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Systemic Acquired Resistance Induced in Cucumber Plants Against Powdery Mildew Disease by Pre-inoculation with Tobacco Necrosis Virus

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Abstract: Systemic Acquired Resistance (SAR) in cucumber plants against powdery mildew disease, caused by *Sphaerotheca fuliginea* (Schlechtend Fr.) Pollacci, was induced by localized infection in cucumber cotyledons with Tobacco Necrosis Virus (TNV). Inoculation of the two cotyledon leaves significantly reduced powdery mildew severity on cucumber true leaves similar to the effect of fungicide. TNV-local lesions developed plants still protected against powdery mildew for 16 days. The level of protection was higher in the upper true leaves when compared with the bottom ones. Antifungal protein was extracted and partially purified by DEAE-cellulose column chromatography from cucumber TNV-inoculated plants only. Bioassay detection for antifungal activity indicated that, 0.6 M NaCl fraction had the highest activity. SDS-PAGE of partially purified 0.6 M NaCl fraction protein indicated the presence of a single protein band with a molecular weight of about 30 kDa. This protein was extracted from upper TNV-uninoculated true leaves of plants inoculated on the cotyledon leaves, which acquired systemic resistance against powdery mildew challenge inoculation. *In vitro* study of the antifungal activity of these proteins showed that only 0.6 M NaCl fraction has direct antifungal activity towards *S. fuliginea* conidial spores. The induced systemic resistance was not accompanied with the activity of β-1, 3 glucanase.

Key words: Powdery mildew, cucumber, *Sphaerotheca fuliginea*, Tobacco Necrosis Virus (TNV) and β-1, 3 glucanase

INTRODUCTION

Powdery mildew, caused by *S. fuliginea*, is a serious disease on cucumber (*Cucumis sativus* L.) grown both in fields and greenhouses (Sitterly, 1978). Powdery mildew occurs on leaves, stems and fruits. Control methods currently available under commercial conditions include the use of repeated applications of elemental sulphur (Kimati *et al*., 1980) and other fungicides (Kimati *et al*., 1997). The constant use of fungicides, however, can result in environmental contamination and selection of resistant populations of *S. fuliginea* (McGrath, 1996; McGrath *et al*., 1996). For these reasons, alternative control measures are warrant. The infection of plants by necrotizing pathogens, including fungi, bacteria and viruses, induces systemic resistance to subsequent attack by the pathogens. This resistance is called Systemic Acquired Resistance (SAR) (Kessman *et al*., 1994; Ryals *et al*., 1994) and is effective against a wide range of pathogens. Also, it can be activated in numerous plant species by pre-inoculation with biotic inducers including pathogens and non pathogens (Sticher *et al*., 1997). The defense response occurring in plants exhibiting SAR effect genetically fixed mechanisms which are typical for each plant species. In general, defense responses in SAR expressing plants include for example lignifications (Hammerschmidt and Kuc, 1982; Siegrist *et al*., 1994; Dean and Kuc' 1987) formation of cell wall appositions (Papillae) at the sites of attempted penetration of fungal pathogens (Schmele and Kauss, 1990; Görlach *et al*., 1996), hypersensitive reactions (Kogel *et al*., 1994; Siegrist *et al*., 1997) and the accumulation of Pathogenesis-related (PR) proteins (Van loon, 1997), which accumulate both locally and systemically after the contact of plants with microbes. As the appearance of PR-proteins coincides with the induction of local and systemic resistance, these proteins are considered to be important factors of SAR (Van Loon, 1997). Fegla *et al*. (1985) noted that infection with *Erysiphe cichoracearum* and either cucumber mosaic virus or watermelon mosaic virus-2 at 0, 5 and 10 days after fungal inoculation resulted in less powdery mildew symptom severity when inoculation occurred at the first leaf stage. The inoculation of lower leaves of tobacco and tomato with TMV or TNV induced systemic resistance to

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Peronospora tabacina and *Phytopthora infestans*, respectively (Ye *et al*., 1992; Jeun and Buchenauer 2001). This work aimed at controlling powdery mildew disease on cucumber plants by predisposing the host with necrotizing biotic agent such as Tobacco Necrosis Virus (TNV).

MATERIALS AND METHODS

Plants and pathogen: Cucumber plants *(Cucumis sativus* L.) cv. Atlas was grown in the greenhouse at the National Research Centre during 2003 and 2004 summer seasons. Seeds were cultivated in plastic pots (20 cm diameter) containing compost. Powdery mildew fungus (*Sphaerotheca fuliginea*) was maintained on 4 weeks old cucumber plants in isolated cages. Tobacco Necrosis Virus (TNV) was obtained from Dr. Reda M. Taha (Department of Botany, Faculty of Sciences, El-Fayoum University) and maintained on leaves of *Phaseolus vulgaris* cv. Contender.

Induction of systemic resistance: Cucumber plants (10 days old) with the first true leaf were used for the induction of systemic acquired resistance in all experiments. Cotyledons of cucumber plants, were either inoculated with TNV (Fig. 1A) or with 0.1 M phosphate buffer (pH 6.0) alone (control). Carborundum was used as abrasive.

Challenge inoculation: Buffer and TNV-inoculated cucumber plants were kept in a greenhouse without inoculation of *S. fuliginea* until they reached to the development stage of four expanded leaves (20 days). At this stage, plants were transferred to cages with high *S. fuliginea* inoculum potential. On the other hand, untreated cucumber plants were transferred to separate cages and treated with the fungicide Benlate (benomyl, 0.1 g L^{-1}) once a week. First application with the fungicide was made immediately after the plants were transferred to the cages with high *S. fuliginea* inoculum. Experiments were set up in a randomized design with ten replicates per treatment. Each replicate consisted of one pot containing one plant. The temperature in the greenhouse during the experiments ranged between 26 and 37°C.

Disease assessment: The effect of pre-inoculation with either TNV, buffer or the fungicide Benlate on disease severity was assessed 16 days after TNV inoculation by counting the number of powdery mildew colonies developed on the true leaves. The severity of powdery mildew infection was visually evaluated on individual leaves and scored as percentage of area infected on the first, second and third true leaves according to (McGrath and Shiskoff, 1996).

Time course formation of acquired resistance: To determine the production course of powdery mildew protection, buffer and TNV-inoculated cucumber plants were used. The plants were challenge inoculated with *S. fuliginea* as mentioned above by transferring them to cages with high *S. fuliginea* inoculum potential. The transference was done at interval days from 0 to 16 days after buffer and TNV-inoculation.

Extraction and purification of antifungal protein: Ten days after the cotyledons leaves were inoculated with TNV or buffer, the proteins were extracted from true uninoculated leaves and roots by Hydrated Calcium Phosphate (HCP) according to Faccioli and Capponi (1983). A partial purification was achieved by DEAE-column chromatography of the crude preparation as suggested by Sela *et al*. (1966). Columns were stepwise eluted with 10 mL of 0.01 M phosphate buffer (pH 7.6), 10 mL of buffer containing 0.3 M NaCl and 10 mL of buffer containing 0.6 M NaCl. The resulting proteins were dialyzed against distilled water overnight and lyophilized. The lyophilized materials were dissolved in distilled water and then protein concentrations were determined according to (Bradford, 1976).

Detection of antifungal activity *in vitro*: To study the antifungal activity against *S. fuliginea*, eluted proteins (0.3 and 0.6M NaCl fractions), which extracted from TNV or buffer inoculated plants were used. Volumes of 25 μL of freshly harvested conidiospores by rinsing from leaves, were placed on glass slides and mixed with 25 μL of protein fractions containing concentrations 50, 75, 100 μg protein per mL. Slides were incubated overnight at 30°C, then examined under light microscope.

Gel electrophoresis: Electrophoresis of purified active fraction (0.6 M NaCl) was performed on 10% SDS-PAGE according to Laemmli (1970).

Determination of β-1, 3 glucanase activity: By using a pre-chilled pestle and mortar, proteins were extracted from frozen tissue (0.2 g) in 1 mL of 0.05 M sodium acetate buffer, (pH 5.2) containing 2.5 mM EDTA. The homogenate was centrifuged at 8000 rpm for 10 min and the supernatant was dialyzed against distilled water at 4°C for 24 h then against 0.05 M sodium acetate buffer (pH5.2) for 2 h. Total β-1, 3-glucanase activity was

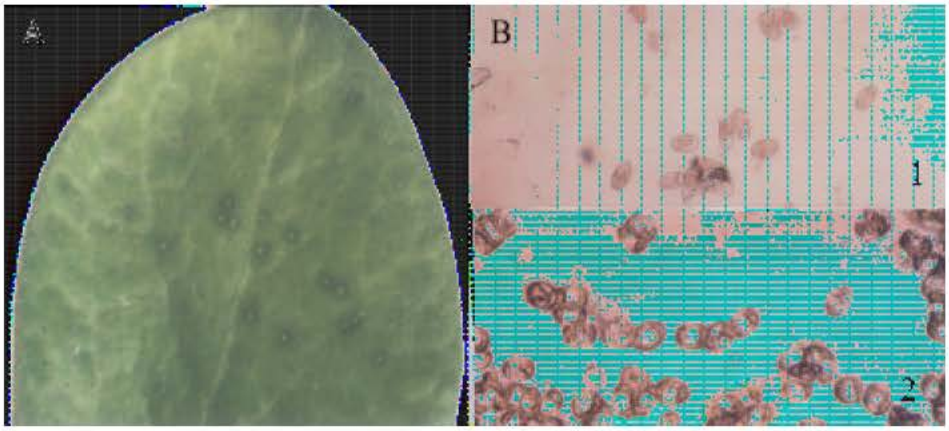

Fig. 1: A), Cotyledon leaf of cucumber plant inoculated with TNV. Note, development of necrotic local lesions.
B), Antifungal activity of protein towards *S. fuliginea*. Effects of 0.6 M NaCl fraction extracted from buffer-inoculated (1) or from the corresponding TNV-inoculated cucumber plants (2), on collapsing of conidiospores

colorimetically assayed by the Laminarin-dinitrosalicylate method according to Abeles and Forrence (1979). Crude enzyme extract was precipitated with 5 volumes of cold acetone at 20°C for 30 min and centrifuged at 6000 rpm for 10 min. The pellet was vacuum dried and suspended in sodium acetate buffer (pH 5.0). The suspension 62.5 µL was added to 62.5 µL of 4% laminarin and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µL of dinitrosalicylate reagent and heated for 5 min in a boiling water bath. The resulting colored solution was diluted with 4.5 mL of distilled water, vortexed and its absorbance at 500 nm was determined. The plank was the suspension mixed with the dinitrosalicylate reagent to which the solution containing laminarin was added (Pan *et al*., 1989). One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 mole of glucose equivalent per second under the above conditions.

Statistical analysis: Data obtained were statistical analysis and means were compared according to (Snedecor and Cochran, 1980).

Table 1: Effect of TNV inoculation on symptoms of powdery mildew infection in relation to leaf position on cucumber plants

	No. of colonies			Infected leaf area per infected leaf (%)		
	True leaf position			True leaf position		
Treatments	1	2	3	1	2	3
TNV-inoculated	10.3(54)a	7.4(55)a	0.0	13.2(65)a	8.4(75)a	0.0
Benlate	9.6(58)a	2.6(84)c	0.0	9.7(82)a	5.4(84)a	0.0
Buffer-inoculated	22.6b	16.5b	0.0	52.9b	33.5b	0.0

Data are presented as means from two separate experiments; each is consisted of 10 plants per treatment. Means in the same column followed by the same letter are not significantly different at 0.05. The number in parenthesis is the percentage of disease control in relation to buffer-treatment (control)

Table 2: Antifungal activity of protein preparations obtained from cucumber TNV or buffer-inoculated plants

	Collapsed conidiospores					
	TNV-inoculated[1]			Buffer-inoculated[2]		
Fractions	50[3]	75	100	50	75	100
Crude protein	-[4]	-	+	-	-	-
DEAE-Cellulose						
Buffer	-	-	-	-	-	-
Buffer + 0.3 M NaCl	-	-	-	-	-	-
Buffer + 0.6 M NaCl	+	+	+	-	-	-

(1) Protein fractions were obtained from TNV-inoculated plants. (2) Protein fractions were obtained from buffer-treated plants. (3) Protein concentrations as µg mL^{-1}. (4) - = spores not collapsed. + = spores collapsed

RESULTS

Induction of systemic acquired resistance against *S. fuliginea*: Inoculation of the two cotyledon leaves of cucumber with TNV induced systemic acquired resistance against *S. fuliginea*. The resistance was evident as a significant reduction in number of colonies caused by the fungus per leaf in case of TNV-inoculated plants compared with buffer treated plants. Data presented in Table 1 shows that the percentage of infected leaf area per infected leaf in the first, second and third true leaves of plants treated with buffer alone were 52.9, 33.5 and 0.0 compared with 13.2, 8.4 and 0.0 in TNV-inoculated plants, respectively. The level of protection by TNV inoculation was 75%. TNV-inoculated plants give higher controlled against powdery mildew on cucumber plants at least as conventional fungicide. The time course study presented in Fig. 2 shows that the resistance was evident at day 7

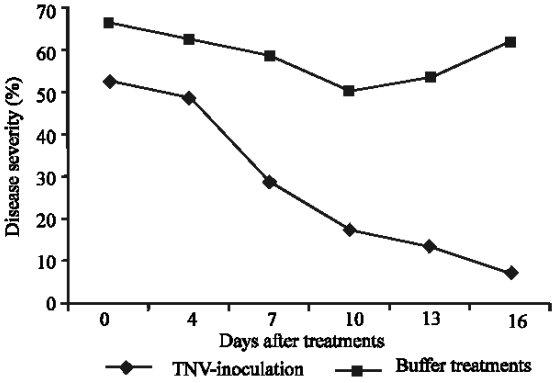

Fig. 2: Disease severity of powdery mildew induced by challenge inoculation with *S. fuliginea* at different time intervals after TNV or buffer inoculation on cotyledons stage

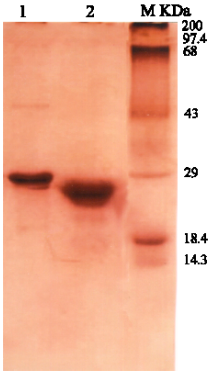

Fig. 3: Electrophoresis of purified antifungal proteins in 10% SDS-PAGE. Lane (1): 0.6 M NaCl fraction from roots, Lane (2): 0.6 M NaCl fraction from leaves, Lane (M): protein mol. wt. marker

and the level of protection increased with time. Maximum protection reached at day 16 after TNV-inoculation.

Antifungal activity: The inhibitory effect of protein fractions obtained from TNV-inoculated and buffer-treated plants were tested *in vitro*. As shown in Fig. 1B, incubation of conidiospores with 0.6M NaCl fraction obtained from TNV-inoculated plants, destroyed the conidispores of *S. fuliginea*. This activity was not detected in the 0.3 M NaCl or washing buffer fractions or in fractions obtained from buffer-treated plants (Table 2).

Gel electrophoresis: Because the 0.6 M NaCl fraction had the most activity against *S. fuliginea*, some characters was done such as molecular weight. As shown in Fig. 3, 0.6 M NaCl fractions from roots (Lane, 1) and leaves

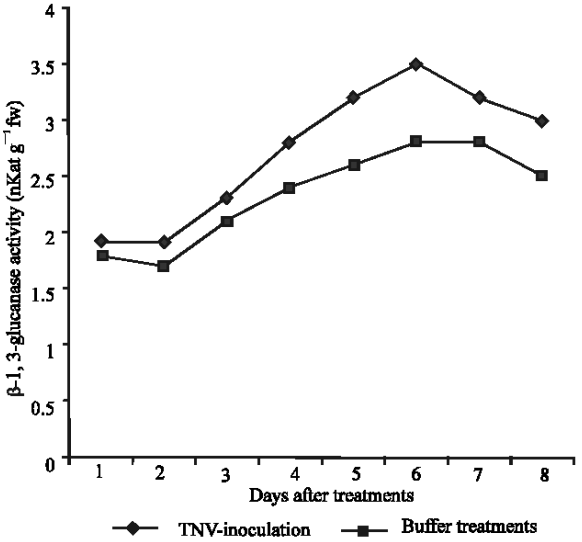

Fig. 4: The time course of β-1, 3-glucanase activity in cucumber plants after TNV or buffer treatments

(Lane, 2) as a result of TNV-inoculation at cotyledons stage. The molecular weight was estimated about 30.0 KDa.

Induction of β-1, 3-glucanase: Low difference in β-1, 3-glucanase activity was observed as a consequence of inoculation of cotyledon leaves with TNV (Fig. 4). A gradual increase in the activity was observed until 6 days post inoculation, (the time at which the necrotic lesions caused by TNV became evident).

DISCUSSION

In Egypt cucumber is much of the crop shipped to the wholescale market. Despite the crops value, induce many growers ponder whether they should commit much money to pest control program. Economically powdery mildew of cucumber is among the most important diseases. Powdery mildew disease attacks every year cucumber plants and rapidly defoliates veins. It's increasingly evident that the use of certain pesticides in plant production will become restricted at the same time that the productivity, profitability and competitiveness of agriculture must increase. Pesticides contribute on the problem of environmental pollution which influence on the economy, health and quality of life. Beside that, the generation of new pesticides is becoming more difficult and expensive. Resistance strains of pathogens rapidly arise to many new systemic pesticides and the difficulty of developing "environmentally-sound" pesticides has increased their cost (McGrath *et al*., 1996). Advances in biotechnology,

including the development of transgenic plants, biocontrol, inducing systemic resistance (plant immunization) and disease resistant plants providing alternative means of disease control, which are effective and reduce the dependence on pesticides (Kuc', 1990; Russel, 1965; Siegrist *et al*., 1994). Plant immunization regulates genes for defense compounds present even in susceptible plants. It's safe for the environment as disease resistant mechanism in plants (Kuc', 1990). The interaction between virus and cells of the organized host may vary between two extremes, from complete susceptibility (in systemic infection) to immunity (in local infection). Defense phenomena resulting after development of local lesions are known. The restriction of virus movement from local lesion to adjacent tissues in hypersensitive hosts (localization) is one of the most efficient resistance mechanisms. No infected parts of local lesion host become resistant to attack by any microorganisms. In recent years, research in this field has focused on molecular aspects believed to be associated with localization and resistance. Some evidence has been gathered, indicating the involvement of various specific and defined substances with the build-up of resistance and with the mechanisms which restrict the pathogen (Faccioli and Capponi, 1983).

The association of the inducible cucumber antifungal proteins with necrotizing infection by Tobacco Necrosis Virus (TNV) in this study provides supporting evidence for this point.

In this study, data showed that powdery mildew infection of the upper leaves of cucumber can be reduced by prior infection with TNV at the cotyledon leaves. This is in agreement with Ehara *et al*. (1982), Russel (1965), Pan *et al*. (1993), Ivanovic *et al*. (1994) and Anfok and Buchenauer (1997). The results of this study provide further evidence that the infection of plants by necrotizing viruses have post-infection curative activity against cucumber powdery mildew. The results showed that the period between the initial inoculation and challenge inoculation is required to develop systemic protection in the true leaves. These finding are in agreement with general suggestion that this period is required for the putative signal to be transported from the site of induction to other plant parts (Dean and Kuc', 1986; Jeun and Buchenauer, 2001). Leaf age affected the susceptibility of cucumber leaves to *S. fuliginea* in both TNV-inoculated and buffer-treated plants. The younger leaves being more resistant than older leaves. A similar observation has been made on cucumber and tomato with respect to resistance to *S. fuliginea* and *Phytophthora infestans* (Enkerli *et al*., 1993; Mosa, 1997). Cucumber plants inoculated with TNV on cotyledon leaves produced an antifungal protein in the true leaves. This protein was found in one elute of DEAE-column chromatography (0.6 M NaCl fraction), while no antifungal activity was found in the buffer and 0.3 M NaCl fractions. The 0.6 M NaCl fraction caused conidiospore collapsing. Thus antifungal activity was practically all concentrated in 0.6 M NaCl fraction. Data indicated that the antifungal activity was not dependent on protein concentration. SDS-PAGE indicated that, the antifungal protein have molecular weight about 30 KDa. Results revealed that induction of SAR to *S. fuliginea* was not correlated with the increase of β-1, 3-glucanase activity. These results are in contrast with Cheng and Kuc' (1995), who demonstrated that there is a positive correlation between SAR and β-1, 3-glucanase activity in cucumber plants. β-1, 3-glucanase activity was not enhanced, therefore, it is postulate that the extracted protein from TNV-inoculated plants may play a major role in the defense mechanism of cucumber plants to *S. fuliginea*.

Systemic acquired resistance provides protection against *S. fuliginea* and it does not require the introduction of foreign genes. Protection of cucumber plants may be achieved by compounds which are released as the immunizing agents, then systemically protect the plants against powdery mildew disease. The reduction in powdery mildew of cucumber plants by localized infection induced by necrogenic TNV may be is associated with the systemic accumulation of Salicylic Acid (SA) and certain pathogenesis-related proteins (PR-protein). SA levels have been shown to increase in the inoculated tissue as well as in uninoculated tissue prior to the establishment of induced disease resistance according to Kessmann *et al*. (1996).

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