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SCREENING FOR EFFECTS OF PHYTOCHEMICAL VARIABILITY ON CYTOPLASMIC PROTEIN SYNTHESIS PATTERN OF CROP PLANTS

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Abstract—Crop plants have to cope with phytochemical variability along with other environmental stresses. Allelochemicals affect several cellular processes. We tested the effect of toxic aqueous leachates from *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, and *Lantana camara* on the radicle growth and cytoplasmic protein synthesis patterns of *Zea mays* (maize), *Phaseolus vulgaris* (bean), *Cucurbita pepo* (squash), and *Lycopersicon esculentum* (tomato). 2D-PAGE and gel scan densitometry analysis were used to detect differences in cytoplasmic root protein pattern expression. High-, medium-, and low-molecular-weight cytoplasmic proteins were affected by the different aqueous leachates. Crop plant responses were diverse, but in general, an increase in protein synthesis was observed in the treated roots. Maize was the least affected, but both the radicle growth and also the protein pattern of tomato were severely inhibited by all allelopathic plants. The changes observed in protein expression may indicate a biochemical alteration at the cellular level of the tested crop plants.

Key Words—Allelochemical stress, allelopathy, mode of action, *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, *Lantana camara*, *Zea mays*, *Cucurbita pepo*, *Phaseolus vulgaris*, *Lycopersicon esculentum*, protein synthesis, phytochemical variability.

INTRODUCTION

Secondary plant metabolites that mediate interactions among different organisms are known as allelochemicals. These metabolites are released into the environment

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by different routes and belong to several chemical groups, including terpenes, alkaloids, phenolics, and flavonoids (Larcher, 1995). Plants, as sessile organisms, encounter an array of biotic and abiotic environmental constraints such as phytotoxic compounds, which can affect different cellular processes on target organisms. Effects on physiological processes are well known (Einhellig, 1995) and include disruption of membrane permeability (Galindo et al., 1999), ion uptake (Yu and Matsui, 1997), inhibition of electron transport in both the photosynthesis and respiratory chains (Calera et al., 1995a; Rimando et al., 1998; Abrahim et al., 2000), alteration of enzymatic activity (Calera et al., 1995b; Friebe et al., 1997; Politycka, 1999; Romagni et al., 2000), and inhibition of cell division (Cruz-Ortega et al., 1988; Anaya and Pelayo-Benavides, 1997).

Environmental stresses (biotic and abiotic) have also been shown to induce the synthesis of new proteins in plants. These proteins might have evolutionary value for survival under adverse environmental situations. Synthesis of such stress-induced proteins has been well documented under stress by salinity, drought, heat shock, cold, anaerobiosis, metal toxicity, and pathogenicity (Cruz-Ortega et al., 1997; Riccardi et al., 1998; Seki, 2001), but there are few reports about allelochemical stress (Baziramakenga et al., 1997; Koitabashi et al., 1997).

The plants from El Eden Ecological Reserve (Quintana Roo, Mexico) tested in this study were previously evaluated in our laboratory for their phytotoxic effects on other plants (Anaya and del Amo, 1999). Currently, we are conducting biodirected fractionation studies to determine the chemical nature of the active metabolites. As a part of this project, we screened the changes induced in root cytoplasmic protein synthesis pattern of maize, bean, squash, and tomato by the aqueous leachates, with the aim of contributing to the knowledge of the modes of action of the stress produced by the phytotoxic compounds.

METHODS AND MATERIALS

Phytotoxic Plants. Leaves from *Acacia sedillense* L. Rico (Fabaceae), *Sebastiania adenophora* Pax & K. Hoffm. (Euphorbiaceae), and *Lantana camara L*. (Verbenaceae) were collected at El Eden Ecological Reserve in Quintana Roo, Mexico. *Sicyos deppei* G. Don (Cucurbitaceae) leaves were collected in some crop fields in the Mexico basin.

Plant Material. Seeds of maize (*Zea mays* L., cv. Chinampero, Poaceae), squash (*Cucurbita pepo* L. cv. Criolla, Cucurbitaceae), and bean (*Phaseolus vulgaris* L. var. Flor de mayo, Fabaceae) were obtained from a local market at Tulyehualco, Mexico, D. F. Tomato seeds (*Lycopersicon esculentum,* var. Pomodoro, Solanaceae) were obtained from Sun-Seeds, Parma, Idaho, USA.

Bioassays. Seeds were germinated in Petri dishes containing aqueous leachate (1% w/v) of each phytotoxic plant with 1% agar as substrate. Aqueous leachates were prepared by soaking dried leaves (2 g/100 ml) in distilled water for 3 hr

and were mixed with agar (2%) for a final aqueous leachate concentration of 1%. Leachates were filtered through Whatman No. 4 paper and then through a Millipore membrane (0.45 μm). Osmotic pressure was measured by using a freezing-point osmometer (Osmette A, Precision System, Inc.); all values ranged from 15 to 17 mosm/liter. Bioassays were conducted under sterile conditions in a laminar hood. Controls contained only distilled water and agar. Ten seeds were placed on each Petri dish and kept in the dark at 27°C. Twenty-five Petri dishes were used per treatment and per crop plant. The terminal 0.5 cm of the primary root was excised after 48 hr from maize, squash, and beans seedlings and after 72-hr from tomato seedlings. Roots were frozen in liquid nitrogen and kept at −70°C until use. For root growth response, experiments were conducted in a complete randomized experiment design with four replicates. Primary root lengths were measured after 48 and 72 hr, and data were analyzed by ANOVA.

Cytoplasmic Protein Extraction, Gel Electrophoresis, and Densitometry Analysis. Cytoplasmic proteins were extracted and purified from roots of control and treated plants. The terminal 0.5 cm of the primary root from 300 treated and control seedlings was homogenized in liquid nitrogen with a mortar and pestle and suspended (1:4 w/v ratio) in a cold homogenization buffer [50 mM K_2HPO_4 and 50 mM of KH_2PO_4, pH 6.8, 1 mM phenylmethylsulphonyl fluoride (PMSF)]. The homogenate was centrifuged at 300*g* for 10 min to pellet the nuclear fraction, and the supernatant was centrifuged at 12,000*g* for 10 min at 4°C. Proteins from the supernatant were extracted into phenol, precipitated with methanol, and redissolved in an isoelectric focusing (IEF) medium (ampholyte solution at pH 4–7) as described by Hurkman and Tanaka (1988). Protein content was determined by the Bradford method (1976).

Two-dimensional gel electrophoresis (2D-PAGE) was performed according to O'Farrell (1975). For the first dimension, 10 μg of protein was loaded at the basic end of the gels (capillary tubes, Bio-Rad). Isoelectric focusing was conducted for 30 min at 300 V, and then for 4 hr at 750 V. After extrusion, gels were either frozen at −70°C or loaded onto a second dimension of 12% polyacrylamide resolving gel. Gels were run in a mini-protein apparatus (Bio-Rad), fixed, and silver stained according to Morrisey (1981). Controls and treated gels were analyzed by GelScan XL (release 2.1) densitometry. Total area and absorbance of protein spots on the gels were determined, adjusted for background, and assessed for any change in size.

RESULTS

Aqueous leachates (1%) of the four phytotoxic plants affected maize (*Zea mays*) radicle growth differently, with *S. deppei* leachate causing 39% ($P < 0.001$) growth inhibition, while *A. sedillense*, *S. adenophora*, and *L. camara* had no

TABLE 1. RADICLE GROWTH AND CYTOPLASMIC PROTEINS ALTERED IN *Zea mays*, *Cucurbita pepo*, *Phaseolus vulgaris*, AND *Lycopersicon esculentum* BY AQUEOUS LEACHATES (1%) OF *Acacia sedillense*, *Sebastiania adenophora*, *Lantana camara*, AND *Sicyos deppei*

Crop plant	Allelochemical plant	Radicle growth (%)	Proteins (Number)				
			Increased	Decreased	Repressed	Induced	Total
Z. mays	*A. sedillense*	112	18	1	2		21
	S. adenophora	115	9	2		3	14
	L. camara	107	3	7			10
	S. deppei	61[a]					0
C. pepo	*A. sedillense*	80	16	3			19
	S. adenophora	149[a]	4	5			9
	L. camara	70[a]	12	4	2		18
	S. deppei	104	6	4	3		13
P. vulgaris	*A. sedillense*	75[a]	16				16
	S. adenophora	83	7	3			10
	L. camara	59[a]	5	9		1	15
	S. deppei	30[a]					0
L. esculentum	*A. sedillense*	40[a]	12		1	1	14
	S. adenophora	30[a]	4	6	1		11
	L. camara	19[a]	6	5			11
	S. deppei	19[a]	4	6	1		11

[a] $P < 0.001$.

significant effect (Table 1). Although the leachate of *S. deppei* inhibited maize root growth, no change was observed in cytoplasmic protein synthesis (results not shown). In contrast, *A. sedillense*, *S. adenophora*, and *L. camara* caused several changes in its cytoplasmic protein synthesis pattern (Figure 1). Twenty-one proteins were altered by *A. sedillense* leachate, with 18 increased (proteins 1–11, 14–18, 20, and 21), 1 decreased (protein 19), and 2 repressed (proteins 12 and 13). *S. adenophora* leachate affected 14 proteins, with 9 increased (proteins 1, 2, and 6–12), 2 decreased (13 and 14), and 3 induced (3–5). Ten proteins were altered by *L. camara*, with 7 decreased (proteins 1, 3, and 6–10) and only 3 increased (2, 4, and 5) (Table 1, Figure 1). These results indicate that maize responds differently to various phytochemical stresses caused by different leachates. Analysis of cytoplasmic proteins revealed that different aqueous leachates affected the same proteins differently (Table 2). For example, a protein with apparent molecular weight of 57 kDa, and pI of 5.5 was repressed with *A. sedillense* leachate but decreased with *L. camara*. Another protein (27 kDa, pI 6.4) increased with *A. sedillense*, *S. adenophora*, and *L. camara* leachates. Three low-molecular-weight proteins, 18 (pI 6.1), 16 (pI 5.8), and 15 (pI 5.7) kDa, were induced only with *S. adenophora* leachate (Table 1, Figure 1D, proteins 2–4).

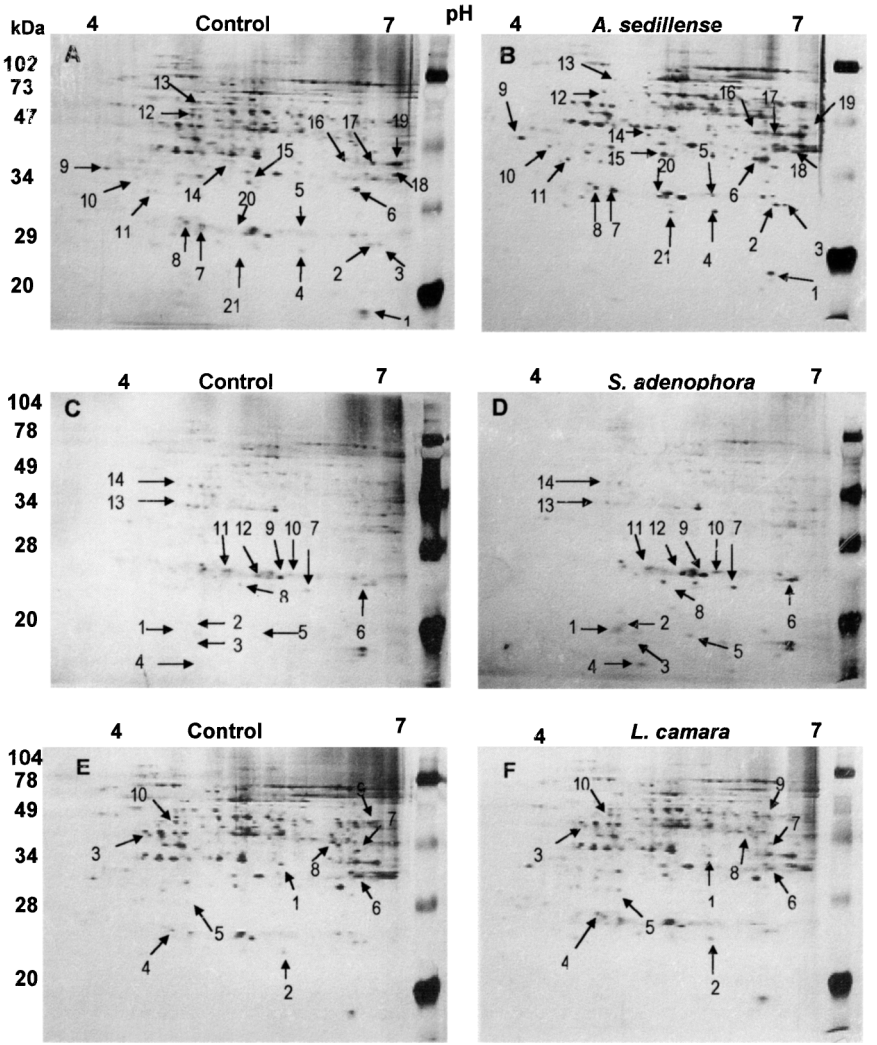

FIG. 1. 2D-PAGE of cytoplasmic root proteins from control maize seedlings (A, C, and E) and those subjected to the aqueous leachates: *A. sedillense* (B); *S. adenophora* (D), and *L. camara* (F). Ten micrograms of the proteins were loaded into each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

TABLE 2. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Zea mays* BY AQUEOUS LEACHATES OF *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*A. sedillense*	*S. adenophora*	*L. camara*
57.0	5.5	E		D
30.0	5.5	I		I
29.0	6.0	I	I	
28.0	6.8	I	I	
27.0	6.4	I	I	I

[a] I = increased; D = decreased; E = repressed.

Table 1 shows that *L. camara* inhibited squash (*Cucurbita pepo*) radicle growth by 30% ($P < 0.001$), while *S. adenophora* had a stimulatory effect (49%) ($P < 0.001$). No significant effect was observed with *A. sedillense* and *S. deppei* leachates. All four leachates also differently affected the protein synthesis in squash (Figure 2). *A. sedillense* and *L. camara* caused the most changes (a total of 19, and 18 proteins, respectively; Figure 2D, H, Table 1). Of the 19 proteins altered by *A. sedillense*, 16 increased (proteins 1, 4–8, and 10–19) and three decreased (2–4) (Table 1). Of the 18 altered by *L. camara*, 12 increased (6, and 8–18), 4 decreased (1, 3, 5, and 7), and 2 were repressed (2 and 4). In contrast, *S. adenophora* leachate, which significantly stimulated radicle growth, induced fewer changes in cytoplasmic proteins: 9 were altered with 4 increased (1–3, and 7) and 5 decreased (4–6, 8, and 9). *S. deppei* altered 13 proteins with 6 increased (5, 8–11, and 13), 4 decreased (3, 6, 7, and 12) and 3 repressed (1, 2, and 4) (Fig. 2b, F, Table 1). Table 3 shows that the same protein was differently affected by the different aqueous leachates. A16-kDa protein (pI 6.2) decreased with *S. deppei* and *L. camara* but increased with *A. sedillense*. The expression of a 15-kDa (pI 6.0) protein was repressed, increased, and decreased by *S. deppei*, *A. sedillense*, and *L. camara*, respectively.

As in maize and squash, aqueous leachates affected *P. vulgaris* radicle growth differently. *S. deppei*, *L. camara*, and *A. sedillense* leachates inhibited it 70%, 41%, and 25% ($P < 0.001$), respectively, while *S. adenophora* did not have any effect (Table 1). Although *S. deppei* strongly inhibited root growth in beans, it did not affect the expression of proteins. *A. sedillense* increased the expression of 16 proteins, and *L. camara* leachate altered 15 proteins: 5 proteins increased (2, 8–11), 9 decreased (3–7, 12–15), and one induced (1). *S. adenophora* altered 10 proteins, including 7 that increased (3, 5–10) and 3 that decreased (1, 2, and 4) (Table 1, Figure 3B, D, F). Two cytoplasmic proteins were differently affected by leachates. The expression of a 24-kDa (pI 5.8) protein was increased by *A. sedillense* but decreased by *S. adenophora*, while a 26-kDa (pI 5.9) protein was increased by both *A. sedillense* and *L. camara* (Figure 3B, F).

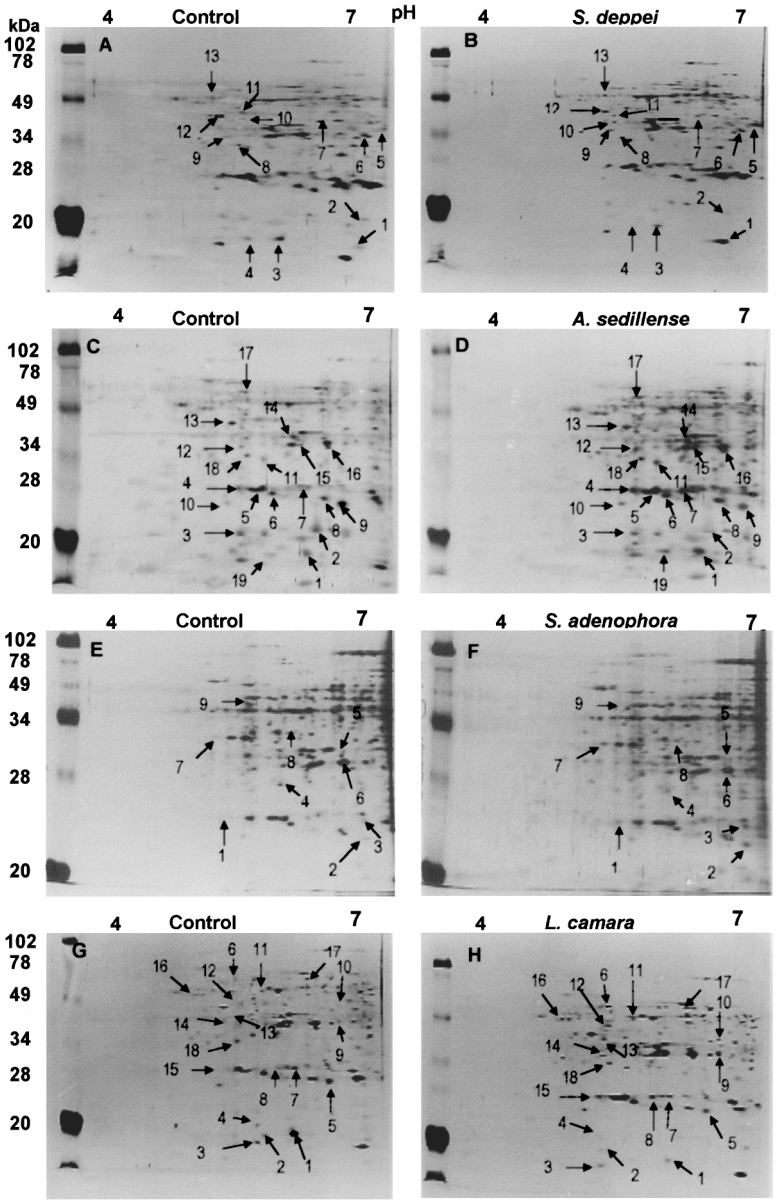

FIG. 2. 2D-PAGE of cytoplasmic root proteins from control squash seedlings (A, C, E, and G) and those subjected to the aqueous leachates: *S. deppei* (B), *A. sedillense* (D); *S. adenophora* (F); and *L. camara* (H). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

TABLE 3. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Cucurbita pepo* BY AQUEOUS LEACHATES OF *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*S. deppei*	*A. sedillense*	*S. adenophora*	*L. camara*
63.0	6.7		I		I
49.0	5.8	D	I		
47.0	5.9	I			I
40.0	5.9	I	I		I
39.0	6.0	I	I		I
27.0	5.7		I		I
25.0	6.0		I		I
22.0	5.7		D	I	D
16.0	6.2	D	I		D
15.0	6.0	E	I		D

[a] I = increased; D = decreased; E = repressed.

Tomato (*Lycopersicon esculentum*) seedlings were the most sensitive to phytochemical stress. All aqueous leachates significantly ($P < 0.001$) inhibited radicle growth, ranging from 81% for *S. deppei* and *L. camara*, to 70% for *S. adenophora* and 60% for *A. sedillense* (Table 1). The aqueous leachates also similarly affected the number of protein expressed in tomato, with a total of 11 each for *S. deppei*, *S. adenophora*, and *L. camara*; and 14 for *A. sedillense* (Table 1, Figure 4), with nearly an equal number of proteins increasing and decreasing for each leachate. Eight proteins were differently affected by leachates in tomato. For example, a high-molecular-weight protein (70 kDa, pI 5.5) was repressed by *A. sedillense* but was decreased by *L. camara*. A 34-kDa (pI 6.9) protein decreased under *S. deppei* but was increased by *A. sedillense* and *S. adenophora* (Table 4, Figure 4).

TABLE 4. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Lycopersicon esculentum* BY AQUEOUS LEACHATES OF *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*S. deppei*	*A. sedillense*	*S. adenophora*	*L. camara*
70.0	5.5		E		D
34.0	6.9	D	I	I	
30.0	6.3	D			
29.0	6.2			I	I
26.0	6.7			I	I
25.0	6.8		I		I
23.0	7.0	D		D	
17.0	6.0	E		E	

[a] I = increased; D = decreased; E = repressed.

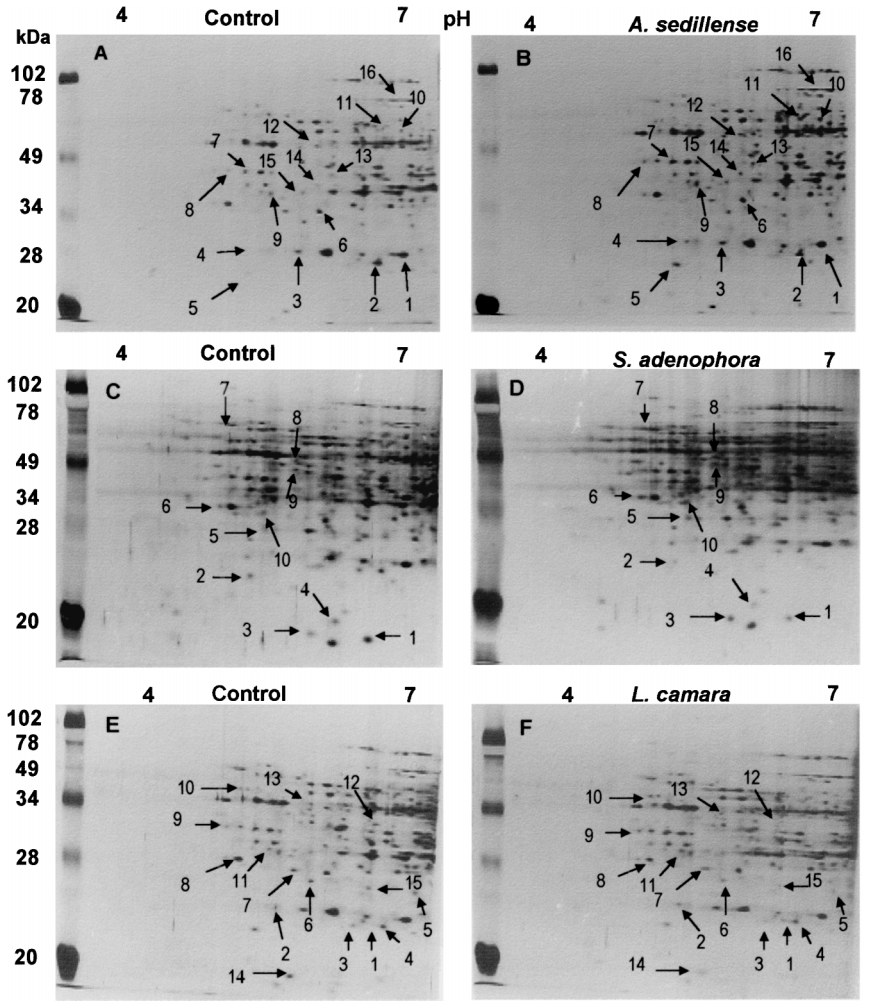

FIG. 3. 2D-PAGE of cytoplasmic root proteins from control bean seedlings (A, C, and E) and those subjected to the aqueous leachates: *A. sedillense* (B), *S. adenophora* (D), and *L. camara* (F). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

DISCUSSION

The metabolic stress response is an ubiquitous defense mechanism activated when cells are confronted with unfavorable environmental conditions. Induction leads to the expression of proteins known as stress proteins, which are thought

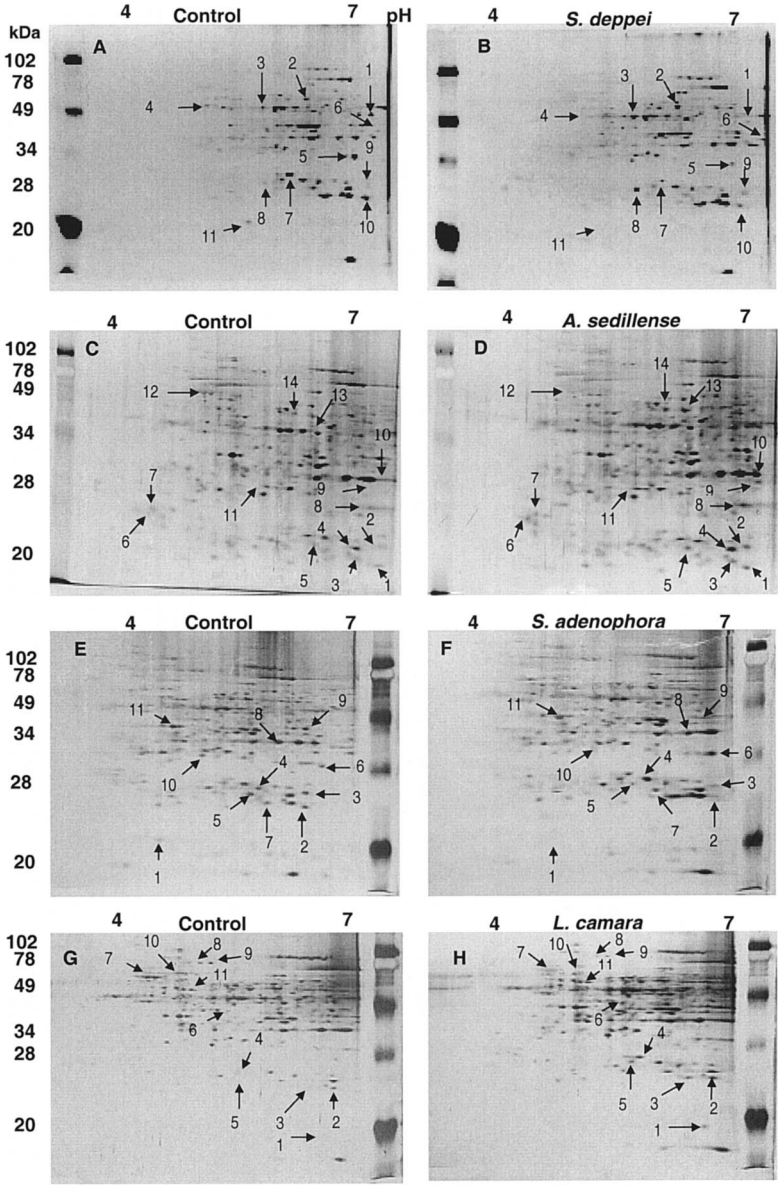

FIG. 4. 2D-PAGE of cytoplasmic root proteins from control tomato seedlings (A, C, E, and G) and those subjected to the aqueous leachates: *S. deppei* (B), *A. sedillense* (D); *S. adenophora* (F); and *L. camara* (H). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

to protect the cell. Stress and signaling inside the cell lead to protein expression changes, the activation of new biochemical pathways, and repression of others that are characteristic of the unstressed state (Bohnert and Sheveleva, 1998). The allelochemicals produced by the plants reported in this study, most of them of unknown chemical nature, may cause stress. Dayan et al. (2000) asserted that determination of the mode of action of allelochemicals is a challenging endeavor due to the multitude of potential molecular targets. However, this seemingly overwhelming task also presents an opportunity to discover new sites of action. In this sense, the present study adds to our understanding.

The effects of the leachates on the crop plants investigated varied in both radicle growth and cytoplasmic protein synthesis. Maize was the most chemical stress tolerant crop plant (inhibited by only *S. deppei*), and tomato the least (inhibited by all allelochemical plants). All the crop plants exhibited changes in the expression of their root cytoplasmic proteins. Overall there was a general increase in protein expression, some reduction, and little induction or repression. *A. sedillense* and *L. camara* leachates affected the largest number of proteins, resulting in a general increase in protein expression. *S. deppei* affected the least and produced a significant change only in tomato. *L. camara* caused a significant inhibition of radicle growth of *C. pepo*, but resulted in the highest decrease of proteins in *P. vulgaris*.

It is possible that the proteins that increased or were induced in response to the treatments are stress proteins that are related to defense mechanisms. They may be formed as a response to allelochemical stress, as are heat shock proteins (HSPs), pathogenic-related proteins (PR), or salinity and dehydration proteins (osmotin, LEA, dehidrins, among others) (Vierling, 1991; Bray, 1993; Van Loon et al., 1994). Proteins whose expression either decreased or were repressed may be those involved in "housekeeping processes," such as cell division or elongation, cell wall formation, or vesicle trafficking. Riccardi et al. (1998) reported a decrease in the expression of certain proteins when maize leaves were subjected to water stress. Sequencing of these peptides showed they were enzymes of the Krebs cycle and glycolysis, such as triose-phospho isomerase, enolase, and NAD-malate-dehydrogenase. Baziramakenga et al. (1997) found that some phenolic acids alter the incorporation of ^{32}P into DNA and RNA and reduce the uptake of [^{32}S] methionine into root soybean seedlings proteins.

S. deppei inhibited radicle growth significantly in maize and beans, but interestingly it did not modify the protein synthesis pattern. In a previous study, we observed that *S. deppei* caused plasma membrane disruption in root tips and plasmolized cells in the peripheral zone of beans and bottle gourd roots (Cruz-Ortega et al., 1998), suggesting that it may be altering membrane processes. It would be interesting to study the effects of this plant on electrolyte leakage and enzyme activities.

This study is one of the first describing changes in protein synthesis patterns caused by allelochemicals from phytotoxic plants. The proteins that were

significantly affected are currently being microsequenced to understand their function. In addition, isolation and identification of the major phytotoxic compounds are underway so that we can test the effects of individual compounds.

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