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Allelochemical stress produced by the aqueous leachate of *Callicarpa acuminata*: effects on roots of bean, maize, and tomato

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The in vitro effects of an aqueous leachate (1%) of *Callicarpa acuminata* Kunth. (Verbenaceae) on radicle growth, protein expression, catalase activity, free radical production and membrane lipid peroxidation in roots of bean, maize, and tomato were examined. Aqueous extract of *C. acuminata* inhibited the radicle growth of tomato by 47%, but had no effect on root growth of maize and beans. 2D-PAGE and densitometry analysis showed that *C. acuminata* aqueous leachate modified the expression of various proteins in the roots of all treated plants. In treated bean roots, microsequencing analysis of an 11.3-kDa protein, whose expression was enhanced by leachate treatment, revealed a 99% similarity with subunits of α-amylase inhibitor of other beans. A 27.5-kDa protein induced in treated tomato showed 69–95% similarity to glutathione-*S*-transferases (GST) of other Solanaceae. Spectrophotometric analysis and native gels revealed that catalase activity was increased by 2.2-fold in tomato roots and 1.4-fold in bean roots. No significant changes were observed in treated maize roots. Luminol chemiluminescence levels, a measure of free radicals, increased 3.8-fold in treated tomato roots and 2.1-fold in treated bean roots. Oxidative membrane damage in treated roots was measured by lipid peroxidation rates. In tomato we observed a 2.4-fold increase in peroxidation, however, no effect was observed in maize or beans.

Introduction

Plants produce diverse secondary metabolites (allelochemicals) that are released into the environment. Some of them have a biological activity on other plants and microorganisms, a phenomenon called Allelopathy (for reviews see Rice 1984, Anaya 1999). Consequently, plants must cope with allelochemical stress as well as with other environmental stresses (i.e. drought, temperature, salinity, and pathogens). Like many other stress factors, allelochemicals have several molecular targets, and some of their physiological processes or modes of action have been described (Einhellig 1995, Reigosa et al. 1999). Allelochemical compounds have been shown to affect many different cellular processes in target organisms, including disruption of membrane permeability (Galindo et al. 1999), ion uptake (Lehman and Blum 1999), inhibition of electron transport in both photosynthesis and the respiratory chain (Calera et al. 1995, Peñuelas et al. 1996, Abrahim et al. 2000), alterations of some enzymatic activities (Cruz-Ortega et al. 1990, Silva et al. 1996, Politycka 1998), and inhibition of cell division (Cruz-Ortega et al. 1988, Anaya and Pelayo-Benavides 1997). Currently, we are performing a bioprospecting study on the abundant shrub or small tree (1–6 m height) *Callicarpa acuminata* Kunth. (Verbenaceae) from the secondary plant communities of the ecological reserve El Eden, Quintana Roo, Mexico. Anaya and del Amo (1999) reported that the aqueous leachates from leaves and stems of this plant exhibit a strong allelopathic effect on other plants such as *Amaranthus hypochondriacus* and *Echinochloa crus-galli*. The present study investigates the effects of a 1% (w/v) aqueous leachate of *C. acuminata* on the radicle growth, protein expression, catalase activity, free radical production and membrane lipid peroxidation in roots of *Phaseolus vulgaris* L. (Fabaceae), *Lycopersicon esculentum* L. (Solanaceae), and *Zea mays* L. (Poaceae), with the aim of contributing to

Abbreviations – CHAPS (3[3-cholamidopropyl) dimethylammonio]-1-propanesulphonate); DTT, dithiotreitol; LUMINOL, 5-amino-2,3-dihydro-1,4-phthalazinedione; PVDF, polyvinylidene difluoride.

the knowledge of the modes of action of this allelopathic plant.

Materials and methods

Plant material

Live leaves and stems of *C. acuminata* Kunth. (Verbenaceae) were collected at El Eden Ecological Reserve in Quintana Roo, Mexico. Plant materials were air-dried and used to prepare an aqueous leachate. This leachate was prepared by soaking dried-leaves and stems of *C. acuminata* (2 g per 100 ml) in distilled water for 3 hours. The leachate was first filtered through a Whatman #4 paper and then through a Millipore membrane (0.45 μm). To avoid osmotic effects due to leachate concentration, osmotic potential of the leachate was measured with a freezing-point osmometer (Osmette A, Precision System, Inc., Natick, MA, USA); and was found to be between 15 and 17 mosm l^{-1}, which do not cause an osmotic effect on any of the test crop plants (data not shown).

Test seeds of maize (*Z. mays* L. cv. Chinampero) and beans (*P. vulgaris* L. var. Flor de Mayo) were obtained from a local supplier in Mexico, D.F. Tomato seeds (*L. esculentum*, var. Río Grande) were obtained from Sun-Seeds, Parma, ID, USA.

Bioassays

Seeds were germinated in the aqueous leachate (2%) mixed with agar (2%) in Petri dishes, to yield a final concentration of 1%. The bioassays were prepared under sterile conditions in a laminar flow hood. For controls, seeds were germinated in agar and sterile distilled water. Ten or 12 seeds of each crop were placed in a Petri dish at 27°C in the dark. Twenty-five Petri dishes were used per treatment for each crop. The terminal 0.5 cm of the primary root was excised from maize and bean seedlings after 48 h, and after 72 h from tomato seedlings. Samples were frozen in liquid nitrogen and stored at −70°C until use. For root growth response, a complete randomized design experiment was performed with four replicates. Primary roots length were measured after the above indicated times, and data were analysed by the statistical test ANOVA.

Root-tip homogenization, cytoplasmic protein extraction, 2D-gel electrophoresis, and gel scan analysis

Cytoplasmic proteins were extracted and purified from *C. acuminata*-treated and control seedling roots. The terminal 0.5 cm of the primary root of 300 seedlings per treatment was homogenized in liquid nitrogen with a mortar and pestle and resuspended in a 1:4 w/v ratio in cold homogenization buffer (700 m*M* sucrose; 50 m*M* Trizma-base, 30 m*M* HCl, 100 m*M* KCl, 5 m*M* Na_2EDTA, and 1 m*M* DTT) (Barent and Elthon 1992). The homogenate was centrifuged at 300 *g* for 10 min to pellet residues. The supernatant was then centrifuged at 12 000 *g* for 10 min at 4°C. Proteins in the supernatant were phenol-extracted, precipitated with 0.1 *M* ammonium acetate-methanol, and resuspended in isoelectric focusing (IEF) medium (9 *M* urea, 4% CHAPS, 0.5% DTT, 2% 5/7 ampholyte, and 0.75% 3/10 ampholyte-solution at pH 4–7) as described by Hurkman and Tanaka (1988). Protein content was determined by the method of Bradford (1976).

Two-dimensional gel electrophoresis (2D-PAGE) was carried out according to O'Farrell (1975). For the first dimension, 10 μg of protein were loaded at the basic end of the focusing gels (capillary tubes, Bio-Rad Laboratories Inc., Hercules, CA, USA). Isoelectric focusing was conducted for 30 min at 300 V, and then for 4 h at 750 V. After extrusion, the gels were either frozen at −70°C or loaded onto a second dimension 12% polyacrylamide resolving gel. The gels were run in a mini-protean gel (Bio-Rad), fixed and silver stained according to Oakley et al. (1980). Gels were scanned with a GelScan XL (release 2.1) to determine the total volume and absorbance of protein spots.

Protein microsequencing

Amino acid sequence analysis was performed according to Barent and Elthon (1992) and Dunbar et al. (1997). Briefly, 400 μg of cytoplasmic protein were used for the first dimension, and then electrophoresed in a 12% acrylamide gel and blotted onto a PVDF membrane using a TE-semidry system (Hoefer Scientific Instruments, San Francisco, CA, USA). Proteins were sent for N-terminal microsequencing to the Protein Chemistry Laboratory of the University of Texas, Medical Branch, at Galveston, TX, USA. Protein induced in bean was trypsin-digested because of N-terminal blocking. Amino acid sequences obtained were compared with sequences in the non-redundant peptide sequences database of the National Center for Biotechnology Information by using the BLAST program (Altschul et al. 1990).

Catalase activity (EC 1.11.1.6)

For analysis of enzyme activity in native gels, total protein was extracted from control and *C. acuminata*-treated root tips under native conditions. Roots were homogenized with 50 m*M* potassium phosphate, pH 7.2 and 5 m*M* DTT. Insoluble materials were removed by centrifugation at 14 000 *g* for 15 min at 4°C. Protein concentration was determined according to Bradford (1976). Twenty-five μg of protein in *β*-mercaptoethanol-free loading buffer was loaded into a 6.5% non-denaturing polyacrylamide gel (Laemmli 1970), and electrophoresed at 40 V for 6 hours at 4°C. Gels were soaked for 5 min in 5% methanol, 15 min in 0.1% H_2O_2, with 1× washing in distilled water between and after soakings, and then stained in a solution of 1% (w/v) potassium ferricyanide, 1% (w/v) ferric chloride (equal volumes of 2% w/v of each component, added sequentially) according to Harris and Hopkinson (1976) and modified by Chary

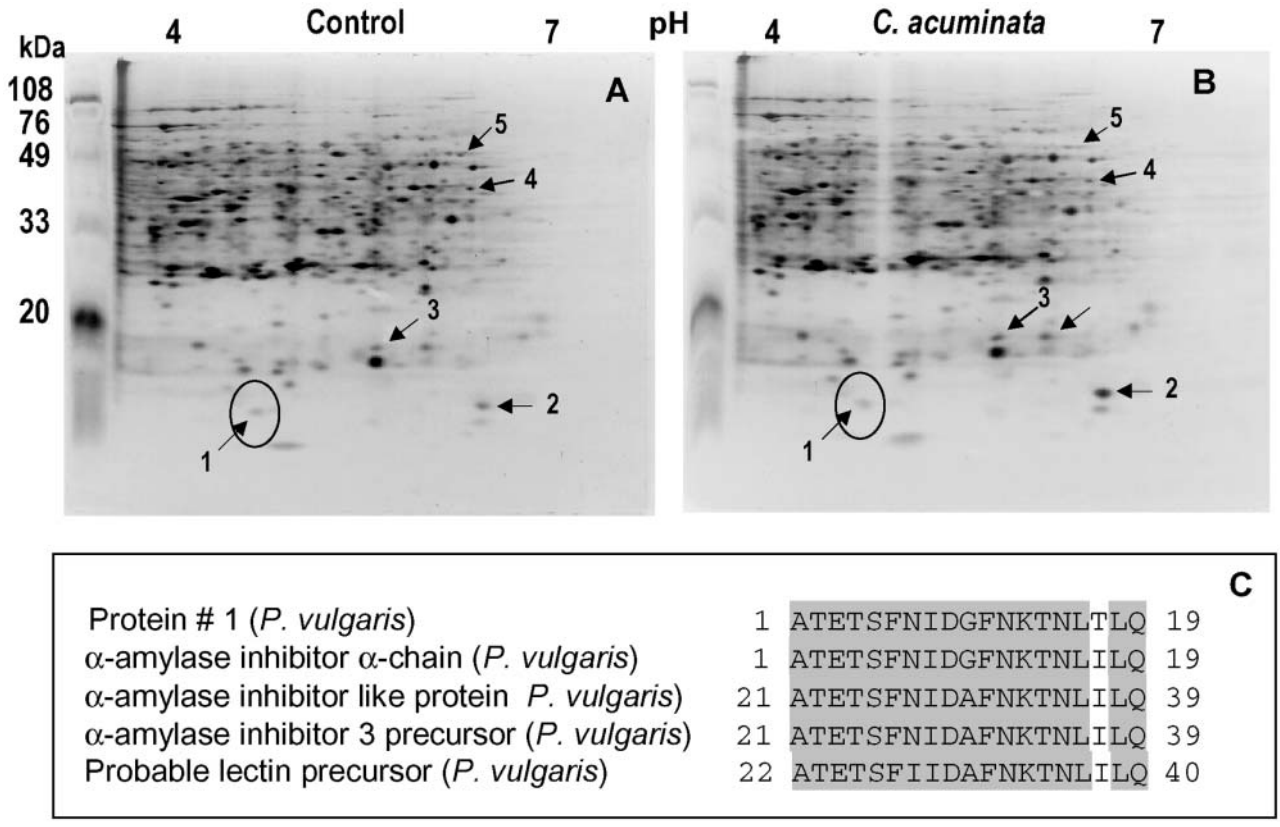

Fig. 1. 2D-PAGE of cytoplasmic root proteins from control (A) and *C. acuminata* aqueous leachate treated bean roots (B). Ten µg of proteins were loaded per gel. Arrows indicate proteins whose expression was modified and detected by gel-scanned. Oval symbols indicate the sequenced protein (#1). (C) Amino acid residues alignment with other subunits of bean α-amylase inhibitors. Gels represent the mean of at least three replicates. Molecular masses (kDa) are indicated.

and Natving (1989). The reaction was stopped with 10% acetic acid: 30% methanol.

For the spectrophotometric assay, total protein was extracted as described above in a buffer containing 0.1 m*M* EDTA. The homogenate was passed through Sephadex G-25 columns (1 ml bed volume) at 4°C and eluted with 100 m*M* potassium phosphate, pH 7.8 in order to remove low-molecular-weight compounds that interfere with the assay (Anderson et al. 1995). Catalase activity was determined according to Beers and Sizer (1952). Experiments were repeated three times and in triplicates.

Free radicals and lipid peroxidation

To determine if *C. acuminata* aqueous leachate induced the production of free radicals, we measured luminol-mediated chemiluminescence. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is employed to amplify chemiluminescence signals. Luminol is oxidized by several oxygen intermediates, i.e. O_2^-, H_2O_2, HO, $^1O_2$ to an electronically excited aminophthalate anion, that, upon relaxation to the singlet ground state, emits photons (Cadenas and Sies 1984). The reaction medium contained 0.5 *M* ammonium acetate, pH 10.5, 0.250 m*M* cobalt chloride, 1 m*M* sodium carbonate, and 0.06 mg of protein from control or *C. acuminata*-treated roots. Emmittance was measured in a scintillation counter. An initial reading of cpm with no sample was taken, and then, after the protein-sample was added, at least 10 readings per sample were taken and the highest value of cpm was used as a final value (Cadenas and Sies 1984). The number of free radicals was calculated as follows: (Final value of cpm − initial value of cpm)/0.06 mg of protein-sample.

To determine if the free radicals generated by the aqueous leachate treatment caused membrane damage, the presence of conjugated dienes as a product of membrane lipid peroxidation was measured by their absorption in the ultraviolet range (233 nm), according to the method of Recknagel and Glende (1984). Experiments were repeated three times and in triplicates. Briefly, 1 mg of protein from control or leachate-treated was dissolved in 1 ml of H_2O and 4 ml of a mixture of chloroform-methanol (2:1 v/v). This mixture was placed in ice for 30 min and then centrifuged at 260 *g* for 5 min. The chloroform layer was removed and transferred to a clean tube and placed in a water bath at 40–50°C to remove the chloroform. The extracted chloroform-free lipids were dissolved in 1.6 ml cyclohexane and optical density was recorded at 230 nm.

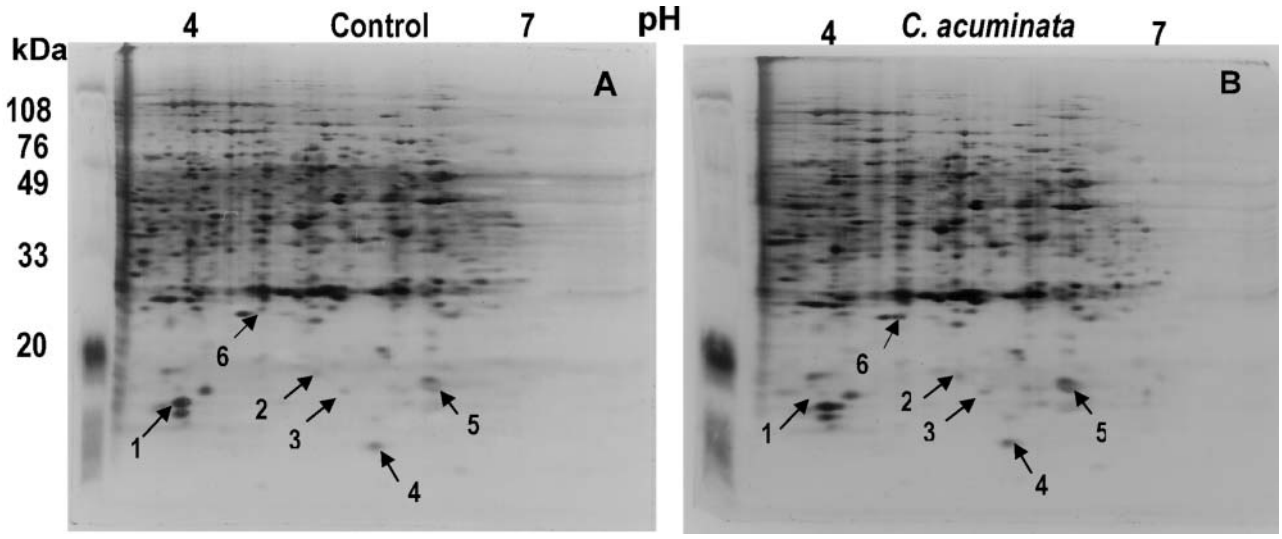

Fig. 2. 2D-PAGE of cytoplasmic root proteins from control (A) and *C. acuminata* aqueous leachate treated maize roots (B). Ten µg of proteins were loaded per gel. Arrows indicate proteins whose expression was modified and detected by gel-scanned. These gels represent the mean of at least three replicates. Molecular masses (kDa) are indicated.

Results

Effects on radicle growth and cytoplasmic protein expression

C. acuminata aqueous leachate (1%) inhibited radicle growth of tomato seedlings (47%, $P < 0.001$), but had no effect on radicle growth of either bean or maize seedlings (90.4 and 100% of control growth, respectively). Protein expression, however, was modified in all three crop plants. In roots of bean seedlings, densitometry analysis of 2D-PAGE gels showed that 5 proteins were significantly modified. Three proteins (proteins 1–3) increased and two decreased (4,5) (Fig. 1 and Table 1). A protein of 11.3 kDa (protein 1, Fig. 1), which absorbance increased 2-fold in treated roots, was selected for internal amino acid sequence. Nineteen-amino acid residues were obtained from a trypsin digestion, since its N-terminal was blocked. Amino acid sequence comparisons showed 94% similarity with α-amylase inhibitor from another *P. vulgaris* (kidney bean) (pir IJC4855; Kasahara et al. 1996), 89% with α-amylase inhibitor-like protein (dbj BAAA86927; Ishimoto et al. 1999); 89% with α-amylase inhibitor 3 precursor of *P. vulgaris* (kidney bean) (pir S51830; Mirkov et al. 1994), and 84% with probable lectin precursor of *P. vulgaris* (kidney bean) (ITI2036; Lee et al. submitted Jan-1997 to the EMBL Data Library).

In maize, expression of six proteins was significantly increased by the aqueous leachate of *C. acuminata* (Fig. 2 and Table 1). On the other hand, we found 12 proteins that were modified in treated tomato roots. Of these, the amount of 10 was decreased (proteins 1–4; 6–9; 11 and 12), one was increased (protein 10), and one was induced (protein 5) (Fig. 3 and Table 2). The identity of the induced-protein, with a molecular weight of 27.5 kDa, was determined by amino acid sequencing of 23 amino acids at the N-terminal end. Protein database search showed 95% similarity to the enzyme glutathione-*S*-transferases (EC 2.5.1.18) (GST), class-phi from *Solanum commersonii*, pir T07906 ([q9']Seppanen 1997; direct submission); 90% similarity with GST from *Hyoscyamus muticus*, pir

Table 1. Maximum absorbance and relative molecular weights (kDa) of proteins, whose expression was modified in bean and maize by *C. acuminata* aqueous leachate. Sequenced protein (*). Protein number corresponds to that on the 2D-gels. I = increased; D = decreased.

Crop plant	Protein	Absorbance (kDa)	Control	*C. acuminata*	Increase/Decrease Factor
Bean	1	11.3	0.06	0.12 (I)*	2.0
	2	8.5	0.06	0.27 (I)	4.5
	3	13.5	0.13	0.24 (I)	1.8
	4	43.8	0.33	0.09 (D)	2.7
	5	52.0	0.27	0.09 (D)	3.0
Maize	1	12.9	0.19	0.38 (I)	2.0
	2	15.0	0.06	0.13 (I)	2.2
	3	13.9	0.06	0.12 (I)	2.1
	4	8.0	0.16	0.34 (I)	2.1
	5	12.7	0.02	0.13 (I)	6.5
	6	21.7	0.14	0.38 (I)	2.7

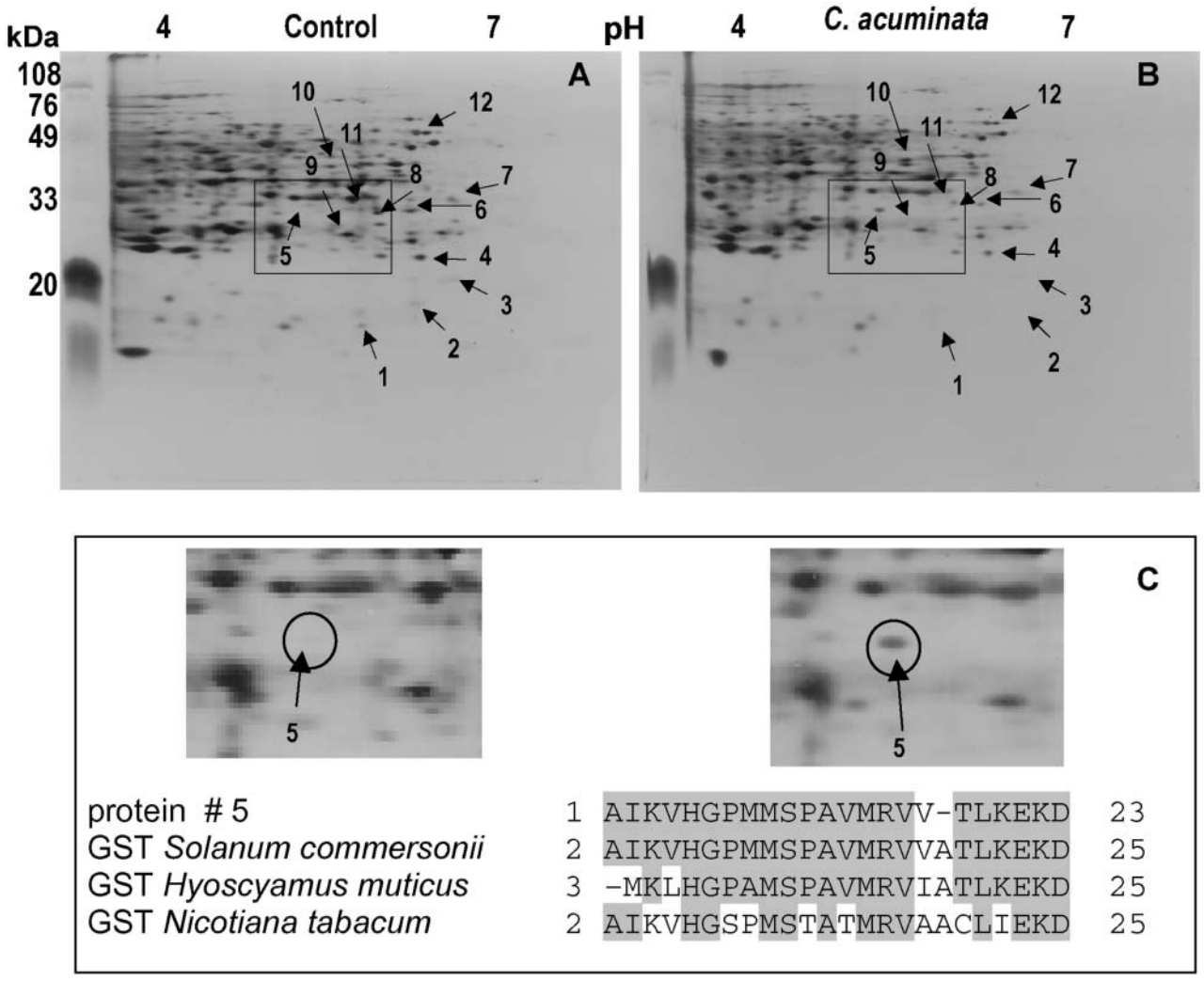

Fig. 3. 2D-PAGE of cytoplasmic root proteins from control (A) and *C. acuminata* aqueous leachate treated tomato roots (B). Ten µg of proteins were loaded per gel. Arrows indicate proteins whose expression was modified and detected by gel-scanned. The square indicates the magnified location of the microsequenced protein #5. (C) Amino acids residues alignment with selected GSTs proteins from Solanaceae. These gels represent the mean of at least three replicates. Molecular masses (kDa) are indicated.

PQ0744 (Bilang et al. 1993); and 69% with GST from *Nicotiana tabacum*, P46440 (Ezaki et al. 1995) (Fig. 3C).

Effects on catalase activity, free radicals and lipid peroxidation

To determine whether the allelochemical stress produced by *C. acuminata* aqueous leachate caused oxidative stress in the three crop plants, we measured the activity of the important antioxidant enzyme catalase. We also assayed lipid peroxidation by measuring the conjugated dienes, and production of free radicals by luminol chemoluminescence. The effect of the aqueous leachate of *C. acuminata* on catalase activity was measured by both native gels and by spectrophotometry. Catalase activity increased in treated bean roots (1.5-fold, 47%), and in tomato roots (1.9-fold, 80%). In the case of maize, roots catalase activity decreased 2.3-fold (56%) (Fig. 4 and Table 3). Maize has three isoenzymes of catalase: CAT1, CAT2, and CAT3. The first and the third form can be expressed in roots (Scandalios et al. 1984), however, in the present study we could only visualize CAT1, since

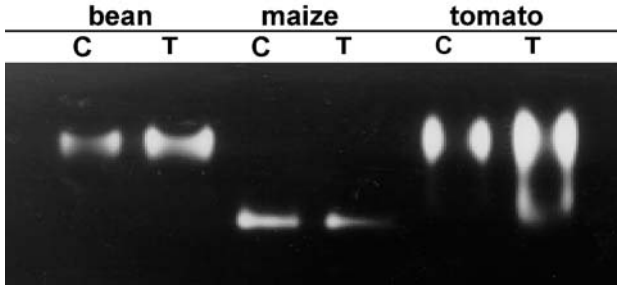

Fig. 4. Effects of *C. acuminata* aqueous leachate on catalase activity of control and roots of treated bean, maize and tomato. Twenty-five µg of protein were loaded per well and per treatment.

Table 2. Maximum absorbance and relative molecular weights (kDa) of proteins, whose expression was modified in tomato roots by *C. acuminata* aqueous leachate. Sequenced protein (*). The number of protein corresponds to that on the 2D-gels. I = increased; D = decreased.

		Absorbance		
Protein	(kDa)	Control	*C. acuminata*	Factor
1	12.16	0.14	0.04 (D)	2.9
2	13.56	0.10	0.03 (D)	3.3
3	17.02	0.26	0.06 (D)	2.1
4	20.89	0.47	0.22 (D)	4.7
5	27.54	0.00	0.21 (I)*	–
6	29.24	0.26	0.12 (D)	2.2
7	27.54	0.14	0.07 (D)	2.0
8	30.20	0.30	0.12 (D)	2.5
9	25.70	0.12	0.02 (D)	6.0
10	40.74	0.06	0.27 (I)	4.5
11	28.97	0.16	0.03 (D)	5.3
12	62.23	0.26	0.10 (D)	2.6

Table 3. Effects of *C. acuminata* aqueous leachate on catalase activity of bean, maize, and tomato roots.

	Catalase Activity (A_{240}) (nmol mg^{-1} min^{-1})	
Crop species	Control	*C. acuminata*
Bean	1.7 ± 0.4	2.5 ± 0.2
Maize	3.2 ± 1.7	1.4 ± 0.4
Tomato	1.9 ± 0.4	3.7 ± 1.2

CAT3 is susceptible to oxidation on the gel (Anderson et al. 1995).

Free radicals increased in both treated root of bean (3.8-fold) and tomato (2.1-fold), but decreased in maize (1.3-fold). With regard to membrane lipid peroxidation rates we found no differences between control and treated roots of bean and maize, but in tomato this rate was increased 2.4-fold (Table 4). The lack of correlation between free radicals, lipid peroxidation and catalase activity in all test plants could be due to different activity of other antioxidant systems.

Discussion

The aqueous leachate of *C. acuminata* differentially affected the radicle growth of the three crop test plants. Tomato seedlings were the most sensitive, while bean and maize were the most tolerant. *C. acuminata* aqueous leachate also affected expression of diverse cytoplasmic root proteins in the three crop plants. We have previously observed that different aqueous leachates of other phytotoxic plants also caused diverse effects on radicle growth and protein expression of crop plants (Romero-Romero et al. 2002).

Root growth of bean was not inhibited by the aqueous leachate of *C. acuminata*, and of the five proteins, whose expression was altered, all were increased (Fig. 1 and Table 1). Microsequencing analysis of one of these proteins (11.3 kDa, protein 1) showed 99% similarity with subunits of α-amylase inhibitor of other *P. vulgaris* varieties. These α-amylase inhibitors are defense storage proteins, also induced during insect attack (Ishimoto et al. 1999). The fact that α-amylase inhibitor increased 2-fold during treatment with *C. acuminata* aqueous leachate suggests that the induction of this type of protein can be a general defense response, not specifically for insect attack. Some environmental stresses can induce expression of proteins not specially related to a particular stress, but as a reaction to cell damage. These include some classes of heat shock proteins (Heikkila et al. 1984), thiol proteases (Williams et al. 1994); proteinase inhibitors (Reviron et al. 1992); osmotin and other PR proteins (Cruz-Ortega and Ownby 1993, Kononowicz et al. 1993, Cruz-Ortega et al. 1997). On the other hand, catalase activity increased 1.4-fold in treated-bean-roots, as did the amount of free radicals (3.7-fold), but we did not observe an increased in the rate of lipid peroxidation (Tables 5 and 6). These results suggest that defense mechanisms in beans might be turning on during the allelochemical stress produced by the mixture of secondary metabolites present in the aqueous leachate of *C. acuminata*.

The response of maize to *C. acuminata* aqueous leachate was similar to that of bean. Maize root length was not inhibited by the treatment, and expression of six proteins was increased. Riccardi et al. (1998) found that in maize leaves exposed to a progressive water stress, proteins that were induced corresponded to enzymes involved in basic metabolic pathways such as glycolysis and the Kreb cycle, or to the phenylpropanoid pathway. It appears that maize also responds to allelochemical stress by increasing the expression of specific proteins. Moreover, in treated maize roots, catalase activity seems to decrease, but we did not observe any change in free radical production or in lipid peroxidation rates (Tables

Table 4. Effect of *C. acuminata* aqueous leachate on free radicals and membrane lipid peroxidation of control and treated roots of bean, maize and tomato.

	Free radicals (cpm mg^{-1} protein min^{-1}		Lipid peroxidation (ΔA_{233})	
Crop species	Control	*C. acuminata*	Control	*C. acuminata*
Bean	2.2 ± 0.5	8.2 ± 1.0	0.25 ± 0.07	0.24 ± 0.02
Maize	8.1 ± 1.7	6.2 ± 1.1	0.36 ± 0.05	0.39 ± 0.05
Tomato	4.2 ± 1.2	8.6 ± 3.1	0.20 ± 0.03	0.48 ± 0.00

3 and 4). These results most likely mean that in maize, antioxidant defense mechanisms other than catalase might be working to cope with this stress.

Tomato was the most sensitive crop plant tested in this study. Its radicle growth was significantly inhibited, and of the 12 proteins whose expression was altered by *C. acuminata* treatment, 10 were decreased. This decreased might be a manifestation of cell damaged caused by allelochemical stress. One of the two induced proteins appears to be a glutathione-*S*-transferases (GST). In plants, GSTs play roles in both normal cellular metabolism as well as in detoxification of a wide variety of toxic compounds. GSTs have been implicated in numerous stress responses, including pathogen attack, oxidative stress, and others (Marrs 1996, Dixon et al. 1998, Edwards et al. 2000). On the other hand, the aqueous leachate of *C. acuminata* increased by 2.2-fold the activity of catalase in tomato roots. Moreover, in treated roots of tomato, free radicals increased 3.8-fold and lipid peroxidation rate increased 2.4-fold (Tables 3 and 4). These results suggest that the allelochemical stress produced by *C. acuminata* aqueous leachate is causing an oxidative stress. The number of free radicals formed in treated-tomato roots, the rate of lipid peroxidation, the increase in catalase activity, and the induction of a GST-like protein can be a signal of this cellular damage. Additional studies are currently in progress to clone the GST from tomato to confirm if its expression increases during this particular allelochemical stress.

Results of this study show that each plant species responded in a particular way to the same allelochemical stress. Current biodirected fractionation studies on *C. acuminata* indicate that this plant species contain various terpenoids that are toxic to plants, insects, fungi, and brine shrimps (Anaya et al., in preparation). It is known that other *Callicarpa* species contain diterpenoid compounds (Talapatra et al. 1994, Agrawal et al. 1996, Xu et al. 2000). Tellez et al. (2000) have described the composition and some biological activities against plants, fungi, and algae of the essential oil of *Callicarpa americana.*

One of the goals of bioprospection studies is to discover new natural products that could be used to control pests and to solve some disease problems, as well as to know the mode of action of these compounds. Bioprospection studies must be greatly encouraged particularly in tropical zones, which possess a great, and so far unknown, biodiversity.

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