

Assessment of genetic variation in lucerne (*Medicago sativa* L.) using protease inhibitor activities and RAPD markers

Author Details

Amaresh Chandra
(Corresponding author)

Crop Improvement Division, Indian Grassland and Fodder Research Institute,
Jhansi-284003, India
e-mail: amaresh62@yahoo.com

Krishna Chandra Pandey

Crop Improvement Division, Indian Grassland and Fodder Research Institute,
Jhansi - 284 003, India

Publication Data

Paper received:
11 April 2009

Revised received:
03 March 2010

Accepted:
19 April 2010

Abstract

The aim of the present study is to identify and characterize lucerne lines resistance to weevil infestation. After three years of field screening for resistance to weevil infestation, 13 lines of lucerne were selected to assess the genotypic variations for lucerne weevil (*Hypera postica* Gyll.) at biochemical and molecular levels. Total phenols varied from 0.15 to 0.91 mg g⁻¹ (DM) in these genotypes. The highest trypsin (11.11 unit mg⁻¹ protein) and chymotrypsin (93.0 unit mg⁻¹ protein) inhibitors activities were recorded in G-1-02 and B-4-03 lines respectively, whereas highest α -amylases inhibitor activity (14.2 unit mg⁻¹ protein) in C-6-01. Zymogram patterns for trypsin inhibitor activity showed quantitative variations among the lines. In total 262 DNA fragments were generated when 45 deca-mer random primers were employed. Genetic variation in terms of genetic distance ranged from 0.65 to 0.85. Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using the Un-weighted Pair Group Method with Arithmetic mean (UPGMA) algorithm yielded two clusters (cluster I and II) which converged at 72% similarity level. Cluster I contained most of the lines having low level of weevil infestation. High bootstrap values (>40) indicated the significance of nodes embodied in these two clusters. However, SDS-PAGE analysis of the leaf proteins of these 13 lines showed no major variations except minor difference in the protein bands of molecular weights between 14 to 20 kD.

Key words

Genotypic variation, Heat stable proteins, Insect resistance, Lucerne, Proteinase inhibitor, RAPD, SDS-PAGE

Introduction

Lucerne or alfalfa (*Medicago sativa* L.) is a perennial forage legume, often called 'Queen of the forages'. It is cultivated throughout the world in diverse environment ranging from temperate regions to the equator. In India, lucerne occupies more than 1 million ha area and provides 60 to 130 tones of green forage ha⁻¹ (Hazra, 1995). The alfalfa weevil (*Hypera postica* Gyll.), a coleopteron insect, is one of the primary defoliators of alfalfa. Largely, this insect is estimated to reduce yields by 10 to 15% annually (forage quality not taken into account). The pest remains active during December to March in parts of India where temperature is low and the incidence is highest in mid of February. Majority of the non-dormant Indian cultivars are susceptible to this insect pest (Pandey and Faruqui, 1990). Due to the involvement of complex mechanism that includes avoidance, antibiosis and tolerance, identification of pest resistant plants is time

consuming and difficult. In general screening and selection of such plants poses critical intrinsic problem as the population of insect in the field is varying and fluctuating in climatic conditions.

Cultivated lucerne is an out crossing auto-tetraploid plant developed by combining different *M. sativa* and *M. falcata* germplasm sources in order to maximize heterosis and to secure multiple pest resistance (Gherardi *et al.*, 1998). In addition, cultivated lucerne varieties exist as synthetic varieties developed by random inter-mating of selected parents from 9 original germplasm sources (Segovia-Lerma *et al.*, 2003; Muller *et al.*, 2005). As a consequence, the genetic structure of lucerne cultivars is not definite compared to fixed cultivated forms like inbred lines or hybrids. Therefore, screening lucerne lines for resistance under natural growing conditions and optimal insect pressure is the efficient method for plant selection and results thus obtained would be the most reliable as

compared to screening done under laboratory and greenhouse conditions. However, efficiency of screening for insect pest resistance in the field is determined by: (a) the fluctuation of pest population during growing season, (b) uneven distribution of the insect population in the field, and (c) occurrence of wide variety of insect-pests under natural conditions. In view of this, attempts have been made to identify some of the biochemical components and allelochemical factors such as antifeedants, growth retardants or toxins responsible for the insect pest resistance (Laskowski and Kato, 1988; Ryan, 1990; Prasad *et al.*, 1996; Yang *et al.*, 2002).

Information on genetic diversity and relationships within and among crop species is essential for the efficient utilization of plant genetic resources, their management and identifying the lines possessing distinct characters when evaluated for different agronomical attributes. Different types of markers have been used for genetic analysis and characterization. The DNA markers are considered the best tools for determining genetic relationships/diversity, as they are unlimited in number, highly polymorphic in nature and are independent of environmental interaction. Among the DNA based markers, random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990) data can be generated faster and with less labor than others. It employs single short oligonucleotide with an arbitrary sequence to generate genome specific 'finger print' of multiple amplification products. Polymorphism found between RAPD profiles can serve as genetic marker (Williams *et al.*, 1990).

The present study was undertaken to examine the role of trypsin, chymotrypsin and α -amylase inhibitors activities in field selected lines of lucerne both resistant and susceptible to lucerne weevil (*Hypera postica* Gyll.). Zymogram pattern for trypsin inhibitor activity was also developed to assign any change in the isoforms of inhibitor proteins. Genetic relationship among the selected lines was also attempted by performing RAPD analysis to identify genetically diverse lines having improved levels of resistance against lucerne weevil.

Materials and Methods

Plant materials and selection of lines: For the present study plant materials were selected and identified following four consecutive steps: (i) initially 86 single plants were selected from 2 ha area of the lucerne production field at Indian Grassland and Fodder Research Institute (IGFRI) research farm, Jhansi in 2003-04 (among these lines level of weevil infestation varied from nil to 40%); (ii) those 86 selected lines were further vegetatively established in three replications and were assessed for field resistance to lucerne weevil during 2004-06; (iii) on the bases of the level of infestation caused by the weevil larvae (infestation index), yield loss per plant as well as leaf shape, thirteen heterogeneous lines were selected and categorized into resistant and susceptible categories; (iv) finally progenies of 13 lines each having 8 to 14 plants were again tested for resistance to lucerne weevil infestation. Progenies were raised from the seeds of thirteen selected plants obtained by selfing them individually. Hence, intra-line variation of the resistant / susceptible

trait was taken into account. Screening of lines was performed during January and February when insect population was highest in field. Infestation loss regression equation of weevil damage was calculated by putting infestation index on x-axis and yield loss per plant on y-axis. All biochemical study was carried out in triplicates using leaves of the plant materials representing 8 to 10 plants of each line.

Isolation and determination of total phenols: The total phenol was determined as per the methods described in AOAC (1980) using leaf samples representing ten plants of each line. The total phenol was expressed in mg g^{-1} (DM) using catechol as standard.

Protein extraction and enzyme inhibitor analysis: Fresh leaves (500 mg) collected from 10 plants were extracted with 25 ml of pre-chilled sterile distilled water and incubated at 4°C for 2 hr with occasional shaking. Homogenate was centrifuged at 12,000 g for 20 min at 4°C and resultant supernatant was used as a source for enzyme inhibitor analysis after dilution (1:10) with distilled water. The trypsin, chymotrypsin and α -amylase inhibitor activities (TIA, CIA and AIA respectively) were determined following the procedure of Kakade *et al.* (1972). Enzyme activity was expressed in inhibitory unit mg^{-1} protein where one unit corresponded to the amount of inhibitors in μg protein which gave 50% inhibition of enzyme activity under the experimental condition.

Native PAGE of heat stable proteins: Part of the protein extract was incubated at 70°C for 10 min and the supernatant containing heat stable proteins (HSPs) was used for construction of zymogram pattern for trypsin inhibitor activity (TIA). Polyacrylamide gel electrophoresis (10 % polyacrylamide gel) was carried out for HSPs at 5°C. The gel was stained for TIA using N-acetyl phenyl alanyl naphthyl ester as substrate and fast blue RR as coupling dye (Prasad *et al.*, 1996). The resultant isoform of trypsin inhibitor protein was photographed.

SDS-PAGE of soluble proteins: Fresh and young leaves from 10 plants were ground ($1 \text{ gm } 2 \text{ ml}^{-1}$, w/v) in chilled extraction buffer containing 3% SDS and 20 mM phenyl methyl sulphonyl fluoride (PMSF). The extract was centrifuged at 12,000 g for 20 min and supernatant was used as protein source to perform SDS-PAGE following the procedure of Laemmli (1970). The gel was stained with staining solution containing brilliant blue (0.01% w/v), ethanol (40% v/v) and acetic acid (10% v/v). After 2 hr of incubation at room temperature gel was photographed for analysis.

Isolation of DNA: For molecular analysis, DNA was extracted from approximately ten plants of each line and they were equally mixed (bulked). ADNA sample bulked from ten individuals is reported to provide good representation of variations that exist in a particular cultivar or population of lucerne as demonstrated based on RAPD markers (Yu and Pauls, 1993; Chandra, 2007). Total genomic DNA was isolated following the method of Iqbal *et al.* (1997) with suitable modifications (Chandra *et al.*, 2004). The concentration was adjusted to $5 \text{ ng } \mu\text{l}^{-1}$ for use in PCR analysis. DNA from ten individual plants of each line was mixed in equal amount (bulked) to carry out the PCR.

RAPD analysis: Based on clear reaction and reproducibility of the amplified bands with lucerne genomic DNA, 45 random primers were selected and used in the present investigation. Each PCR amplification was performed in a final volume of 20 ml of reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.45% (v/v) Triton X-100, 4 μg BSA, 3.5 mM MgCl_2 , 150 μM of each of dATP, dCTP, dGTP and dTTP, 7.5 pmol (15 ng) primer, 25 ng bulked genomic DNA template and 0.5 unit *Taq* Polymerase (Bangalore Genei, India). Amplifications were performed on a DNA thermal cycler PTC-200 (MJ Research, USA) with the cycling program consisting of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min for 40 cycles followed by 41st cycle at 94°C for 1 min, 37°C for 1 min and finally at 72°C for 10 min extension. Amplification products were separated by electrophoresis on 1.6% agarose gel in 0.5X TBE buffer (pH 8.0) to which ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) was added for visualization with UV light.

Data analysis: The binary data generated on the basis of presence (1) or absence (0) of the bands in RAPD was analyzed for genetic similarity among the lines based on Dice's similarity coefficients (s). Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using UPGM algorithm. The reliability of the dendrogram was evaluated with 1,000 bootstraps using the WinBoot software (Yap and Nelson, 1996).

To evaluate the discriminatory power of RAPD in the present study with lucerne, the polymorphic information content (PIC) and marker index (MI) were calculated across assay units, assuming that each primer with 13 lucerne lines was an assay unit, and each polymorphic DNA fragment within an assay unit was a single dominant

marker locus as reported earlier for AFLPs marker in celeriac (Muminovic *et al.*, 2004). The PIC value was calculated employing the formula of Roldan-Ruiz *et al.* (2000): $\text{PIC}_i = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i . MI was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.*, 1996). Other statistical analysis like CV, SEM and CD were calculated following the standard statistical method (Gomez and Gomez, 1984).

Results and Discussion

In total 86 lucerne lines were selected from large lucerne growing areas which showed significant variations ($p = 0.05$) in the levels of weevil infestation. Present reports as well as earlier work (Pandey and Faruqi, 1990) have indicated Jhansi as a hot-spot for screening of lucerne for resistance to weevil infestation. All screening work was carried out under natural conditions when the pressure of insect was highest. In two consecutive years initially selected 86 lucerne lines were further evaluated for insect pest resistance by measuring the percentage of leaf damage (infestation index) caused by lucerne weevil and yield loss per plant as well as leaf shape. Based on infestation loss regression equation of weevil damage, 13 significantly correlated ($r > 0.75$ at $p = 0.05$) lines were selected and categorized into resistant and susceptible categories. Infestation among these lines ranged from 0 to 40%. Seeds from these 13 lines were collected after bagging them to avoid cross pollination. In the consecutive years, all plants (8 to 14) emerging from seeds of these selected 13 lines were further evaluated against weevil. Hence, 13 heterogeneous populations comprising 8 to 14 plants were categorized into resistant (A-1-01, H-2-02, A-2-03, C-6-01, D-3-01,

Table - 1: Total phenols, enzyme inhibitory activities, infestation index and yield loss caused by weevil infestation in 13 lucerne lines.

Lucerne lines	Details of lines (Leaf shape) Resistance/Susceptible	Total phenols (mg g ⁻¹ dry wt.)	Enzyme inhibitory units*			Infestation index	Yield loss/Plant (%)
			TIA	CIA	AIA		
1	A-1-01 (Broad) (R)	0.33	3.13	9.5	3.3	Nil	5-8
2	C-1-01 (Narrow) (S)	0.44	3.43	14.8	2.5	17.6	20-30
3	H-2-02 (Broad) (R)	0.44	3.03	ND	4.0	Nil	4-8
4	B-4-03 (Narrow) (S)	0.91	ND	93.0	5.3	29.6	30-45
5	A-2-03 (Broad) (R)	0.48	3.52	ND	1.3	Nil	6-10
6	C-6-01 (Broad) (R)	0.38	7.14	71.0	14.2	Nil	4-8
7	B-1-03 (Narrow) (S)	0.39	ND	28.0	5.7	25.6	20-30
8	D-3-01 (Narrow) (R)	0.15	6.67	47.0	7.3	4.5	8-12
9	G-2-02 (Narrow) (S)	0.33	6.54	21.0	3.1	34	35-50
10	D-2-01 (Narrow) (S)	0.32	7.37	61.0	2.2	9.5	10-15
11	G-1-02 (Broad) (R)	0.42	11.11	30.0	4.5	Nil	4-8
12	H-1-02 (Broad) (R)	0.36	5.00	59.0	3.8	Nil	4-8
13	B-3-03 (narrow) (S)	0.53	4.64	3.7	0.37	38	40-60
	SEM	0.002	2.35	3.93	1.24	3.23	-
	CD	0.04	1.47	2.12	1.54	2.45	-
	Cv	3.25	3.08	4.56	2.35	4.34	-

ND = not detected ; R = Resistance, S = Susceptible, *inhibitory units mg⁻¹ protein, TIA-Trypsin inhibitor activity, AIA- α -amylase inhibitor activity, CIA-Chymotrypsin inhibitor activity, Each value is a mean of three replications (SEM). Critical difference (CD) denoted the significance of values at $p=0.05$. Coefficients of variants (Cv) was less than 10 in all cases indicated appropriateness of data

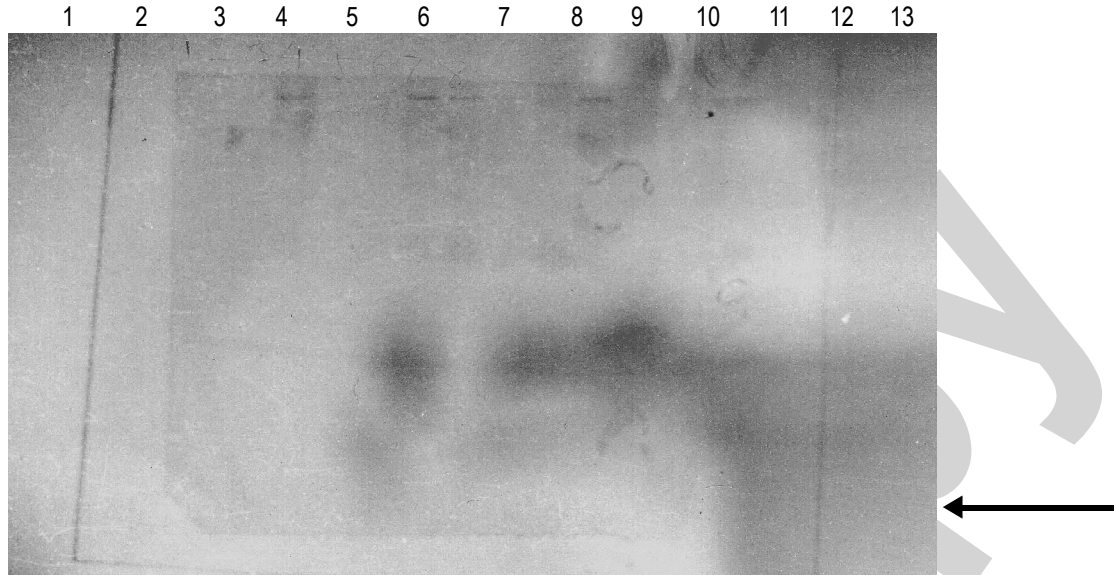


Fig. 1: Specific staining for heat stable trypsin inhibitory enzyme activity (arrow) in 13 lines of lucerne. Lane 1 = A-1-01 (Broad) (R), lane 2 = C-1-01 (Narrow) (S), lane 3 = H-2-02 (Broad) (R), lane 4 = B-4-03 (Narrow) (S), lane 5 = A-2-03 (Broad) (R), lane 6 = C-6-01 (Broad) (R), lane 7 = B-1-03 (Narrow) (S), lane 8 = D-3-01 (Narrow) (R), lane 9 = G-2-02 (Narrow) (S), lane 10 = D-2-01 (Narrow) (S), lane 11 = G-1-02 (Broad) (R), lane 12 = H-1-02 (Broad) (R) and lane 13 = B-3-03 (narrow) (S). Alphabets with numbers indicate the accessions identification number. R = Resistance, S = Susceptible

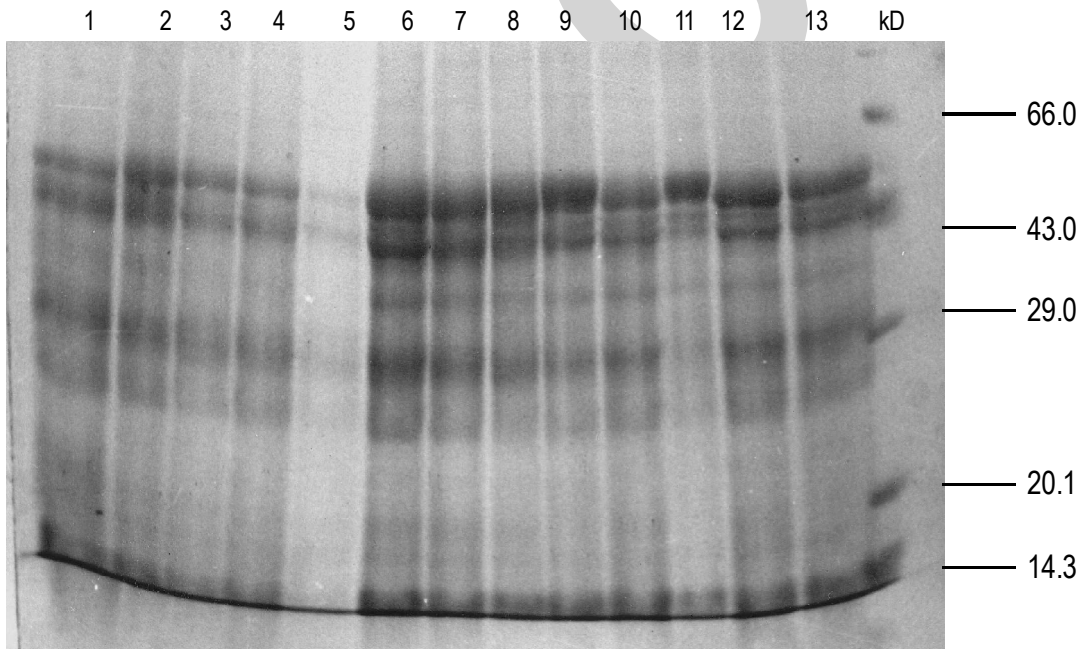


Fig. 2: SDS-PAGE profile of total leaf proteins of 13 lucerne lines. Samples in lanes were as per Fig. 1

G-1-02 and H-1-02) and susceptible (C-1-01, B-4-03, B-1-03, G-2-02, D-2-01 and B-3-03) types and henceforth referred as 13 lines (Table 1). Intra-line variations (0-10%) in level of weevil infestation were observed in all 13 lines. This was expected as lucerne is a highly cross-pollinated crop. The present and earlier observations (Pandey and Faruqui, 1990) showed larvae as the most damaging stage of insect. In general, lines bearing broad leaves showed low levels of infestation (Table 1). It has been reported that high crown

girth and low stem thickness are more prone to weevil infestation (Pandey and Faruqui, 1990). Total phenol content in these 13 lines was in the range of 0.15 to 0.91 mg g⁻¹ (DM) (Table 1), possibly indicated the variations in expression of plant resistance involving hypersensitive reaction against the insect. It has been reported that phenolics and tannins are the important allelo-chemicals factors present in plant system which act as antifeedant, growth retardant and toxicants for insect-pests (Goodman *et al.*, 1986). The protease

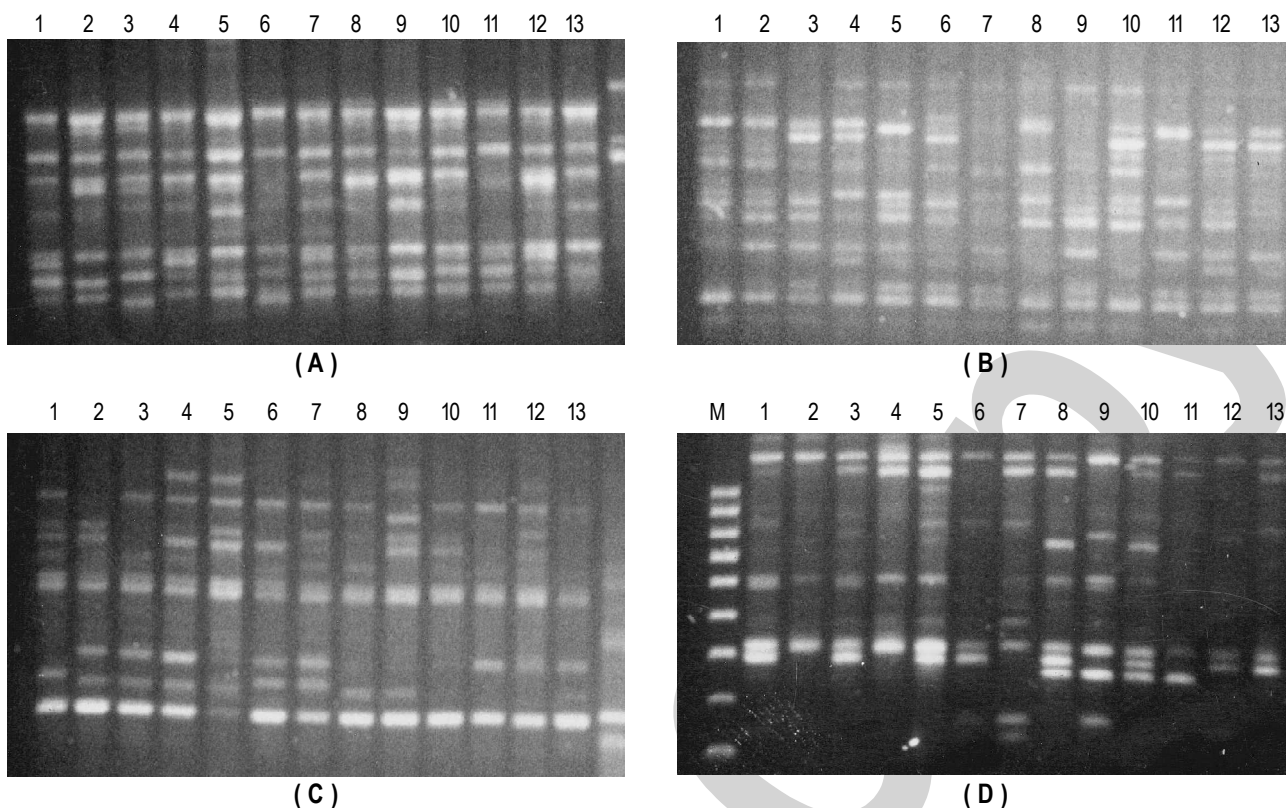


Fig. 3: RAPD profile of 13 lucerne lines with four primers. A = OPAH02, B = OPU14, C = OPV08 and D = OPN01, M = 100bp DNA ladder as molecular weight marker. The details of the samples in lanes are as per Fig. 1

inhibitors (PIA) namely trypsin (TIA) and chymotrypsin (CIA) and α -amylase inhibitor activity (AIA) also showed wide variations among the lines (Table 1). The highest TIA (11.1 units) and CIA (93.0 units) activities were recorded in G-1-02 and B-4-03 respectively, whereas highest AIA (14.2 units) activity was observed in C-6-01. The results also indicated that both TIA and CIA were absent in two different lines. The absence of inhibitors activities have been also reported in cowpea (Prasad *et al.*, 1996). Most of the insect-pests larval gut proteases, for example, serine- and metallo-proteases in lepidopterans (*Heliothis* and *Spodoptera*); cysteine and acidic proteases in coleopterans and hemipterans (Bruchids), have been found to play a very important role in digestion and utilization of food proteins (Ryan, 1990). In case of weevil, specifically cysteine proteinase inhibitors have been reported most effective in controlling the rate of infestation, growth of larva and pupa development (Elden, 1995; Chandra and Pandey, 2008).

Two lucerne lines namely C-6-01 and D-3-01 showed better resistance (0 to 4.5% infestation) against lucerne weevil under field condition. They also showed high levels of inhibitors activities (PIA, 7.14 to 71.0 and AIA from 7.3 to 14.2). However, B-3-03 line showed highest percentage of weevil infestation and low levels of activities of all three enzymes (Table 1). The inhibition of larval growth caused by enzyme inhibitors, especially proteases and amylases, would not only reduce the damage

caused by larvae feeding on the plant but also result in the extension of the larval development period during which the larva would be exposed to their natural predators (Prasad *et al.*, 1996). Selected proteinase inhibitors namely of the class of cysteine, serine and aspartic indicated that alfalfa weevil larvae uses cysteine proteinase as major digestive enzymes and that other proteinases are present as well (Elden, 1995). Though there are as such no reports available indicating the presence of protease inhibitors in the lucerne seeds, however other legumes like cowpea seeds have been reported to contain several trypsin and chymotrypsin inhibitors and some of them have been purified, characterized and classified under the Bowman-Bink family of double headed inhibitors of low molecular weight proteins (Hilder *et al.*, 1987; Ryan, 1990).

Cationic gel electrophoresis for the analysis of thermostable proteins revealed variations in the protein patterns among 13 lines (each was bulk of 10 plants) of lucerne. Specific staining for TIA showed variation in the trypsin inhibitory banding patterns among the lines. Namely C-6-01, D-3-01, G-2-02, D-2-01 and G-1-02 lines showed strong TIA zones indicating more activity in these lines in comparison to the other (Fig. 1). Of the five lines showing high TIA activity, three lines namely C-6-01, D-3-01 and G-1-02 showed good correlation at $p = 0.05$ with level of weevil infestation as these lines depicted low level of infestation. The SDS-PAGE analysis of the leaf proteins from all

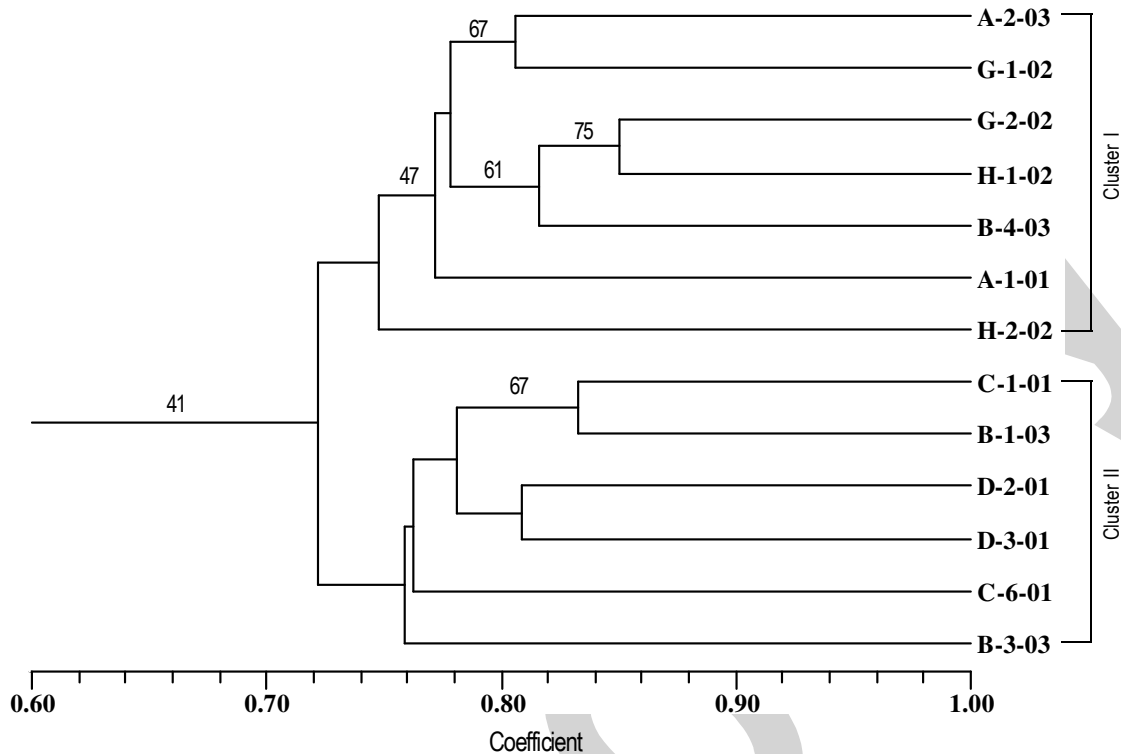


Fig. 4: Dendrogram developed using 262 RAPD markers generated from 45 primers with 13 lucerne lines using UPGMA module. Bootstrap values (>40) are indicated at major nodes showing significance of the nodes

13 lines (bulked samples) showed no major variations in the protein bands except for minor bands in the range of 14 to 20 kD molecular weights (Fig. 2). The protein bands of higher molecular weights (43 to 66 kD) were present in all the lines analyzed. No major variations in banding patterns among these lines indicated less impact of it on variability that exists in these lines for weevil infestation.

As lucerne is a cross-pollinated crop, the heterogeneous nature of the lines was evident from the dendrogram developed with 45 RAPD markers developed from the bulked genomic DNA of 10 individual plants of each of 13 lines (Fig. 3). In total 262 bands were scored. Of these, 213 were polymorphic indicating a high level (81%) of polymorphism among the lines. Variations in terms of genetic distance was also recorded which ranged from 0.65 to 0.85. Polymorphic information content (PIC) of the primers when used with the 13 lines of lucerne ranged from 0 to 0.5 whereas marker index (MI) from 0 to 4.8 indicated usefulness of these primers in discriminating the lines based on DNA bands. However, two types of lines (susceptible and resistant) selected based on the level of weevil infestation were not clustered distinctly as two different types of lines, namely H-1-02 (no infestation) and G-2-02 (34% infestation) showed 85% similarity to each other. Similarly, three different lines having maximum level of trypsin, chymotrypsin and α -amylase inhibitor activities clustered in two different clusters. Nevertheless, RAPD analysis divided 13 lines into two clusters having 72% similarity to each other wherein cluster

I contained five out of seven lines having no infestation, indicating dominance of resistant lines in this cluster (Fig. 4). Though cluster II embodied one line showing no infestation, rest of the lines were having infestations in the range of 4.5 to 38% indicating most of the lines in this cluster were susceptible type. Six major nodes were supported with high bootstrap values (> 40) indicating strong clustering (Fig. 4).

The field evaluation of these 13 lucerne lines for weevil resistance indicated variations in the degree of resistance which has partly corresponded with variations in enzyme inhibitors activities. Though, the level of activities of these enzyme inhibitors in leaves was observed low in comparison to those reported in seeds of other crops (Prasad *et al.*, 1996), significant differences in the level of activities among the lines supported the observed variations in weevil infestation. Lines C-6-01 and D-3-01 having higher activities for TIA, CIA and AIA showed variations in the level of weevil infestation over two years in the field. The other lines showing high level of weevil infestation can be classified as susceptible lines.

In conclusion, it can be suggested that these parameters can be utilized in screening the lines for better resistance to the insect-pest and for identification of specific proteinase inhibitor. The lucerne lines namely C-6-01 and D-3-01 are presently being targeted in developing the precise genetic stocks (homozygous lines) treating them as weevil resistant source. Thus, the developed stocks will

precisely decipher the nature of the segregation of traits associated with weevil tolerance.

Acknowledgments

Authors are thankful to the Head Crop Improvement Division and Director of the institute for providing the necessary facilities to carry out the present work.

References

- AOAC: Official methods of analysis, Association of Official Agricultural Chemist, 13th Edn., Washington, DC (1980).
- Chandra, A. and K.C. Pandey: Effect of proteinase inhibitors on Indian alfalfa weevil (*Hypera postica* Gyll.) growth and development. *Acta. Physiol. Plant.*, **30**, 501-505 (2008).
- Chandra, A., R. Saxena, A.K. Roy and P.S. Pathak: Estimation of genetic variation in *Dichanthium annulatum* genotypes by RAPD technique. *Trop. Grasslands*, **38**, 245-252 (2004).
- Chandra, A.: Bulk genomic DNA PCR analysis-A rapid method to estimate genetic relatedness among heterogeneous lucerne (*Medicago sativa* L.) cultivars. *Cytologia*, **72**, 363-368 (2007).
- Elden, T.C.: Selected proteinases inhibitor effects on alfalfa weevil (Coleoptera: Curculionidae) growth and development. *J. Econ. Entomol.*, **88**, 1586-1590 (1995).
- Gherardi, M., B. Mangin, B. Goffinet, D. Bonnet and T. Huguet: A method to measure genetic distance between allogamous populations of alfalfa (*Medicago sativa*) using RAPD molecular markers. *Theor. Appl. Genet.*, **96**, 406-412 (1998).
- Gomez, K.A. and A.A. Gomez: Statistical procedure for agricultural research, J. Willey and Sons, New York, USA (1984).
- Goodman, R.N., Z. Kirlay and K.R. Nool: Biochemistry and physiology of plant diseases. University of Missouri Press, Columbia (1986).
- Hazra, C.R.: Advancement in forage production technology. IGFRI, Jhansi, 4 (1995).
- Hilder, V.A., A.M.R. Gatehouse, S.E. Sheerman, R.F. Barker and D. Boulter: A novel mechanism of insect resistance engineered into tobacco. *Nature*, **330**, 160-163 (1987).
- Iqbal, M.J., N. Aziz, N.A. Saeed, Y. Zafar and K.A. Malik: Genetic diversity evaluation of some elite cotton varieties by RAPD analysis. *Theor. Appl. Genet.*, **94**, 139-144 (1997).
- Kakade, M.L., N.R. Simmons, I.E. Liener and J.W. Lamber: Biochemical and nutritional assessment of different varieties of soybeans. *J. Agric. Food Chem.*, **20**, 87-90 (1972).
- Laskowski, M. and I. Kato: Protein inhibitors of proteinases. *Annu. Rev. Biochem.*, **49**, 593-626 (1988).
- Laemmli, V.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).
- Muller, M.H., C. Poncet, M. Prospero, S. Santoni and J. Ronfort: Domestication history in the *Medicago sativa* species complex: Inferences from nuclear sequence polymorphism. *Mole. Ecol.*, **15**, 1589-1602 (2005).
- Muminovic, J., A.E. Melchinger and T. Lubberstedt: Prospect form celeriac (*Apium graveolens* var. *rapaceum*) improvement by using genetic resources of *Apium*, as determined by AFLP marker and morphological characterization. *Plant. Genetic. Resour.*, **2**, 189-198 (2004).
- Pandey, K.C. and S.A. Faruqi: Preferential response of lucerne weevil to crude extracts of some *Medicago* spp. *Ind. J. Entomol.*, **52**, 610-612 (1990).
- Prasad, D.T., N.S. Umpathy and R. Veeranna: Genotypic variation in cowpea (*Vigna unguiculata*) cultivars in relation to insect resistance. *J. Plant Biochem. Biotech.*, **5**, 47-49 (1996).
- Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski: The comparison of RFLP, RAPD, AFLP and SSR (microsatellites) marker for germplasm analysis. *Mole. Breed.*, **2**, 225-238 (1996).
- Roldan-Ruize, I., E. Calsyn, T.J. Gilliland, R. Coll, M.J.T. Vaneijk and M. De Loose: Estimating genetic conformity between related ryegrass (*Lolium*) varieties. 2. AFLP characterization. *Mole. Breed.*, **6**, 593-602 (2000).
- Ryan, C.A.: Proteinase inhibitor in plants: Genes for improving defenses against insect and pathogens. *Ann. Rev. Phytopathol.*, **28**, 425-449 (1990).
- Segovia-Lerma, A., R.G. Cantrell, J.M. Conway and I.M. Ray: AFLP-based assessment of genetic diversity among nine alfalfa germplasm using bulk DNA templates. *Genome*, **46**, 1-58 (2003).
- Yang, G.D., Z. Zhu, Y.E. Li, Z.J. Zhy, G.F. Xiao and X.L. Wei: Obtaining transgenic plants of Chinese cabbage resistant to *Pieris rapae* L. with modified CpTI gene. *Acta Horticulture Sinica*, **29**, 224-228 (2002).
- Yap, I.V. and R.J. Nelson: WinBoot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRRI, Philippines (1996).
- Yu, K. and K.P. Pauls: Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by random amplification of bulked genomic DNA samples. *Theor. Appl. Genet.*, **86**, 788-794 (1993).
- Williams, J.G.K., A.R. Kubehk, K.I. Livak, J.A. Rafalski and S.V. Tingey: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, **18**, 6531-6535 (1990).