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Full Length Research Paper

Protection of melon plants against *Cucumber mosaic virus* infection using *Pseudomonas fluorescens* biofertilizer

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This study was carried out to characterize a virus causing severe mosaic, yellowing, stunting and leaf deformation on melon (*Cucumis melo* L.), and evaluate the capacity of *Pseudomonas fluorescens* as biofertilizer to improve plant growth and restrict the accumulation of the virus in the plant. The virus was identified as an isolate of *Cucumber mosaic virus* (CMV) by means of symptoms on indicator plants, serological characteristics using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and immunochromatography, and molecular weight of coat protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The source of the virus infection was also determined by the same means. *P. fluorescens* was used as seed treatment and soil applications. The seedling grown from bacterial treated seeds in non-treated soil and those grown from non-treated seeds in bacterial treated soil were mechanically inoculated with CMV extract at the primary leaf stage. Two proteins representing CMV coat protein appeared on 10% SDS-PAGE of 24 and 26 kD. It has been found that 9 of 35 weed plants harbor CMV. Treatment seeds and soil with *P. fluorescens* suspension induced significant reduction in virus accumulation in the plants as proved by absorbance values of ELISA-reactions. Minimum absorbance values of ELISA reactions at 405 nm were found to be 0.160 and 0.298 for seed and soil treatments, respectively when compared with 1.190 for samples from CMV-inoculated plants (control). The inhibition activity of *P. fluorescens* against CMV continued to be significant up to 20 days of virus inoculation with absorbance values of ELISA-reactions (0.460 and 0.930) for seed and soil treatment, respectively. The results indicate that *P. fluorescens* is able to induce systemic resistance against CMV in the plants.

Key words: *Cucumber mosaic virus* (CMV), *Cucumis melo*, melon, plant growth promoting microorganisms (PGPM), *Pseudomonas fluorescens*, viral diseases management.

INTRODUCTION

Melon (*Cucumis melo* L.) is one of the most cultivated cucurbits vegetables in Iraq. It has been reported that melon is infected by several viruses; among these viruses, *Cucumber mosaic cucumovirus* (family: Bromoviridae) is the most destructive (Alonso-Prados et al., 2003; Grafton-Cardwell et al., 1996; Ko et al., 2007. This virus has been reported to infect more than 1200 plant species worldwide belonging to 100 families from monocotyledons to dicotyledons (Palukaitis and Garcia-Arenal, 2003; Adhab and Al-Ani, 2011). The virus induced mosaic patterns, leaf distortion, plant stunting and yellowing associated with heavy losses in yield in melon (Alonso-Prados et al., 1997).

Cucumber mosaic virus (CMV) is transmitted by more than 80 species of aphid in non-persistent manner; of these species, *Myzus persicae* is the most efficient (Palukaitis and Garcia-Arenal, 2003). The broad host range of CMV and its ability to be transmitted by aphids

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in non-persistent manner, rendered the control of this virus very difficult. The use of insecticides was found to be ineffective in preventing CMV dissemination due to brief probes for the insect to acquire and inoculate the virus from infected to healthy plants. The virus is acquired too quickly for the insecticide to prevent the viruliferous aphids from entering to the plants.

Recent studies indicated that microorganisms that colonize plant root improved plant growth and yields through facilitating nutrient uptake and producing phytohormones, termed as plant growth promoting rhizobacteria (PGPR) and suppressing soil borne pathogens (Pieterse and Van Loon, 2007; Saharan and Nehra, 2011).

The suppression of pathogens by PGPR may be due to direct effect on the pathogens (Antibiosis) and competition or indirectly through inducing systemic resistance in the plants. Several studies indicated that plant growth promoting microorganisms (PGPM) act as inducers of systemic resistance in the plants (Van Peer et al., 1991; Wei et al., 1991; Walters, 2010). The resistance is characterized by restriction of pathogen and suppression disease symptoms (Hammerschmidts, 1999). Systemic resistance can be induced in aerial parts of plants by PGPM application to the root (Van Loon et al., 1998).

Pseudomonas fluorescens, a plant growth promoting rhizobacteria, is reported to induce systemic resistance in the plants against several fungal and bacterial diseases (Pieterse et al., 1996; Van Wees et al., 1997). These bacteria were tested in this study for its capacity to protect melon plants from CMV infection.

MATERIALS AND METHODS

The virus

Symptomatic melon plants showing mosaic, yellowing, and leaf distortion were collected from different locations of Abu-Ghraib area, Iraq. The samples were homogenized with 0.05 M sodium phosphate buffer, pH = 7.0 in mortar and pestle. The homogenate was filtered through double layer of muslin and the filtrate was used as virus inoculum. The presence of CMV was confirmed by symptoms on test plant, immunochromatography and double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA).

Test plants and virus inoculations

Seeds of *Cucurbita pepo*, *Cucurbita maxima*, *Vigna unguiculata*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Datura stramonium*, *Datura metel*, *Gomphrena globosa*, *Vigna sinensis* var Black, and *Nicotiana tabacum* var Xanthi, were sown in mix soil and peat moss (3:1) in plastic pots (25 × 18 cm) in glasshouse (25 to 30°C). The seedlings were transferred to small plastic pots (12 × 10 cm) containing mix soil. Leaves of test plants were dusted with carborandum powder (600 mesh) and gently rubbed with extracts of infected plants. The inoculated plants were maintained in insect protected greenhouse for two to four weeks and the symptoms were evaluated.

Detection of virus by immunochromatography

This test was carried out using polyclonal anti-CMV antibodies purchased from Agdia biofords, France as flashkits. A piece of infected melon and test plants leaves (0.15 g) was homogenized in plastic sac containing phosphate buffer saline (PBS), pH 7.0, provided with flashkits, with a pestle. The immunochromatography end was dipped to 0.5 cm in the extract for 3 to 5 min. The development of a band on the strip indicates positive reaction.

Samples of weed plants were collected from melon and vicinity fields and were tested for the presence of the virus by the same technique to determine virus infection source in the field.

DAS-ELISA

CMV was detected by DAS-ELISA. Young leaves of infected and non-infected melon plants were homogenized with a mortar and pestle in PBS (10 mM Na_2HPO_4, 0.1 M NaCl, pH = 7.0) 1:10 (g:ml). The homogenate was filtered through 2 layers of muslin and the filtrate was collected. ELISA plate wells were coated with anti-CMV immunoglobin g (IgG) at 1.5 µg/ml in coating buffer (35 mM Na_2HCO_3, 15 mM Na_2CO_3, 0.2% bovine serum albumin (BSA), and 2% polyvinyl pyrolidone, pH 9.6, 100 µl/well), and incubated at 4°C for 12 h. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST). The wells were then loaded with leaves extracts (100 µl/well) and incubated at 4°C for 12 h and were washed three times as before. The alkaline phosphatase conjugated IgG at dilution of 1:2000 in conjugate buffer was added (100 µl/well). The plates were incubated at 37°C for 1 h. The plates were washed as previously described and the substrate P-nitrophenyl phosphate at 1 mg/ml in 10% diethylamine pH 9.8 was added (100 µl/well) and the absorbance values at 405 nm were recorded after 15 to 60 min of incubation at room temperature in micro plate auto reader.

Polyacrylamide gel electrophoresis

Cucumber mosaic cucumovirus was extracted and purified according to the procedure descried by Scott (1963) for CMV. Total proteins from infected and healthy plants were obtained as described by Da Rocha et al. (1986). The proteins in 0.125 M Tris-HCl buffer containing 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, 15% glycerol and 0.05% bromophenol blue as tracking dye were incubated at 100°C for 3 min and were analyzed by electrophoresis on 10% polyacrylamide vertical slab gel.

Activity of *P. fluorescens* against CMV multiplication

An isolate of *P. fluorescens* was obtained from Plant Protection Department, College of Agriculture, University of Baghdad, Iraq. The bacteria were activated on Kings B agar medium [20 g peptone, 2.5 g potassium phosphatase (K_2HPO_4), 6 g $MgSo_4$, 15 ml glycerol, and 17 g agar in 1 L water]. Well isolated colony was inoculated to King's B (KB) broth medium in 250 ml flasks, incubated at 28 ± 2°C for 48 h and was used in this study. The colony forming units (CFU)/ml was determined by plate count method on KB agar medium.

Seeds of melon were dipped in *P. fluorescens* suspension at 4 × 10^9 CFU/ml for 24 h and were sown in sterilized mixed soil and peat moss (3:1) in pots (3 seeds/pot). Non-treated seeds were sown in other pots as control. The seedling grown from bacterial treated and non-treated seeds were mechanically inoculated with CMV extract at primary leaf stage. In other tests, 100 ml of *P. fluorescens* suspension was poured into each pot immediately after seeding and the seedlings were inoculated with virus in primary leaf stage.

Figure 1. Symptoms induced on cucurbit plants that are mechanically inoculated by sap from melon showing mosaic symptoms, symptoms of mottle and mosaic on melon leaves (A and B) and mosaic on cucumber leaves (C).

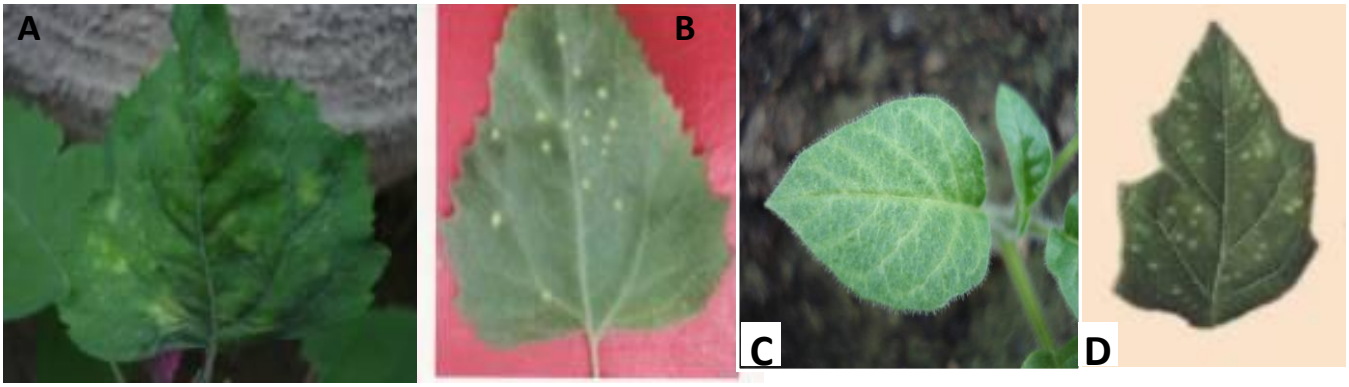

Figure 2. Symptoms induced on indicator plants that are mechanically inoculated by sap from melon showing mosaic symptoms, local chlorotic lesions on *C. quinoa* and *C. amaranticolor* inoculated leaves (A and B), vein clearing and yellowish mottle on *D. metel* L. new leaves (C), and local chlorotic spots on *D. stramonium* L. inoculated leaves (D).

Seedlings from seeds sown in bacterial treated soil, non-inoculated with the virus, were used as control. In addition, seedling from non-bacterial treated seeds in non-treated soil and non-inoculated with the virus were considered as control.

RESULTS

Virus characterization

Symptomatology

Symptoms of mosaic associated with yellowing and distortion on the leaves of *C. melo, C. sativus, C. pepo*, and *C. maxima* following mechanical inoculation by an extract of symptomatic melon leaves were developed within 15 days of inoculation (Figure 1).

Sap inoculation of *C. amaranticolor* and *Chenopodium quinoa* by virus extract induced chlorotic local lesions on the inoculated 4 and 10 days leaves, respectively. *D. stramonium* responded to the virus by forming chlorotic local lesions on the inoculated 6 days leaves followed by systemic mosaic on the new leaves (Figure 2).

The virus caused vein clearing followed by mosaic symptoms on *N. tabacum* cv. Turkish and *N. tabacum* cv. Xanthi mechanically inoculated by virus extract. The virus caused vein clearing, mottling, and deformation of growing new leaves of *Nicotiana glutinosa*. Vein clearing followed by mottling were developed on *V. unguiculata* within 20 days of sap inoculation with the virus (Figure 3).

Serological characteristics

A precipitation line was developed on the immunochromatographic strip containing anti-CMV polyclonal antiserum dipped in an extract from symptomatic melon leaves. No reaction was observed when the immunochromatographic strip was dipped in extract from healthy melon leaves (Figure 4).

The anti-CMV antibodies gave positive reaction with extracts from infected melon plants as shown by

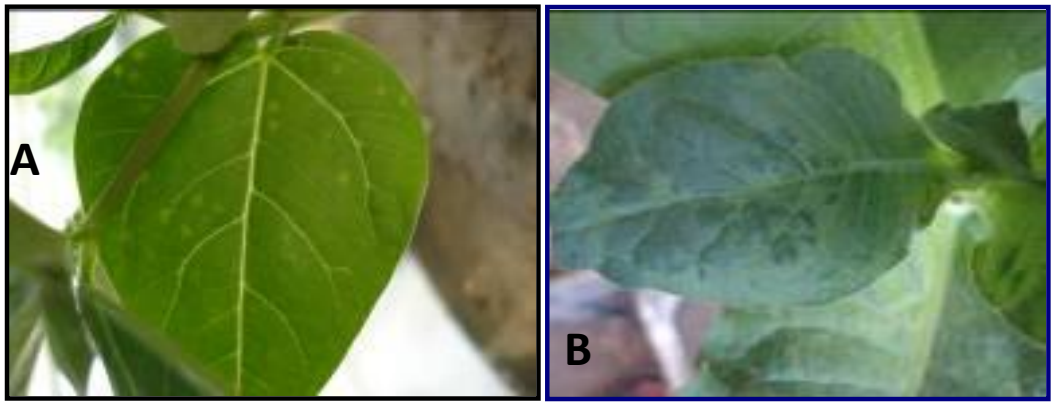

Figure 3. Symptoms induced on indicator plants that are mechanically inoculated by sap from melon showing mosaic symptoms, local chlorotic spots on *V. unguiculata* L. inoculated leaves (A), and severe mosaic on *N. tabacum* cv. Turkish new leaves (B).

Figure 4. Polyclonal antibodies to CMV on immunochromatography which reacted with extracts from infected melon plants showing band (arrow) indicates the presence of CMV in the extract (A), compared with a healthy plant (control) (B).

development of obvious yellow color in ELISA micro plate wells. The mean absorbance values at 405 nm were 1.635 when compared with 0.027 with extracts from non-infected plants.

Proteins analysis

The analysis of the structural proteins of CMV, and the total proteins extracted from infected melon plants revealed the presence of two proteins of 24 and 26 kD. These proteins were absent in the profile of the protein extracted from healthy melon plants (Figure 5). These two proteins represented the coat protein of CMV.

Based on symptoms on test plants, serological and molecular means, it is concluded that the virus infecting melon plants could be a strain of CMV.

Source of virus infection

Of the 35 plant species tested for CMV by biological and serological means, nine species including, *Portulaca oleraceae*, *Sisymbrium irio*, *Beta vulgaris*, *Chenopodium murale*, *Chenopodium album*, *Amaranthus retroflexus*, *Solanum nigrum*, *Sonchus oleraceus*, and *Withania samnifera*, were found to harbor the virus. Some of these hosts harbor the virus in asymptomatic infection (Table 1).

Antiviral activity of P. fluorescens

The treatment of seed and soil with *P. fluorescens* suspension induced high inhibitory activity against CMV multiplication in the plant as demonstrated by absorbance values of ELISA reactions (Table 2). The higher activity of *P. fluorescens* was registered after 10 days of the virus inoculation with absorbance values of ELISA reactions at 405 nm as 0.160 and 0.298 for seed and soil treatments, respectively when compared with 1.190 for samples from CMV inoculated plants only (the percent of inhibition is 86.55 and 74.14%, respectively). The inhibition activity of *P. fluorescens* continued to be significant up to 20 days of virus inoculation with absorbance values of ELISA reactions as 0.460 and 0.930 for seed and soil treatment,

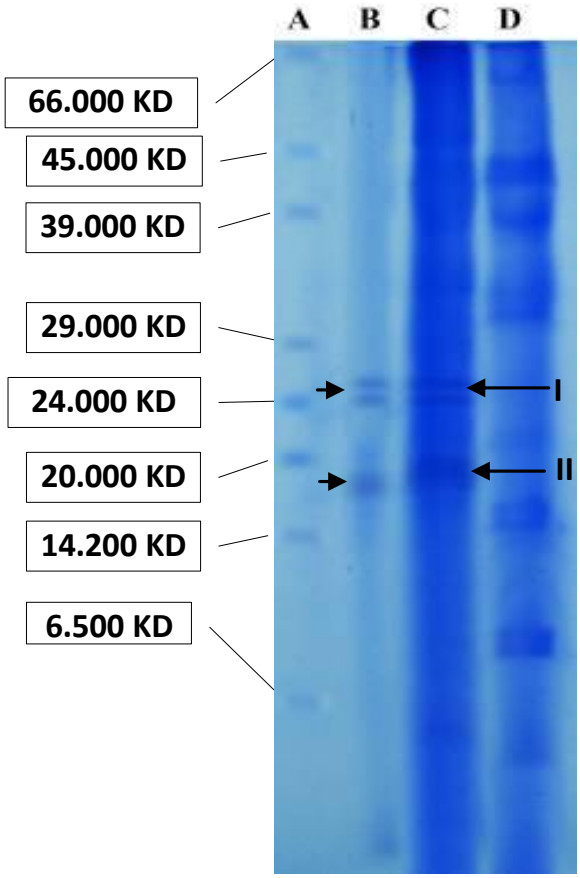

Figure 5. Profile of slab SDS-gel electrophoresis for samples of CMV on 10% polyacrylamide gel with 0.1% SDS. Arrows referred to principle protein band of the virus observed in samples from infected plants only. (A) Protein markers of known molecular weight used to determine the molecular weight of virus proteins. (B) Sample of partially purified CMV (control). (C) Total proteins extracted from virus infected plants. (D) Total proteins from healthy plant (control).

Table 1. Detection of CMV in weed plants.

S/N	Weed name	Family	Serological test
1	*Aster subulatus* Michx.	Compositae	-
2	*Sonchus oleraceus* L.	Compositae	+
3	*Xanthium strumarium* L.	Compositae	-
4	*Plantago lanceolate* L.	Plantaginaceae	-
5	*Portulaca oleracea* L.	Portulaceae	+
6	*Cardaria draba* (L.) Desv.	Cruciferae	-
7	*Raphanus raphanistrum* L.	Cruciferae	-
8	*Sisymbrium irio* L.	Cruciferae	+
9	*Cuscuta* spp.	Cuscutaceae	-
10	*Rumex dentatus* L.	Plygonaceae	-
11	*Polygonum aviculare* L.	Plygonaceae	-
12	*Corchorus olitorius* L.	Malvaceae	-
13	*Malva parviflora* L.	Malvaceae	-
14	*Malva rotundifolia* L.	Malvaceae	-

Table 1. Continued.

15	*Withania samnifera* L.	Solanaceae	+
16	*Solanum nigrum* L.	Solanaceae	+
17	*Euphorbia helioscopia* L.	Euphorbiaceae	-
18	*Chrozophora verbascifolia* (Wild) Juss	Euphorbiaceae	-
19	*Chenopodium murale* L.	Amaranthaceae	+
20	*Chenopodium album* L.	Amaranthaceae	+
21	*Beta vulgaris* L.	Amaranthaceae	+
22	*Schanginia aegyptiaca* (Hasselq) Aellen	Amaranthaceae	-
23	*Kochia eriophora* Schrad.	Amaranthaceae	-
24	*Amaranthus retroflexus* L.	Amaranthaceae	+
25	*Amaranthus albus* L.	Amaranthaceae	-
26	*Sorghum halepense* (L.) Pers.	Poaceae	-
27	*Echinochloa colonum* (L.) Link.	Poaceae	-
28	*Digitaria sanguinalis* (L.) Scop.	Poaceae	-
29	*Phragmites communis* Trin.	Poaceae	-
30	*Ammi majus* L.	Apiaceae	-
31	*Cyperus rotundus* L.	Cyperaceae	-
32	Prosopis farcta (Banks & Sol.) Macbr.	Leguminosae	-
33	*Melilotus indicus* (L.) All.	Leguminosae	-
34	*Alhagi maurorum* Medik.	Leguminosae	-
35	*Convolvulus arvensis* L.	Convolvulaceae	-

Table 2. Effect of seeds and soil treatment with *P. fluorescens* suspension on CMV multiplication in melon plants as estimated by ELISA reaction absorbance at 405 nm.

Treatment	Periods (days) of virus inoculation								
	4	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Seed treatment	0.430	0.300	0.180	0.160	0.220	0.300	0.420	0.460	0.460
Soil treatment	0.435	0.480	0.400	0.298	0.490	0.585	0.890	0.925	0.930
Virus only (control)	0.490	0.930	1.050	1.190	1.380	1.440	1.580	1.630	1.635
Bacteria only (control)	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027
Without treatment (control)	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027

Values represent means of three replicates for ELISA readings at 405 nm.

respectively.

DISCUSSION

A virus infected melon plants causing severe mosaic with yellowing, leaf distortion and stunting was characterized by biological, serological and molecular means. The symptoms developed on the test plants used in this study indicated that the virus isolated from symptomatic melon plants closely corresponded to CMV. This conclusion was confirmed by serological immunochromatography and ELISA test. The cross reactivity with anti-CMV antibodies by immunochromatography and ELISA suggest that the virus infecting melon is a strain of CMV. Similar results were previously reported by other workers concerning the response of test plants to CMV inoculation (Palukaitis and Garcia-Arenal, 2003).

A part of our results show that CMV can infect wide range of weeds (9 of 35) in the melon and neighboring fields; *P. oleraceae*, *B. vulgaris*, and *C. murale* were more abundance and persisting round the year in both protected and open field. Since CMV is easily transmitted by several species of aphids in non-persistent manner (Palukaitis and Garcia-Arenal, 2003), the virus is introduced into the field primarily by aphids from its sources. The wide spread of CMV from plant to plant in the field and from vicinity may be attributed to the wide distribution of weed plants which acts as virus reservoir in the presence of aphid vectors. It was reported that aphids acquire the virus during brief probes on weeds carrying the virus in the field (Zehnder et al., 2000).

Various strategies, including avoidance of virus sources of infection and control of aphids vectoring the virus, were carried out to manage CMV using specific insecticides which were revealed to be ineffective. Therefore, our research was oriented to test the efficiency of inducing systemic resistance in the plants against CMV using plant growth promoting rhizobacteria (*P. fluorescens*). Results show that seeds and soil treatments with bacterial suspension induced high reduction in virus accumulation in the plants as proved by decrease of ELISA absorbance at 405 nm for samples from treated plants when compared with those from plants grown from non-treated seeds or in non-treated soil. Since there is no direct contact between *P. fluorescens* cells and the virus, the effect of the bacteria against the virus is probably indirect through inducing certain compounds that have antiviral activity.

It has been reported that treatment of plants with variety of agents (biotic and non-biotic) induced systemic resistance against plant pathogens, locally and systemically (Walters et al., 2005; Al-Ani et al., 2011). The resistance induced is characterized by restriction of pathogen growth and suppression of disease symptoms development. Reduction in disease incidence and severity of symptoms of CMV was reported in greenhouse following treatment of tomato plants by strains of *Bacillus* species.

The bacteria induced a signi-ficant reduction in disease severity based on symptoms development, and decreased the accumulation of the virus in the plants based on ELISA-reactions absorbance at 405 nm when incorporated into potting mix (Zehnder et al., 2000; Murphy et al., 2003).

Induce systemic resistance (ISR) may occur as a result of the activation of the defense gene in the plants encoding for proteins that may act directly as antiviral agents, or could be enzymes such as peroxidase and phenylalanine ammonia lyase, which may play a role in phenolic compound metabolism which in turn lead to the production of antiviral substances. It has been reported that increase in peroxidase correlated with resistance in plant against pathogens (Young et al., 1995). Other studies reported that ISR is related with peroxidase and phenylalanine ammonia lyase (Chen et al., 2000; Zheng et al., 2005).

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