Genetic characterization of pea (*Pisum sativum*) germplasm from Turkey using morphological and SSR markers

G. Sarıkamış[1], R. Yanmaz[1], S. Ermiş[2], M. Bakır[3] and C. Yüksel[3]

[1]Department of Horticulture, Faculty of Agriculture, Ankara University, Ankara, Turkey
[2]Variety Registration and Seed Certification Centre/MARA, Yenimahalle, Ankara, Turkey
[3]Biotechnology Institute, Ankara University, Ankara, Turkey

Corresponding author: G. Sarıkamış
E-mail: golges@yahoo.com

Genet. Mol. Res. 9 (1): 591-600 (2010)
Received December 10, 2009
Accepted January 15, 2010
Published March 30, 2010

ABSTRACT. The need for the conservation of plant genetic resources has been widely accepted. Germplasm characterization and evaluation yield information for more efficient utilization of these valuable resources. The aim of the present study was to characterize the pea germplasm conserved at the Aegean Agricultural Research Institute of Turkey using morphological and simple sequence repeat (SSR)-based molecular approaches. Genetic characterization of 30 pea genotypes collected from different regions of Turkey and 10 commercial pea cultivars was performed using the criteria of the International Union for the Protection of New Varieties of Plants (UPOV) (TG 7/9 *Pisum sativum*), and with 10 SSR markers. We originally tested 15 SSR markers; 10 of these markers were selected on the basis of high polymorphism information content in the molecular assays. Sixty-one alleles were detected at the 10 loci. The number of alleles per SSR locus ranged from 3 (PVSBE2) to 12 (AB53), with a mean of 6.1 alleles. The most informative loci were AB53 (12 alleles), AA355 (9 alleles), AD270 (8 alleles), A9 (7 alleles), AD61 (7 alleles), and AB25 (6 alleles). The UPGMA dendrogram defined by

SSR markers revealed genetic relatedness of the pea genotypes. These findings can be used to guide future breeding studies and germplasm management of these pea genotypes.

Key words: Pea; *Pisum sativum*; Simple sequence repeat; Morphology; Genetic characterization

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 legume after common bean (*Phaseouls vulgaris* L.) in Turkey with a total production of 88,828 tons (FAO, 2008).

Endowed with a rich diversity of families (163), genera (1225) and species (9000) of plants, Turkey is one of the centers of origin and/or diversity of several crop plants, and many plant species (Tan, 1998; Özgen et al., 2000). Turkey is also the center of origin and genetic diversity of many wild, transitional, and cultivated forms of annual and perennial, herbaceous and woody plants such as the cultivated species of *Allium*, *Amygdalus*, *Avena*, *Beta*, *Cicer*, *Hordeum*, *Lens*, *Linum*, *Pisum*, *Prunus*, *Secala*, *Triticum*, and *Vitis* (Tan, 1998).

In order to preserve these genetic resources, several crop species from different geographical regions of Turkey are collected and preserved. One such institution is the Aegean Agricultural Research Institute of the Ministry of Agricultural and Rural Affairs, which has a collection of plant genetic resources belonging to several crop species. This collection includes pea genotypes obtained from different geographical regions of Turkey.

The aim of the present study was to assess the level of genetic diversity within this collection of pea genotypes to aid in the selection and more efficient utilization of this germplasm in breeding programs.

Several studies have been carried out to study genetic diversity within the pea germplasm and wild and cultivated species using different approaches (Samec and Našinec, 1995; Hoey et al., 1996; Zong et al., 2008a,b). In addition to morphological and biochemical traits, molecular markers (Smýkal et al., 2008) have been used for the identification of genetic relationships and exploring diversity. Molecular markers have enormous potential to explore genetic diversity by detecting polymorphisms to improve the efficiency and precision of conventional plant breeding. Using random amplified polymorphic DNA (RAPD) markers, significant differences were identified between and within wild and cultivated pea species (Samec and Našinec, 1995; Hoey et al., 1996). Amplified fragment length polymorphism (AFLP) markers were also used for detecting polymorphisms within pea genotypes (Simioniuc et al., 2002). Among the most widely used markers in crop species are simple sequence repeats (SSRs) or microsatellites (Blair et al., 2007; Sarıkamış et al., 2009). They are highly reliable because they are reproducible, co-dominant in inheritance and generally highly polymorphic.

However, SSR markers require a substantial investment of time and money to develop, and hence, adequate numbers for high-density mapping are not available for some

crop species. Significant effort has been dedicated to their development in various species during the last decade. They are now widely used for investigating genetic diversity among cultivars and genetic resources, and for developing genetic maps suitable for quantitative trait loci (QTL) detection and marker-assisted selection programs. In pea, comprehensive consensus linkage maps integrating linkage relationships from multiple maps and linkage studies (Weeden et al., 1998; Ellis and Poyser, 2002; Loridon et al., 2005) located several anonymous SSRs and their map positions for broad application of these markers as a common set for genetic studies in pea.

In this study, genetic diversity of pea genotypes collected from different regions of Turkey and preserved at the Aegean Research Institute Gene Bank was performed using morphological and SSR markers. This information could greatly assist in the identification, breeding and preservation of the pea germplasm.

MATERIAL AND METHODS

Plant material

The germplasm used in this study consisted of 30 pea (*Pisum sativum* L.) genotypes obtained from the Gene Bank of the Aegean Research Institute of the Turkish Ministry of Agricultural and Rural Affairs. The collection site of each pea genotype is shown in Figure 1. Ten commercial cultivars (Rondo, Utrillo, Sprinter, Spring, Television, Karina, Bolero, Sorgun, Jof, Senador Cambados) were used as reference cultivars in SSR analysis. Pea genotypes assessed by morphological and molecular markers in the present study were grown in the experimental plots of the Department of Horticulture, Faculty of Agriculture, Ankara University. The seeds were sown directly in the field when soil temperature increased to 7°C, leaving 60 cm between rows and 15 cm between plants. Standard cropping practices were used during the growth period. The experiment was performed during 2006 and 2007.

Figure 1. Map of Turkey indicating pea collection sites. Each genotype is labeled on the map.

Morphological characterization

Morphological characteristics of 30 pea genotypes were determined on 10 randomly selected plants per genotype. A total of 72 morphological traits including plant, stem, leaf and leaflet, wing, stipule, flower, pod characters, and the occurrence of pests and diseases were evaluated according to the guidelines provided by the International Union for the Protection of New Varieties of Plants (UPOV, 1990) (TG 7/9 *Pisum sativum* L.).

However, only certain characters regarding plant, stipule, flowering time, pod, and seed traits required for variety discrimination according to UPOV and that revealed polymorphisms among genotypes were selected and presented in the current study (Table 1). Morphological characteristics of the commercial pea cultivars were obtained from cultivar catalogue information.

Molecular characterization

Genomic DNA was extracted from young leaf tissues using Promega® DNA Extraction Kit. The extraction was carried out according to the protocol of the manufacturer. Subsequently, the eluted DNA samples were treated with RNAse. DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide. Quantity was then checked using a NanoDrop® ND-1000 Spectrophotometer. A total of 15 SSR markers were selected to detect polymorphisms and assess genetic diversity of the germplasm: 7 originated from a pea genetic map constructed by Loridon et al. (2005) (http://dx.doi.org/10.1007/s00122-005-0014-3) from the Pea Microsatellite Consortium, and 8 originated from common bean, which were previously used to characterize common bean genotypes (Sarıkamış et al., 2009) and hence already at hand to enrich the study. The selection of 7 pea SSRs was based on their high polymorphism information content and the quality scores reported (Loridon et al., 2005). Primers revealing clear single-band patterns were preferred. However, only 10 SSR markers (7 pea SSRs and 3 bean SSRs) were considered for molecular characterization of genotypes. The list of primer pairs used and the relevant information are presented in Table 2.

Polymerase chain reaction (PCR) mixture contained 15 ng DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5 U GoTaq DNA polymerase (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 2 µL 5X buffer in a volume of 10 µL. The forward primers were labeled with WellRED fluorescent dyes D2 (black), D3 (green) and D4 (blue) (Sigma). Reaction mixtures without DNA were included as negative controls. PCR amplification was performed using the Biometra® PCR System. The amplification conditions involved an initial step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52-56°C and 2 min at 72°C, with a final extension at 72°C for 10 min. PCR products were first separated on a 3% (w/v) agarose gel stained with ethidium bromide at a concentration of 10 mg/mL and run at 80 volts for 2 h. DNA Ladder (100 bp) (Promega) was used for the approximate quantification of the bands. The products were visualized under UV light and sized relative to the ladder. For further determination of polymorphisms, the PCR products were run on a CEQ 8800 XL capillary DNA Analysis System (Beckman Coulter, Fullerton, CA, USA). The analyses were repeated at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using the Beckman CEQ fragment analysis software.

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Table 1. Collection site and the morphological traits regarding plant, stipule, flower, pod and seed, that revealed polymorphisms.

Genotype	Collection site	Plant		Stipule			Flower	Pod							Seed
		Height	Anth. color	Type of development	Rabbit eared	Flecking	Flowering time	Length	Max. width	Ground color	Parchment	Type of curvature	Degree of curvature	Black color of hilum
G1	Aydın	Very short	Present	Rudimentary	Absent	Absent	Early	Very short	Medium	L. green	Absent	Concave	Weak	Absent
G2	Muğla	Short	Present	Rudimentary	Present	Present	Early	Very short	Medium	L. green	Absent	Concave	Absent	Absent
G3	Edirne	Medium	Present	Rudimentary	Present	Absent	Early	Short	Medium	Green	Absent	Concave	Absent	Absent
G4	Denizli	Medium	Absent	Rudimentary	Absent	Present	Early	Medium	Broad	Green	Absent	Concave	Weak	Absent
G5	Artvin	Medium	Present	W developed	Present	Present	Medium	Tall	Broad	Green	Absent	Concave	Weak	Absent
G6	Zonguldak	Medium	Present	W developed	Present	Present	Very late	Medium	Broad	Green	Present	Concave	Weak	Absent
G7	İstanbul	Medium	Absent	Rudimentary	Absent	Absent	Very late	Short	Medium	L. green	Absent	Concave	Absent	Absent
G8	İzmit	Tall	Present	Rudimentary	Absent	Absent	Early	Medium	Medium	Green	Absent	Concave	Weak	Absent
G9	Adapazarı	Medium	Present	W developed	Present	Present	Medium	Very tall	Broad	Green	Absent	Convex	Absent	Absent
G10	Sivas	Tall	Absent	W developed	Absent	Present	Late	Tall	Medium	Green	Absent	Concave	Weak	Absent
G11	Ordu	Very tall	Present	W developed	Absent	Present	Early	Tall	Broad	Green	Absent	Concave	Weak	Absent
G12	Artvin	Tall	Present	W developed	Absent	Absent	Medium	Very tall	Medium	D. green	Absent	Concave	Medium	Absent
G13	Bursa	Medium	Present	W developed	Absent	Present	Medium	Very tall	Broad	Green	Absent	Concave	Weak	Absent
G14	Tekirdağ	Very short	Absent	Rudimentary	Present	Present	Very early	Short	Narrow	L. green	Present	Concave	Absent	Absent
G15	Çanakkale	Very short	Absent	Rudimentary	Present	Present	Very early	Medium	Broad	Green	Present	Concave	Absent	Absent
G16	Hakkari	Short	Absent	Rudimentary	Present	Absent	Very early	Short	Narrow	Green	Absent	Concave	Absent	Absent
G17	Mardin	Tall	Present	W developed	Absent	Present	Early	Medium	Medium	Green	Absent	Convex	Absent	Present
G18	Çorum	Short	Absent	Rudimentary	Absent	Absent	Early	Medium	Broad	Green	Absent	Concave	Absent	Absent
G19	Sakarya	Tall	Present	W developed	Absent	Absent	Medium	Medium	Broad	Green	Absent	Concave	Medium	Absent
G20	Giresun	Medium	Present	Rudimentary	Absent	Present	Medium	Medium	Broad	Green	Absent	Concave	Absent	Absent
G21	Hatay	Medium	Present	W developed	Absent	Absent	Medium	Medium	Broad	Green	Absent	Concave	Weak	Present
G22	İzmir	Medium	Absent	W developed	Absent	Present	Medium	Medium	Broad	Green	Absent	Concave	Absent	Absent
G23	Kastamonu	Short	Present	W developed	Absent	Present	Late	Medium	Narrow	D. green	Absent	Concave	Strong	Absent
G24	Kütahya	Very tall	Absent	W developed	Absent	Absent	Medium	Medium	Medium	Green	Absent	Concave	Weak	Absent
G25	Muğla	Medium	Present	W developed	Absent	Present	Medium	Medium	Medium	Green	Absent	Concave	Weak	Absent
G26	Zonguldak	Short	Present	W developed	Present	Present	Late	Medium	V broad	L. green	Absent	Concave	Weak	Present
G27	Çanakkale	Medium	Absent	W developed	Present	Present	Late	Medium	Medium	L. green	Absent	Concave	Absent	Absent
G28	Tekirdağ	Medium	Absent	W developed	Present	Present	Late	Medium	Medium	Green	Absent	Concave	Absent	Absent
G29	Hatay	Short	Absent	W developed	Present	Present	Medium	Tall	Broad	L. green	Absent	Concave	Weak	Absent
G30	Bursa	Short	Present	W developed	Present	Present	Late	Very tall	Broad	Green	Absent	Concave	Absent	Absent

Anth. = anthocyanin; L. green = light green; D. green = dark green; W developed = well developed.

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Genetic analysis

The genetic analysis "IDENTITY" 1.0 program (Wagner and Sefc, 1999) according to Paetkau et al. (1995) was used for the calculation of number of alleles, allele frequency, expected and observed heterozygosity, estimated frequency of null alleles, and probability of identity per locus. Genetic dissimilarity was determined by the "MICROSAT" program, version 1.5 (Minch et al., 1995) using proportion of shared alleles, which was calculated using ps (option 1 - (ps)) as described by Bowcock et al. (1994). The results were then converted to a similarity matrix and a dendogram was constructed with UPGMA (unweighted pair-group method with arithmetic mean) (Sneath and Sokal, 1973), using the NTSYS-pc software (Numerical Taxonomy and Multiware Analysis System, version 2.0 (Rohlf, 1988).

RESULTS

Morphological characterization

The origin (collection site) and a summary of the morphological traits that revealed polymorphisms are presented in Table 1. When seeds were considered, black color of hilum was only present in G17, G21 and G26. In terms of plant height, 10% of genotypes were categorized as very short, 23% as short, 43% as medium, 17% as tall, and 7% as very tall plants. The presence of anthocyanin coloration was evident in 60% of genotypes. Variation in terms of pod characters such as pod length, maximum width, ground color, parchment, degree of curvature, and type of curvature was observed. Pod ground color was mostly green (70%), but in some, it was light green (G1, G2, G7, G14, G26, G27, and G29) and in one case (G23) dark green. Parchment was present only in three genotypes (G6, G14 and G15). Degree of curvature varied being mostly concave except the genotypes G9 and G17. The stipule was mostly well developed, but in some it was rudimentary. Rabbit-eared stipules were present in 47% and was absent in 53% of the genotypes. Flecking was present in the majority (67%) but absent in some genotypes (33%). Variation in terms of flowering time was observed. However, no differences in leaf color and pod shape were observed among the genotypes, and hence, these characters are not presented in Table 1.

Molecular characterization

Seven microsatellite markers selected on the basis of high polymorphism information content (Loridon et al., 2005) revealed successful amplifications of expected allele sizes (Table 2). Among a total of eight common bean SSR markers (Yu et al., 2000) selected on the basis of high polymorphism information content, only three markers (PV-at002, PV-at006 and PVSBE2) were used for characterization due to their reliable amplification patterns. Therefore, genetic diversity within the collection of 30 pea genotypes together with 10 commercial pea cultivars was assessed by 10 SSR markers. In all, a total of 61 alleles were detected at the 10 SSR loci analyzed. The number of alleles per SSR locus ranged from 3 (PVSBE2) to 12 (AB53) with an average of 6.1 alleles, while one of the primers was monomorphic (PV-at006).

Table 2. Allele range values (bp), number of alleles, expected heterozygosity, observed heterozygosity, frequency of null alleles and probability of identity values of pea genotypes calculated at 10 simple sequence repeat loci.

Locus	Allele range (bp)	Number of alleles	Expected heterozygosity	Observed heterozygosity	Frequency of null alleles	Probability of identity
AA205 (*P. sativum*)	180-238	4	0.629	0.100	0.324	0.306
AD270 (*P. sativum*)	283-313	8	0.787	0.000	0.440	0.128
AB25 (*P. sativum*)	182-232	6	0.671	0.000	0.401	0.235
AB53 (*P. sativum*)	88-148	12	0.820	1.000	-0.098	0.099
A9 (*P. sativum*)	360-384	7	0.651	0.050	0.364	0.229
AA355 (*P. sativum)*	184-232	9	0.760	0.175	0.332	0.157
AD61 (*P. sativum*)	113-135	7	0.820	0.050	0.423	0.106
PV-at002 (*P. vulgaris*)	247-367	4	0.601	0.450	0.094	0.354
PV-at006 (*P. vulgaris*)	368	1	0.000	0.000	0.000	1.000
PVSBE2 (*P. vulgaris*)	113-133	3	0.324	0.350	-0.019	0.591

The number of alleles suggested that the most informative loci were AB53 (12 alleles), AA355 (9 alleles), AD270 (8 alleles), A9 (7 alleles), AD61 (7 alleles), AB25 (6 alleles). Contrarily, the least informative loci were AA205 (4 alleles), PV-at002 (4 alleles) and PVSBE2 (3 alleles). One of the primers (PV-at006) was monomorphic (Table 2). Probability of identity values were generally greater than 0.05 (Sefc et al., 2001).

The closest genetic relationship was observed between the two genotypes G13 and G25 (0.900), followed by G9-G23 (0.850) and G30-Sprinter (0.800). While the genetic similarity between the cultivars Utrillo and Senador was 0.950, the similarity of these two to Sorgun was 0.900.

The UPGMA dendrogram (Figure 2) as defined by SSR markers revealed two main groups. While genotypes G2 and G27 clustered as one major group (group 1), pea cultivars and the rest of genotypes formed a separate group (group 2). A single genotype (G30) originating from Bursa was closely related to Sprinter, a commercial pea cultivar. Subsequently, group 2 was further divided into several subgroups containing the remaining genotypes and cultivars. Genotypes G3, G12 and G22 were related to Rondo and Bolero with a similarity index of around 0.45.

DISCUSSION

The present study revealed the genetic diversity within a collection of pea germplasm representing different geographical regions of Turkey, using morphological and molecular (SSR) approaches (Figure 2).

Assessment of genetic variability within a germplasm is of interest for practical applications such as conservation of genetic resources and for breeding purposes, to predict the ability to combine or to rapidly verify the breeding material. Hence, it is crucial for genetic improvement and elite gene exploitation, such as tolerance genes to abiotic stresses.

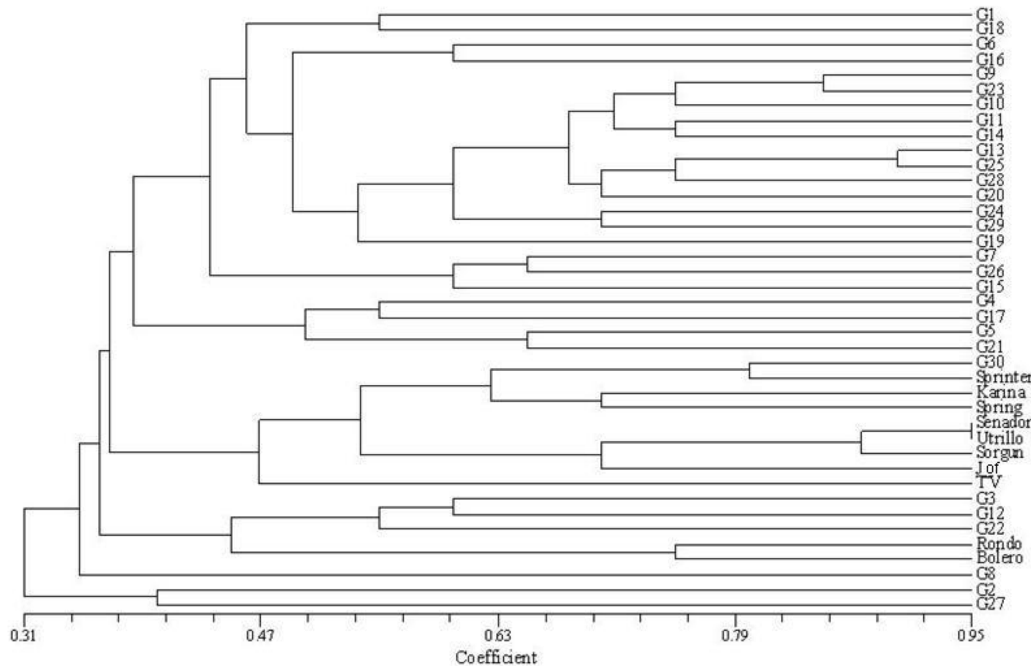

Figure 2. Dendrogram showing the genetic relationships of 30 pea genotypes together with 10 commercial pea cultivars based on the UPGMA cluster analysis of 10 simple sequence repeat marker data.

Within the collection studied, variation was observed in terms of seed, plant, pod, stipule, flower characteristics, and flowering time, 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.

Several different marker systems (isozymes, restriction fragment length polymorphisms (RFLPs), AFLPs) have been used for either mapping studies or diversity assessment in pea. However, few studies have used SSR markers (Weeden et al.,1998; Burstin et al., 2001; Pilet-Nayel et al., 2002; Baranger et al., 2004; Prioul et al., 2004; Tar'an et al., 2005; Zong et al., 2008a,b). SSR markers were reported as superior in terms of high information content and discrimination power owing to high allelic variation, allowing clear identification of all varieties compared to most DNA as well as biochemical and morphological markers (Smýkal et al., 2008). Primers used in the present study were selected among a set of microsatellite markers developed for the Pea Microsatellite Consortium (Loridon et al., 2005).

All 7 primers consistently revealed a clear banding pattern in all samples analyzed. However, when common bean primers were used to amplify pea genomic DNA, only three primers displayed clear banding patterns, while the rest of the primers either revealed a multiple band pattern, non-reproducible bands or no product and discarded after several attempts

to improve PCR conditions. Overall, 10 successful SSR loci yielding bands of expected sizes were utilized for characterization and provided precise identification of all genotypes. The average number of alleles obtained in the present study is in agreement with results obtained by Loridon et al. (2005). Observed heterozygosity was generally lower than expected, except for AB53 and PVSBE2. This is probably due to the inbreeding nature of pea but not due to null alleles. The UPGMA dendrogram clearly demonstrated the genetic relationship among genotypes and reference cultivars. Taken together, the closely linked genotypes were also closely related in terms of the morphological traits presented.

However, no ecogeographical distribution was observed within the subgroups.

CONCLUSION

The need for the conservation of plant genetic resources has been widely accepted. Germplasm characterization and evaluation complemented by molecular studies generate the information base for more efficient utilization of these valuable resources by gene bank managers, plant breeders and research scientists to focus on the search for desirable new characters. Utilization of plant genetic resources following a network approach with effective connections between the national gene bank and researchers must be encouraged. The present study aimed to characterize the pea germplasm maintained at the Aegean Research Institute Gene Bank of Turkey using morphological and molecular approaches. The results may offer scope for pea breeding programs aimed to generate new improved cultivars in the future.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Ali Ergül from the Biotechnology Institute, Ankara University for his kind help and support and the Aegean Agricultural Research Institute for kindly providing the seeds for this research.

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