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ENZYMATIC ACTIVITY AS BIOCHEMICAL MARKERS FOR DETECTING CUCUMBER AND SQUASH TOLERANCE TO NEMATODE INFECTION

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Abstract

Classical host suitability designations of three cucumber and three squash cultivars were used for the plant-parasitic nematode Meloidogyne javanica infection. Enzyme assays were implemented concerning three systems of enzymes peroxidase, esterase and acid phosphatase in M. javanica-infected and non-infected plant roots in order to contrast such designations with a novel approach of using enzymatic activity as biochemical markers for their rating. Under uninfected conditions, the cucumber cultivar Royal Sluis revealed peroxidase activity higher than the two other cultivars. After nematode infection, however, such an activity reached as high as 103.8% at Alzaeem cultivar which might confirm the classical rating of its tolerance to nematode infection. The enzyme activity increased after infection but to a less degree in the other two susceptible cultivars of cucumber as compared to the uninfected plants. On the other hand, the uninfected squash cultivar Arlika, rated as nematode tolerant because there was no significant difference between growth parameters of uninfected and infected plants which had high nematode reproduction capability, manifested the highest peroxidase activity. Yet, the enzyme activity generally decreased in the three squash cultivars after infection as compared to the untreated controls except esterase enzyme in Arlika cultivar where a 75.7% increase in its activity occurred after nematode infection.

Key Words: ***Meloidogyne javanica, Relative susceptibility, Enzymes, Electrophoresis.***

Introduction

Increasing yield of cucurbits can be obtained through reducing losses in yield due to nematode infection. Plants generally respond to pathogen invasion by activation of a series of local and systemic defense mechanism. Plant resistance is determined by genes of the host and enzymes are gene products which can be detected using electrophoresis (Reuveni *et al* 1992). Koraiem (1979) reported that *Meloidogyne incognita* and *M. javanica* were the dominant knotting nematode species isolated from the infected roots in fields of cucumber in nine localities of the northern region of Egypt. Inducible defenses of plants against nematodes include accumulation of peroxidase and superoxide dismutase (Zacheo *et al* 1995). Halliwell and Gutteridge (1989) speculated that hydrogen peroxide, formed under biotic

and abiotic stress, exhibited cytotoxicity and its removal is accomplished by the action of catalase. Peroxidase is a key enzyme in several stages of disease development in many host-pathogen interactions and its high activity is often related to resistance expression (Johnson and Lee, 1978).

Shukla and Chakraborty (1988) found that *M. incognita*-resistant varieties of tobacco and tomato had significantly higher peroxidase enzyme specific activity than susceptible varieties. In a pot experiment with seedlings of tomato cv. VFN8 (resistant to *M. incognita*) and cv. Roma VF (susceptible) inoculated with 60 *M. incognita*-infective juveniles, Zacheo *et al* (1988) found that the peroxidase activity increased in all cell fractions of plant roots but less for the susceptible cultivar. Sharma (1991) stated that the pre-infectional activities of acid phosphatase were found to be lower in the roots of isogenic resistant plants than isogenic susceptible ones. After nematode inoculation, the activity of this enzyme increased in the roots of isogenic resistant plants. Bisztray *et al* (1997) found that polyacrylamide gel electrophoresis (PAGE) system was evaluated for the separation of protein products of the Aps-1 (acid phosphatase) gene, an allele of which is a biochemical marker for the nematode resistance allele Mi used in tomato breeding. In this respect, various breeding lines of tomato were tested for resistance to the root-knot nematode *M. incognita* and clear band resolution of products of the Aps-1 alleles was obtained (Medina-Filho and Tanksley, 1983).

When susceptible (S-12) and resistant (NR-7) tomato cultivars were inoculated with *M. incognita* in a pot experiment, resistant plants had an increase in peroxidase activity following inoculation compared with susceptible plants (Sharma 1993). Mohamed *et al* (1999) reported that three tomato cultivars reacted differently, based on root gall index and significant plant damage, to *M. incognita* infection under greenhouse conditions where the activity of the enzymes peroxidase and polyphenol oxidase increased within infected roots over their controls in the order Dual Large > Castle Rock > Strain B, indicating consistency with the cultivar reaction to the nematode. Mohamed and Abd-Elgawad (2003) found that the peroxidase enzyme activity in *M. incognita*-resistant tomato cultivar was highly induced 2-8 days after nematode inoculation, while it slightly increased in infected susceptible cultivar. The enzymatic activity was mostly induced in the cytoplasm fractions of the resistant cultivars Bark and Nematode-1400 with 73.7 and 81% increase over their non-infected check after 8 days of nematode infection, respectively. Thus, the flexibility and swiftness of using such enzyme activity in rating plant genotypes for nematode resistance may enhance their potential utility as biochemical markers especially to expedite breeding programs. Currently, many of the available germplasm resources

remain to be characterized concerning their resistance to nematodes (Starr and Roberts 2004).

The objective of this study was determination of some enzyme activities in some cucumber and squash cultivars after infection with nematodes using electrophoresis analysis to detect biochemical markers to assist selection for nematode-resistance.

Materials and Methods

Plant material

Seeds of cucumber, *Cucumis sativus*, cultivars Alzaeem, Royal Sluis and Alnems as well as squash (*Cucurbita pepo* var. Melopepo) cultivars Arlika, Alexander and Alexander Hybrid were used in this study.

Growth, inoculation and rating procedures

The procedures were reported elsewhere (Abd-Elgawad *et al* 2007)

Enzymes electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was used to study enzymes of esterase, peroxidase and acid phosphatase. Enzyme electrophoresis was performed according to Stegemann *et al* (1980). Esterase and acid phosphatase analyses were performed according to Jonathan and Wendel (1990) and peroxidase analysis was carried out using method of Graham *et al* (1965). Gels were photographed and scanned by Gel Doc Bio-Rad System (Gel - Pro analyzer V. 3), to determine the enzymatic activities.

Extraction of isozymes

One gram roots from 60-day old plants representing each of the above-mentioned *Meloidogyne javanica*-infected and non-infected cultivars were extracted with 1 ml extraction buffer (pH 7.5). Each sample was vortexed for 15 seconds by electric vortex and centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant was transferred to new eppendorf tube and kept at deep-freeze until use for electrophoretic analysis.

Extraction buffer

50 mM Tris-HCL buffer, pH 7.5 (0.61 g); 5% Glycerol (5 ml), 14 mM mercaptoethanol, 0.1% V/V (100 µl) and H_2O (d.w) up to 100 ml.

Application of samples and electrophoresis conditions

A volume of 50 µl extract of each sample was mixed with 10 µl bromophenol blue, a volume of 60 µl from this mixture was applied to each well of the gel. The gel was completely covered with electrode buffer (tris-

glycine buffer). The electrodes were connected to power supply and adjust at 200 V for two hours.

Enzymatic assays

The gels were stained after electrophoresis according to its system and incubated at 37°C in dark for complete staining after adding the appropriate substrate for each enzyme and staining solution as follow;
Peroxidase enzyme: 1M Na-acetate, pH 4.7 (50 ml); Methanol (50 ml); 3, 3`, 5, 5`-tetramethylbenzidine (50 ml); 30% H_2O_2 (2 ml).

Esterase enzyme: 100 mM Na-phosphate, pH 6.0 (50 ml); α-naphthyl acetate (25 mg); β-naphthyl acetate (25 mg); Fast blue RR salt (50 mg). Acid phosphatase: 50 mM Na-acetate, pH 5.0 (50 ml); Na-α-naphthyl acid phosphate (50 mg); $MgCl_2$ (50 mg); Fast Garnet GBG salt (50 mg).

RESULTS AND DISCUSSION

The relative susceptibility of the cucumber and squash cultivars to *M. javanica* was investigated in greenhouse tests by Abd-Elgawad *et al* (2007). In general, as a result of infection by *M. javanica,* the weight and length of the infected plants were decreased contrary to the healthy plants (Table 1). Yet, damage of cucumber and squash cultivars varied, within each species, as a result of nematode infection. For example, growth parameters of cucumber cultivar Al-Zaeem did not significantly differ between *M. javanica*-infected and non infected plants whereas such a difference was significant concerning the other cucumber cultivars. Likewise, squash cultivar Arlika was not significantly affected by the nematode invasion contrary to the others (Table 1). These variations in the growth parameters of the tested cultivars may reflect the extent of plant tolerance to *M. javanica* infection. Hence, it is recommended to cultivate tolerant cultivars (Alzaeem and Arlika) in nematode-infested soil whereas susceptible cultivars (e.g., Royal Sluis and Alexander) should be planted in nematode-free soil.

Peroxidase activity

The peroxidase enzyme extracted from roots of the three cucumber and three squash cultivars under control and infection with *M. javanica* conditions showed a considerable variation in its activity as shown in Figure (1-A) and Table (2). Under uninfected conditions, the cucumber cultivars Royal Sluis and Alnems revealed peroxidase activity higher than Alzaeem. Under infected conditions, the enzyme activity increased in the three cultivars of cucumber but Alzaeem demonstrated the highest enzymatic activity (103.8%) which suggests that this cultivar may be tolerant to *M.*

Table 1. Number of galls, gall index and growth parameters of three cultivars of cucumber and three cultivars of squash grown in sterilized and *M. javanica*-infested soils[#].

Crop and Cultivar	No. of Galls	Gall Index[†]	Plant growth			
			Shoot		Root	
			Length (cm)	Weight (g)	Length (cm)	Weight (g)
Cucumber						
Alzaem (control)	-	-	44	7.5	24	1
Alzaeem+ Nematode	44	3	40	6	20	0.8
Royal Sluis (control)	-	-	48.6	6.5	17.7	0.6
Royal Sluis +Nematode	31	3	36**	2.5**	15.5	0.5
Alnems (control)	-	-	54	4.8	22	0.5
Alnems+ Nematode	19	2	41*	3*	16.5*	0.3*
Squash						
Arlika (control)	-	-	28	26.5	30	5
Arlika + Nematode	90	4	29	26.7	30.5	5
Alexander (control)	-	-	34	33	33	8
Alexander+ Nematode	159	5	36	27	26*	6**
Alexander Hybrid (control)	_	_	36.5	26	29	5.5
Alexander Hybrid+ Nematode	120	5	32 **	23.6	35	8.6

#: The values of the Table were taken from a study by the author (Abd-Elgawad *et al* 2007), each value is the mean of ten replicates.

†: Plants were rated on a 0-5 scale where 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100 and 5 = > 100 galls per root system.

*, **: Indicate significant differences between nematode-treated cultivar and its non-treated check at 0.5 and 0.1 probability levels, respectively.

javanica infection since its growth parameters were not affected by nematode attack (Table 1). Such a suggestion was confirmed by the number of nematode galls on all cucumber cultivars (Table 1) as well as the fact that the other two cultivars suffered from the nematode infestation as soon as 60 days after inoculation in the environmental conditions used. Similar

Table 2. Enzyme activity[+] as estimated from electrophoretic patterns for three cucumber and three squash cultivars.

Crop and Cultivars	Peroxidase activity			Esterase activity			Acid phosphatase activity		
	uninfected	infected	% change	uninfected	infected	% change	uninfected	infected	% change
Cucumber									
Alzaeem	26.1	53.2	+ 103.8	7.4	12.1	+ 63.5	11.5	14.3	+ 24.3
Royal Sluis	43.9	48.0	+ 9.3	16.2	23.7	+ 46.3	12.5	17.7	+ 41.6
Alnems	37.6	43.5	+ 15.6	13.4	14.5	+ 8.2	11.3	13.8	+ 22.1
Squash									
Arlika	49.5	5.0	- 89.8	23.5	41.3	+ 75.7	22.3	16.3	- 27.0
Alexander	-	-	-	48.6	20.2	- 58.4	20.5	17.9	- 12.6
Alexander Hybrid	23.0	19.0	- 17.3	52.8	42.7	- 19.1	41.9	17.8	- 57.5

+: The density area of enzyme used to measure its activity is considered as the percentage of the total area of the lane from start to end points of migration.

suggestion was reported for "NemaSol" tomato against *M. incognita* (Molinari and Abd-Elgawad, 2007). Such a reaction of Alzaeem cultivar should further be explored to investigate whether this cultivar can demonstrate tolerance to a virulent biotype of *M. javanica*.

On the other hand, the peroxidase activity decreased in the squash cultivars after infection with nematode as compared to the control (Fig. 1-A and Table 2) which might agree with the number of nematode galls recorded. The uninfected squash cultivar Arlika manifested higher peroxidase activity than Alexander Hybrid, while the enzyme activity was not observed in the lanes of squash cultivar Alexander. Arlika cultivar is considered more tolerant than the two other cultivars of squash especially because its growth parameters were not affected by nematode infection.

The previous results indicated that peroxidase activity can be used as biochemical marker related to tolerance of cucumber cultivars against infection by *M. javanica*. These results are in accordance with those of Shukla and Chakraborty (1988) who showed that *M. incognita* resistant varieties of tobacco and tomato had significantly higher peroxidase enzyme specific activity than susceptible varieties.

Esterase activity

The esterase enzyme extracted from roots of three cucumber and three squash cultivars under uninfection and infection with *M. javanica* is illustrated in Figure (1-B). The esterase activity increased in *M. javanica*-infected plants of the three cucumber cultivars Alzaeem, Royal Sluis and Alnems (63.5%, 46.3% and 8.2%, respectively) as compared to the controls (Table 2). The cultivar Alzaeem appeared more tolerant than the two other cucumber cultivars. Concerning the squash cultivars, the cultivar "Arlika" revealed high esterase activity (increase of 75.7%) under infection conditions as compared to the uninfected plants. On the contrary, the esterase activity decreased after infection in the other two squash cultivars, i.e. Alexander (58.4%) and Alexander Hybrid (19.1%). These results coincided with those of plant growth parameters, where the cultivar "Arlika" was not significantly damaged after infection as compared to the control (Table 1). These results revealed that the squash variety "Arlika" was tolerant against nematode infection.

Acid phosphatase activity

Activity of the acid phosphatase enzyme extracted from roots of the three cucumber and three squash cultivars under uninfection and *M. javanica*-infection conditions is shown in Figure (1-C). The acid phosphatase activity increased in infected plants of the three studied

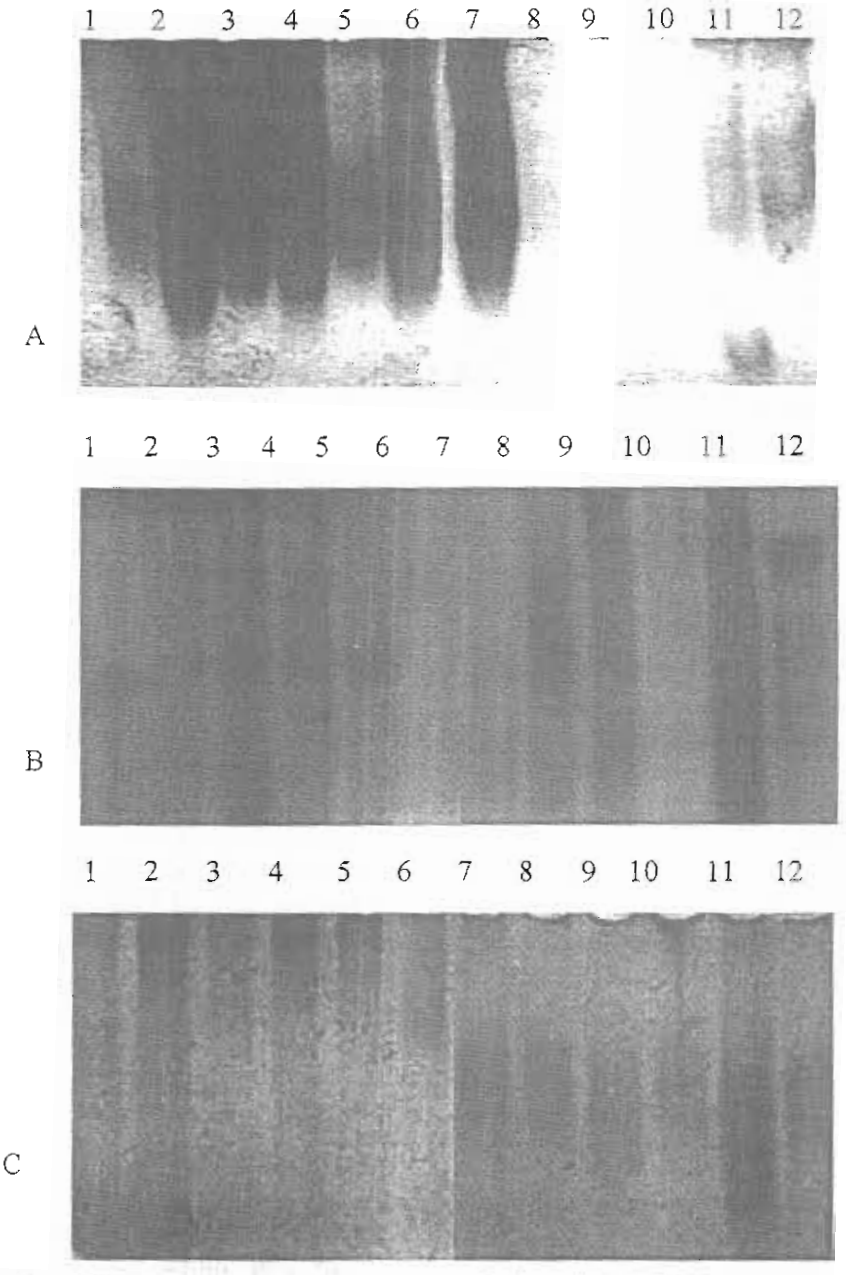

Fig. 1 (A). Peroxidase, (B) Esterase and (C) Acid phosphatase patterns for three cucumber cultivars Alzaeem, Royal Sluis and Alnems (1-6 lanes) and three squash cultivars Arlika, Alexander and Alexander Hybrid (7-12 lanes). Numbers 1, 3, 5, 7, 9 and 11 are controls, and 2, 4, 6, 8, 10 and 12 were infected with *M. javanica*.

cucumber cultivars Alzaeem (24.3%), Royal Sluis (41.6%) and Alnems (22.1%) as compared to the uninfected plants (Table 2). In contrast, the acid phosphatase activity decreased after infection with nematode in the three squash cultivars Alrika, Alexander and Alexander Hybrid by 27%, 12.6% and 57.5%, respectively. These results are in agreement with those of Sharma (1991) who found similar activity of acid phosphatase isozymes in the roots of cucumber plants after inoculation with nematode.

In General, the three studied enzymes can be used as biochemical markers to detect the injury with nematode, and related positively to tolerance against nematode infection especially in cucumber cultivars. Instead of the routine work which consume a lot of efforts, time and materials, e.g. determining peroxidase activity spectrophotometrically by measuring the increase in absorbency at 470 nm due to oxidation of guaiacol (Mohamed *et al* 1999), the gel was directly scanned and analyzed in the present study by Gel Doc Bio-Rad System (Gel-Pro Analyzer V. 3).

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النشاط الإنزيمي بوصفه دلالات بيوكيميائية لإستبيان تحمل العدوى بالنيماتودا . في الخيار والكوسة

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تم عدوى ثلاثة أصناف خيار هى الزعيم ، رويال سليس والنمس وثلاثة أصناف كوسة هى أرليكا ، الكسندر والكسندر هيبرد بنيماتودا ***Meloidogyne javanica*** – في دراسة سابقة للباحث - حيث زادت أعداد هذه النيماتودا فى أخر التجربة عن أعدادها فى بدايتها (معدل التكاثر أكثر من واحد) مما يعنى أن جميع الأصناف المختبرة عوائل جيدة لتكاثر النيماتودا. وقد اختلفت الأصناف فى مدى الضرر الحادث لها بسبب الإصابة بالنيماتودا فمثلا تحمل صنف الخيار الزعيم الإصابة ولم تظهر فروق معنوية فى مؤشرات نموه بين النباتات المعدية بالنيماتودا وغير المعدية بها في حين تأثر الصنفان رويال سليس والنمس بالإصابة ، وبالمثل لم يتأثر صنف الكوسة أرليكا جوهريا بالعدوى بالنيماتودا فى حين تاثر الصنفان الكسندر، الكسندر هيبرد. تم عمل تحليل لإنزيمات البيروكسيديز، الأستيريز والأسيد فوسفاتيز في جذور نباتات سليمة ومصابة بالنيماتودا لمقارنة نتائج التجربة الحقلية مع نتائج النشاط الإنزيمي بوصفها دلالات بيوكيماوية لتحديد مدى تحمل العدوى. أوضح صنف الخيار رويال سليس قبل العدوى نشاط أعلى لإنزيم البيروكسيديز عن الصنفين الآخرين. بعد العدوى بالنيماتودا أظهر الصنف الزعيم أعلى نشاط لإنزيم البيروكسيديز وصل الى ١٠٣,٨% زيادة عن الكنترول (بدون عدوى) ، مما يؤكد الى حد ما نفس تصنيف تحمل الأصناف المذكورة للعدوى بالإعتماد على مؤشرات النمو التقليدية في التجربة الحقلية. كذلك زاد نشاط إنزيم البيروكسيديز بعد العدوى في الصنفين الآخرين ولكن بنسبة أقل. أعطى صنف الكوسة أرليكا – قبل العدوى - نشاط أكثر لإنزيم البيروكسيديز عن صنفي الكوسة الآخرين. قل نشاط الإنزيمات الثلاثة في الأصناف الثلاثة للكوسة محل الدراسة بعد العدوى مقارنة بالكنترول ، ما عدا الصنف أرليكا حيث زاد نشاط إنزيم الأستيريز بنسبة ٧٥,٧% مقارنة بالكنترول.

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