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ELECTROPHORETIC ANALYSIS OF EXPRESSION OF TRYPSIN INHIBITORS DURING CHICKPEA (*CICIER ARIETINUM* L.) SEED DEVELOPMENT

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ABSTRACT

Chickpea (*Cicer arietinum* L.) is one of the important pulse crops in India which provides high quality proteins for vegetarians. India shares 75% of the world's chickpea supply. Investigation on accumulation of protease inhibitors during chickpea seed development was carried out. A total of eight genotypes were investigated for expression of trypsin inhibitors during seed development by using polyacrylamide gel-X-ray film contact print method. Electrophoretic profiles of trypsin inhibitors (TI) revealed the results in which a total of 6 isoinhibitors of trypsin were detected in the mature seeds of chickpea. All the eight genotypes exhibited no expression of trypsin inhibitors at 15 days after flowering (DAF). Six genotypes (ICCL-86111, Digvijay, ICC-4958, Chafa, WR-315 and PG-9758) showed no expression of trypsin inhibitors up to 30 DAF. Two genotypes (ICCV-10 and 3137) started expressing trypsin inhibitors around 30 DAF.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important pulse crops of the world that provides quality proteins for vegetarians. Chickpea is also used as feed for livestock. India is the largest producer of chickpea as it shares 75% of world production. (Saxena and Singh 1987). Chickpea production is restricted due to many biotic and abiotic stresses. The insect pest *Helicoverpa armigera* is the most important biotic stress to chickpea. It is a polyphagous pest of developing seeds of several legume species. *H. armigera* feeding on chickpea begins at flowering stage. Typically a single larva damages five pods per day leading to heavy losses in the crop yield. Plants synthesize various compounds like protease inhibitors, amylase inhibitors, lectins against insect attack or for imparting insect tolerance (Jouanin *et al* 1998, Schuler *et al* 1998). Protease inhibitors is the most studied class of plant defense proteins. According to Casaretto and Corcuera (1995) protease inhibitor content is about 10% of total protein present in seed storage tissues. Gatehouse and Boulter (1983) demonstrated that the resistance of a cowpea variety to the bruchid beetle was due to the elevated trypsin inhibitor (TI) levels in the seeds.

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Very few studies have been done on accumulation of protease inhibitors in developing seeds of chickpea or other legumes. Therefore it was felt necessary to investigate expression of trypsin inhibitors during seeds development of chickpea. The present investigation was carried out to determine the trypsin inhibitor profiles in developing seeds of chickpea by using electrophoretic technique.

MATERIALS AND METHODS

Chickpea seeds of eight genotypes (ICCL-86111, Digvijay, ICC-4958, Chafa, WR-315, PG-9758, ICCV-10 and 3137) were cultivated under irrigated conditions at Pulse Research Station, Mahatma Phule Krishi Vidhyapeeth (MPKV), Rahuri, Ahmednagar, Maharashtra, India. Samples of developing seeds were collected from the same crop. The X-ray films were bought from Laser Selvasa, India. Chemicals and reagents required for electrophoresis and trypsin were procured from SRL, India. Other reagents used were of analytical grade.

Sample collection: The opened chickpea flowers were tagged and the developing pods from such flowers were collected on 15,th 30,th 45,th and 60th day after flowering (DAF). The seeds of these different developmental stages were homogenized in chilled acetone in mortar and pestle. Acetone was removed by

filtration using whatman filter paper no. 1 and residues were air dried.

Extraction of trypsin inhibitors: The dried seed powder was extracted in distilled water at 5 ^{o}C for 12 hours and centrifuged at 10000 rpm for 15 min at 4 ^{o}C. The supernatants were collected and stored at 0 ^{o}C until used.

Electrophoretic separation and detection of trypsin inhibitors: Electrophoresis was carried out on discontinuous polyacrylamide gel (10%) using Davis buffer system (Davis, 1964). Equal volumes (50 µl) of the seed extracts were loaded. The PAGE was carried out at 30 mA at constant current mode. After electrophoresis the gels were processed for activity staining of PIs by Gel-X-ray film contact print method (Pichare and Kachole, 1994). After run the gel was removed from glass plates and equilibrated with 0.1 M Tris-HCl buffer pH 7.8 for 10 minutes. The gel was incubated in 0.1 mg/ml trypsin solution in equilibration buffer for 10-15 minutes. Gel was then washed briefly in equilibration buffer and subsequently placed on an undeveloped X-ray film for 5-15 minutes. The X-ray film was removed and washed in a tray containing warm water to visualize trypsin inhibitor (Ti) bands as unhydrolyzed gelatin. The X-ray film showing optimum bands was then photographed.

RESULTS AND DISCUSSION

Electrophoretic profile of trypsin inhibitor isoforms in chickpea genotypes: Figure 2 shows the electrophoretic profile of TIs in mature seeds of eight different chickpea cultivars. A total of 6 isoinhibitors of trypsin were detected by the method employed. These trypsin iso-inhibitors are named as Ti1, Ti2, Ti3, Ti4, Ti5 and Ti6. Among these isoinhibitors Ti1 was found to be located in the stacking gel while remaining separated in resolving gel. Ti1, Ti2 & Ti3 could be observed in all the eight chickpea genotypes investigated, with significant variations in their band intensities. In regard to Ti4, Ti5 and Ti6 significant variation was observed among the genotypes studied. Ti6 was present only in Digvijay genotype. Ti5 was seen only in ICCL-86111 and variety Digvijay. Ti4 with strong activity was seen in ICCL-86111, Digvijay and Chafa while others have very weak activity band. Ti 6 with very weak intensity could be seen in genotype Digvijay only.

Electrophoretic profiles of trypsin inhibitor isoforms in developing seeds of chickpea genotypes

Figures 3, 4 and 5 depict the electrophoretic patterns of TIs in eight different chickpea genotypes through four stages of seed development.

Figure 1. Chickpea pods and seeds at different developmental stages

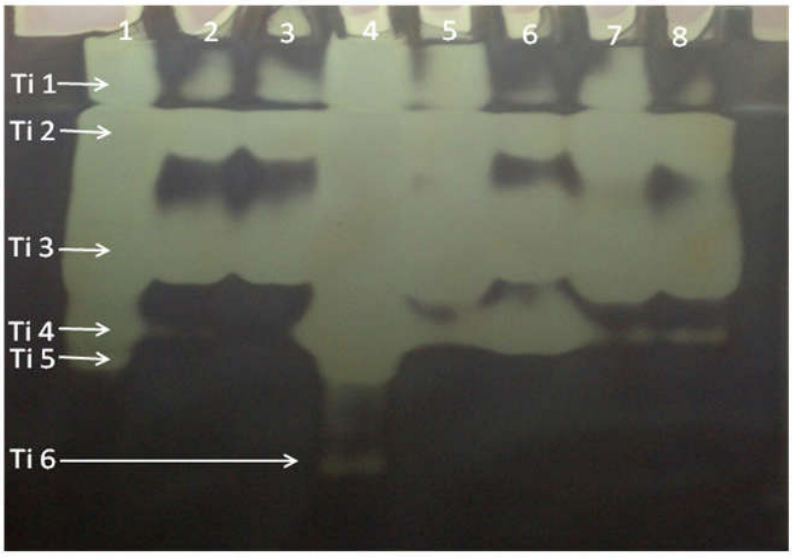

Figure 2. Trypsin inhibitor profile of eight Chickpea genotypes (mature seeds). Lane no. 1. ICCL-86111, 2. ICCV-10, 3. 3137, 4. Digvijay, 5. ICC-4958, 6. Chafa, 7. WR-315 and 8. PG-9758

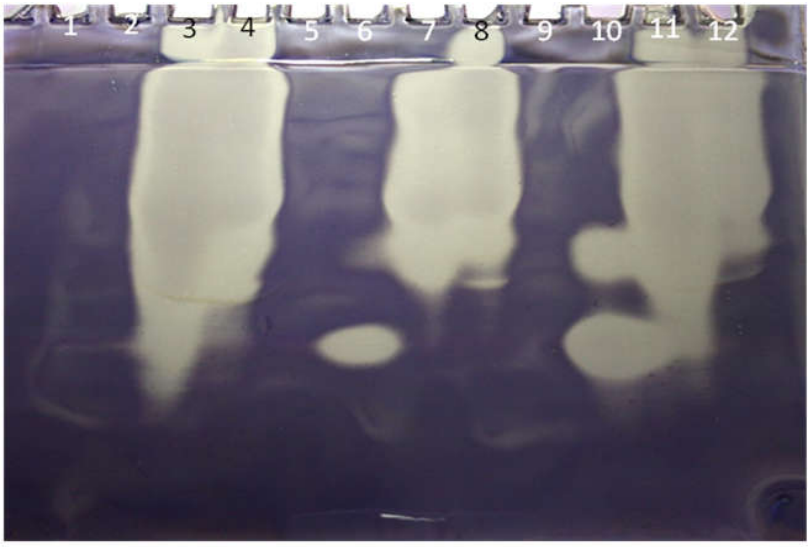

Figure 3. Trypsin inhibitor profile of three chickpea genotypes during developing stages -Lane no 1-4: ICCL-86111, lane no 5-8: ICCV-10 and lane no 9-12: 3137). Lane No. 1,5,9. -15 DAF, lane no. 2,6,10- 30 DAF, lane no. 3,7,11-45 DAF and lane no. 4,8,12-60 DAF

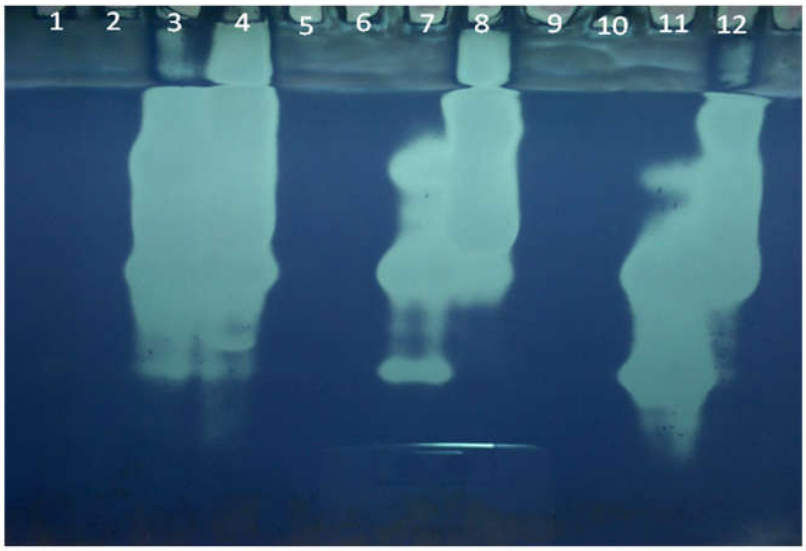

Figure 4. Trypsin inhibitor profile of three chickpea genotypes during developing stages- Lane no 1-4: Digvijay, lane no 5-8: ICC-4958 and lane no 9-12: Chafa Lane no. 1,5,9-15 DAF, lane no. 2,6,10-30 DAF, lane no. 3, 7, 11-45 DAF and lane no 4, 8, 12-60 DAF

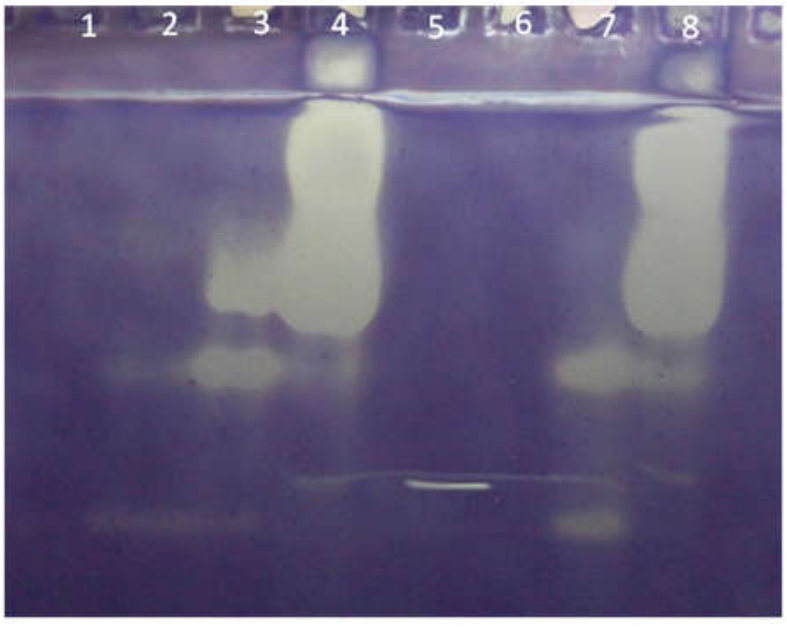

Figure 5. Trypsin inhibitor profile of two chickpea genotypes during developing stages- Lane no 1-4 : WR-315 , lane no 5-8: PG-9758. Lane no. 1, 5- 15 DAF, 2, 6- 30 DAF, 3, 7- 45 DAF and 4, 8- 60 DAF

Table 1. Summarization of Ti expression at different stages of seed development

Genotypes	Stages of Seed Development (Days after flowering)															
	15 DAF	30 DAF			45 DAF						60 DAF					
ICCL-86111					Ti1	Ti2	Ti3	Ti4	Ti5	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	Ti6
ICCV-10			Ti4	Ti6		Ti2	Ti3	Ti4	Ti5	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	
3137			Ti4	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	
Digvijay					Ti1	Ti2	Ti3	Ti4	Ti5	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	Ti6
ICC-4958							Ti3	Ti4	Ti5	Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	
Chafa							Ti3	Ti4		Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	
WR-315							Ti3	Ti4			Ti1	Ti2	Ti3	Ti4	Ti5	
G-9758								Ti4		Ti6	Ti1	Ti2	Ti3	Ti4	Ti5	

No expression of any TI isoform was observed on 15th DAF in all the investigated chickpea genotypes. Except cultivar ICCV-10 and 3137 no expression of TI isoforms at 30th DAF could be seen in the chickpea genotypes studied. Ti 4 and Ti 6 were seen expressed at 30 DAF in ICCV-10 and 3137 varieties (Figure 3, lanes 6 & 10). Ti 6 disappeared in the matured seeds (Figure 3, lanes 8 & 12). At 45 DAF, Ti1did not express in genotypes ICCV-10, ICC-4958, Chafa, WR-315 and PG-9758. Ti 2 was not observed in genotypes ICC-4958, Chafa, WR-315 and PG-9758. Ti3 could not be detected in genotype PG-9758 only, while all other genotypes exhibited strong expression of the same at 45 DAF. At 45 DAF Ti4 was seen strongly expressed in all the genotypes. Ti 6 was seen intensely expressed at 45 DAF in genotypes ICC-4958, Chafa and PG-9758. Summary of TI expression during chickpea seed development is presented in Table 1. Varietal difference with respect to the trypsin inhibitors and chymotrypsin inhibitors in chickpea has been reported by Sastry and Murray (1987). Patankar *et al*. (1999) found lesser extent of variability in TI isoforms in chickpea cultivars as compared to its wild relatives. Similar reports have been published in pigeonpea. TIs and chymotrypsin inhibitors are conserved in mature seeds of the cultivated pigeonpea whereas high level of variation exists in the uncultivated species of *Cajanus* (Kollipara *et al*. 1994, Pichare and Kachole 1996). Expression of TIs in chickpea seeds begins around 30 DAF and different electrophoretic forms of TIs go on accumulating during seed development. In the present study it was found that Ti4 and Ti6 were found to express during mid period of the seed development, as seed matures these TIs disappeared. This differential appearance of TIs may be related to temporal expression of different genes or may be due to post-translational modifications of the inhibitors (Domoney *et al*. 1995, Harsulkar *et al*. 1997, Giri *et al*. 1998). Giri *et al*. (1998) demonstrated that Ti5 is the proteolysis product of Ti1. According to Giri *et al*. (1998) to confirm the differential expression of TI isoforms, northern analysis with specific Ti genes or the measurement of protein synthesis at different stages during seed development is necessary. Results of this investigation clearly indicate that TI isoforms are not early and major proteins expressing in the developing seeds. Late expression of TIs (30 to 45 DAF) during chickpea seed development can be correlated with heavy infestation of young pods/seeds by pod borer *Helicoverpa armigera*.

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