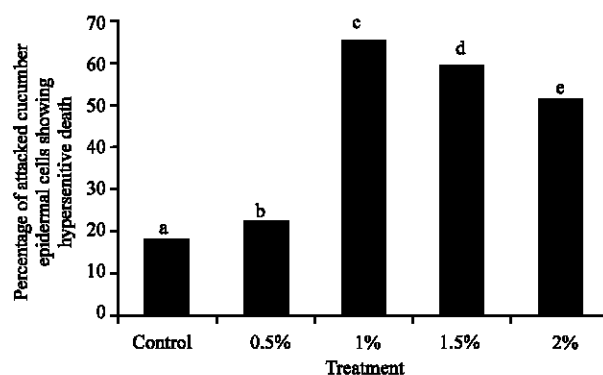


Fig. 1: Percentage of attacked cucumber epidermal cells showing hypersensitive browning in cucumber leaves treated with different concentrations of Trilogy and fixed 72 h after inoculation. Bars capped with the same letter do not differ significantly ($p<0.05$)

Intercellular Protein Concentration of Leaves

Compared with the control, the protein concentration in the intercellular fluid of leaves treated with Trilogy was higher (Fig. 2). Intercellular fluids from 1.5% treated leaves had maximum protein concentration ($p<0.01$) followed by concentrations, estimated in leaves treated with 1 and 2% of Trilogy ($p<0.05$). There was an increase in intercellular fluid protein content with longer incubation periods in Trilogy treatment, while intercellular fluid from leaves floated on water did not increase with prolonged incubation (Fig. 2).

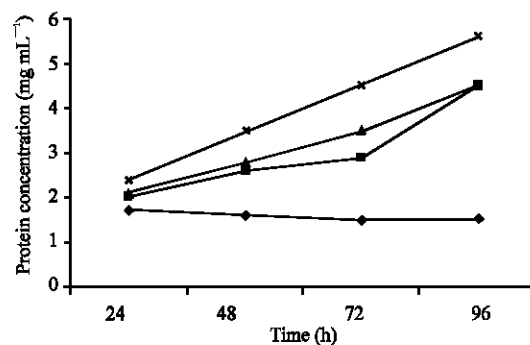


Fig. 2: Effect of Trilogy treatment on protein content of intercellular fluids. Control (♦) ; 1% (■) ; 1.5% (x) and 2% (▲) Trilogy

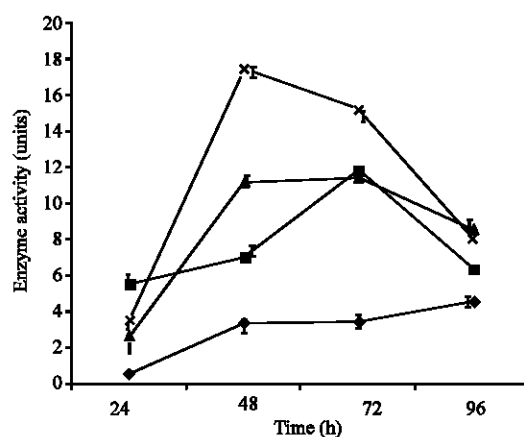


Fig. 3: Effect of Trilogy treatment on phenylalanine ammonia-lyase activity in cucumber leaves. The vertical bars represent standard deviation, Control (♦) ; 1% (■) ; 1.5% (x) and 2% (▲) of Trilogy

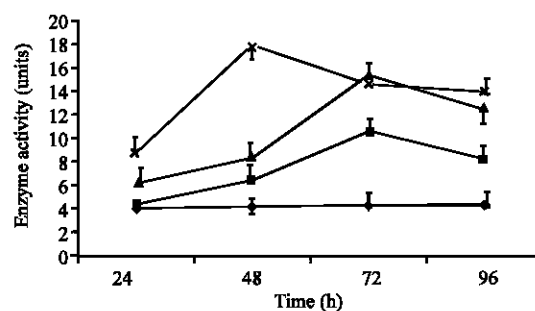


Fig. 4: Effect of Trilogy treatment on TAL activity in cucumber leaves. The vertical bars represent standard deviation, Control (♦) ; 1% (■) ; 1.5% (x) and 2% (▲) of Trilogy

Phenylalanine Ammonia Lyase and Tyrosine Ammonia Lyase Activity

The activity of both, PAL and TAL was higher in Trilogy- treated cucumber leaves (Fig. 3 and 4). However, maximum activity of both the enzymes was recorded in the leaves treated with 1.5% followed by 1 and 2% treatments ($p < 0.05$).

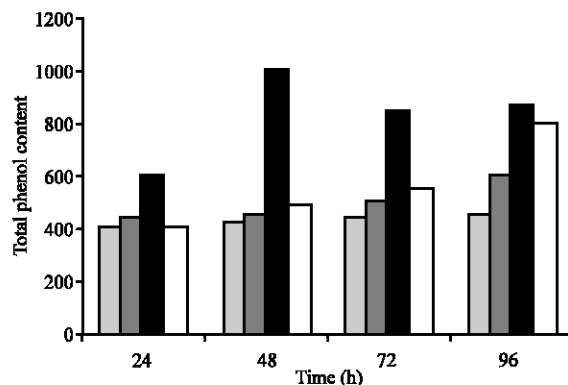


Fig. 5: Effect of Trilogy treatment on total phenol contents of cucumber leaves. Control (□), 1% (▤), 1.5% (▥) and 2% (■) of Trilogy

PAL and TAL activities were increased in all Trilogy-treated leaves with either peaking at 48 h (1.5%) or at 72 h (1 and 2%) (Fig. 3 and 4).

Total Phenol Content

It is evident from Fig. 5 that 1 and 2% Trilogy had no significant effect on the total phenol content of cucumber leaves $p > 0.05$. However, 1.5% dilution of Trilogy could significantly increased phenols of cucumber leaves ($p > 0.01$). Maximum phenol content in cucumber leaves treated with 1.5% of Trilogy was recorded after 48 h of treatment.

Identification of Phytoalexins

HPLC analysis of extracts prepared from carefully selected leaf tissues of control (E₋ I₋), inoculated plants (E₊ I₋) and elicited inoculated plants (E₊ I₊) harvested 48 h post the first eliciting Trilogy application revealed important differences among the treatments for the hydrolyzed extract (Fig. 6).

For inoculated plants, E₊ I₋, the presence of the pathogenic fungus *P. xanthii* alone induced the production of at least 12 identifiable compounds within the leaves of (E₊ I₋) cucumber plants (listed in order of increasing elution time): Caffeic acid, isoorientin, orientin, p-coumaric acid, isovitexin, vitexin, sinapinic acid, ferulic acid, vitexin-6 (4-hydroxy-1-ethylbenzene) or cucumeric A, isovitexin 8 (4-hydroxy-1-ethylbenzene) or cucumeric B, p-came and trans cinnamic acid (Fig. 6B), compared with control (E₋ I₋) which have 5 common identified phenolic compounds: p-coumaric acid, isovitexin, vitexin, sinapinic acid and p-came (Fig. 6A).

These same compounds were detected within the hydrolyzed leaf extracts of Trilogy-elicited inoculated plants (E₊ I₊), albeit in much greater quantities with the exception of caffeic and ferulic acid (Fig. 6C). For instance, p-coumaric acid, p-came and especially the C glycosyl flavonoid compounds isoorientin, orientin, isovitexin, vitexin, cucumerin A and B were systematically more concentrated within the leaves of elicited inoculated plants (E₊ I₊) compared with non elicited inoculated plants (E₊ I₋) (Fig. 6B and C). Of particular interest, the C glycosylflavones cucumerin A and B (compounds 9, 10) were dominant within the leaves of E₊ I₊ plants compared with E₊ I₋ plants (Fig. 6B and C). Based on area under-curve calculations, there was approximately 30 times more cucumerin A and B within leaves of E₊ I₊ plants compared to E₊ I₋ plants.

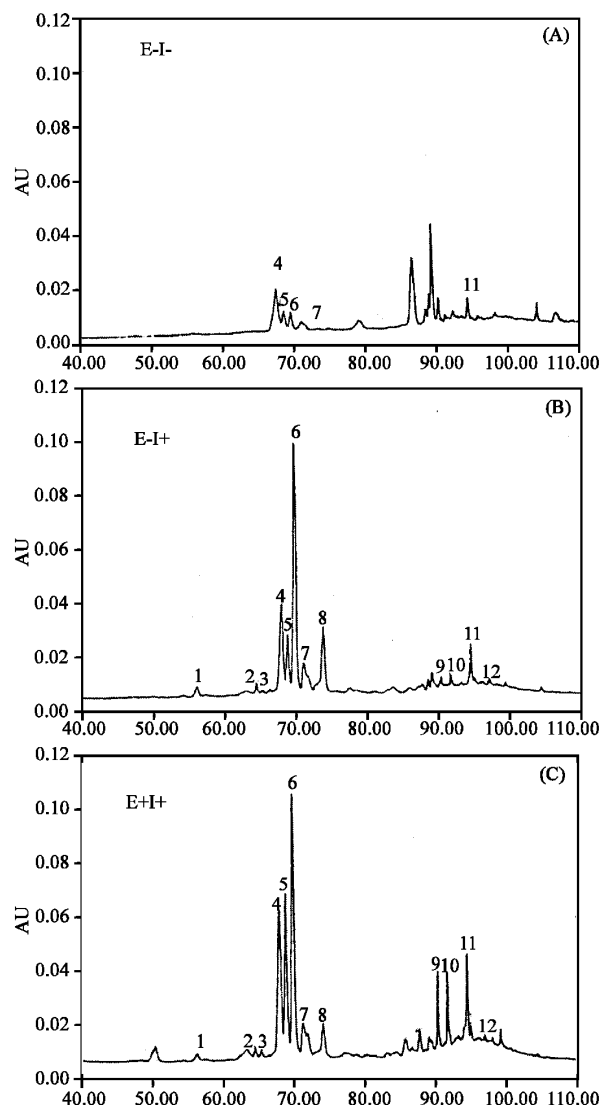


Fig. 6: HPLC analysis (272 nm) of hydrolyzed cucumber leaf extracts prepared from, A- control plants (E₋ I₋), B- inoculated plants (E₊ I₋) and C- elicited/inoculated plants (E₊ I₊) harvested 48 h after the first eliciting Trilogy® treatment. Identified compounds are numbered in order increasing elution time of: (1) caffeic acid, (2) isorientin, (3) orientin p-coumaric acid, (5) isovitexin, (6) vitexin, (7) sinapinic acid, (8) ferulic acid, (9) cucumerin A, (10) cucumerin B, (11) p-came, (12) trans cinnamic acid

DISCUSSION

Pre-inoculation treatments of cucumber with formulated Trilogy had a good efficiency against powdery mildew. At a concentration of 0.5% Trilogy, the pustule number was reduced by ~ 83.5%. At the highest applied concentration, i.e., 2% the efficiency of Trilogy ranged between 56.2-85.9%, dose dependent.

The effect of Trilogy on powdery mildew development on intact cucumber plants showed that the pre-inoculation treatment with Trilogy was significantly more effective than post-inoculation application.

To explain these differences, two modes of action can be proposed. In the pre-inoculation treatment, the oil component irrespective of Trilogy ingredients forms a barrier on the cucumber leaves. Most of the conidia on the leaf cannot develop, only few pustules are formed. The pustules produced are of normal size. This is in line with the observation by Häberle and Schlösser (1993), who used Telmion, formulated rape seed oil, against powdery mildew of cucumber. The oil component, irrespective of neem ingredient seems to be most important for a protection against powdery mildew in the very sensitive stage of conidia germination.

In the post-inoculation at the stage of the powdery mildew fungus has already established itself on the leaves, sometimes young developing pustules were seen. Compounds with fungicidal activity in Trilogy can stop further development of the mildew pustules. The pustules that still develop are smaller in size and the form is not circular. It can introduce systemic acquired resistance.

Rovesti *et al.* (1992) reduced the affected leaf surface with *S. fuliginea*, *Erysiphe graminis* f. sp. *tritici* and *E. graminis* f. sp. *hordei* to 6.8, 0 and 25%, respectively by a postinoculation treatment (four days after inoculation) with aqueous neem seed extract. *Phytophthora infestans*, *Cercospora beticola* and *Septoria apiicola* were not affected by a treatment one day before inoculation.

The curative effect of formulated neem products against powdery mildew of cucumber is most important for a practical use of these extracts in green houses. It seems to be possible to control developing powdery mildew epidemic at stage where first pustules become visible. The recommended concentration should be 1% Trilogy. In addition to its good protective effect, Trilogy is distinguished by a good curative effect against powdery mildew of cucumber even at rather low concentration.

Secondary growth parameters of the fungus, like the number of germ tubes emerging from a conidium was greatly affected by Trilogy, whereas primary germ tube formation and appressorium development appeared unaffected. The host tissues reacted hypersensitively in the form of browning of cell following treatment with this chemical. Tissue browning in rice plants following treatment with 2, 2-dichloro-3-dimethyl-cyclopropane carboxylic (DCP) in response to picolinic acid (The Pathotoxin of *Pyricularia oryzae*) has also been reported (Langcake and Wickins, 1975).

From the results, it is suggested that treatment of cucumber leaves with Trilogy induced an increase in the activity of PAL and TAL enzymes which led to an increased phenol biosynthesis rendering the leaves resistant to *P. xanthii*.

An increase in the concentration of PAL and TAL enzymes is reflected by enhanced protein levels in intercellular fluid of treated cucumber leaves. Increased activities of PAL and TAL, the key enzymes in phenol biosynthesis by neem products have also been demonstrated earlier by Singh and Prithiviraj (1997). These defended enzymes were selected because they represent parts of different physiological host response to pathogen. PAL activity is required for biosynthesis of phenyl propanoids, which are required for penetration and all death responses to pathogen in barely to powdery mildew (Shiraishi *et al.*, 1995).

In this study, Trilogy elicited plants inoculated with *P. xanthii* (E₊ I₊) maintained a low level of infection that never exceeded 5% for the duration of the experiment (3 weeks). In contrast, the level of infection for non elicited inoculated plants (E₋ I₋) progressed rapidly and covered approximately 80% of the leaf surfaces. These results are consistent with those obtained by Fofana *et al.* (2002) who examined the prophylactic properties of Milsana.

The production of phytoalexin compounds has in recent times been proposed to explain induced resistance for cucumber on the basis of correlative evidence (Fawe *et al.*, 1998). McNally *et al.* (2003) established a clear link between the production of these compounds, viz., C-glycosyl flavonoids and induced resistance against powdery mildew. During the present studies, phytochemical analyses of

targeted leaf tissues harboring fungal colonies eliminated the effects of whole leaf extraction and led to the identification of six structurally related C-glycosyl flavonoid phytoalexins, i.e., vitexin, isovitexin, orientin, isoorientin, cucumerin A and B. At the same time, phenolic acids involved in lignification, such as sinapinic acid, were detected in approximately equal concentration within the leaves of E. I₊, E. I₊ plants, thereby negating this process as an important form of resistance against powdery mildew in cucumber. In fact, high concentration of ferulic and caffeic acids were detected within the leaves of E. I₊ plants.

Expectedly, higher concentration of p-coumaric acid and its derivative, p-came, were observed within E. I₊ plants, since these phenolic acids are biosynthetic precursors of all six identified C-glycosyl/flavone phytoalexins (Burbulis and Winkel-Shirly, 1999). The accumulation of approximately 30 times more cucumerin A and B within the leaves of E. I₊, compared to E. I₋ is consistent with the increase in phytoalexin concentration. This agreed with the previous studies reported by Essenberg *et al.* (1992). Moreover, the induction of C-glycosyl flavonoids as phytoalexins and the existence of p-coumaric acid and its methyl ester (p-came) within E. I₊ plants supports the previous studies that associated these compounds with induced resistance against powdery mildew in cucumber (Fawe, 1997).

Phytoalexin synthesis was triggered by the combination of an eliciting treatment and fungal penetration and seemed to be closely synchronized with germination of fungal conidia. Moreover, elicited plants became sensitized to the presence of the pathogen by producing phytoalexins more rapidly in response to subsequent infection.

Based on the chemical analyses, flavonoid (glycosylates and aglycones) compounds were found to accumulate in larger quantities in elicited plants than in controls. These findings are particularly interesting since they suggest that the increased activity of flavonoid biosynthetic enzymes following elicitation leads to a strong accumulation of defensive compounds in elicited cucumber, highlighting the correlation between phytoalexin accumulation and induced resistance. These results are in agreement with those mentioned by Fawe *et al.* (1998) who described the presence of fungitoxic flavonoids in cucumber tissues following an eliciting treatment. Previously, flavonoid phytoalexins had been found only in a few plant species such as sorghum, rice and barley (Christensen *et al.*, 1998).

In conclusion, resistance of cucumber plants to powdery mildew following an eliciting treatment with Trilogy appears to be associated with accumulation of the flavonoid biosynthetic enzymes. Both flavonoids and phenolic acids have specific role in the complex defence system of cucumber.

REFERENCES

- Avdiushko, S.A., S.X. Ye and J. Kuć, 1993. Detection of several enzymatic activities in leaf prints of cucumber plants. *Physiol. Mol. Plant Pathol.*, 42: 441-445.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, 72: 248-254.
- Braun, U. and S. Takamoro, 2000. *Podosphaera xanthii* (Castagne) a synonym for *Sphaerotheca Fusca* (Fr.) S. Blumer 1933, *Schlechtendalia* Veröffentlichungen aus dem Institut für Geobotanik und Botanischer Garten der Martin-Luther-Universität Halle-Wittenberg, 4: 31.
- Bray, H.G. and W.V. Thorpe, 1954. Analysis of phenolic compounds of interest in metabolism. *Methods Biochem. Anal.*, 1: 27-52.
- Burbulis, I.E. and B. Winkel-Shirley, 1999. Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. *Proc Natl. Acad. Sci., USA.*, 96: 12929-12934.
- Carver, T.L.W. and M.E. Adaiybe, 1990. Effects of oat host genotype, leaf age and position and incubation humidity on germination and seedling development by *Erysiphe graminis* f.sp. avenae. *Mycol. Res.*, 94: 18-26.

- Chen, C., R.R. Bélanger, N. Benhamou and T.C. Paulitz, 2000. Defense enzymes induced in cucumber roots by treatment with Plant Growth-promoting Rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiol. Mol. Plant Pathol.*, 56: 13-23.
- Christensen, A.B., P.L. Gregersen, J. Schröder and D.B. Collinge, 1998. A chalcone synthase with an unusual substrate preference is expressed in barley leaves in response to UV light and pathogen attack. *Plant Mol. Biol.*, 37: 849-857.
- Daayf, F., A. Schmitt and R.R. Bélanger, 1995. The effects of plant extracts of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. *Plant Dis.*, 79: 577-580.
- Das, N.D., 1987. Study of the comparative efficacy of saprol with some other fungicides against powdery mildew (*Erysiphe polygoni* DC) of pea. *Pesticides*, 21: 40-42.
- De Wit, P.J.G.M., M.B. Buurlages and K.E. Hammond, 1986. The occurrence of host, pathogen and interaction specific proteins in the apoplast of *Cladosporium fulvum* (Syn. *Fulvia fulva*) infected tomato leaves. *Physiol. Mol. Plant Pathol.*, 29: 159-172.
- Dercks, W. and H. Buchenauer, 1986. Untersuchungen zum Einfluss von Aluminiumfosetyl auf den pflanzlichen Phenolstoffwechsel in den Pathogen-Wirt Beziehungen *Phytophthora fragaria* Erdbeere und *Bermia Lactuca*-Salat. *J. Phytopathol.*, 115: 37-55.
- Essenberg, M., M.L. Pierce, E.C. Cover, B. Hamilton, P.E. Richardson and V.E. Scholes, 1992. A method for determining phytoalexin concentrations in fluorescent, hypersensitively necrotic cells in cotton leaves. *Physiol. Mol. Plant Pathol.*, 41: 101-109.
- Fawe, A., 1997. Étude du mode d'action du silicium dans l'accroissement de résistance du concombre au blanc poudreux: Intervention de composés à activité antifongique et identification. Ph.D. Thesis, Québec City, Qc: Université Laval.
- Fawe, A., M. Abou-Zaid, J.G. Menzies and R.R. Bélanger, 1998. Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. *Phytopathology*, 88: 396-401.
- Fofana, B., D.J. McNally, C. Labbe, R. Boulanger, N. Benhamou, A. Séguin and R.R. Bélanger, 2002. Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase. *Physiol. Mol. Plant Pathol.*, 61: 121-132.
- Häberle, R. and E. Schlösser, 1993. Protective and Curative Effects of Neem Seed Extracts Against *Sphaerotheca Fuliginea* on Cucumber. In: Proc. 2nd Workshop. Practic Orientated Results on Use and Production of Neem Ingredients. Kleeberg, H., M. Trifolio GmbH, Druck and Graphic, Giessen (Eds.), Germany, pp: 107-112.
- Hammerschmidt, R., 1999. Phytoalexins: What have we learned after 60 years? *Ann. Rev. Phytopathol.*, 37: 285-306.
- Havir, E.A. and K.R. Hanson, 1970. In: *Methods in Enzymology*. Tabor, H. and C.W. Tabor (Eds.), Volume XXVII. New York: Academic Press, pp: 575-581.
- Kowalewski, A., 1993. Investigations about the Chemical Nature and the Mode of Action of the Resistance Inducing Ingredients from *Reynoutria sachalinensis* (F. Schmidt) Nakai. In: *Mechanisms of Plant Defense Responses*. Fritig, B. and M. Legrand (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp: 182.
- Langecake, P. and S.G.A. Wickins, 1975. Studies on the action of the dichloropropanes on the host-parasite relationship in rice blast disease. *Physiol. Plant Pathol.*, 7: 113-126.
- Lyon, G.D., T. Reglinski and A.C. Newton, 1995. Novel disease control compounds: The potential to "immunize" plants against infection. *Plant Pathol.*, 44: 407-427.
- McNally, D.J., K. Wurms, C. Labbé and R.R. Bélanger, 2002. A standardized methodology for the study of induced glycosylated plant phenolics. *Can. J. Plant. Pathol.*, 24: 429-436.
- McNally, D.J., K.V. Wurms, C. Labbé, S. Quideau and R.R. Bélanger, 2003. Complex C-glycosyl flavonoid phytoalexins from *Cucumis sativus*. *J. Nat. Prod.*, 66: 1280-1283.

- Paloukidou, E., 2005. Biological control of *Powdery Mildew*, *Podosphaera Xanthii*, on Cucumber using Plant Extracts Neem and Allicin, Honours Thesis, University of Aberdeem.
- Paul, P.K. and Sharma, 2002. *Azadirachta indica* leaf extract induces resistance in barley against leaf stripe disease. *Physiol. Mol. Plant Pathol.*, 61: 3-13.
- Prithiviraj, B. and U.P. Singh, 1995. Biological control of plant pathogens: A key to a serene agroecosystem. *J. Recent Adv. Applied Sci.*, 10: 99-110.
- Remoero, D., A. Pérez-García, M.E. Rivera, F.M. Cazorla and A. de Vicente, 2004. Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Applied Microbiol. Biotechnol.*, 64: 263-269.
- Rovesti, L., DiMarco and D. Pancaldi, 1992. Effect of neem kernel extract on some phytopathogenic fungi under greenhouse conditions. *Z. PflKrankh. PflSchutz.*, 99: 293-296.
- Shiraishi, T., T. Yamada, R.L. Nicholson and H. Kunoh, 1995. Phenylalanine ammonia-lyase in barley: Activity enhancement in response to *Erysiphe graminis* f. sp. *hordei* (race) a pathogen and *Erysiphe pisi*, a nonpathogen. *Physiol. Mol. Plant. Pathol.*, 46: 153-162.
- Siegrist, J., W. Jeblick and H. Kauss, 1994. Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. *Plant Physiol.*, 105: 1365-1374.
- Singh, U.P., V.N. Pandey, K.G. Wanger and K.P. Singh, 1990. Antifungal activity of ajone, a constituent of garlic (*Allium sativum*). *Canadian J. Bot.*, 68: 1354-1356.
- Singh, U.P. and B. Prithiviraj, 1997. Neemazal, a product of neem (*Azadirachta indica*), induces resistance in pea (*Pisum sativum*) against *Erysiphe pisi*. *Physiol. Mol. Plant Pathol.*, 51: 181-184.
- Yarwood, C.E., 1964. Detached leaf culture. *Bot. Rev.*, 12: 1-56.