ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 6 (4): 573-577, 2007
ISSN 1682-296X

Augmenting the Genetic Base in Pea (*Pisum sativum* L.)

[1]M.A. Espósito, [1]L. Milanesi, [2]E. Martin, [2]V. Cravero, [1]F.S. López Anido and [1]E. Cointry
[1]Department of Genetics and Plant Breeding, Rosario National University,
CC14 (S2125ZAA), Zavalla-Santa Fe, Argentina
[2]CONICET

Abstract: In Argentina, pea is cultivated predominantly in Buenos Aires and Santa Fe provinces. Market requirement and decrease of the grown area caused a severe reduction of the genetic diversity in this species. To reverse this, mutagenesis was proved as a tool to generate new and superior genotypes. The aim of this study was determine the ability of chemic mutagens to generate genetic variability. Seeds of two varieties (Sprut and Eric II) were imbibed in water overnight. Then were treated with Ethyl Methane Sulfonate (EMS) 4 mM for 5 h at room temperature and decontaminated whit sodium tiosulphate 0.2 M. Plants obtained were harvested and seeds of each plant sowed as a family progeny. To determine the mutagen effect, six quantitative traits were evaluated: number of pods, seeds per plot, seeds per pod, weight of 100 seeds; days to flowering and yield. Furthermore, molecular markers, SRAP (Sequence-Related Amplified Polymorphism) were used. Gels were stained with $AgNO_3$ and SRAP fragments were scored as present or absent. Cluster analysis was performed with morphological and molecular data. Three molecular clusters and four morphological clusters were obtained for Sprut and four molecular clusters and three morphological clusters for Erik II. The consensus between both data was 53 and 71%, respectively. Through mutagenesis, new genotypes were generated in both cultivars by EMS action, extending their genetic bases and facilitating new cultivar obtainment.

Key words: Variability, SRAP marker, mutant

INTRODUCTION

Pea (*Pisum sativum* L.), a native specie of Southwest Asia, was among the first crops cultivated by man. Wild field pea can still be found in Afghanistan, Iran and Ethiopia. It is the second most important food legume worldwide after common bean. 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). The importance of hybridization in crop improvement varies greatly from one crop to another and this is particularly true when dealing with autogamous species as is the case for most grain legumes. However, the relatively narrow gene pool (Heath and Hebblethwaite, 1985) and the frequent use of a low number of parental varieties by breeding programs, have led to a reduced genetic diversity among actual pea cultivars (Gantotti and Kartha, 1986; Simioniuc *et al*., 2002; Baranger *et al*., 2004). With the application of scientific methods to plant breeding, the world's agricultural output has increased immensely, however, the substitution of traditional local landraces by widespread genetically homogeneous varieties, had accounted for a substantial loss of diversity. In this context, the extensive use of closely related cultivars by producers could result in vulnerability to pests and diseases (Duvick 1984; Cox *et al*., 1986). The lack of genetic diversity also fixes the plateau in the future genetic improvements of yield in pea. Mutation breeding has been used worldwide for improvement of grain legumes through increased genetic variation and creation of novel alleles. Several pea cultivars resulting from mutagenesis have been released with improvements including increased yield, lodging resistance (afila leaf trait), larger seed, increased protein concentration and modified maturity. Mutation breeding of pea has been centered in many European countries including Sweden, the Netherlands, Germany, Italy as well as several eastern European countries and Russia (McPhee, 2003).

The current pea variety identification system is based mainly on morphological and phenological characters. Although these descriptors are useful, they are limited in number and may be affected by environmental factors. 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. The use of molecular markers for

Corresponding Author: M.A. Espósito, Department of Genetics and Plant Breeding, Rosario National University, CC14 (S2125ZAA), Zavalla-Santa Fe, Argentina

diversity analysis can also serve as a tool to discriminate between closely related individuals from different breeding sources (Lombard *et al*., 2000; Métais *et al*., 2000; Sun *et al*., 2001). For these purposes, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) had been used in pea (Simioniuc *et al*., 2002; Baranger *et al*., 2004; Tar'an *et al*., 2005). We proposed the use of SRAP (Sequence-Related Amplified Polymorphism), (Espósito *et al*., 2007) because is simpler than AFLP and more reliable than RAPD.

The objective of this study was to determine the ability of chemic mutagens to generate genetic variability and to characterize pea mutants through molecular markers and their association with morphology and productive traits.

MATERIALS AND METHODS

Seeds of two varieties (Sprut and Erik II) were imbibed in water overnight. Then were treated with Ethyl Methane Sulfonate (EMS) 4 mM for 5 h at room temperature and decontaminated whit sodium tiosulphate 0.2 M. Plants obtained were harvested and seeds of each plant sowed as a family progeny in plots of twenty plants in a complete randomized design with two replications in the Experimental Field of Rosario University (33° 1' S y 60° 53' W) during 2006. To determine the mutagen effect, six quantitative traits were evaluated: number of pods (NP), seeds per plot (NS), seeds per pod (SP), weight of 100 seeds (WS); days to flowering (DF) and yield (Y).

For DNA extraction and SRAP procedure, about 100 mg of fresh leaf was ground in liquid nitrogen and the total genomic DNA was extracted using the commercial kit PureLink™ Plant Total DNA Purification Kit (Invitrogen). The amplifications were carried out in a thermo-cycler MyCycler™ (BIO-RAD). At the beginning of the PCR reaction, the annealing temperature was set at 35°C and run for 5 cycles. Then the annealing temperature was raised to 50°C for another 35 cycles. Denaturing was done at 94°C for 1 min, while extension was carried out at 72°C for 1 min in all cycles. The amplified fragments were separated by denaturing acrylamide sequencing gels and reveled with silver (Espósito *et al*., 2007). SRAP fragments were scored for presence or absence in each sample. Cluster analysis was performed with morphological and molecular data and a comparison between both data was carried out with the Procrustes Generalized method using InfoGen program (Balzarini and Di Renzo, 2003). Previously a Principal Component Analysis (PCA) and Principal Coordinates Analysis (PCoA) was carried out for morphological and for molecular data respectively.

RESULTS AND DISCUSSION

All the mutants genotypes presented a cycle to flowering similar to Erik II. The PCA for Erik II cultivar showed that the two principal components explained 85% of the total variation. The first component (PC1) was highly correlated with yield, NP, WS and NS meanwhile PC2 was correlated with DF and SP (Table 1).

Relationships between the 10 families revealed by cluster analyses based on PC1 and PC2 are presented in

Table 1A: Mean values and standard errors for each morphological trait in the Erik mutants

Cultivars	DF	NP	Y(g)[a]	WS (g)[b]	NS[a]	SP
E_1	71±0.9	393±33.4	221.0±13.8	16.0±0.8	1380±234.8	3.5±0.5
E_{16}	85±0.3	490±25.5	432.4±31.0	11.2±0.3	3860±156.9	4.8±0.4
E_{17}	69±1.3	570±13.6	298.2±23.9	13.2±0.5	2740±138.5	4.8±0.4
E_4	94±0.8	176±35.7	166.0±18.4	15.2±1.1	1160±230.1	6.6±0.2
E_6	89±0.5	701±12.9	507.0±11.3	12.5±1.3	4060±152.1	5.8±0.6
E_{10}	86±2.2	720±14.7	386.0±21.6	11.0±1.6	3500±276.2	4.9±0.2
E_{12}	92±1.5	673±28.6	424.0±20.4	13.8±1.1	3180±189.5	4.7±0.5
E_7	80±0.6	753±23.8	432.3±18.2	11.3±0.9	3820±145.9	5.1±0.4
E_8	78±0.5	664±31.9	399.2±23.5	11.2±0.4	3560±286.3	5.4±0.3
ERIK II	76±0.4	683±16.9	456.7±12.8	12.0±0.7	4000±122.5	5.9±0.2

[a]: Measures per plot. [b]: Weight of 100 seeds

Table 1B: Mean values and standard errors for each morphological trait in the Sprut mutants

Cultivars	DF	NP	Y(g)[a]	WS(g)[b]	NS[a]	SP
SP_2	97±1.3	597±11.8	494.5±19.9	19.2±0.7	2580±162.1	4.0±0.5
SP_3	102±1.6	278±17.3	325.0±20.0	23.5±0.4	1380±139.0	5.0±0.4
SP_5	89±2.3	311±27.5	249.3±15.5	21.4±1.4	1160±167.3	3.7±0.3
SP_7	93±0.2	165±24.9	170.1±16.0	18.2±1.6	940±108.5	5.7±0.4
SP_9	89±0.6	215±16.7	161.8±14.2	20.2±0.5	800±111.7	3.7±0.5
SPRUT	81±0.8	440±10.1	487.0±13.2	24.0±0.6	940±106.9	4.0±0.3

[a]: Measures per plot. [b]: Weight of 100 seeds

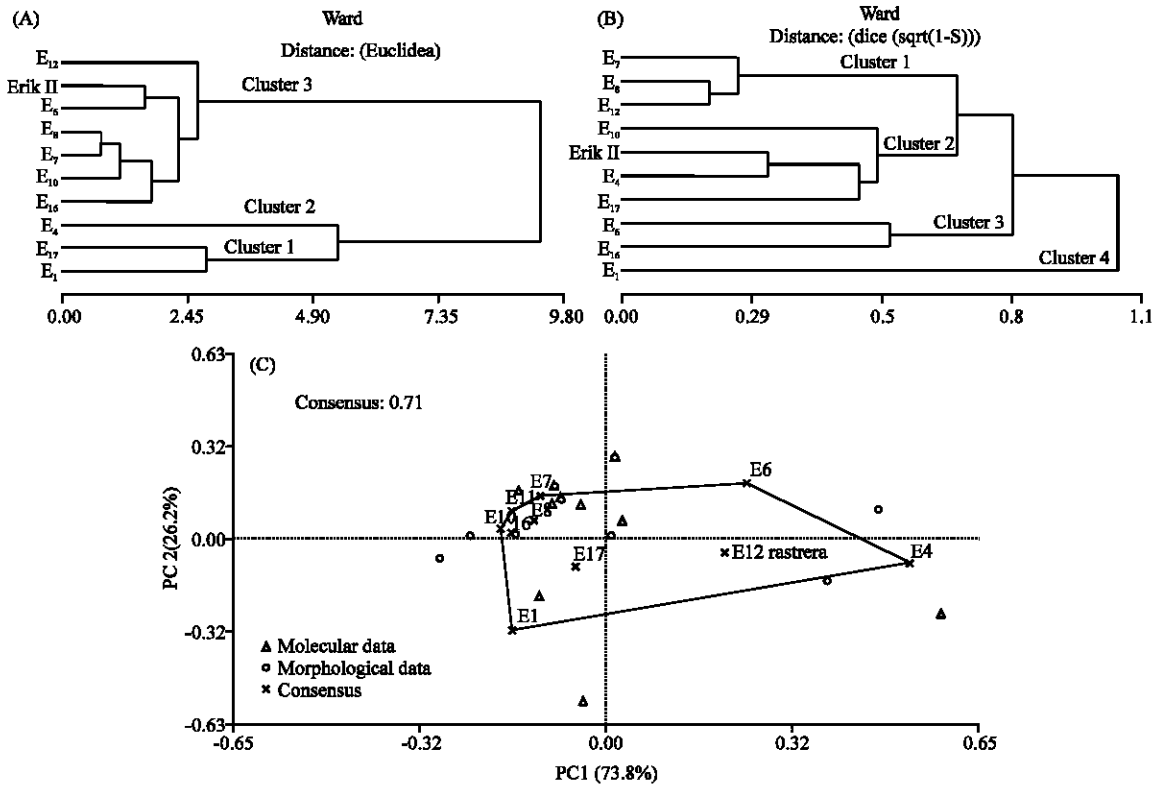

Fig. 1: A: Dendrogram of Erik materials based on morphological data using Euclidean distance matrix. B: Dendrogram of some materials based on molecular data using Dice distance matrix. C: Consensus diagram of de first two principal components (PC1 and PC2) of Erik material based on SRAP fragments and morphological characters

Fig. 1A. Three main clusters can be observed. Cluster 3 included 6 new genotypes similar to Erik II, however E_6 presented higher yield (12%). Cluster 2 included only one genotype (E_4) with the lowest yield and their components, while cluster 1 was intermediate between both.

To verify if the phenotypic differences observed corresponded to genotypic changes the SRAP analysis was a powerful tool (Fig. 3). This analysis presented a level of polymorphism of 65.6%. The relationships between the 10 families revealed by cluster analyses based on Dice distance are presented in Fig. 1B. Four main clusters can be observed with 3, 4, 2 and 1 accessions, respectively.

Comparison between SRAP and morphological data were done using the Procrustes Generalized Analysis of the InfoGen program. The correlation between Dice similarity index (SRAP data) and Euclidean distance (morphological data) matrices was 0.71 indicating good correspondence between both data set (Tatineni *et al*., 1996; Espósito *et al*., 2007). Accessions distribution for this analysis is showed in the Fig. 1C.

EMS showed an inferior effect on Sprut cultivar, since only 5 new genotypes were observed. The PCA also showed that two principal components explained 77% of the total variation. The first PC was highly correlated with yield, NP and NS; meanwhile PC2 was correlated with DF, WS and SP. Only one mutant, genotype (SP_2) (cluster 1), presented a yield similar to cultivar Sprut and an increased (75%) NS and a lower seed weight. Cluster 2 and 3 behaved differently (Table 1B and Fig. 2A).

SRAP analysis presented a level of polymorphism of 23%. The relationships between the 6 families revealed by cluster analyses based on Dice distance are presented in Fig. 2B. Three main clusters can be observed with 1, 4 and 1 element, respectively. The consensus between morphological and SRAP data was 53% (Fig. 2C).

These results shown that through EMS mutagenesis, new genotypes were generated in both cultivars, extending their genetic bases and facilitating new cultivars obtainment.

These genotypes could be useful in a hybridisation program to improve the performance of actual cultivars. E_4 and SP_3 mutants can be used for commercialization in fresh for its bigger size grain.

The variation originate by EMS at DNA level was superior in comparison at the observed at phenotypic

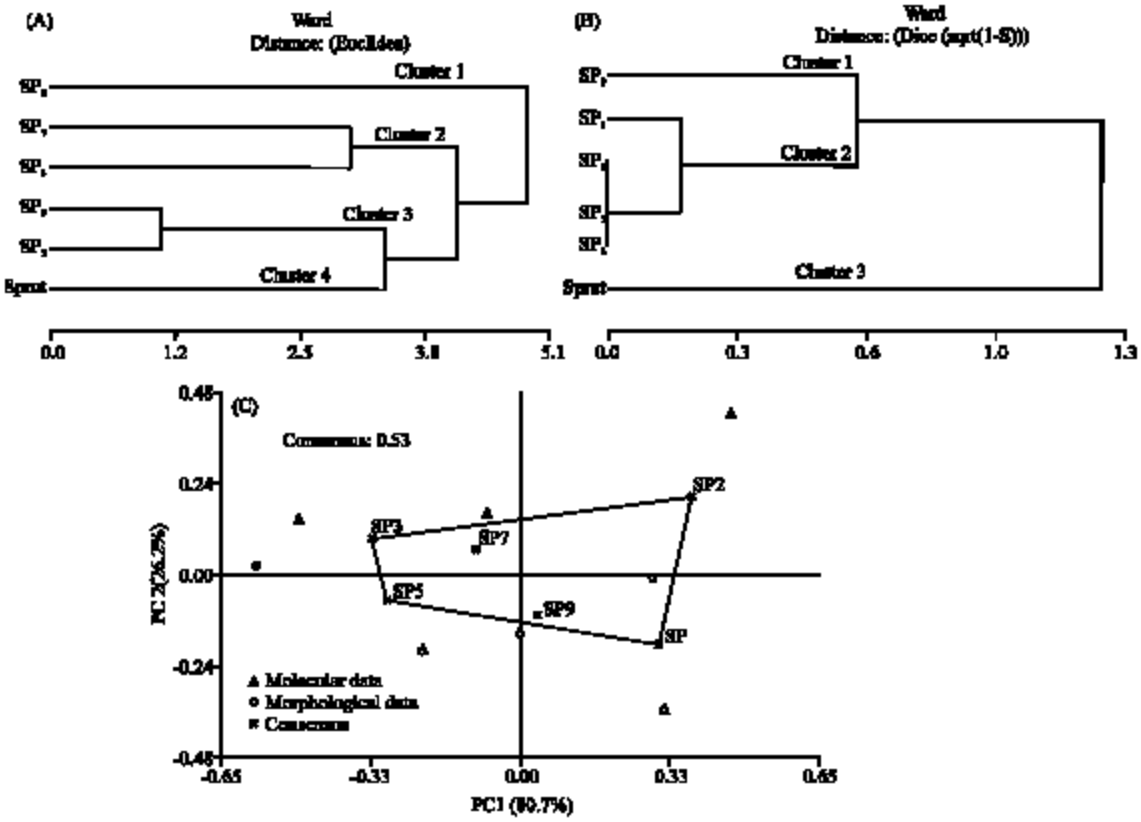

Fig. 2: A: Dendrogram of Sprut materials based on morphological data using Euclidean distance matrix. B: Dendrogram of some materials based on molecular data using Dice distance matrix. C: Consensus diagram of de first two principal components (PC1 and PC2) of Sprut material based on SRAP fragments and morphological characters

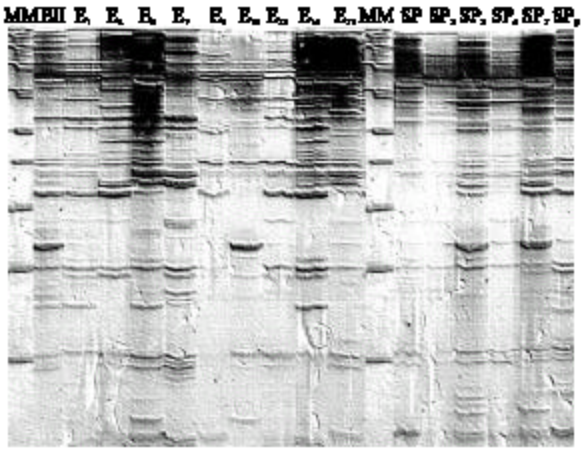

Fig. 3: SRAP profile in pea. Arrow indicates polymorphic bands. EII: Erik cv, E1-E17: Mutants for Erik cv. SP: Sprut cv, SP2-SP9: Mutants for Sprut cv.

level in both cultivars. SRAP markers preferentially amplify ORFs (Open Reading Frame), which in turn may be involved in the morphological traits that characterise the different materials. Nevertheless the percentage of polymorphic fragments, as well as gene diversity, was greater in Erik than in Sprut.

In future improve programs hybridizations will carried out with normal cultivars for understand the inherence of these modifications.

ACKNOWLEDGMENT

Financial support for this research work was provided by PICT N° 08-14645 from the Agencia Nacional de Inverstigaciones Científicas y Técnicas (ANCYT).

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