IJCRR
Vol 05 issue 10
Section: General Sciences
Category: Research
Received on: 06/04/13
Revised on: 28/04/13
Accepted on: 19/05/13

EVALUATION OF DIVERSITY IN PEA (PISUM SATIVUM L.) GENOTYPES USING AGRO-MORPHOLOGICAL CHARACTERS AND RAPD ANALYSIS

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ABSTRACT

Analysis of the extent and distribution of genetic diversity in crop plants is essential for optimizing sampling and breeding strategies. Germplasm characterization and evaluation yield information for more efficient utilization of these valuable resources. Morphological characters and Randomly amplified polymorphic DNA (RAPD) was used to estimate diversity among 5 genotypes of Pea (*Pisum sativum*). Selected four RAPD primers generated 24 bands, 10 of which were found to be polymorphic. All the primers produced polymorphic amplification products, however the extent of polymorphism varied with each primer .The percentage of polymorphism generated by primer was P-03 (83.33%), P-05 (25%), P-06 (16.66%) and P-17 (50%). Great variation among morphological characters viz., plant height, number of nodes, internode distance, number of branches, length of branch from main axis, number of leaves, stem diameter, germination percentage and radicle length was observed. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram obtained from cluster analysis using Jaccard's similarity coefficient resulted into two major clusters. Cluster I comprised of two genotypes KPMR526 and KPMR569; Cluster II can be further divided into two sub clusters, Sub cluster I comprised two genotypes KPMR571 and KPMR583 while as sub cluster II consists of a single genotype KPMR594. All the genotypes could be discriminated from one another using combined profiles of 4 primers.

Keywords: *Pisum sativum*; cultivars; Morphology; RAPD; genetic diversity.

INTRODUCTION

Pea (*Pisum sativum* L.) is an important legume grown and consumed extensively worldwide. As a rich source of proteins, carbohydrates and vitamins, peas are important in human nutrition. Consumed mostly as green peas, total production worldwide is around 8.3 million tons (FAO, 2008). Pea is the fourth leading legume in terms of consumption in the world and is the second most important food legume worldwide after common bean (*Phaseolus vulgaris* L.). The increasing demand for protein-rich raw materials for animal feed or intermediary products for human nutrition have led to a greater interest in this crop as a protein source (Santalla *et al*., 2001). However, the relatively narrow gene pool (Heath and Hebblethwaite 1985) and the heavy use of a small number of varieties as parents by competing breeding programs have led to low genetic diversity among pea cultivars (Singh, 1990; Simioniuc *et al*., 2002; Baranger *et al*., 2004). Extensive use of closely related cultivars by producers could result in vulnerability to pests and diseases (Duvick, 1984; Cox *et al*., 1986). Previous studies about genetic diversity in the *Pisum* genus were developed using

morphological characters and (or) molecular techniques (Hoey *et al*., 1996; Ellis *et al*., 1998; Tar'an *et al*., 2005). There is increasing number of reports where RAPD has been used to estimate genetic variability in common wheat **(**Liu *et al*. 1999 and Sivolap *et al*. 1999), *Brassica* **(**Demeke *et al*. 1992, Dulson *et al*. 1998) and barley (Hamza *et al*. 2004). RAPD markers have been used successfully for identification and phylogentic relationship among and within the species **(**Ren *et al*. 1995). DNA-based molecular markers have been proposed as an excellent tool for identifying geographical variation, genetic diversity, phylogenetic relationship, authentication of plant species, pharmacognostic characterization, species characterization and genetic mapping in medicinal plants (Joshi *et al*. 2004) Recently, RAPD and simple sequence repeat (SSR) were performed to estimate correlations between secondary metabolite contents and genetic profile of *H. perforatum* and other *Hypericum* species, which demonstrated variable rates of correlation (Smelcerovic *et al*. 2006 and Verma *et al*. 2008). Random amplified polymorphic DNA (RAPD) markers are used as a powerful tool for the identification of species or strains, the estimation of genetic variability between isolates, and the construction of dendrograms out of the computed distances (Welsh and McClelland 1990, Williams *et al*. 1990 and Bruns *et al*.1991). Molecular markers are useful complements to morphological and phenological characters because they are plentiful, independent of tissue or environmental effects, and allow cultivar identification in the early stages of development. They reveal polymorphism at DNA level which has been shown to be a very powerful tool for characterization and genetic diversity estimation. Tinker *et al*. (1993) suggested that diversity estimates based on molecular markers are better suited than pedigree data for parental selection. The use of molecular markers for diversity analysis can also serve as a tool to discriminate between closely related individuals from different breeding sources (Lombard *et al*., 2000; Me´tais *et al*., 2000; Sun *et al*., 2001) and may help to eliminate redundancy in phenotype base germplasm collections. Different markers (RFLP, RAPD, and AFLP) had been used in pea (Simioniuc *et al*., 2002; Baranger *et al*., 2004; Tar'an *et al*., 2005. The objective of present study was to characterize Pea cultivars both at morphological and molecular level so that the information thus yielded can be potentially utilized for selection of better parents for effective breeding programmes.

MATERIAL AND METHODS

Five Pea cultivars viz. KPMR526, KPMR569, KPMR571, KPMR583 and KPMR594 used for present investigation were collected from Chandrasekhar, Azad Agricultural university; Kanpur (U.P), India. The seeds were washed three times with distilled water and placed in sterile Petri dishes containing wet filter paper. The germination of seeds was determined by observing radicle formation. The germinated seeds were transplanted in pots carefully filled with top soil. The soil was sandy loam type.

Morphological traits:

In order to characterize the plant morphology diversity, several traits were analyzed. Data were collected for nine traits. Plant height, number of nodes, internode distance, number of branches, length of branch from main axis, number of leaves, stem diameter, germination percentage and radicle length was observed. The results obtained for morphological characters were analyzed statistically like arithmetic mean, coefficient of Range, standard error etc.

Molecular Analysis:

Seeds of all five cultivars were sown separately in pots and leaf samples pooled from all plants of each cultivar were collected into labeled bags and stored in $-96^{O}C$ in liquid N_2 prior to genomic DNA Isolation. DNA was extracted by CTAB method (Saghai Maroof *et al*., 1984) followed by

amplification in PCR. DNA amplification was carried out in a sterile 0.2ml thin- wall PCR tubes containing sterile water 18.5µl, Buffer 2.5µl, dNTPs composed of 0.5µl (200 µm) each, RAPD primers (1µl 300 ng), Enzyme 1µl (1 unit) and DNA template 2 µl (100ng). RAPD analysis was done individually with 4 random decamer primers. The PCR reaction was performed in Thermal Cycler at 94^0C, 94^0C, 37^0C, 72^0C and 72^0C, respectively for 5 min, 1 min, 1 min, 2 min and 10 min. The four primers used were P-03, P-05, P-06 and P-17 with Sequence (5´-3´) CTGATACGCC, CCCCGGTAAC, GTGGGCTGAC and TGACCCGCCT respectively. The amplification product along with 2µl of loading dye (bromophenol blue), was separated on 1.5% agarose gel at 50 volts using 0.5 x TBE buffer at pH 8.0 containing ethidium bromide (0.5µg/ml) of gel. The gel was viewed under UV trans-illuminator and photographed using gel documentation. All the selected 4 primers used, produce recognizable bands. Scorable bands for a primer in each genotype were compared and allotted 0 (absence) or 1 (presence values). Band pattern (0/1 matrix) was tabulated for individual primers separately and the data pooled to obtain a combined matrix for five genotypes of 4 primers. Diversity coefficient for each primer (no. of polymorphic bands/total no. of bands) and pair wise genetic distance was calculated.

RESULTS

The germination percentage after 96 hours was highest in KPMR569 and KPMR583 (100%) followed by KPMR526 and KPMR594 (90%) and KPMR571 (80%). The highest percentage (100%) of seed viability was found in variety KPMR526 followed by (90%) KPMR583 and KPMR594, (80%) KPMR569 and (70%) KPMR571. Mean values and standard errors for each morphological trait are present in (Table 1) The extent of standard error (SE) varied from 0.72 to 2.48 (Plant height), 0.16 to 0.44 (Internode distance), 0.33 to 1.20 (number of nodes), 0.88 to 1.55 (number of leaves), 0.66 to 0.88 (number of branches), 0.37 to 0.76 (length of branch from main axis) and 0.03 to 0.15 (stem circumference). This showed that there was variability for various traits. A wide range of variation was noticed for plant height (24.5-33 cm) in KMPR594, while for internode distance (4.5-6cm) in KMPR569, number of nodes (5-8) in KMPR526, number of leaves (9-12) in KMPR594, number of branches (5-8)
in KPMR583, length of branch from main axis (1.2-2.6cm) and stem circumference (1.1-12cm) in KMPR526 as shown in (Table 2). The coefficient of range for all characters studies showed highest coefficient of range for plant height (0.18cm) in KPMR526, number of nodes (0.33) in KPMR571, number of leaves (0.38) in KPMR526, number of branches (0.33) were equal in three varieties KPMR526, KPMR569, and KPMR594, length of branch from main axis (0.45cm) in KPMR571 and KPMR583 and stem circumference (0.66cm) in KPMR526 as shown in (Table 3). The greatest value of mean was found for radicle length (4.39) in KPMR526, (4.22) in KMPR569, (4.71) in KPMR571, (2.78) in KMPR583, and (3.04) in KPMR594 after four days. The extent of standard error (SE) for radicle length varied from 0.07 to 0.63 (KPMR526), 0.15 to 0.51 (KPMR569), 0.04 to 0.44 (KPMR583), 0.07 to 0.47 (KPMR571) and 0.16 to 0.63 (KPMR594).

The present study revealed the genetic diversity within a collection of pea germplasm representing different geographical regions of India, using molecular (RAPD) approaches. Out of total 12 primers tried in the PCR amplification, 4 primers showed clear and unambiguous amplification while rest of the primers did not give amplification in several reactions tried .A total of 24 scorable bands were generated in 5 genotypes with 4 primers ranging from 4 to 8 corresponding to an average of 6 bands per primer (Table 4). These data were utilized for

further computations. Out of 24 bands, 10 bands were found to be polymorphic and 14 were found to be monomorphic. The highest percentage polymorphism was generated by primer P-03 (85.33%) and the lowest percentage polymorphism was generated by primer P-O6 (16.66%). Maximum number of 8 amplified products was obtained by primer P-05 followed by P-03, P-06 produced 6 bands each and P -17 4 bands. Further, the similarity index revealed the maximum similarity between varieties KPMR526 with KPMR569 and KPMR571 with KPMR583 (SI 0.82) while distantly related varieties were KPMR526 and KPMR594 (SI 0.74). Dendrogram was constructed based on UPGMA. Cluster analysis of data placed five accessions of Pea into two major clusters. first Cluster comprised two genotypes (KPMR526 and KPMR569); second Cluster can be further divided into two sub clusters, Sub cluster I comprised two genotypes KPMR571 and KPMR583 while as sub cluster II consists of a single genotype KPMR594 (Figure 1).

DISCUSSION

Within the collection studied, great variation was observed in terms of plant height, number of nodes, internode distance, number of branches, length of branch from main axis, number of leaves, stem diameter, germination percentage and radicle length which are important traits for the identification, characterization and grouping of genotypes. Morphological characterization has long been performed in many plant species either on its own or in combination with biochemical and/or molecular assays (Duran *et al*., 2005; Smýkal *et al*., 2008). However, for the majority of traits, interactions between genotype and environment complicate the evaluation process. Molecular markers have the potential to facilitate this procedure, increase the reliability of decisions, and substantially save time. Out of total 12 primers tried in the PCR amplification, 4 primers showed clear and unambiguous amplification while rest of the primers did not give amplification in several reactions tried. Out of 24 scorable bands, 41.66% bands were found to be polymorphic and 58.33% were found to be monomorphic, as compared to 55.7% obtained by (Simioniuc *et al*., 2002) in 21 released German cultivars by employing 20 RAPD primers. (Choudhury *et al.*, 2007) this percentage located in the 55.9% they worked with *Pisum sativum* using 60 RAPD primers. Almost similar results were observed by (Malvia *et al.*, 2010) She observed a total of 14 bands and out of them 11 bands were polymorphic with a total of 78.57% polymorphism and 3 bands were monomorphic with a total of 21.42% monomorphism. The little variation observed in polymorphism percentage might be due to the cultivar change. (Mahmood *et al*., 2011) got sixty three bands from seven random primers. Out of sixty three bands fifty were polymorphic in all genotypes. Similarly (Ahmad *et al*.,2010) a total of 16 bands were scored for the 4 RAPD primers ranging from 3 to 5 corresponding to an average of 4 bands per primer and 6 bands (37.5%) of these were polymorphic. Polymorphic bands for each primer ranged from 0-75%. The change is might be due to the number of primers used. The GS calculated using the pooled marker data showed that estimates from pair-wise comparisons among the 5 cultivated varieties ranged from 0.74 to 0.84. The current results are in agreement with the previous report by (Simioniuc *et al.*, 2002), who found high GS values 0.84 to 0.94 between pairs of pea genotypes from 21 varieties that were released in Germany.

Baranger *et al.*, (2004) also demonstrated the narrow genetic diversity among the varieties released in Western Europe, especially within the spring-sown feed pea market class. Most recently Ahmad *et al.*, (2010) revealed the maximum similarity values 0.7692 to 0.963 among five pea varieties. From the clustering pattern and genetic relationships obtained using RAPD makers, breeders can identify the diverse cultivars from

different clusters and employ them in their future breeding programmes or the results obtained in this study can be used for the selection of parents to generate the mapping populations.

CONCLUSION

Assessment of the extent and distribution of the genetic variation in a crop species and its relatives is essential in understanding pattern of diversity and has important implications in breeding programmes and for conservation of genetic resources. Large numbers of cultivars utilizing limited genetic resource is grown and are being released. Often plant breeders limit their efforts to narrow range of adapted lines of genetic improvement which were more likely to produce economic gains in the short term but have enhanced vulnerability to insect pests and other biotic stresses. Therefore, it is concluded that KPMR526 and KPMR594 all the experimental accessions showed much genetic diversity in central India region than the remaining three varieties. It is recommended that KPMR526 and KPMR594 genotypes should be used in future breeding program for improving yield and quality characteristics of *Pisum*. Although there has been great advancement in the marker technology with the advent of different DNA markers like Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Single Nucleotide Polymorphism (SNPs) and Diversity arrays technology (DArt), still RAPD is quite convenient to apply provided the problem of reproducibility is minimized. The only option left over is to validate by using maximum number of primers for the samples provided. The preliminary work carried out with four random primers selected from literature revealing the genetic diversity among 5 pea cultivars could be exploited further by increasing the number of random primers and validating it with other available DNA markers.

Competing Interests

The authors declare that they have no competing interests.

ACKNOWLEDGEMENT

The authors are thankful to Indian Grassland and Fodder Research Institute (IGFRI), Jhansi, India, for providing lab facilities and support throughout the work.

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Table 1: Mean and Standard error of Morphological data

Characters	KPMR526 Mean ± SE	KPMR569 Mean ±SE	KPMR571 Mean ± SE	KPMR583 Mean ±SE	KPMR594 Mean ± SE
Plant Height(cm)	22.33±2.33	20.8±0.72	23.23±0.95	24.0±1.73	29.16±2.48
Internode distance(cm)	3.16±0.16	5.16±0.44	4.56±0.29	4.16±0.16	4.66±0.16
No. of Nodes	6.33±0.88	3.66±0.66	5.66±1.20	4.6±0.33	5.33±0.66
No. of Leaves	10.33±1.45	9±1.55	7.66±1.20	11.0±1.52	10.16±0.88
No. of Branches	3.33±0.66	4.33±0.88	3.66±0.66	6.33±0.88	4.33±0.88
Length of branch from main axis (cm)	2.1±0.45	1.66±0.44	3±0.76	1.7±0.43	1.26±0.37
Stem Circumference (cm)	0.93±0.06	1.03±0.03	0.63±0.15	1.3±0.08	1.58±0.03

Table 2: Range of Morphological data

Characters	KPMR526	KPMR569	KPMR571	KPMR583	KPMR594
Plant Height(cm)	18-26	19.5-22	21.7-25	21-27	24.5-33
Internode Distance (cm)	3-3.5	4.5-6	4-5	4-4.5	4.5-5
No. of Nodes	5-8	3-5	4-8	4-5	4-6
No. of Leaves	8-13	7-11	6-10	8-13	9-12
No. of Branches	2-4	3-6	3-5	5-8	3-6
Length of Branch from main axis (cm)	1.2-2.6	1-2.5	1.5-2.4	0.9-2.4	0.8-2
Stem Circumference(cm)	0.8-4	1-1.1	0.7-1.2	0.9-1.2	1.1-1.2

Table 3 : Coefficient of range of Morphological data

Characters	KPMR526	KPMR569	KPMR571	KPMR583	KPMR594
Plant Height(cm)	0.18	0.06	0.07	0.12	0.14
Internode Distance (cm)	0.07	0.14	0.11	0.05	0.05
No. of Nodes	0.23	0.25	0.33	0.11	0.02
No. of Leaves	0.38	0.22	0.25	0.23	0.14
No. of Branches	0.33	0.33	0.25	0.23	0.33
Length of Branch from main axis (cm)	0.36	0.42	0.45	0.45	0.42
Stem Circumference (cm)	0.66	0.04	0.26	0.14	0.39

Table 4: Primer names, polymorphic band size range, total number of amplified bands, monomorphic and polymorphic bands and percentage of polymorphic bands for each primer

Name of primer	Nucleotide sequence (5´ -3´)	Polymorphic Bands	Monomorphic Bands	% G+C Content	Total Bands	% of polymorphism
P-03	CTGATACGCC	5	1	60%	6	83.33%
P-05	CCCCGGTAAC	2	6	70%	8	75%
P-06	GTGGGCTGAC	1	5	70%	6	16.66%
P-17	TGACCCGCCT	2	2	70%	4	50%

Figure 1: UPGMA- based dendrogram showing genetic relationship among five *Pisum* genotypes based on Jaccards similarity, estimates for RAPD data