Theor Appl Genet (1994) 88: 986–993

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Genetic variation of trypsin and chymotrypsin inhibitors in pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild relatives

Received: 10 September 1993 / Accepted: 21 December 1993

Abstract Variation in the trypsin inhibitors (TIs) and the chymotrypsin inhibitors (CIs) among 69 pigeonpea [*Cajanus cajan* (L.) Millsp.] strains from a wide geographical distribution and among 17 accessions representing seven wild *Cajanus* species was studied by electrophoretic banding pattern comparisons and by spectrophotometric activity assays. The TI and CI electrophoretic migration patterns among the pigeonpea strains were highly uniform but varied in the inhibitor band intensities. The migration patterns of the inhibitors in the wild *Cajanus* species were highly species specific. The mean TI activity of pigeonpea strains (2279 units) was significantly higher than that of the wild *Cajanus* species (1407 units). However, the mean CI activity in the pigeonpea strains (62 units) was much lower than that in the wild species (162 units). Kenya 2 and ICP 9151 were the lowest and the highest, respectively, in both the TI and CI activities among all the pigeonpea strains used in this study. A highly-significant positive correlation was observed between the TI and CI activities. The Bowman-Birk type inhibitors with both TI and CI activities were identified in all the pigeonpea strains and also in the accessions of all the wild species except *C. volubilis* (Blanco) Blanco. The *C. volubilis* accession ICPW 169 was found to be 'null' for both CI bands and CI activity. Environment, strain, and environment × strain interaction showed highly-significant effects on both the TI and CI activities. Growing the pigeonpea strains at a different environment from their area of adaptation increased TI and CI activities and also altered the maturity period.

Key words Pigeonpea · Wild *Cajanus* species
Trypsin inhibitors · Chymotrypsin inhibitors
Non-denaturing PAGE · Activity staining · Environment

Communicated by A. L. Kahler

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Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is cultivated in the semi-arid tropics of Asia, Africa, and the Caribbean, under a wide variety of cropping systems. Besides its main use as *dhal* (dry, dehulled, split seed used for cooking), pigeonpea's tender green seeds are used as a vegetable, while crushed dry seeds serve as animal feed, green leaves as fodder, and stems as fuel wood and to make huts, baskets, etc. (Nene and Sheila 1990).

Exploration for genetic variability and diversity is very important for improving both the agronomic and nutritional traits of pigeonpea. The trypsin and chymotrypsin inhibitors are widely distributed among many plant families and are considered to be involved in regulating endogenous proteases and protecting plants against insect and pathogen attack, and also to function as storage proteins (Liener and Kakade 1980; Ryan 1990). Protease inhibitors have been studied extensively in several grain legume species. Examination of these inhibitors in soybean, the most-thoroughly studied of all legume species, showed that they are anti-nutritional and that their residual activities, even in processed human foods, are a cause of concern to human health (Gumbmann et al. 1986; Liener 1986; Brandon et al. 1991). Protease inhibitors in pigeonpea were also considered to be anti-nutritional factors that affected protein quality and can be reduced by cooking, germination, or fermentation (Singh and Eggum 1984; Singh 1988). Studies of protease inhibitors in pigeonpea and its wild relatives have so far been very limited (Singh and Jambunathan 1981; Mulimani and Paramjyothi 1992; Pichare and Kachole 1992). Furthermore, the effect of environmental conditions on the levels of these protease inhibitors in the seeds of any grain-legume species has not been previously reported.

In this study, we examine the genetic variation of trypsin and chymotrypsin inhibitors among various land races and cultivars of pigeonpea and its wild relatives in the genus *Cajanus* (previously classified in the genus *Atylosia*; Maesen 1990) as estimated by their electrophoretic banding patterns in non-denaturing polyacrylamide gels and by their inhibitor activities using spectrophotometry. The effect of environment on the levels of trypsin and chymotrypsin inhibitor activities was also studied by growing the selected strains of pigeonpea at various locations.

Materials and methods

Plant material

A total of 69 strains, including land races and cultivars, of pigeonpea (*C. cajan)* from India, East and West Africa, and the Caribbean, two accessions of *C. cajanifolius* (Haines) van der Maesen, ten accessions of *C. scarabaeoides* (L.) Thouars, and one accession each of *C. acutifolius* (F.v. Muell.) van der Maesen, *C. albicans* (W. & A.) van der Maesen, *C. lineatus* (W. & A.) van der Maesen, *C. platycarpus* (Benth.) van der Maesen, and *C. volubilis* (Blanco) Blanco, were obtained from the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India. Thirty of the sixty-nine pigeonpea strains were selected to study the effect of environment. Seeds of these strains were harvested from their area of adaptation in Tanzania and Kenya at 20–1 500 meters above sea level (masl), considered as location I in this study, grown during November 1990 to August 1991 (Table 1). These seeds were then grown in the following season (November 1991–August 1992) at two different locations (environments): at Kibwezi in Kenya, 700 msal, 2°S latitude (location II) and at Katumani in Kenya, 1 600 msal, 2°S latitude (location III). Lists of the strains of pigeonpea and its wild relatives with their identification number, source, and code number are given in Tables 1 and 2, respectively.

Polyacrylamide-gel electrophoresis (PAGE) and inhibitor activity staining

After removal of the seed coat, 2–5 seeds from each strain were crushed to a fine meal using flat-nosed pliers and 50–100-mg seed meal samples were placed in 1.5-ml microcentrifuge tubes. Tris-$CaCl_2$ pH 8.1 buffer, containing 0.023 M $CaCl_2$ and 0.092 M Tris-HCl [tris (hydroxymethyl) aminomethane, pH adjusted to 8.1 with HCl], was used to extract the inhibitors. Final concentrations of the samples were precisely adjusted to 50 mg of seed meal per ml of the buffer in the case of pigeonpea and 100 mg of seed-meal per ml of the buffer in the case of the wild *Cajanus* species. The samples were incubated in the refrigerator (4 °C) overnight for protease-inhibitor extraction. The samples were thoroughly mixed 2–3 times during the extraction period using a vortex-mixer and the supernatant was clarified by centrifugation at 10 000 g for 2–5 min at 4 °C. The supernatant from each sample was used for both the electrophoretic analysis and the spectrophotometric assays.

PAGE was based on the Laemmli (1970) method without SDS as described by Kollipara et al (1991). This gel system was primarily non-denaturing, discontinuous, and uniform. PAGE was conducted using a Mini-PROTEAN® II Cell from Bio-Rad Laboratories, Richmond, Calif.. All the solutions were filter-sterilized (0.45 μm nitrocellulose filters, Micron Separation Inc). In brief, the resolving gel consisted of 12% acrylamide:bis (30 : 0.8) and 0.375 M Tris-Cl, pH 8.8, and the stacking gel consisted of 3.9% acrylamide:bis (30:0.8) and 0.125 M Tris-Cl, pH 6.8. Both gels were polymerized by adding ammonium persulfate and TEMED (N,N,N′,N′-tetramethylethylenediamine) to a final concentration of 0.05 and 0.1%, respectively. The running buffer consisted of an 0.025 M Tris base and 0.193 M glycine (pH not adjusted). All the gels were cast with the stacking gels about 1–1.5 cm high from the bottom of the wells to the top of the separating gels. A dye solution consisting of 20 or 60% glycerol with 0.005% bromophenol blue in distilled water was mixed with the seed extract in the ratio of 1:1 or 1:5, respectively, and an aliquot of 2–12.5 ml was applied to each sample well. The gels were run at a constant potential difference of 200 V until the bromophenol blue dye-front migrated to the end of the gel (approximately 45 min).

At the end of electrophoresis, the stacking gels were removed and the separating gels were stained for TI and CI activities based on Uriel and Berges (1968) with some modification (Kollipara and Hymowitz 1992). The gels were first washed in 0.1 M sodium phosphate buffer (pH 7.5) in a glass tray on an orbital shaker for three 5-min washes to equilibrate the gel with phosphate buffer. The gels were then incubated in phosphate buffer containing 15 mg/ml of bovine trypsin or α-chymotrypsin for 15–20 min on the shaker at room temperature. At the end of the incubation period gels were rinsed three times for 2 min each rinse in distilled water before incubating in staining solution. The staining solution was prepared by dissolving 20 mg of N-acetyl-DL-phenylalanine β-naphthyl ester (APNE) in 8 ml of N, N-dimethylformamide and 40 mg of o-dianisidine, tetrazoitized (zinc chloride complex) in 80 ml of distilled water separately. These solutions were mixed immediately before pouring on the gel. The gels were stained for 4–10 h without shaking in the dark. The stained gels were rinsed in distilled water and stored in 7% acetic acid.

Spectrophotometric assays of protease inhibitors

The TI and CI activity assays were conducted based on Hummel's (1959) method using p-toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE), respectively, as substrates according to the procedure described by Kollipara and Hymowitz (1992). A trypsin or α-chymotrypsin unit (TU or CU) is defined as the amount of trypsin or α-chymotrypsin that catalyzes the hydrolysis of 1 μmole of the substrate (TAME and BTEE, respectively) per min. A trypsin or α-chymotrypsin inhibitor unit (TIU or CIU) is the reduction in the activities of the respective enzymes by one unit, i.e., TU and CU, respectively (Friedman et al. 1991).

Results

The TI and CI banding patterns

All pigeonpea strains contained several TI bands with highly-uniform migration patterns (Fig. 1). At least two high-intensity and three low-intensity bands with Rf values of 0.55–0.60 (band 5 and 6) and 0.30–0.50 (band 1, 2, and 4), respectively, were observed across all the strains (Fig. 1). Three strains from West Africa (ICP 8194, ICP 13574, and ICP 13575) contained one additional faint band, each with an Rf value of about 0.45 (band 3 in Fig. 1). Variations in the TI band intensities were also observed among the pigeonpea strains. Some of the strains exhibited slower migrating bands (bands 1–4) with extremely-low intensities and could only be observed in the gel (not obvious from the photograph, see Fig. 1). Accessions of all the wild *Cajanus* species contained TI bands (Fig. 2). A high degree of variation in the migration pattern, number, and intensities of the TI bands was observed among the wild species of the genus *Cajanus*. However, the migration patterns of these bands were uniform among the accessions within a species in the case of both of *C. cajanifolius* and *C. scarabaeoides* (Fig. 2). The migration pattern of TIs in *C. cajanifolius* was strikingly similar to that in *C. cajan*. *Cajanus lineatus* and *C. albicans* also showed one major band each with a migration distance similar to that observed in *C. cajan* and *C. cajanifolius*.

The CI-activity staining revealed the presence of only a single band in pigeonpea as shown in Fig. 3. This band

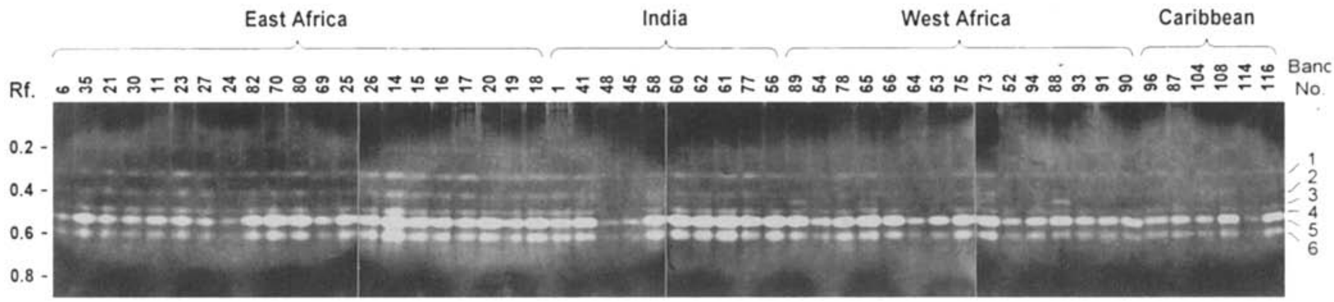

Fig. 1 Trypsin inhibitors in pigeonpea strains resolved by non-denaturing 12% PAGE and stained for TI activity. Crude extract from 125 μg of seed-meal (i.e., 2.5 μl of 50 mg/ml seed extract +2.5 μl of 20% dye solution) was applied per lane in each strain. The code numbers of the strains (see Table 1) and their geographical distribution are shown above the corresponding lanes. The scale of band migration distance (Rf scale) and the band numbers are indicated on either side of the gel

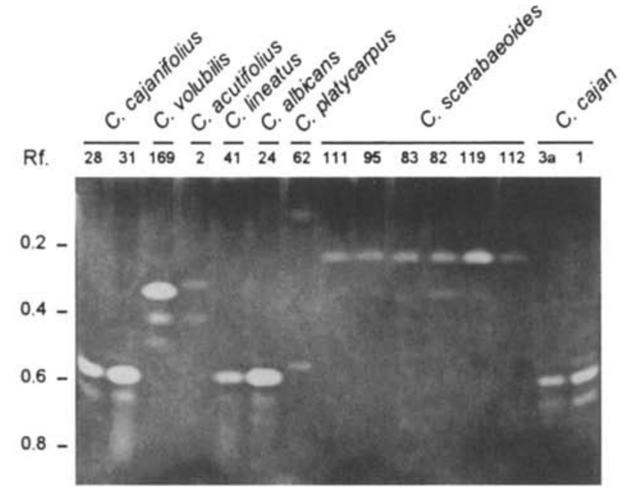

Fig. 2 Trypsin inhibitors in the wild *Cajanus* species resolved by non-denaturing 12% PAGE. Crude extract from 100 μg of seed-meal (i.e., 1 μl of 100 mg/ml of seed extract +1 μl of 20% dye solution) was applied per lane. Species names and accession numbers (ICPW numbers) are shown above the lanes (see Table 2). *Cajanus cajan* (pigeonpea) strains: 3a and 1 are same as Tr 3a and T7, respectively, as shown in Table 1

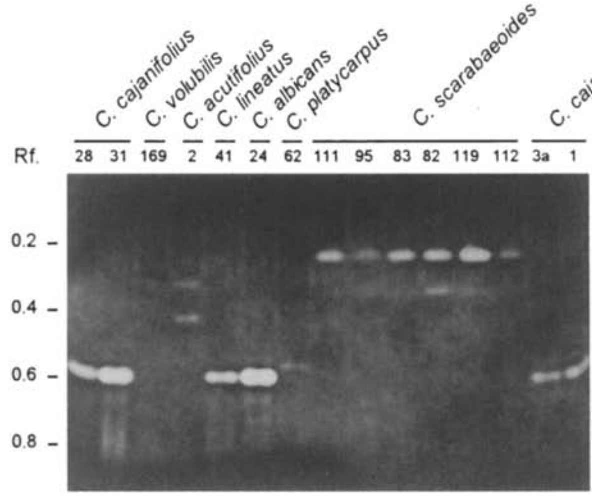

Fig. 4 Chymotrypsin inhibitors in the wild *Cajanus* species separated by non-denaturing 12% PAGE. Crude extract from 170 μg of seed-meal (i.e., 1.7 μl of 100 mg/ml seed extract +0.3 μl of 60% dye solution) was applied per lane. Species names and accession numbers (ICPW numbers) are shown above the lanes (see Table 2)

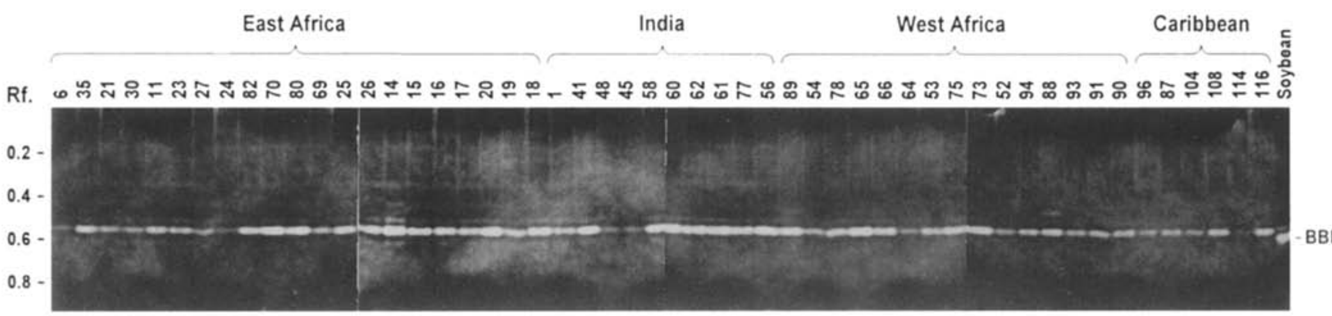

Fig. 3 Chymotrypsin inhibitors in pigeonpea strains separated by non-denaturing 12% PAGE and stained for CI activity. Crude extract from 250 μg of seed-meal (i.e., 5 μl of 50 mg/ml seed extract +5 μl of 20% dye solution) was applied per lane in each strain. The code numbers of the strains (see Table 1) and their geographical distribution are shown above the corresponding lanes. The Rf scale is indicated on left and the soybean BBI (Bowman-Birk Inhibitor) is shown on the right side of the gel

was observed in all pigeonpea strains with the same Rf value but with varying intensities and was identified to be the same as one of the major TI bands in pigeonpea (band 5 in Fig. 1). Similar observations were recorded in the wild species where the CI bands were same as the major TI bands in most of the species (Figs. 2 and 4). *Cajanus volubilis* accession (ICPW 169) used in this study was found to be "null" for CIs. When an excess amount of sample was loaded per lane, *C. volubilis* still did not exhibit CI bands but several new bands were resolved in the other species (Fig. 5). Some new bands were highly diffused and the others showed a weaker reaction (lower intensity).

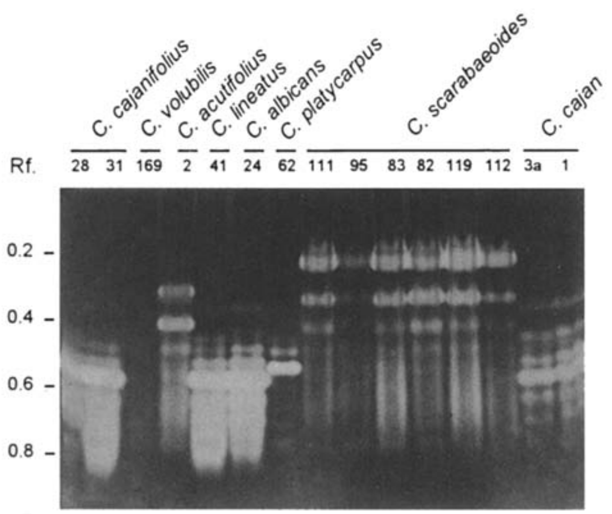

Fig. 5 The profiles of chymotrypsin inhibitors when excess amounts of samples, i.e., crude extract from 1 040 μg of seed-meal (10.4 μl of 100 mg/ml seed extract +2.1 μl of 60% dye solution), was loaded in each well of a 12% non-denaturing gel. The corresponding samples in each lane are identified above and the Rf scale is given on the left side of the gel

The TI and CI activities

Highly-significant variation was observed in the TIU/g seed values among the pigeonpea strains (Table 1). The TIU/g seed values ranged from 1 222±111.1 units in the strain Kenya 2 to 3 722±55.6 units in the strain ICPW 9151. No specific pattern was observed in the TI activities among the pigeonpea strains based on their geographical distribution. Significant variations in TIU/g seed values were also observed among the wild *Cajanus* species. Accessions of *C. albicans, C. cajanifolius,* and *C. volubilis* showed higher TI activities (TIU/g seed) compared to those in the accessions of *C. acutifolius, C. platycarpus,* and *C. scarabaeoides* (Table 2). *Cajanus platycarpus* accession (ICPW 62) contained the lowest TI activity (383±12.4 TIU/g seed) of all the pigeonpea and the wild *Cajanus* species strains used in this study. The mean TIU/g seed value of the wild species (1 407±119.8 units) was also much lower than that of the pigeonpea strains (2 279±49.9 units; Tables 1 and 2). In general, the TIU/g seed values of pigeonpea and its wild relatives corroborated well with the band intensities observed in the gels stained for TI activities (Tables 1–2; Figs. 1–5).

The CI activity (CIU/g seed) was also found to be significantly variable among pigeonpea strains, ranging from 31±0.0 units in Kenya 2 to 87±0.0 units in ICP 9151 (Table 1). The accessions of wild *Cajanus* species exhibited an even higher degree of variation in their CI activities (Table 2). As expected, *C. volubilis* accession (ICPW 169) did not contain any detectable CI activity, confirming the lack of CIs observed in the activity-stained gels (Figs. 4 and 5). The mean CIU/g seed value of wild *Cajanus* species (162±12.8 units) was significantly higher than that of the pigeonpea strains (62±1.0 units; Tables 1 and 2). The CIU/g seed values in both pigeonpea and its wild relatives (except *C. volubilis*) also correlated positively with the TIU/g seed values (Table 1 and 2).

Effect of environment on the protease inhibitor activities

Environment, strain, and environment × strain interaction all showed statistically significant effects on the TI and CI activities (data not shown). The mean values of all the seed traits measured, such as 100 seed weight, seed-protein content, TIU/g seed, TIU/g protein, CIU/g seed, and CIU/g protein, in the 30 selected pigeonpea strains are given in Table 3. The seed weight was observed to have increased when the pigeonpea strains were grown at the higher altitude (location III, at 1 600 masl). The mean TIU/g seed, TIU/g protein, CIU/g seed, and CIU/g protein values were highest when the pigeonpea was grown at 700 masl, followed by those at 1 600 masl. These values were lowest in the pigeonpea strains grown at the area of their adaptation (Table 3).

The correlation coefficients between various traits measured across the 30 pigeonpea strains are shown in Table 4. Statistically-significant positive correlations were observed among TIU/g seed, TIU/g protein, CIU/g seed, and CIU/g protein values. However, significantly-negative correlations were observed between seed weight and protein content and also between the protein content and CIU/g protein. The seed weights were not found to be correlated significantly with the TI and CI activities (Table 4).

Discussion

Highly-uniform banding patterns of TIs and CIs among the pigeonpea strains suggest that these proteins are strongly conserved (Figs. 1 and 3). The conservative nature of these proteins has made them very useful markers to study the biosystematics of several plant species, including those of *Glycine* (Weder 1985; Kollipara et al. 1993). This is also evident from the migration patterns of these proteins in the wild *Cajanus* species. In the wild species, these inhibitors exhibited highly species specific banding patterns with a fair amount of uniformity among the accessions within a species, as observed in case of *C. cajanifolius* and *C. scarabaeoides* (Figs. 2, 4, and 5). The migration patterns of the two major inhibitors in *C. cajanifolius* were similar to those observed in *C. cajan* (pigeonpea), suggesting that *C. cajanifolius* is genomically closest to the pigeonpea. This observation agrees with the previously reported cyto-morphological, isozyme, and molecular evidence (Krishna and Reddy 1982; Pundir and Singh 1985; Nadimpalli et al. 1993). We found that the major TI and CI band in both *C. lineatus* and *C. albicans* accessions co-migrated with that in *C. cajan,* indicating a closer genomic relationship among these species (Figs. 2, 4, and 5). Based on cytological and electrophoretic analyses, Pundir and Singh (1985) also found that *C. cajan* was closer to *C. lineatus* followed by *C. cajanifolius.*

Table 1 List of the pigeonpea strains with the mean values of trypsin inhibitor units (TIU) and chymotrypsin inhibitor units (CIU) per gram seed from two estimations by spectrophotometric activity-assays

Code no.[a]	Strain identification[b]	Source[c]	TIU/g seed±SE	CIU/g seed±SE
1*	T 7	India Land race Selection	2 222±222.2	56±0.0
2*	ICPL 91055	ICRISAT Pigeonpea Breeding	1 722± 55.6	56±6.2
3*	ICPL 91056	ICRISAT Pigeonpea Breeding	1 778±444.5	53±3.1
4*	ICPL 91058	ICRISAT Pigeonpea Breeding	2 500±167.0	68±6.2
5*	1 Babati	Tanzania (EA) LS Col. 1210 masl	3 333±333.5	78±3.1
6*	4 Babati	Tanzania (EA) LS Col. 1440 masl	1 667±222.2	40±3.1
7*	4 ILCA	GRU ILCA	2 444±333.5	59±3.1
8*	Kat. 777	Kenya (EA) Katumani Selection	2 889±333.0	72±3.1
9*	Kat. 2	Kenya (EA) Katumani Selection	2 889±111.1	75±0.0
10*	Kat. 81/3/3	Kenya (EA) Katumani Selection	2 556±777.5	75±6.2
11*	Kenya 12	Kenya (EA) LS Col. 1530 masl	2 388± 55.6	59±3.1
12*	Kenya 17	Kenya (EA) LS Col. Market Sample	3 222±111.1	75±0.0
13*	Kenya 18	Kenya (EA) LS Col. Market Sample	2 167±166.5	56±0.0
14*	Tanzania 2	Tanzania (EA) LS Col. 590 masl	2 500±166.7	65±3.1
15*	Tanzania 6	Tanzania (EA) LS Col. 270 masl	1 611± 55.6	53±3.1
16*	Tanzania 10	Tanzania (EA) LS Col. 330 masl	1 888±111.1	65±3.1
17*	Tanzania 18	Tanzania (EA) LS Col. 340 masl	1 722± 55.6	56±0.0
18*	Tanzania 20	Tanzania (EA) LS Col. 120 masl	2 111± 0.0	65±3.1
19*	Tanzania 21	Tanzania (EA) LS Col. 20 masl	2 167±166.7	65±3.1
20*	Tanzania 22	Tanzania (EA) LS Col. 20 masl	2 444±111.1	81±6.2
21*	7 Babati 12	Tanzania (EA) LS Col. 30 masl	1 944±166.7	50±0.0
22*	Tanzania 12	Tanzania (EA) LS Col. 340 masl	1 389±278.0	50±0.0
23*	Kenya 1	Kenya (EA) LS Col. 1100 masl	1 944± 55.6	50±0.0
24*	Kenya 2	Kenya (EA) LS Col. 1120 masl	1 222±111.1	31±0.0
25	Kenya 7	Kenya (EA) LS Col. 680 masl	2 500±388.9	75±6.2
26	Kenya 4	Kenya (EA) LS Col. 850 masl	2 222±333.3	65±3.1
27	Kenya 20	Kenya (EA) LS Col. Market sample	1 778±111.1	44±0.0
28*	Gwalior 3	India Land race Selection	2 111±222.2	53±3.1
29*	ICPL 366	ICRISAT Pigeonpea Breeding	2 722±389.0	68±6.2
30	9 Babati	Tanzania (EA) LS Col. 1400 masl	1 611± 55.6	37±0.0
31*	16 Babati	Tanzania (EA) LS Col. 1400 masl	3 277± 55.6	68±6.2
34*	ICP 11820	GRU ICRISAT	1 945±276.5	56±0.0
35	12 Babati	Tanzania (EA) LS Col. 1400 masl	3 111±222.2	72±3.1
36*	Tanzania 23	Tanzania (EA) LS Col. 460 masl	2 333±111.1	72±3.1
41	ICPL 87 B	ICRISAT Pigeonpea Breeding	2 444±222.2	65±3.1
42*	2 ILCA	GRU ILCA	3 389±389.0	81±6.2
45	Dwarf 21	ICRISAT Pigeonpea Breeding	1 500± 55.6	47±3.1
48	Dwarf 4	ICRISAT Pigeonpea Breeding	1 611± 55.6	53±3.1
52	ICP 2811	Nigeria (WA) GRU ICRISAT	2 167± 55.6	56±3.1
53	ICP 4024	Nigeria (WA) GRU ICRISAT	1 556±333.3	47±0.0
54	ICP 4715	Ghana (WA) GRU ICRISAT	1 722± 55.6	47±3.1
56	ICP 6997	India GRU ICRISAT	2 556±222.2	62±0.0
58	ICP 7035	India GRU ICRISAT	2 000±111.1	56±0.0
60	ICP 8006	India GRU ICRISAT	2 278± 55.6	65±3.1
61	ICP 8081	India GRU ICRISAT	2 278± 55.6	62±0.0
62	ICP 8084	India GRU ICRISAT	2 000±111.1	62±6.2
64	ICP 8193	Senegal (WA) GRU ICRISAT	2 222±111.1	65±3.1
65	ICP 8194	Senegal (WA) GRU ICRISAT	2 611± 55.6	75±0.0
66	ICP 8202	Senegal (WA) GRU ICRISAT	2 167±166.7	62±0.0
69	ICP 9150	Kenya (EA) GRU ICRISAT	3 111±111.1	75±0.0
70	ICP 9151	Kenya (EA) GRU ICRISAT	3 722± 55.6	87±0.0
73	ICP 9272	Nigeria (WA) GRU ICRISAT	2 111± 0.0	65±3.1
75	ICP 11479	Nigeria (WA) GRU ICRISAT	2 000±333.3	72±3.1
77	ICP 11934	India GRU ICRISAT	1 889±111.1	59±3.1
78	ICP 12190	Ghana (WA) GRU ICRISAT	2 389± 55.6	65±3.1
80	ICP 13075	Kenya (EA) GRU ICRISAT	3 444±111.1	81±0.0
82	ICP 13082	Kenya (EA) GRU ICRISAT	3 000± 0.0	72±3.1
87	ICP 13556	Grenada (WI) GRU ICRISAT	2 278±277.8	50±6.2
88	ICP 13574	Sierra Leone (WA) GRU ICRISAT	2 444±333.3	75±0.0
89	ICP 13575	Sierra Leone (WA) GRU ICRISAT	3 167±277.8	78±3.1
90	ICP 13576	Sierra Leone (WA) GRU ICRISAT	1 889±111.1	59±3.1
91	ICP 13640	Cape Verde (WA) GRU ICRISAT	2 056± 55.6	59±3.1
93	ICP 13642	Cape Verde (WA) GRU ICRISAT	1 889±111.1	65±3.1
94	ICP 13643	Cape Verde (WA) GRU ICRISAT	2 000±111.1	62±6.2
96	ICP 13820	Grenada (WI) GRU ICRISAT	2 500± 55.6	56±0.0

Table 1 (continued)

Code no.[a]	Strain identification[b]	Source[c]	TIU/g seed±SE	CIU/g seed±SE
104	ICP 13856	St.Vincent (WI) GRU ICRISAT	2 167±277.8	62±12.5
108	ICP 13860	St.Vincent (WI) GRU ICRISAT	2 222± 0.0	56±6.2
114	Tr 3a	Trinidad (WI) LS Col.	1 444±111.1	37±6.2
116	Tr 3c	Trinidad (WI) LS Col.	2 667±111.1	56±0.0
Mean			2 279	62
SE ±			49.9	1.0
CV%			13.2	8.6

[a] Temporary numbers assigned at the University of Illinois, * strains selected to study the effect of environment
[b] Identification numbers designated by International Crops Research Center for Semi-Arid Tropics (ICRISAT)
[c] EA, East Africa; GRU, Genetic Resource Unit; ILCA, International Livestock Center for Africa; LS Col., collected by Laxman Singh; masl, meters above sea level; WA, West Africa; WI, West Indies

Table 2 Wild relatives of pigeonpea (*C. cajan*), accession number, and mean values of trypsin inhibitor units (TIU) and chymotrypsin inhibitor units (CIU) per gram seed from three estimations by spectrophotometric activity-assays

Species	Accession number[a]	TIU/g seed±SE	CIU/g seed±SE
C. acutifolius (F.v. Muell.) van der Maesen	ICPW 2	741± 14.8	91± 4.2
C. albicans (W. & A.) van der Maesen	ICPW 24	2 852± 98.0	398±25.0
C. cajanifolius (Haines) van der Maesen	ICPW 28	2 333±111.1	124± 0.0
	ICPW 31	3 370±225.3	282±22.0
C. lineatus (W. & A.) van der Maesen	ICPW 41	1 481±196.0	216±41.5
C. platycarpus (Benth.) van der Maesen	ICPW 62	383± 12.4	46± 4.2
C. scarabaeoides (L.) Thouars	ICPW 82	1 086± 98.8	167±15.0
	ICPW 83	1 062± 65.3	145±11.0
	ICPW 87	1 185±154.2	162±26.0
	ICPW 95	913± 24.7	50± 0.0
	ICPW 101	667±113.2	162±36.0
	ICPW 111	889± 0.0	124± 0.0
	ICPW 112	790± 24.7	100± 0.0
	ICPW 117	963± 42.8	158±11.0
	ICPW 119	1 383± 65.3	199± 7.2
	ICPW 122	1 160± 49.4	166± 4.2
C. volubilis (Blanco) Blanco	ICPW 169	2 667± 64.2	0± 0.0[b]
Mean		1 407	162
SE ±		119.8	12.8
CV%		12.5	19.6

[a] Accession numbers are assigned by ICRISAT
[b] CIU/g seed value is excluded from the estimation of the mean, SE, and CV

Table 3 Mean, standard error (SE), and coefficient of variability (CV) of 100 seed weight, total seed protein[a], and the trypsin and chymotrypsin inhibitor units (TIU and CIU, respectively) of the selected 30 pigeonpea strains grown at three locations (environments)

Parameters (traits) measured	Location (environment)[b]								
	(I) Area of adaptation			(II) 700 masl			(III) 1600 masl		
	Mean	SE	CV (%)	Mean	SE	CV (%)	Mean	SE	CV (%)
100 seed wt. (g)	14.0	± 0.42	4.3	13.5	± 0.43	6.1	15.7	± 0.44	7.9
Total protein (%)	22.5	± 0.43	3.1	22.2	± 0.21	2.4	22.6	± 0.19	1.6
TIU/g seed	2 211	± 98.4	19.9	2 909	±101.3	14.4	2 531	±129.7	22.1
TIU/g protein	9 846	±443.7	19.6	13 133	±451.8	14.4	11 205	±568.6	23.1
CIU/g seed	60	± 1.6	12.5	71	± 1.2	7.6	65	± 1.9	8.5
CIU/g protein	269	± 7.3	13.1	322	± 5.8	7.8	290	± 8.6	8.3

[a] Total protein was estimated using the micro-Kjeldahl method as described by Mulvaney (1993)
[b] Plants grown in three locations: (I), at the area of adaptation (see Table 1); (II), at Kibwezi, Kenya, at 700 m above sea level (masl); and (III), at Katumani, Kenya, at 1 600 masl

Table 4 Correlation coefficients among various seed quality parameters measured in 30 selected pigeonpea strains grown in three different environments

Parameter	% Protein	TIUS[a]	TIUP[b]	CIUS[c]	CIUP[d]
100 seed wt.	–0.28*	0.00	0.05	0.00	0.09
% Protein		0.07	–0.13	0.03	–0.31*
TIUS			0.98*	0.82*	0.75*
TIUP				0.81*	0.81*
CIUS					0.93*

[a] TIUS, trypsin inhibitor units/g seed
[b] TIUP, trypsin inhibitor units/g protein
[c] CIUS, chymotrypsin inhibitor units/g seed
[d] CIUP, chymotrypsin inhibitor units/g protein
* Correlation coefficients are significant at α=0.05 level

The variation in TI and CI band intensities may simply be quantitative or could be the result of a weaker inhibition due to lower affinity of these inhibitors to the substrate (trypsin or chymotrypsin). Similar observations were previously recorded in soybean and its wild perennial relatives by Kollipara and Hymowitz (1992). The quantitative variations in the TI and CI activities found among the pigeonpea strains corroborated fairly well with the intensity variations of the respective inhibitor activity bands in the gels (Table 1, 2; Figs. 1–5). A maximum of six TI bands (of which only two were prominent), but only one CI band, was observed among pigeonpea strains (Figs. 1 and 3). Multiple TI bands were also recorded by Pichare and Kachole (1992) upon subjecting the crude extracts of pigeonpea seed to isoelectric focussing. Up to six CI bands were seen when excess quantities of samples were analyzed per lane in the gel (Fig. 5). This was most likely due to a weaker and non-specific binding of these proteins (which appear to be TIs from their migration distances) to chymotrypsin. Such a weaker reaction was also observed in the case of the soybean Kunitz trypsin inhibitor (KTI), a specific inhibitor of trypsin (Kollipara 1992). The diffused and faster-migrating bands in some of the lanes might be due to specific and non-specific proteolytic degradation of the inhibitors. Specific proteolytic degradation of the KTI and the Bowman-Birk inhibitor (BBI), producing derivatives with inhibitor activities, was previously recorded in soybean (Madden et al. 1985; Wilson et al. 1988).

The TIU/g seed was at least two orders of magnitude higher than the CIU/g seed in pigeonpea (Table 1). These results conflict with the observations of Mulimani and Paramjyothi (1992) who found that CI activity was higher than TI activity in several pigeonpea strains. Furthermore, the TI and CI values reported by Singh and Jambunathan (1981) and Mulimani and Paramjoythi (1992) were much lower than those observed in the present study. One of the reasons for this may be because of the difference in the inhibitor extraction procedure. These previous authors presoaked the seeds in distilled water for over 12 h and only then were the seeds used for extracting the inhibitors. This could cause a partial loss of the inhibitors due to leaching. Significant portions of the protease inhibitors (Kunitz type and Bowman-Birk type) were shown to be localized in the extracellular spaces in soybean seed (Horishberger and Tacchini-Vonlanthen 1983 a, b) and are released (by leaching) within a few hours after imbibition (Hwang et al. 1978; Tan-Wilson and Wilson 1982). Another reason for the disagreement in the inhibitor activity values could be due to the difference in defining the unit-enzyme (trypsin or chymotrypsin) and unit-inhibitor activities.

In order to improve the nutritional quality of pigeonpea for both human and animal consumption, the levels of these protease inhibitors must be reduced. Heat-processing (cooking by moist heat or microwave) of the seeds was shown to help denature most of these proteins in soybeans (Sakla et al. 1988; DiPietro and Liener 1989). However, it is also possible to eliminate or reduce these inhibitors by transferring the null or low-expressing alleles of these inhibitors (natural variants) to the cultivated strains through breeding. A good example of such an effort was the release of soybean cultivar 'Kunitz', an isoline of cv Williams 82 lacking the functional *Ti* allele (i.e., containing ti allele), which was shown to be nutritionally superior to cv Williams 82 in animal feeding studies (Bernard et al. 1991; Friedman et al. 1991; Han et al. 1991; Zhang et al. 1991). Pigeonpea strains with the lowest TI and CI activities identified in the present study, such as Kenya 2, Tr 3a, etc., could potentially be used in a breeding program to reduce the inhibitor contents of pigeonpea cultivars (Table 1). On the other hand, protease inhibitors are also considered to improve the plants defense against insect and pathogen attack (Ryan 1990). The high TI and CI strain, ICP 9151, identified in our study, could be used for breeding new cultivars that contain high levels of these inhibitors.

The TI and CI activities of pigeonpea strains increased when they were grown in environments (locations II and III) different from their area of adaptation (location I; Table 3). Highest activities were found when pigeonpea was grown at a lower altitude (700 masl). The mean maximum temperature was recorded to be about 3°C lower in high altitude (location III, 1600 masl) compared to that at low altitude (location II, 700 masl). The maturity periods were also altered due to the change in environment. All the 30 pigeonpea strains were of the long-duration type (210 days from planting to flowering) when they were grown in their area of adaptation (location I). When grown in a different environment (location II or III) most of the strains flowered earlier showing a medium- or short-duration type of behaviour (100–150 days from planting to flowering; data not shown). Therefore, the highly significant effect of environment on the TI and CI activities observed in this study might, in part, be due to the change in temperature and maturity period.

As expected from the band-intensity comparisons, highly-significant positive correlations were observed between the TI and CI activities (Figs. 1–5; Table 4). This could be due (as in soybean) to the presence of Bowman-Birk type inhibitors which inhibit both trypsin and chymotrypsin simultaneously (Odani and Ikenaka 1973; Birk 1985). In the present study, we have, for the first time, identified the presence of such Bowman-Birk type inhibitors in pigeonpea and its wild relatives. However, further characterization needs to be done through purification and protein sequencing to gain a better understanding of these inhibitors.

Acknowledgements The authors would like to thank Dr. R. L. Mulvaney for providing facilities to perform the micro-Kjeldahl procedure. One of the authors, Laxman Singh, was supported by ICRISAT during the course of this investigation on his sabbatical leave.

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