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Changes of Amino Acids and Protein Patterns in some Cucumber Cultivars Infected by *Pseudoperonospora cubensis*

M.A. Swelim*, S.M. El-Desouky, S.M. Mahmoud and E.M. Hussein

**Bot., Dept., Fac., Sci., Benha, Zagazig University and Plant Path., Res., Instit., Agric., Res., Center, Giza, Egypt.*

AN APPROACH to determine the changes of free amino acids and protein patterns in different healthy cucumber cultivars and infected with downy mildew caused by *Pseudoperonospora cubensis* under greenhouse conditions was made using sodium dodecyle sulphat-polyacrylamide gel electrophoresis (SDS-PAGE). Results revealed that, total free amino acids were higher in the highly susceptible cultivar than in the least susceptible ones. Downy mildew infection increased total free amino acids in the infected plants compared with the healthy ones. Data obtained also showed that downy mildew infection caused an increase in protein bands from the infected leaves compared with the healthy ones. New types of proteins bands were found in the infected plants using finger print for both healthy and infected susceptible and least susceptible cultivars.

Changes in proteins and total free amino acids in different infected plants have been reported by many investigators (Ali, 1984; Mohamed *et al.*, 1995 and El-Kafrawy, 1997). Infection of cucumber with powdery mildew increased total free amino acid contents in leaves and stems of different cucumber cultivars and the magnitude of increase was more pronounced in samples of the highly susceptible cutlivars (Ali, 1984). Also, downy mildew infection of cucumber increased total free amino acid in both resistant and susceptible cultivars (Mohamed *et al.*, 1995).

Gel electrophoresis is a powerful technique with numerous applications in plant pathology. Plant pathologists have adopted this technique to settle some taxonomic disputes, identify "unknown" cultures, "fingerprint" patentable fungal lines and plant cultivars, trace pathogen spread and determine ploidy levels of fungi and other plant pathogens (Bonde *et al.*, 1993). Antoniwa *et al.* (1980) reported that proteins accumulated in the pathogen-infected plant tissues in response to infection, are termed "pathogenesis-related" (PR) proteins. Sang and Joo (1992) mentioned that total soluble proteins isolated from rice plants infected with leaf blight disease, caused by *Xanthomonas oryzae pv. Oryzae,* showed polypeptide changes. Marititano *et al.*, (1994) recorded that during conservation, the ascorbate oxidase enzyme (extracted from *Cucurbita pepo* fruits) becomes progressively dissociated in molecular form with a molecular weight of 70 000

Da. Gouvea *et al.*, (1995) studied total, soluble and microsomal fraction proteins in leaves of healthy and virus-infected cucumber and tomato plants by DSA-PAGE, and reported the accumulation of a microsomal fraction protein in severely infected plants. The proteins that accumulated in the host, showing severe disease symptoms, exihibited the same electrophoric mobility and had a MW of 39 Kda. Bonfitto *et al.*, (1999) mentioned that the use of capillary electrophoresis (CE) in conjunction with SDS-PAGE increased the possibility of its use for cultivar identification.

The present investigation aimed to study the effect of downy mildew disease on the concentration of total free amino acids and protein patterns in some cucumber cultivars grown under greenhouse conditions.

Material and Methods

Cucumber cultivars

Three susceptible cucumber cultivars (Pasandra, Betostar and Rawa) and two least susceptible ones (Premoand Marmar) were tested in this study during the growing seasons (autumn and spring) of the two successive years 1998 and 1999. Fresh cucumber leaves previously naturally infected with downy mildew were collected from the greenhouse, 7 days after the first symptoms appeared at each age of sampling. Disease severity was estimated according to the grading system adopted by Horsfall and Barrtt (1945).

Determination of total free amino acids

Free amino acids were determined in the ethanolic extract of naturally infected and fresh cucumber leaves at different growth stages during the two seasons (autumn and spring) according to the method described by Rosein (1957).

Reagents

a- Stock of sodium cyanide (NacN) 0.01 M (0.49 mg/ml).
b-Acetate buffer (pH 5.3 – 5.4) : 270 g sodium acetate.
$3H_2O$ + 2 L. H_2O ÷ 500 ml galacial acetic acid and made up to 7.5 L with H_2O.
c-Cyanide acetate buffer: 0.0002 M sodium cyanide in acetate buffer (20 ml of stock solution) and made up to one liter with acetate buffer).
d-Ninhydrin 3% in acetone.

Procedure

Five ml of ethanolic extract, 0.5 ml of cyanide acetate buffer and 0.5 ml 3% Ninhydrin were added and the mixture was then heated in boiling water bath for 10 min. After cooling of the reaction mixture under running water, 5 ml of isopropyl alcohol : water mixture (1:1 v/v) were added and the developing colour was measured using spectronic 20 at 570 nm. Free amino acids type in different samples (three replicates) were calculated as mg/g per g fresh weight sample.

Electrophoretic studies

Electrophoretic detection of protein was carried out by sodium dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE).

Polyacrylamide gel electrophoresis (PAGE) was used to determine the quantiative changes that occur in the soluble proteins of the least susceptible *c.v.* (Premo) and highly susceptible one (Betostar) of both inoculated and uninoculated plants (Borglie *et al.*, 1986).

Protein extraction

Leaves showing different degrees of disease severity (0 to 7 scores) were taken. Two g. from each sample were ground in .05 M sodium acetate buffer + sea sand in a mortar in liquid nitrogen at 4°C. After that, 50 mg of the extract plus 0.7 ml of ES (4% SDS, 5% Sucrose, 50% mercaptoethanol) were shaken for 10 min at room temperature with gentle steering. The extract was centrifuged at 18,000 rpm for 30 min and the clear supernatant was heated at 100°C for 2-5min and then cooled to room temperature. Proteins were precipitated by adding cold (20°C) acetone (8x volume of the supernatant). Protein content of the supernatant was determined using the method described by Ekramoddaullah and Davidson (1995). Bovine serum albumin (BSA) was used as a standard.

Staining of protein bands

The silver staining method for protein described by Hochstrasser *et al.*, (1988) was used, where the membrane was stained with 0.1% coomassie blue R-250 in 50% methanol for 48 min and then destained in 50% methanol for 8 min at room temperature. The membrane was then rinsed with water for 10 min. and the stained membrane was scanned by a lasar scanner. Proteins separated by SDS-PAGE were electrophoretically transferred to immobilon-P membrane, as described by Matsudaira (1987).

Results

Total free amino acids

(a) Autumn season

Data in Table 1 demonstrated that, after 35 days from planting the healthy cultivars Pasandra and Premo contained (6.95 and 5.67 mg free amino acids / g. fresh weight, respectively), which is higher than that in Marmar and Betostar (4.04 and 3.25 mg/g. fresh weight, respectively). After 55 and 75 days, the highest total free amino acids were recorded in Marmar and Pasandra (4.67 and 3.11 mg, 5.5 and 7.99 / g fresh weight, respectively.

The infected *c.v.* Betostar and Pasandra contained higher total free amino acid (8.91 and 7.73 mg /g fresh weight, respectively) at 35 days from planting. The same results were also recorded at 75 days from planting, while at 55 days the infected *c.v.* Marmar and Pasandra recorded the highest amounts of total free amino acids (7.25 and 6.53 mg /g. fresh weight, respectively).

TABLE 1. Effect of downy mildew disease on the concentration of total free amino acids (mg/g fresh weight) in healthy and infected different cucumber cultivars during autumn and spring seasons.

Days after planting	Cultivars	Season / total free amino acids (mg/g fresh weight)					
		Autumn			Spring		
		H	I	Increase %	H	I	Increase %
35	Premo	5.67	7.59	33.86	2.56	3.74	46.09
55		2.75	3.78	37.45	2.91	4.45	52.92
75		3.19	5.95	86.52	4.59	7.26	58.16
35	Marmar	4.04	6.10	50.99	1.47	1.99	35.37
55		4.67	7.25	55.24	2.69	4.56	69.51
75		5.5	5.75	4.16	5.15	8.56	66.21
35	Pasandra	6.95	7.73	11.22	2.51	3.83	25.58
55		3.11	6.53	109.96	4.67	8.13	47.08
75		7.99	8.75	9.51	5.44	9.40	72.79
35	Betostar	3.25	8.91	174.15	1.17	2.82	141.02
55		2.56	5.63	119.92	3.74	6.99	86.89
75		4.10	7.74	88.78	4.90	8.94	82.44
35	Rawa	-	-	-	2.42	9.61	297.1
55		-	-	-	4.94	8.22	66.39
75		-	-	-	4.32	9.65	123.3

H : healthy
I : Infected leaves about 20, 40, 60% disease severity at 35, 55 and 75 days respectively.

In general, the total free amino acids decreased then increased with the increase of the plant age in the healthy and infected cultivars except in Marmar.

It could be also observed that, *P. cubensis* infection increased total free amino acids in leaves of the high susceptible (Betostar, Pasandra and Rawa) and the least susceptible cultivars (Marmar and Premo). The high susceptible cultivars contain higher total free amino acids than the least susceptible ones.

(b) Spring season

Data presented in the same Table show that, 35 days after planting, the healthy leaves of Pasandra and Premo contained higher total free amino acids (2.51 and 2.56 mg/g. fresh, weight, respectively) as compared with c.v. Marmar, Rawa and Betostar (1.47, 2.42 and 1.17 mg /g. fresh, weight, respectively). After 55 days from planting, the healthy cultivars Rawa, Pasandra and Betostar contained the highest total free amino acids (4.94, 4.67 and 3.74 mg / g. fresh weight, respectively) than Premo and Marmar(2.91 and 2.69 mg/g. fresh weight, respectively), while at 75 days higher total free amino acid content was recorded in Pasandra and Marmar (5.44 and 5.15 mg /g. fresh weight, respectively).

Total free amino acids at 35 days from planting date were higher in the infected cultivars Rawa and Pasandra(9.61 and 3.83 mg / g. fresh weight). At 55

days the highest total free amino acids were recorded in Rawa, Pasandra and Betostar (8.22, 8.13 and 6.99 mg / g. fresh weight, respectively), compared with the least susceptible ones Premo and Marmar (4.45 and 4.56 mg/g. fresh weight, respectively), the same result was recorded after 75 days of planting.

From the data presented it could be seen that, downy mildew infection increased total free amino acids and that the susceptible cultivars contained higher amounts of total free amino acids compared with the least susceptible ones especially at 55 and 75 days from planting. Data also showed that the total free amino acids in the infected *cvs.* increased with increasing the plant age in all the tested *cvs.* The same results were recorded in the healthy *cvs.* except for *cv.* Rawa.

Generally, it could be concluded that, downy mildew infection increase total free amino acids in both seasons. The susceptible cultivars contain higher total free amino acids than the least susceptible cultivars and the content of total free amino acid increased with increasing the plant age in both seasons except for healthy *cv.* Rawa at spring season and infected *cv.* Marmar at autumn one.

Electrophoretic studies

This experiment was carried out to document protein patterns of the two cucumber cultivars Primo and Betostar and to develop fingerprinting patterns for the disease severity, since the pathogenic fungi is an obligate parasite. The results revealed the possibility of using electrophoretic patterns to differentiate cultivars and the levels of disease severity. A photograph of the Gel electrophoretic patterns of proteins obtained from the extracts are presented in (Fig. 1).

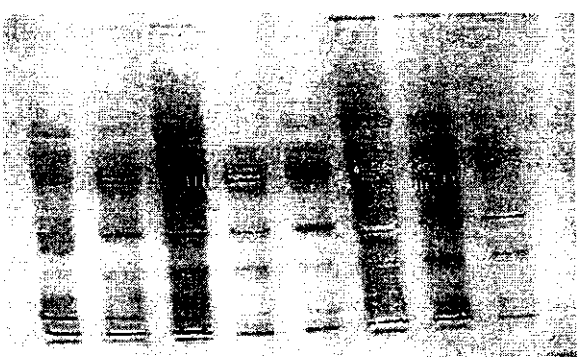

Fig. 1. Photographic protein patterns obtained from leaves extracts from 8 samples of two cucumber cultivars during four different levels of disease severity. Cultivars: Samples 1-4, for Betostar and 5-8 for PremoGrade of disease severity : Healthy (grade 0) = samples 4&5, Diseased (grade 3) = samples 3 & 6, (grade 5) = samples 2& 7 and (grade 7) = samples 1 & 8.

The data presented in Fig. 2 & 3 show denistometer scanning of protein profiles obtained from samples of the two cucumber cultivars Premo and Betostar. The drawn profiles of the protein patterns of all the eight samples are given in Fig. 2,3 for illustration of the position, molecular weight in KD, percentage area and the total scanned area of separated protein bands.

Data presented in Fig. 2, 3 also show that healthy samples of the investigated cultivars Betostar and Premo (lanes 4, 5) gave 9 sharp and equally stained protein bands for each one. A single band of 59-62 KD at R_f value of 0.43 was characteristic to the cultivar Betostar while the cultivar Premo was distinguished with another single band at R_f value of 0.51 with molecular weight of 49-52 KD. Eight bands were completely similar in molecular weight, namely bands No 1 and bands No 3 to 8 (Fig. 2). Such characteristic single band disappeared in case of the cultivar Premo. The characteristic band (No 2) appeared clearly in all diseased samples.

Since the causal pathogen of the disease is an obligate parasite, protein bands of the fungus only could be determined but it seems that the fungus shares the host plant almost in all their 9 protein bands. Moreover, diseased plants contained more bands than healthy samples 4 of the 6 lanes of diseased samples (lanes 1, 3, 6 and 7) as compared with the healthy samples (lanes 4 & 5). In the four grades of diseased samples, the fungus was distinguished by bands separated at R_f value of 0.32, 0.72, 0.33 or 0.26 with molecular weight of 27-30 KD and R_f of 1.46 with molecular weight of 6-9 KD.

The fungal host protein could be more clearly monitored in Fig. 3 percentage area of scanned protein bands in the healthy samples (lanes 4 & 5%) was generally reduced in most bands with few exceptions. These excepted bands may refer to the fungal protein.

Data presented in Fig. 3 show the total area of stained protein area in each band. The sum of the total protein areas shown in the last row, clearly indicate that the least total area was recognized in Premo cultivar either in healthy sample or the diseased sample with high severity. On the other hand, the least total area of stained protein in case of Betostar was recognized in the healthy sample, followed by the diseased samples with moderate and high severity. In both cultivars the maximum total area was recognized in diseased samples with low severity.

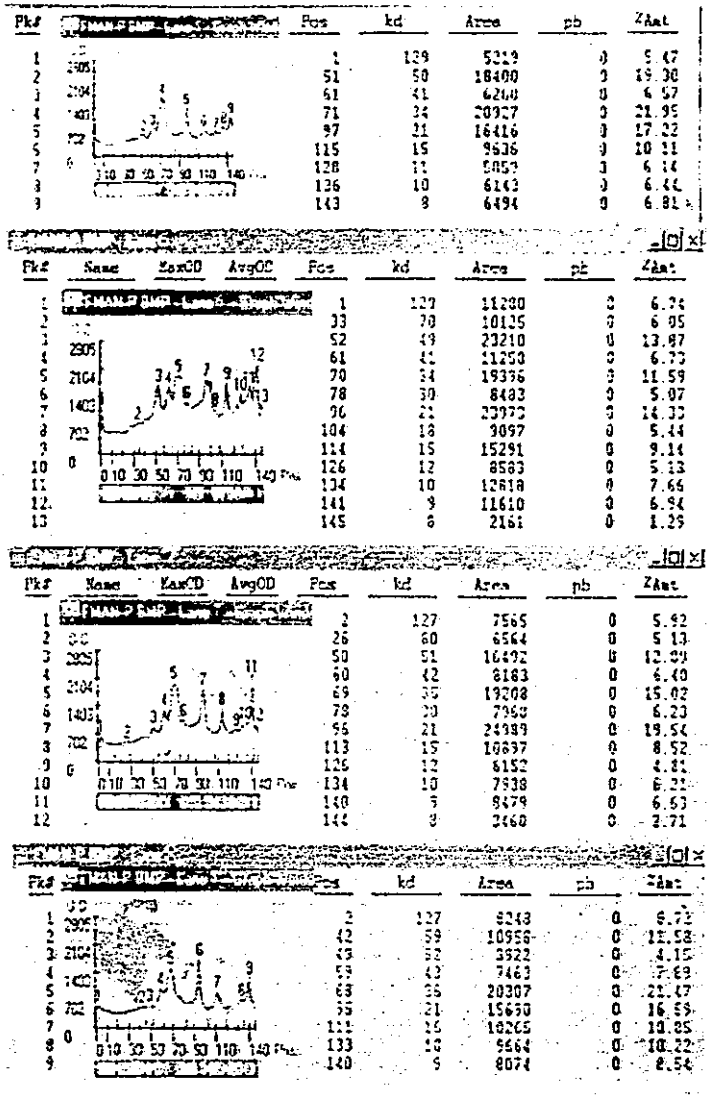

Fig.2. Densitometer scanning of protein patterns obtained from samples extracts from the cucumber cultivar Betostar with four different levels of disease severity. Grade 0 = 0% , Grade 3 = 6.12%, Grade 5 = 12.50% Grade 7 = 50.88% of infected leaf area.

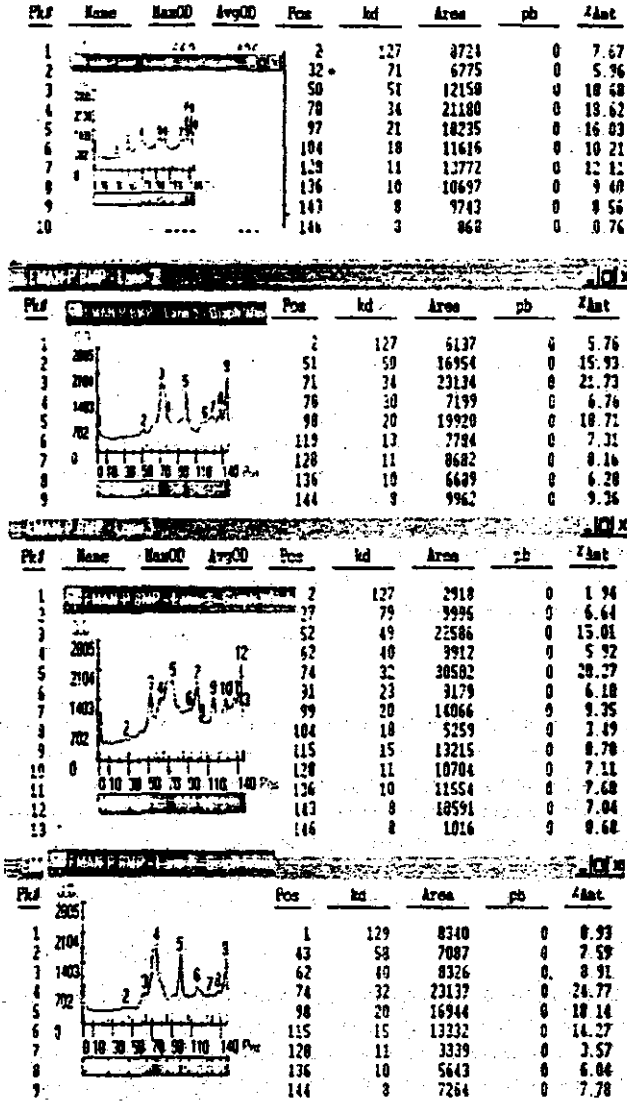

Fig. 3. Densitometer scanning of protein patterns obtained from samples extracts from the cucumber cultivar Premo with four different levels of disease severity. Grade 0 = 0% , Grade 3 = 6.12%, Grade 5 = 12.50% Grade 7 = 50.88% of infected leaf area.

Discussion

The results obtained show that, higher amount of total free amino acids were detected in the infected leaves of highly susceptible cv. Than in the healty least susceptible one. It could be noted that *P. cubensis* infection increased total free amino acids in the leaves of both susceptible and least susceptible *cvs.* The results are in agreement with those obtained by El-Korachy (1991) and Mohamed *et al.* (1995). The increase in amino acids or the synthesis of new amino acids may be due to the host – pathogen interaction. Amino acids may accumulate in the infected leaves due to the blockage of protein synthesis or protein lysis caused by the pathogen as previously reported by Singth and Shukla (1987).

The results provide clear evidence for changes in protein pattern of cultivars after infection by *P. cubensis* and that the changes were higher in susceptible cultivar. These results may be explained on the basis that susceptible cultivar is more suitable for growth and reproduction of the pathogen causing some disappearance of proteins and formation of new proteins in infected plants. Infection with *P. cubensis* resulted in the induction of new polypeptides. These results may indicate the formation of pathogen-related protein (PR-Protein) as mentioned by Antoniw *et al.* (1980). Generally it could be concluded that (PR-protein) reflected a particular type of stress protein which are induced during the infection. The present results are in accordance with those reported by Sang and Joo (1992). Further studies are needed using more varities with different degrees of resistance and susceptibility to evaluate finger printing technique for differentiation of various cultivars.

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التغيرات فى الأحماض الأمينية والبروتين فى بعض أصناف الخيار المصابة بفطرة *سيدوبيرونوسبورا كيوبنسس*

محمود عبد المحسن سويلم*، شوقى محمد الدسوقى، سامى محمد محمود وإيمان محمود حسين

*قسم النبات – كلية علوم بنيا – جامعة الزقازيق و معهد بحوث أمراض النبات – مركز البحوث الزراعية – الجيزة – مصر .

فى تجربة لتحديد التغيرات فى البروتين والأحماض الأمينية الحرة فى بعض أصناف الخيار المختلفة المصابة بمرض البياض الزغبى تحت ظروف الصوبا باستخدام جهاز التفريد الكيربائى وجد أن :
بمقارنة المحتوى البروتينى للأنواع العالية القابلية للإصابة والأنواع الأقل قابلية للأصابة لأصناف الخيار المختبرة أوضحت النتائج أن محتوى الأوراق من الأحماض الأمينية الكلية كان عاليا فى الأصناف المتوسطة القابلية للإصابة.

أدت الإصابة إلى زيادة محتوى الأوراق من الأحماض الأمينية الكلية فى الأوراق المصابة عنها فى الأوراق السليمة.

كما أوضحت النتائج أيضا أن الإصابة بمرض البياض الزغبى أدت إلى زيادة محتوى أوراق النباتات المصابة من البروتين بالمقارنة بالنباتات السليمة وقد وجدت أنواع جديدة من البروتينات فى النباتات المصابة فى صورة باندات عن طريق تفريد البروتينات لكل من النباتات المصابة والنباتات السليمة وذلك باستخدام البصمة الوراثية.