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Breeding for Adaptation Traits of Wheat in Eastern European Environments the Hungarian Example

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ABSTRACT

The traditional wheat germplasm that have evolved in Eastern Europe exhibit a unique adaptation type due to the ecological conditions. Bread wheat type developed in this region is a valuable source for wheat breeders worldwide. Wheat improvement in the 20th century was carried out using traditional breeding methods and the consideration of environmental effects played a significant role in the germplasm development. Efficient wheat breeding programme for continental climatic environments will require new breeding efforts, including new strategies in gene bank research, to develop new germplasm in pre-breeding programmes and the application of modern breeding technologies. The complex tasks facing wheat breeders in Eastern Europe include the improvement of stress resistance, especially winter hardiness and tolerance of drought and heat at higher levels of yield potential. New challenge is to close the yield gap in the changing climate. The good bread making quality of Eastern European wheat will only represent an advantage if it is associated with better quality stability.

Keywords: Wheat, pre-breeding, adaptability, yield gap, bread making quality stability.

Introduction

Changes in the Eastern European Wheat Type

A significant segment of cereal production takes place in suboptimal agro-ecological environments in the World. This fact is especially true in the Eastern European regions where the tendency to drought, the uneven rainfall distribution, the continental climate and the great variety of soil types have made a traditional contribution to the development of a wheat type suitable for low-input environments. These climatic conditions are mainly characterised by extreme variability and unpredictability. Geographical and climatic factors have had a substantial influence on the genetic background of the germplasm and on agronomic management practices.

Landraces have played an important role in the development of the East European wheat germplasm. Special mention should be made of the landrace Bánáti (or Bánátka), originating from the area which is now on the borders of Serbia, Romania and Hungary, and which was made use of by wheat breeders in Russia (Vavilov 1935), Ukraine, Romania and Hungary. The Polish landraces Sandomierka (Strebeyko 1976) and Galicia (from a region now in West Ukraine) (Lelley 1967) were not only the parental populations for a large number of East European wheats, but were also taken to North America by emigrants in the 19th century. The Galicia landrace was also the ancestor of the Hungarian landrace Tiszavidéki (called Teiskaya in Russia), the progeny of which spread at the end of the 19th century to South Russia, to the Kuban region, and also to parts of the North Caucasians (Jakubziner 1962), while also being one

of the parents of the Bánkúti wheat varieties, known for their excellent breadmaking quality.

In the winter wheat zone, the development achieved by Lukyanenko, who selected the variety Bezostaya 1 in Krasnodar, led to excellent results in the Eastern European region after the second world war. The good adaptability of Bezostaya 1 could have been due in part to the fact that its parents originate from different ecological regions of the World. With the selection of this variety, yields were increased in the Krasnodar region of Russia by 2.5 times (Dorofeev and Udachin 1987). In the northern zone of winter wheat production, the extremely winter-hardy variety Mironovskaya 808 became the dominant variety in the northern part of Ukraine, and also occupied large areas in Poland and Czechoslovakia in the 60s and 70s of the last century. The Krasnodar-bred varieties Avrora and Kavkaz, which carry the 1B/1R rye translocation containing the resistance genes Pm8, Lr26, Sr31 and Yr9, represented a great genetic advance in yield potential (Bedő *et al*., 1993). Similar conclusions were drawn by Javornik *et al*., (1991) when analysing 1B/1R translocation varieties such as Yugoslavia, Balkan and Zvezda in Serbia. However, despite the positive effects of wheat-rye translocations on yield and adaptability traits in Eastern Europe, their deleterious effect on breadmaking quality cannot be completely eliminated. The further use of this germplasm in wheat breeding may be limited, as the new stem rust (*Puccinia graminis tritici*) race Ug99 exhibits broad virulence, being able to overcome the stem rust resistance gene Sr 31, located on the 1B/1R translocation (Singh *et al*., 2007).

Daylength insensitivity, race-specific disease resistance and better lodging resistance contributed to the better adaptability and widespread cultivation of modern types of varieties in the second half of the last century. From the 1970s onwards, Ukrainian wheat breeders endeavoured to reduce plant height (Litvinenko 1998). Russian wheat breeders in Krasnodar aimed chiefly at winter hardiness and favourable technological quality, so they made use of Krasnodarskii karlik, developed by mutation breeding from Bezostaya 1 (Bespalova 1996), while Romanian breeders used Rht1 (Saulescu *et al*., 1988). In Hungary the Rht1 and Rht8 genes proved successful. In Serbia Rht8, originating from the Japanese variety Akakomughi, and Rht1, derived from Saitama 27, can be detected in semi-dwarf wheat varieties (Borojevic 1990). These genes cause less dwarfing than the joint presence of Rht2 and Rht1 in a number of West European varieties, but due to the drier climate and the lower nutrient supply levels, their use was a realistic solution for the development of semi-intensive wheat.

As growing conditions improved and chemical input increased in the second half of the last century, breeders endeavoured to develop genotypes with a higher ratio of reproductive organs. Positive correlation was found between the grain yield and the harvest index (r=0.92) in Martonvásár (Szunics *et al*., 1985). The statistical analysis of data collected over many years proved that the year (environment) had a substantial effect on the grain-straw ratio of the varieties examined, though the varieties responded differently to ecological factors. Lukyanenko (1966) considered a 1:1 grain-straw ratio to be ideal. The harvest index increased in many parts of the East European region as a result of breeding efforts. Szunics *et al*., (1985) found a 47% harvest index due to selection efforts focused on an increase in plant productivity and a decrease in plant height.

Yield progress and improved adaptability traits contributed to an annual yield increase of 0.86% in Russian spring wheat varieties between 1920 and 1999 (Vassiltchouk 1999). The genetic progress for yield in Romanian wheat varieties was estimated by Saulescu (1998) to be 50 kg/ha/year. In Hungary, studies on the period between 1965 and 1985, when national yield averages grew to the greatest extent, reveal that the varieties were responsible for 42–45% of the total yield increment (Szunics *et al*., 1985).

The type of wheat developing in the East European region was not uniform. Despite the similarities, there were also substantial differences as a consequence of the diverse ecological conditions. In general the winter type is dominant, but spring wheat varieties spread in several of the central and East European regions of Russia (e.g. in the Volga and Ural regions), where abiotic stress resistance is a decisive factor. In these regions, variety change progresses at a slower rate. The great extent of diversification is demonstrated by the fact that selection is carried out for four different adaptation types in Krasnodar, the largest Russian wheat breeding centre, including both semi-intensive drought-resistant types and semi-dwarf wheat types (Bespalova 1996). In accordance with the duration of the vegetation period and adaptation to soil and climatic zones, Ukrainian wheat varieties can be divided into three fundamental groups, so breeding is underway for intensive, semi-intensive and widely adapted wheat varieties (Lyfenko 1987).

The yield increases recorded between 1960 and 1990 stagnated the last decade of the 20th century and first decade of the 21st century in many regions of Europe, and this situation was characteristic in most East European regions too. This can be attributed to a number of factors, including

• The low level of fertilizer and pesticide applications, which resulted in a lower yield and quality traits stability. The use of technical inputs declined partly due to growing input costs. The strengthening demand for sustainability and environmental protection have also led to a reduction in the use of chemicals (Figure 1).

• Large differences among growing seasons due to the climate change caused more negative effects on wheat production than in many other regions of the World.

Breeding For Adaptability Traits of Wheat in Martonvásár, Hungary

Wheat breeding programmes initiated in Hungarian plant breeding institutes after the second world war developed a whole series of varieties in Martonvásár and in Szeged. Bread wheat improvement in the 20th century was carried out using traditional breeding methods. However, new genetic, pathological and physiological knowledge have significantly contributed to a better understanding of this crop. It has become evident that the next generation of wheat genotypes will need to be developed for better adaptability traits and that further crop improvement will require new breeding tools. It has become important to select wheat genotypes adapted to changing climatic conditions, as a return to old varieties is not an option for future development. Martonvásár small grain cereal breeding programmes established breeding strategy to improve research efforts, including strategies in gene bank research and research efforts to develop new germplasm in pre-breeding programmes.

Gene Bank Research

The role played in plant breeding by the old varieties and populations stored in gene banks has increased recently. One of the richest collections in the World and in Eastern Europe is located in the Vavilov Institute of Plant Industry in St. Petersburg, Russia. The efficiency of plant gene bank research programmes depends on the accuracy and precision of evaluation techniques. The evaluation of large germplasm materials using only traditional tools such as geographic origin, pedigree information, and botanical and agronomic descriptions has become less efficient.

The establishment of a cost-effective core collection to represent the genetic variability of large collections is of vital interest in Eastern Europe, where traditionally large germplasm collections have been available to breeders. Core collections are useful materials for the association mapping of disease resistance, seed quality and domestication-related traits. A good example is the core collection of 372 accessions based on passport and simple sequence repeat (SSR) marker data selected by Balfourier *et al*., (2007) in the Clermont-Ferrand Genetic Resources Center (INRA) to explore the diversity in wheat accessions. Genetic resources are suitable materials for association mapping when breeders analyse low heritability traits like yield components (Breseghello and Sorrells 2006).

The efficiency of gene bank research can be improved through the joint application of new methods of genotyping and phenotyping, which enable plant breeders to screen the collections for genes important for breeding, identify unique alleles and characterize genetic resources at the gene level, dissect the populations of old landraces and wild relatives to provide insights into the allelic content of potential germplasm for use in breeding.

Screening wheat genetic resources, molecular markers are efficient tools for identifying agronomically important genes. In the course of gene bank research in Martonvásár, pedigree analysis revealed that the variety Bezostaya 1 is frequently present in the wheat germplasm. According to Dyck (1994) Bezostaya 1 carries the Lr34 leaf rust resistance gene, which ensures a medium level durable resistance. When it is combined with other Lr genes, is an efficient component of leaf rust resistance. Vida *et al*., (2009) carried out analysis with molecular markers and they proved that the Lr34 resistance gene is significantly present in the Martonvásár germplasm of the 226 genotypes screened (Table 1).

The identification of unique alleles from genetically heterogeneous old populations play an important role in developing new germplasm for breeding. A unique allele was identified with the help of a gene-specific primer from the Bánkúti 1201, an old Hungarian variety population. In the course of the analysis a fragment characteristic of Bánkúti 1201 was identified and the nucleotide sequence was determined. This showed the presence of a 1Ax2* high molecular weight (HMW) glutenin gene variant which, despite near homology, differed from the original 1Ax2* gene at one important point. Nucleotide exchange involving the exchange of serine for cysteine was observed at 1181 bp in the 1Ax2* sequence (Juhász *et al*., 2003). This change resulted an extra sulphydryl group which facilitates the formation of further disulphide bonds, might lead to an improvement in gluten quality characters.

The dissection of the heterogeneous population and the development of sublines contribute to the exploitation of existing variation for different agronomic traits. For example, the analysis of 216 sublines from the Bánkúti 1201 revealed six HMW glutenin subunit

types and 19 different gliadin types. Significant variation was detected involving the overexpression of the Bx7 HMW glutenin subunit and the unextractable polymeric protein % (UPP%) (Juhász *et al*., 2003). Marchylo *et al*., (1992) reported that at least two types of Bx7 protein existed in different varieties, and designated the Cheyenne type, leading to normal protein production, as 7* and the overexpressed Glenlea type as 7. The quality of the Canadian variety Glenlea can also be attributed in part to the Bx7 subunit (Cloutier and Lukow 1998), though in this case the gene is only present in a single copy. Overexpressed Bx7 HMW glutenin subunits have also been detected in many other wheat genotypes, both in old landraces and in modern varieties (Marchylo *et al*., 1992). Analyses carried out by Butow *et al*., (2004) demonstrated that the allele responsible for 1Bx7 HMW glutenin overexpression in the Canadian prime hard red spring wheat Glenlea is also present in certain sublines of Bánkúti 1201, providing an indirect confirmation of the fact that East European germplasm may also have had an influence on the development of North American varieties. The rheological analysis of lines isolated from old varieties and carrying the 1Bx7 HMW glutenin overexpression gene showed that selection for this trait may contribute to the breeding of varieties with excellent gluten quality in Eastern Europe.

New Germplasm Development in Pre-breeding

One consequence of commercial breeding in the second half of the 20th century is that differences between alleles in modern elite varieties are diminishing, and the development of new germplasm in pre-breeding programmes has become important. Different types of genetic resources are available in East European wheat breeding for the development of new germplasm. In commercial breeding programmes breeders use mainly adapted varieties and lines to achieve fast breeding progress and select new registered varieties. The application of old landraces, wild and cultivated relatives need more time, however the chance of broadening genetic variation is better.

The transfer of useful genes is complicated by crossing barriers in the case of wild or cultivated related species, by the absence of pairing between homologous chromosomes, etc. To avoid embryo or endosperm abortion after successful fertilisation *in vitro* techniques such as ovule culture or embryo rescue are applied. It often happens that the F_1 plants from interspecific crosses are sterile. Many methods have been introduced to overcome incompatibility between the species and achieve successful hybridisation. These include doubling the ploidy level, protoplast fusion, etc. Colchicine treatment was applied to F_1 hybrids to induce chromosome doubling in order to produce synthetic hexaploid wheat. The use of a genome homozygous for the crossability alleles (*kr1kr1kr2kr2*) may contribute to higher seed set when wheat is crossed with rye, barley, etc. The recessive crossability allele *kr1* was transferred from the spring wheat cultivar Chinese Spring (CS) into the winter wheat cultivar Martonvásár 9 (Mv9) by backcrossing Mv9×CS hybrids with Mv9. As a result of five backcrosses with Mv9 and two selfings after each backcross, the selected progenies had over 50% seed set with rye when tested on a large number of individual plants (Molnár-Láng *et al*., 1996*)*.

The introduction of alien gene into adapted wheat germplasm is time-consuming using traditional breeding methods. A good example of this are wheat varieties carrying the 1B/1R rye translocation, which required 33 years from the first cross of parents to the registration of the first variety (Rabinovich 1998). Pre-breeding for adaptational traits is efficient if breeders are able to widen the genetic variation and to shorten the selection time. One method which has been routinely introduced in Martonvásár is the doubled haploid technology 30 years ago to produce homozygous progenies from the F_1 generation in a single step (Bedő *et al*., 1988). This technology is an excellent tool not only for cultivar development, but also for pre-breeding and for the establishment of mapping populations.

Despite the complex nature of the breeding process, wild relatives have frequently been used by East European wheat breeders. In Russia Tzitzin selected winter wheat varieties from *Agropyron* sp. × *T. aestivum* crosses and they were grown in commercial production (Zhukovsky 1957). Interspecific crosses were used to incorporate a number of disease resistance and storage protein genes into the common wheat germplasm in the Odessa breeding programme *(*Litvinenko *et al*., 2001).

Today one of the most promising breeding technologies to accelerate the introduction of alien genes into wheat from wild relatives is cisgenesis. It has great potential to overcome many problems of traditional breeding, such as linkage drag, crossing barriers, the introduction of many deleterious genes linked with one useful gene. This method differs from transgenesis that DNA fragments from cross-compatible species are incorporated into the genome, so cisgenic plants do not contain foreign or modified genetic material. As wheat has many wild and cultivated relatives, the prospects for developing precision breeding via the cisgenesis technology are excellent.

This technology would be a modern alternative to traditional breeding and would offer an ideal way of incorporating genes of adaptation traits from wild and cultivated wheat relatives into common wheat.

The use of molecular markers for the development of new germplasm and to improve the efficiency of pre-breeding is a great step forward in breeding. On the one hand it allows breeders to accelerate the introgression and backcrossing of genes into diverse genetic backgrounds, while on the other hand they can use it to pyramid genes with similar phenotypic effects. This technique is particularly useful for the incorporation of resistance genes. The advantage of marker assisted selection (MAS) in early generations is that heterozygous progenies with valuable recessive genes are not discarded, thus contributing to the fixation of recessive genes. To exploit this advantage, the use of MAS in early generations and phenotypic selection in later generations are suggested. MAS can substitute for phenotypic selection in conditions unfavourable for phenotyping, or against stress factors not present in the breeding location or appear only rarely. This technique is not affected by the environment, which means that it is less season-dependent than phenotypic selection.

Marker-assisted backcross breeding (MABC) is one of useful tools to accelerate the introgression of adaptation traits. Simulating recombination during meiosis proves that breeders can recover recurrent parent with molecular markers more efficiently compared to traditional backcrossing (Frisch *et al*., 2000). MABC provides efficient positive foreground selection for the donor trait, positive background selection for the recurrent parental genome and negative background selection against undesirable donor parent alleles. After three backcross (BC) generations it was possible to select genotypes with useful agronomic trait similar to that of the donor line (Figure 2). MABC will be a useful breeding method to introgress transgenes into elite germplasm, which will permit the rapid deployment of agronomic traits.

Molecular markers offer a precise selection for pyramiding genes for resistance to diseases. They were employed for the transfer of leaf rust resistance genes in the framework of the BIOEXPLOIT FP6 EU project (Vida *et al*., 2009). The choice of the Lr genes was based on their effectiveness and how closely linked PCR markers were available. The agronomic traits of BC_5 and BC_6 lines are very similar to those of the recurrent parents. Eleven pyramided gene combinations have been developed, and a doubled haploid programme has been set up in order to stabilize the gene combinations.

Stem rust is one of the most destructive diseases of cereal crops worldwide. During the last period a new aggressive virulent stem rust group of races Ug99 cause severe losses in some wheat growing regions of the World. However, this race is not present in Eastern Europe until now. We carried out pre-breeding studies about the genetic background of stem rust resistance in the Martonvásár gene pool and we selected new genotypes resistant to these virulent stem rust races. In international experiments Mv Zelma was found to be resistant to Ug99 related pathotypes. Preliminary data indicated that its resistance gene is located on 7A chromosome. Resistance genes Sr15 and Sr22 are located on this chromosome. Infection types and map location suggest that the gene in MV Zelma is not Sr22. It is probable that this gene could be a second allele of Sr15 that provides Ug99 resistance (Nava *et al*., 2012).

One of the most important criteria for germplasm development in Eastern Europe is above-average abiotic stress resistance. This includes winter hardiness, which involves different components in different regions of Eastern Europe. One of these components is late winter frost resistance, which has been studied in the Martonvásár phytotron for several decades (Veisz *et al*., 2001). The analysis of wheat varieties characteristic of diverse regions of Europe revealed (Bedő *et al*., 2005) that East European varieties were the most resistant, South European varieties the most frost-sensitive and West European wheats were intermediate for frost resistance.

The importance of winter hardiness, and especially of frost resistance, is proved by the fact that over the last century a large part of the area previously sown to spring wheat in Russia was gradually occupied by winter wheat. While spring wheat was grown on around 80% of the European region of the country in the first two decades of the 20th century, this ratio had dropped to 62.4% by the 1980s. However, a high level of winter hardiness is required in these areas if production is to be reliable (Dorofeev and Udachin 1987). Due to the negative grain yield/frost resistance correlation, varieties with poorer winter hardiness are spreading in Ukraine, if the frost resistance of the varieties grown in 1985 is compared with the situation 15 years later. By the turn of the millennium none of the varieties could equal the winter hardiness of Odesskaya 51, demonstrating the importance of breeding for winter-hardy germplasm (Litvinenko *et al*., 2001).

The most complex task facing East European wheat breeders is the improvement of drought and heat tolerance, or its maintenance in new germplasm at a higher level of yield potential. In Ukraine

drought-resistant genotypes have high vernalisation requirement and low photoperiod sensitivity. They should have intensive nodal root growth in early spring, active root development during double ridge formation, leaves with high osmotic adjustment and the active remobilisation of non-structural carbohydrates to the kernels during the grain-filling period (Litvinenko 1998). In regions prone to drought, adaptability, drought tolerance and yield are closely related traits. In the southern parts of Romania, selection for earliness is one way of avoiding drought and improving yield stability (Saulescu *et al*., 1998). The role of environmental effects depends on the developmental stage of the plant, and may influence both yield and grain quality properties. Breeding for improved heat and drought stress tolerance during meiosis and anthesis remains a significant challenge.

Selection for better adaptability has become particularly important in the light of climate change, especially with the increasing frequency of extreme weather events (Veisz *et al*., 1996). Greater fluctuation is observed not only between regions, but also between years. Price volatility during the recent period is also influenced by unstable yield.

Regardless the fact that drought and heat are the main abiotic stresses in wheat production, extreme weather conditions, like heavy rainfall during the harvest period, can cause the premature germination of seeds, known as preharvest sprouting (PHS). This phenomenon became more frequent during the climate change and it is a serious problem in Eastern Europe too. Especially wheats with white-colored grain are particularly susceptible. PHS has a negative effect on grain yield but it also reduces bread-making quality through the breakdown of starch reserves in the endosperm by the increased enzyme activity of alpha-amylase.

The high yield gap between the potential yield and farm yield is one of the consequences of the lower level of yield stability. To determine the difference between the potential yield and farm yield we set up a three-year small plot experiment carried out with registered varieties widespread in the commercial production in Hungary. The experimental plots were treated with herbicide and fungicides and 240 kg/ha mineral fertilizer - nitrogen, phosphorus and potassium in 2:1:1 ratio - were applied. The difference between the farm yield - characterized by the national average yields - and the potential yield was 48.2% in average of the 3 year results in Hungary (Figure 3). Investigations made by Litvinenko *et al*., (2001) showed that the potential yield of new varieties rose from 2.73 t/ha in the second decade of the 20th century to 6.74 t/ha in the 1980s, based on the results of small-plot experiments. At the same time, the average wheat yield in Ukraine was only 3 t/ha even in favourable years, and was even less at the turn of the millennium. This is less than half of the potential yield. Among the West European countries, the yield gap in the UK is 30%, indicating that the potential yield of the wheat varieties is better exploited (Fischer and Edmeades 2010).

Technological quality stability is a particularly critical property in Eastern Europe because, in general, wheat with better breadmaking quality and higher protein content can be grown in the region than in Western Europe. The vast majority of wheat varieties belong to the hard red quality group, based on the North American classification, although each country has its own quality standard. Over the last two decades the stability of quality parameters has deteriorated due to the negative effects of reduced mineral fertiliser use, extreme climatic conditions and biotic stress factors (e.g. *Eurigaster* sp.). The concept raised by Canadian plant breeders (DePauw *et al*., 1998), who suggested that endeavours should be made to change the composition of the storage protein for new types of industrial uses rather than increasing the protein content, would definitely be worth considering in the course of new germplasm selection for better quality adaptability.

Wheat breeding programme in Martonvásár has been focussed on bread-making quality and quality stability in order to preserve the good quality traditionally characteristic of Hungarian wheat, which is capable of exploiting Eastern European environmental conditions to give good quality with satisfactory stability. The Pannonia quality wheat R&D system, a complex national project was established in 2000 for the management of research and development on wheat quality. As a result of the programme technological quality categories were established, taking into consideration the major quality classifications used in the global cereal industry and the high protein quality of the hard red type of wheat traditionally grown in Hungary. The Pannonian premium and Pannonian standard categories both have criteria to exploit the advantages of the Hungarian germplasm and environmental conditions to produce wheat of a quality satisfying the demands of both domestic and export markets. In order to validate the new quality categories and the quality level expected from them, a trade-mark has been registered (Bedő 2008).

Despite the fact that genetically modified wheat development is limited because of public concern, especially in case of small grain cereals, well-established

transformation protocols are already available for wheat, involving increasingly efficient *Agrobacterium*-mediated transformation techniques, better integration patterns and improved co-transformation. The rapid development of the transgenic breeding technology for various agronomic traits is proved by the increasing number of traits tested in field trials in North America (Dunwell 2008). Breeders from Martonvásár carried out joint analyses on genetically modified (GM) wheat in cooperation with scientists from Rothamsted. This collaboration is focussed on high-molecular-weight (HMW) glutenin subunits to increase dough strength, which is one of the quality stability traits for breadmaking. According to the results of field experiments on GM spring wheats over-expressing HMW subunits 1Ax1 and 1Dx5, led to significant changes in the structure of the glutenin polymers which were detected in flour functional properties, caused by the altered ratio of x-type to y-type HMW subunits. The expression of the subunit 1Ax1 transgene led to increased dough strength, while the expression of the homologous subunit 1Dx5 transgene led to ''overstrong'' dough with low extensibility and poor water absorption (Rakszegi *et al*., 2008).

Seed Policy and Production Stability

Through the breeding programmes initiated in Hungarian Pant Breeding institutes a whole series of varieties were developed, first in Martonvásár and in Szeged, while other varieties of foreign origin are also grown because of the liberal seed market policy of the European Union. The area sown to Hungarian-bred varieties occupy about 50% of the wheat-growing area in the middle of the second decade of the 21st century.

The number of varieties cultivated gradually rose after entering the European Union in 2004. Beside the wheat varieties registered in Hungary, wheat varieties registered in other countries of the European Union are also allowed to produce in Hungary. The higher number of wheat varieties potentially a useful tool to increase biodiversity in the wheat production, however the agronomic properties of unknown wheats, mainly their adaptation to the Hungarian climatic conditions are questionable. The twenty most popular varieties occupy around 50-60% of the total growing area. Even the most successful varieties are rarely grown on more than 10% of the sowing area. Over the last few decades there has been little change in the 5–6-year average life-span of the varieties, which is 6-7 years when weighted with the sowing area.

Concerning the seed industry, the centralised seed supply system was discontinued in 1990 when the market oriented economy was introduced. Although the land changed hands, the majority of the seed multiplication farms continued their activities regardless of the constant transformations. The restructuring of the seed market was accelerated by the fact that foreign breeding companies, which had for years been contracting farmers in Hungary to grow large quantities of seed, set up their own companies, which then entered the market. Leading breeding institutes in Hungary have adapted to the new market environment by establishing their own seed companies and by signing contracts with new market players. Their survival was greatly helped by the fact that Hungary has been a member of UPOV since 1983, which meant that variety rights were reliably protected even during the transitional period of the market oriented economy.

The structural changes were accompanied by a reduction in the use of certified seed. The state attempted to remedy this by providing discounts on seed purchases. For several years a certified seed level of around 50% was achieved by decreeing that these discounts were only available to farmers who sowed certified seed on at least 40% of their wheat fields. When Hungary joined the EU in 2004, however, all seed subsidies were discontinued, so the use of certified seed dropped to 25-30%. This new situation does not contribute to the yield and quality stability of wheat production.

Figure 1. Applied amounts of fertilizers in Hungary (years 1980-2010)

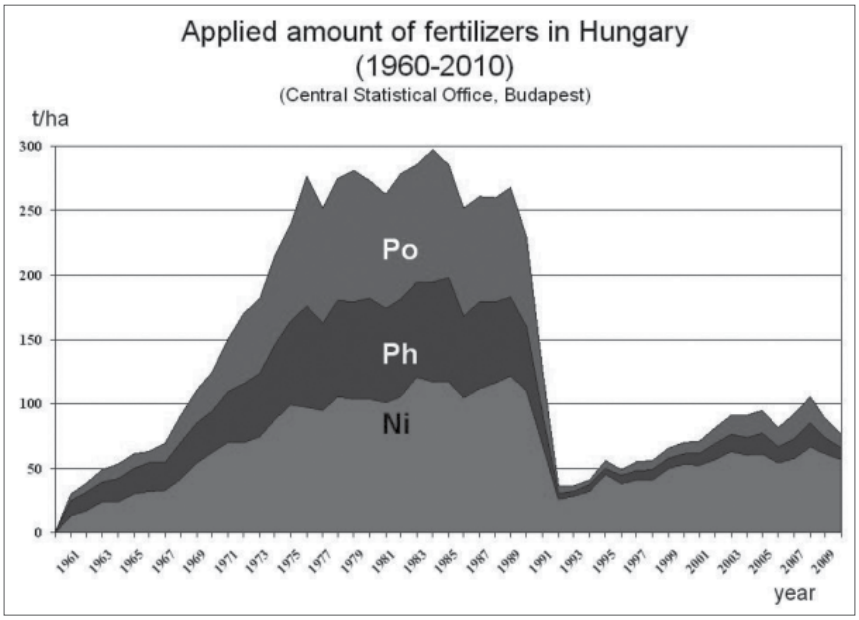


Table 1. Identification of Lr 34 tested in Martonvásár wheat genebank collection

Origin	Total number of genotypes	No. of genotypes with Lr34
Martonvásár	129	35 (27.1%)
Other	97	29 (29.9%)
Total	226	64 (28.3%)

Figure 2. Recurrent parent genome (RPG) recovery using marker-assisted backcrossing (MABC) and traditional backcrossing (Frisch *et al.*, 2000)

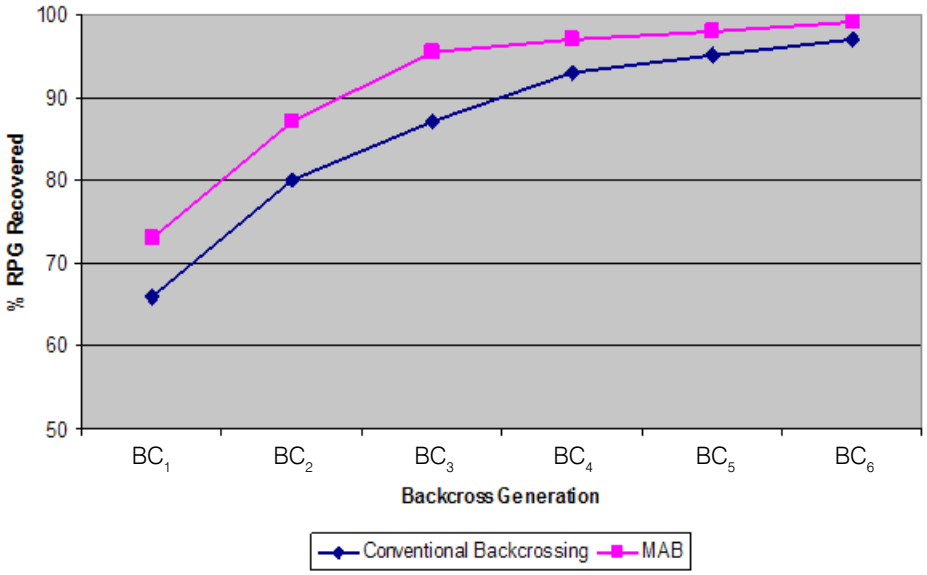
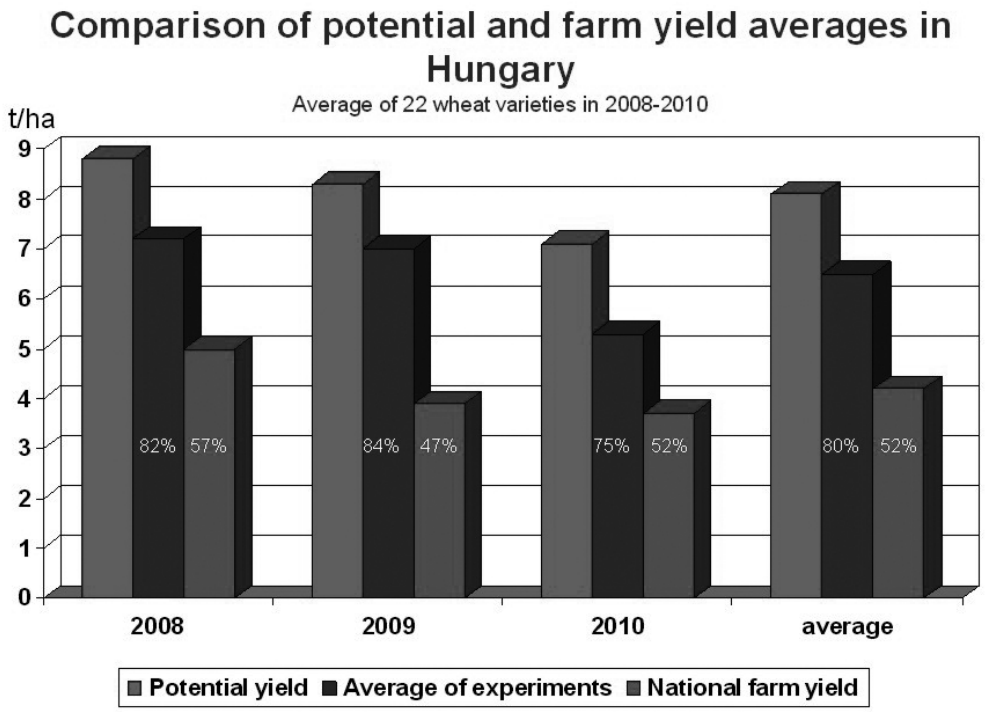


Figure 3. Comparison of potential and farm yield averages in Hungary



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Characterization of Some Agronomic Traits and β-Carotene Contents of Orange Fleshed Altinbas Melon Dihaploid Lines

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ABSTRACT

The objective of this presented study was to characterize orange fleshed Altinbas melon dihaploid lines that we developed in a breeding program. The study was conducted in greenhouse condition in Antalya, Turkey. The DH lines were developed by irradiated pollen technique and morphologically characterized according to modified UPOV descriptor list for 63 characters. Sixteen quantitative characters (3 seedling, 2 plant, 3 leaf, 8 fruit) were also measured. Cluster analysis was performed for both qualitative and quantitiative data. According the research results, orange fleshed melon pure lines showed different level of variation in case of morphological characterization as well as fruit traits and β-carotene contents.

Keywords: Melon, dihaploidization, β-carotene, UPOV.

Introduction

Melon (*Cucumis melo* L.; 2n =2x =24) is member of Cucurbitaceae family with high economic value cultivated extensively in tropical and subtropical regions in the world. It is thought to have originated in East Africa and the centre of diversification is in Asia from the Mediterranean Sea to Eastern Asia (Pitrat 2008).

Besides located in the secondary genetic diversity centre (Pitrat *et al*., 1999), Turkey is the second largest melon producer country after China with 1.7 million tons of production on 102000 ha area (Anonymous 2014). It has been reported that Turkey is rich in melon genetic resources and they are morphologically diverse especially for fruit characters (Sari and Solmaz 2007; Sensoy *et al*., 2007; Solmaz *et al*., 2010; Mancak *et al*., 2014). Melon cultivation is intensively carried out particularly in Central Anatolia; Aegean, Southeastern Anatolia and the Mediterranean regions (Yilmaz 2009). In Turkey, winter melons which belongs to *inodorus* group, are commonly produced in Aegean, Central Anatolia and the Mediterranean regions in open field conditions and it is reported that many genotypes grown localy (Solmaz *et al*., 2010). Among these, Altinbas is one of the the most important winter melons. These melons have a yellow rind with black points and stains. Their fruits vary in size (1.5-3 kg). They have white-green, odourless, sweet and firm fruits with long shelf life.

In recent years, melon breeding programs are focused on improving fruit internal quality traits such as high sugar concentration, beta-carotene concentration and nutritional value. Melon includes Vitamin C, A, B9 and potassium. These four components are very important for human health. β-carotene (provitamin A) is the major carotenoid in orange fleshed melons

and important antioxidant agent that strengthen the human immune system. In addition, it reduces the occurrence of cancer, prevents cardio vascular system disorders, cataracts and night blindness (Hodges and Lester 2011). Esteras *et al.,* (2015) reported that beta-carotene is the most abundant carotenoid and the orange color of the fruit flesh from most cantalupensis correlated with the highest levels of beta-carotene.

The aim of this study was to characterize morphologically 30 Altinbas (*Cucumis melo* var. *inodorus*) melon lines developed by dihaploidization technique according to the modified UPOV descriptor list for 63 features and determine their performance for some quantitative traits.

Material and Methods

Thirty orange fleshed Altinbas melon lines and one green fleshed commercial variety (Edali F_1, Verim Ziraat) were used in this study. These DH melon lines (1-3, 3-6-A, 3-21, 4-91, 4-94, 5-2-2, 5-51-2, 5-54, 5-84, 7-25, 7-40, 9-12, 9-13, 9-24, 9-25-2, 9-36-3, 9-38, 9-48, 9-49-3, 9-61, 9-66-2, 10-5-5, 10-45-3, 12-40, 13-38, 13- 58-2, 19-38, 19-45, L and 1) were developed by dihaploidization technique in a project supported by Ministry of Science, Industry and Technology (SANTEZ programme) and carried out by Antalya Tarim Inc. and Çukurova University during 2010 and 2012. The experiment was conducted in glass greenhouse at Experimental Station of Antalya Tarim Inc. β-carotene content of melons was analyzed in West Mediterranean Agricultural Research Institute (BATEM) of the Ministry of Food Agriculture and Livestock.

The seeds of thirty melon lines and control variety (Edali F1) were sown in plastic multipots in the Nursery of Antalya Tarim Inc. on 20 January 2012. Seedling characterizations and measurements of cotyledon length (CL, cm) cotyledon width (CW, cm) and hypocotyl length (HL, mm) were done 3 weeks after sowing in 15 seedlings at the first true leaf stage on 8th February 2012. These measurements were carried out with a ruler or a digital compass (Mitutoyo CD-15D). Fifteen seedlings of each line at 2-3 true leaf stage were transplanted at spacing of 25 cm within rows and 150 cm between rows on 27th February 2012 in glasshouse. Plants were grown hydroponically in perlite medium. Pruning, irrigation, fertilization and pesticide application were conducted regularly during vegetation period.

Number of nodes on the main stem were counted and leaf blade length (cm), leaf blade width (cm) and petiole length (cm) were measured with a ruler in 10 plants of each line on 25th April 2012. Morphological characterizations were done for 63 characters using a modified UPOV (The International Union for the Protection of New Varieties of Plants) descriptor list at cotyledon, flowering and mature fruit stages. Mature fruits were harvested on 10th, 11th, 13th and 15th June 2012 and four fruits from each line were analyzed. After taking the fruit weight (g); fruit length (cm), fruit diameter (cm), diameter of seed cavity (cm), length of seed cavity (cm), thickness of flesh (cm) were measured with ruler, total soluble solids (%) were analyzed using a hand held refractometer (ATAGO). The β-carotene content of melons (ppm) were determined by spectrophotometric method according to Sasuni and Adebiyi (2009).

For analyzing qualitative and quantitative data, cluster analysis was performed by the unweighted pairgroup method using arithmetic average (UPGMA) method with NTSYS-PC Prograram (Rohlf, 1998).

Results

Thirty orange fleshed melon lines and 1 green fleshed commercial variety (Edali F1) were characterized for morphological characteristics using 63 traits according to UPOV descriptor list. The distribution of the genotypes according to observed characters were given in %.

According to the observations in seedlings, hypocotyl length was observed as medium in 30 lines (96.8%) and long in 1 line (3.2%). Great diversity was found among the accessions regarding cotyledon size, 1 (3.2%) line had very small, 3 (9.7%) lines had small, 21 (67.7%) lines had medium, 4 (12.9%) lines had large and 2 (6.5%) lines had very large cotyledons. The green color of the cotyledons were all (100%) dark.

The number of nodes on the main stem was medium in 23 (74.2%) lines and high in 8 (25.8%) lines. The length of internodes and leaf blade size were observed as medium in all (100%) lines. Green color of leaf blade was dark in most (93.5%) of the lines and medium in the rest (6.5%). Development of the leaf blade lobes was weak in 12 (38.7%) lines, medium in 14 (45.2%) lines and strong in 5 (16.1%) lines. High level of diversity was observed regarding length of the terminal lobes, dentation of margin, undulation of margin and blistering of leaf blade. Attitude of the petiole was semi-errect in 26 (83.9%) lines and horizontal in 5 (16.1%) lines. Regarding petiole length, all (100%) lines had been observed as medium. Considering the sex expression all (100%) lines were found to be andromonoecious.

The ground color of the fruit before maturity was mainly green (90.3%) while grey-green (6.5%) and

white (3.2%) colors were also observed. Intensity of ground color of fruit before maturity was light in 21 (67.7%) lines, medium in 7 (22.6%) lines and dark in 3 (9.7%) lines.

Fruit length and fruit diameter were also quite variable among the lines. Fruit length of the DH lines was observed as short in 4 (12.9%) lines, medium in 21 (67.7%) lines, long in 4 (12.9%) lines and very long in 2 (6.5%) lines. The medium fruit diameter was dominant reaching 71% of all lines, followed by the wide (16.1%) and narrow (12.9%).

Fruit shape showed high diversity among the lines. It was found round in 45.2%, ovate in 9.7%, like-egg in 9.7%, elliptical in 12.9% and flattened-ovate in 22.6% of the lines respectively. Ground color of fruit skin at maturity was white in 3.2% , yellow in 61.3%, yellow-green in 12.9% and green in 22.6% of the lines. Intensity of ground color of skin at maturity was light in 41.9% medium in 32.3% and dark in 25.8% of the lines. Secondary color of skin was present in 21 (67.7%) and absent in 10 (32.3%) lines. The distribution of secondary color of skin was dominantly (95.2%) in dots and in patches. The intensity of the dots and patches were quite variable among the lines. The fruit peduncle length was observed as medium in 30 (96.8%) and short in 1 (3.2%) line. The thickness of fruit peduncle was medium for all (100%) of the lines. Abscission of peduncle was absent in 24 (77.4%) and present in 7 (22.6%) lines. In terms of shape of base and shape of apex of fruits considerable variation was observed. Size of pistil scar was observed as small in 2 (6.5%) lines, medium in 20 (64.5%) lines and large in 9 (29%) lines. Grooves of fruit were absent in 23 and present in 8 of the investigated lines. The width of fruit grooves was narrow in 3 (37.5%) and medium in 5 (62.5%) lines. The depth of the grooves was observed as shallow in 3 (37.5%) and medium in 5 (62.5%) lines. Color of the grooves was green for all (100%) lines. Creasing of surface varied from absent or very week to strong among the lines. Cork formation was present in 8 (25.8%) and absent in 23 (74.2%) of the lines. Thickness of the cork layer, pattern and density of cork formation, showed different level of diversity. Maximum width of outer layer of fruit flesh was thin in 2 (6.5%), medium in 21 (67.7%) and thick in 8 (25.8%) lines. In melon breeding programmes, maximum width of flesh thickness is a desired trait. It was observed to be medium in most of (83.9%) the lines. Regarding main color of flesh orange color was dominant (87.1%), while 3 (9.7%) lines had green and the control variety (Edali F1) had cream flesh. Intensity of main color of flesh was light in 5 (16.1%) lines, medium in 15 (48.4%) lines and dark in 11 (35.5%) lines. In terms of fruit flesh texture in all genotypes (100%) were assessed as grainy. Fruit cavity was full in 17 (54.8%) and medium in 14 (45.2%) lines. Placenta color was observed as highly variable among the lines. Two lines (6.5%) had transparent, 1 (3.2%) line had white, 8 (25.8%) lines had salmon and 20 (64.5%) lines had orange placenta. Considering the number of carpels 10 (32.3%) lines had 3, 20 (64.5%) lines had 4 and 1 (3.2%) line had 5 carpels. Fruit taste was sweet for all lines (100%) and most lines (71%) contained external aroma.

Seed size was medium in 28 (90.3%) lines and large in 3 (9.7%) lines. The shape of hilum end was sharply pointed in all lines. Shape of cross-section of the seed was elliptical in most (96.8%) lines. The seed color was creamy yellow in all lines and the number of seeds were found medium in 21 (71%) and high in 9 (29%) lines.

Means, minimum and maximum values of the seedling, plant, leaf and fruit measurements of 30 DH lines and 1 control variety (Edali F1) are presented in Table 1. Considering hypocotyl length, maximum value (40.64 mm) obtained from Edali F1 while minimum value (17.54 mm) measured in line 3-21. Longest (2.38 cm) cotyledons were determined in line 13-38 and shortest (1.87 cm) in line 9-61. Cotyledon width ranged between 1.32 cm (9-61) and 2.38 cm (Edali F1). Number of internodes on main stem varied between 26 (5-84) and 33 (9-48). Maximum length of internodes on main stem was found 11.42 cm in line 12-40 and the minimum length (7.71 cm) was measured in line 19-45. The longest (16.79 cm) leaf blade was obtained from line 12-40 and the shortest (9.92 cm) from line 9-61. Both for the maximum leaf blade width (19.75 cm) and petiole length (16.75 cm) line 4-94 had the highest values. Control variety Edali F1 was superior than all other lines in terms of fruit weight (3840.42 g), fruit height (22.23 cm), fruit diameter (18.07 cm), length of seed cavity (15.40 cm) and fruit flesh thickness (6.19 cm). Total soluble solid content (SSC) ranged between 6.71% (1-3) and 11.67% (9-49-3). The highest value for the β-carotene content was 38.20 ppm and obtained from 10-45-3, and lowest was 0.47 ppm in green-fleshed Edali F1.

The dendrogram generated by cluster analysis of 63 qualitative characters is presented in Figure 1.

The DH lines were divided into four main clusters. The first cluster (I) included nine lines (7-25, 9-61, 10-45-3, 10-5-5, 7-40, 9-66-2, 9-38, 9-25-2 and 4-91). While line 7-25 was the most distant, line

10-45-3 and 10-5-5 was the most similar lines within this cluster. The second main cluster (II) consisted of 13 lines and Edali F1. Within this cluster green-fleshed Edali F1 formed one individual sub-cluster. Lines 13-38 and 13-58-2 grouped together and showed close relations than any sub-group in the main cluster. The third cluster (III) composed of 7 lines (3-6-A, 9-49-3, 9-13, 9-48, 9-12, 9-24, 9-36-3, 5-84). The fourth cluster (IV) contained only line 1-3 which was the most distant line from other lines.

Based on quantitative data the clustering dendrogram was created and presented in figure 2. The dendrogram was divided into 5 main clusters. While the first cluster (I) consisted of two lines (green-fleshed Edali F1 and line A), the second cluster contained 5 lines (L, 13-38, 19-38, 7-25, 5-84). The third cluster (III) consisted of only 19-45 line. The fourth cluster (IV) included 14 lines, and two main sub-clusters were identified within this cluster. While the first sub-cluster contained lines 9-13, 4-91 and 3-6-A, all other lines in cluster IV were grouped in the second sub-cluster. The fifth cluster (V) was composed of 10 lines and divided into two sub-clusters. Line 9-49-3 was separeted from the other lines and formed the first sub-cluster. The second sub-cluster were divided into new sub-clusters which showed close relations.

Discussion

Thirty Altinbaş DH lines were characterized for morphological traits and high level of diversity was found except green color of the cotyledons, the length of internodes, leaf blade size, petiole length, sex expression, the thickness of fruit peduncle, color of the grooves, fruit texture, fruit taste, shape of hilum end and seed color. Sixteen quantitative characters (3 seedling, 2 plant, 3 leaf, 8 fruit) were also measured and remarkable variation was found among the lines. Our results are compatible with previous studies regarding morphological characterization of melon (Solmaz *et al*., 2004; Sari and Solmaz 2007; Sensoy *et al*., 2007; Lotti *et al*., 2008; Escribano and Lazáro 2009). In a study reported by Szamosi *et al*., (2010), 58 *Cucumis melo* accessions were studied to compare the morphological characteristics of Hungarian and Turkish germplasm. Their results indicated that both Hungarian and Turkish germplasm resources present wide range of diversity for morphological traits for all the traits tested except intensity of green color of cotyledon and color of petals.

Solmaz *et al*., (2010) collected 78 melon accessions from Eastern and Central Anatolia regions of Turkey and characterized them for morphological traits according to the UPOV melon descriptor list. They also measured twenty quantitative characters (3 seedling, 3 plant, 3 leaf, 2 flower, 9 fruit). Results revealed that the Turkish melon accessions have quite diversity for all the traits examined except intensity of green color of cotyledon, attidude of petiole and color of petals. In our study we have obtained more common characters, it was expected because we had characterized DH lines (pure lines) of which all belong to Altinbas (*Cucumis melo* var. *inodorus*) group of melons. The study reported by Killi (2010) also confirmed our results. In this research morphological characterization of 27 Kirkagac and Yuva melon pure melon lines developed by dihaploidization technique were performed for 68 features according to the modified UPOV descriptor. According to the research findings, melon lines showed different level of variation except attidude of petiole, sex expresssion, color of petal, ovary pubescence, intensity of ground color before maturity, fruit diameter, abscission of peduncle, ease of abscission of peduncle, grooves, cork formation, fruit flesh color, fruit taste, color of flesh of outer layer, shape at hilum end and seed shape accessions. It is also stated by Mancak *et al*., (2014), that Kirkagac accessions shared more common characteristics (8 characters) with one another than they did with other accessions.

It can be concluded that different level of variation was obtained among DH Altinbas melon lines in terms of morphological characters. This finding was also supported by the cluster analysis and measurements of quantitative caharacters.

Finally this valuable germplasm must be conserved and used for the future breeding studies of orange fleshed Altinbas melon cultivars.

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Table 1. Means, maximum and minimum values of melon lines obtained by 16 quantitative measurements.

Parameters	Mean	Maximum Value	Line	Minimum value	Line
Hypocotyl length (mm)	26.31	40.64	Edali F1	17.54	1-3
Cotyledon length (cm)	3.11	4.13	13-38	1.87	9-61
Cotyledon width (cm)	1.86	2.38	Edali F1	1.32	9-61
Number of internodes on main stem	28.84	33	9-48	26	5-84
Length of internodes (cm)	9.16	11.42	12-40	7.71	19-45
Length of leaf (cm)	13.09	16.79	12-40	9.92	9-61
Width of leaf (cm)	16.84	19.75	4-94	14.35	9-61
Length of petiole (cm)	13.39	16.75	4-94	10.08	9-24
Fruit weight (g)	1168.36	3840.42	Edali F1	354.17	19-45
Length of fruit (cm)	13.38	22.23	Edali F1	9.52	3-6-A
Diameter of fruit (cm)	12.51	18.07	Edali F1	9	19-45
Length of seed cavity (cm)	8.32	15.40	Edali F1	5.67	3-6-A
Diameter of seed cavity (cm)	6.15	8.36	5-84	4.29	19-45
Thickness of flesh (cm)	3.23	6.19	Edali F1	2.48	3-6-A
Total soluble solids (%)	8.92	11.67	9-49-3	6.71	1-3
β-carotene (ppm)	18.65	38.20	10-45-3	0.47	Edali F1

Figure 1. Dendrogram of melon lines obtained from cluster analysis of 63 qualitative agromorphological traits

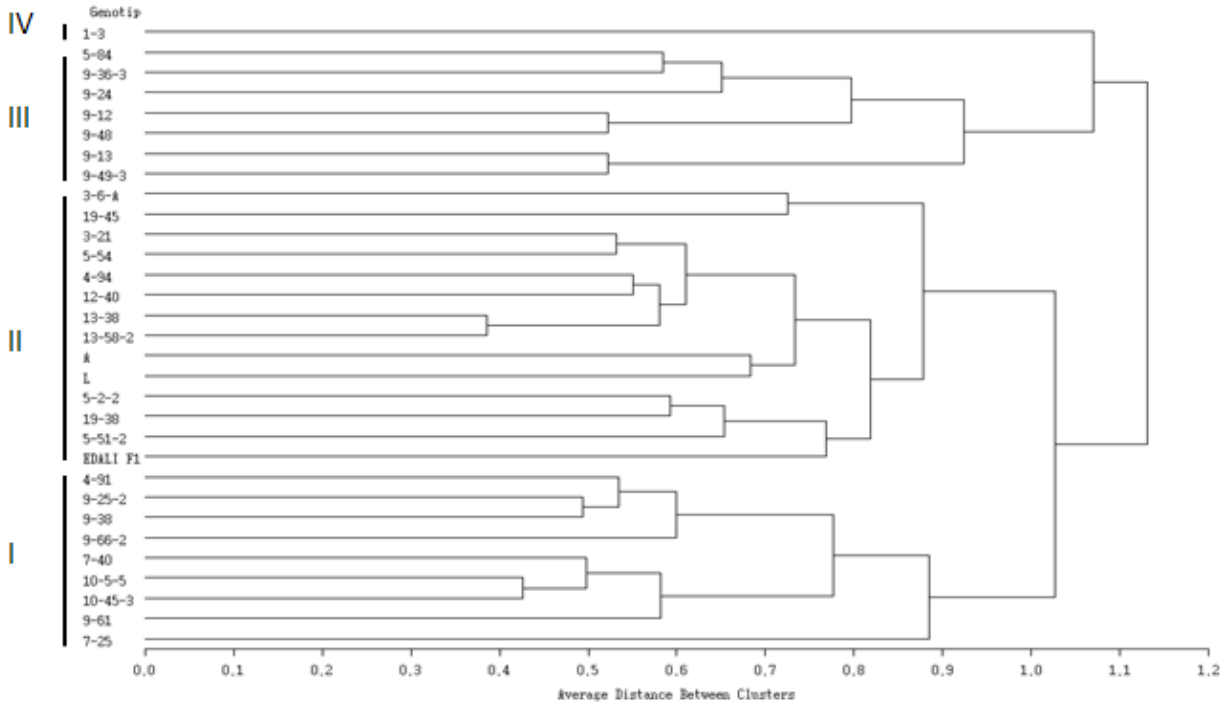
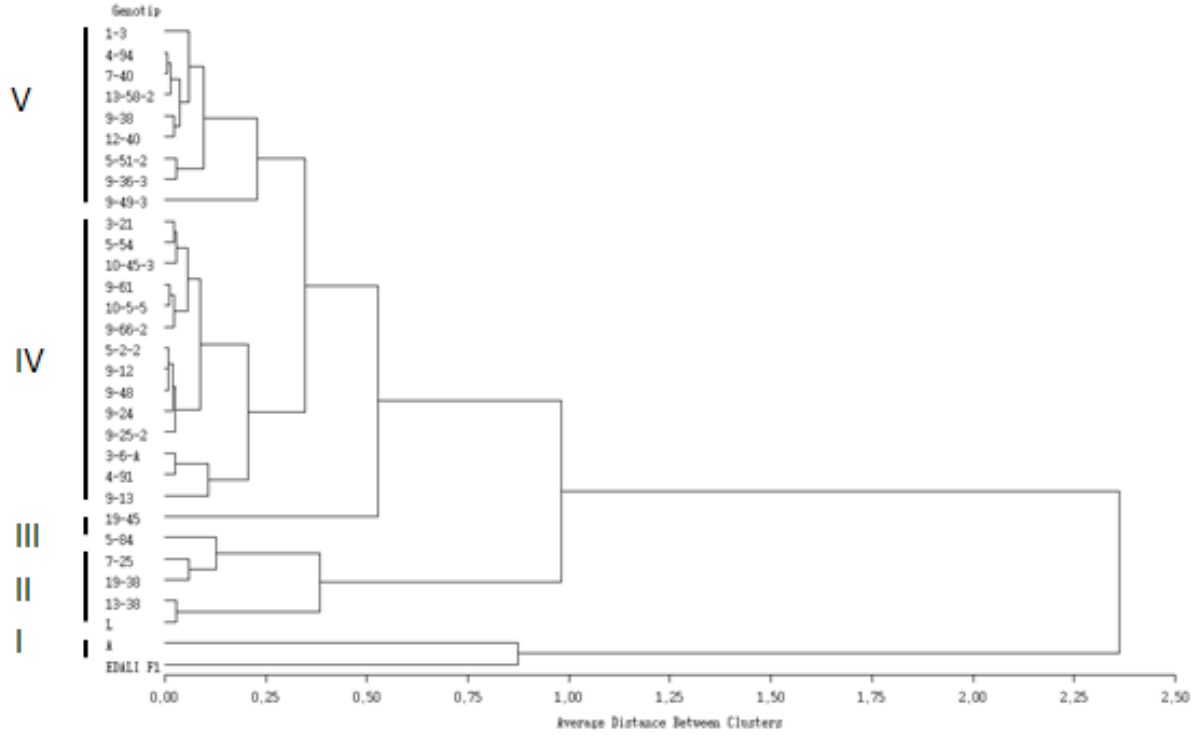


Figure 2. Dendrogram of melon lines obtained from cluster analysis of 16 quantative agromorphological traits



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Determination of Effect of Chemical Mutagen EMS on TAEK A-3 and TAEK C-10 Mutant Soybean Varieties in M_1 Generation

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ABSTRACT

Today beside ionizing radiation, chemical mutagens are also used in crop improvement and Ethylmethanesulphonate (EMS) is one of the most widely used chemical mutagens in the field of plant breeding. This research work was carried out to determine the effect of chemical mutagen (EMS) on seedling height. TAEK A-3 and TAEK C-10 soybean varieties were used as the test plants and effect of presoaking time determined. In greenhouse and field experiments five different EMS doses (0 (Control), 0,025, 0,050, 0,075 ve 1,0 M) and in greenhouse 3 different presoaking time (0, 6 and 18 hours), in the field 2 different presoaking time (0 and 6hours) were applied. After chemical mutagen application in the green house germination percentage and seedling height base EMS dose and presoaking time effect had been determined. Effects of EMS in M_1 germination of soybean for different characterics (no. of plants, plant height, no of pods and seedyield) were determined under field condition. As a result it can be said that in both soybean varieties, seedling height was reduced as the EMS concentration increased. But there was no distinct influence of presoaking time on the seedling height in both soybean varieties. In both soybean varieties the average percentage of germination increased with increasing pre-soaking time. Based on results obtained in field experiment, it was concluded that by 6 hours presoaking the harmful physiolojical effect of EMS could be decreased.

Keywords: Soybean, Induced mutation, mutation breeding, chemical mutagen, EMS.

Introduction

In TAEK, SANAEM, Nuclear Agriculture Division mutation breeding activities have been carried out on wheat, barley, soybean, tobacco, lentil, chickpea, potato, rapeseed, and sunflower between 1982-2014.

Today beside ionizing radiation, chemical mutagens are also used in crop improvement and EMS is one of the most widely used chemical mutagen in the field of plant mutation breeding (Anonymous 1977, Sağel, Z. 1994). As a source of physical mutagen, gamma irradiation source is not always possible to reach easily. When the security and applications are fulfilled, chemical mutagens are easily accessible and appliable. Chemical mutagens are a indispensable tool to create variation in plant breeding (Maluszynski, M, 2000) as chemical mutagen to use base analogs is preferable for plant breeders to induce point mutations and have great capacity. A very high mutation rate invariably causes high sterility so plant breeders aim at the more mutagenic effect and the less physiological damage (Konzak, *et al.,* 1965). Therefore this research Project was planned and conducted.

Material and Methods

This research work was carried out to determine the effect of chemical mutagen (EMS) on seedling height TAEK A-3 ve TAEK C-10 mutant soybean varieties were used as the test plants. Germination percentes of seed of test genotypes was 99% and 50 seed were used for each treatment. For EMS treatments

O (control), 0.025, 0.050, 0.075 ve 0.100 M EMS concentrations were used.After the seeds were pre-soaked for 0, 6 and 18 hours they were treated with EMS for 3 hours at room temperature. Then they were post washed with running tap water for 6 hours (Sağel,1994, Savin, *et al.,* 1968).

In the greenhouse the seeds were planted in 45x30x30 cm wooden cases according to split plot desing with 3 replications .They were placed to grow in optimum conditions. After 12 days, germination percentage was determined. After 14 days of growth seedlings were uprooted and seedling height was determined on the first leaf (Constantin, *et al.,* 1976, Peşkircioğlu, 1995).

For field experiment as the test plants TAEK A-3 ve TAEK C-10 mutant soybean varieties were used. First the seeds of these plants were presoaked for 0 and 6 hours then they were treated with O (control), 0.025, 0.050, 0.075 ve 0.100 M EMS for 3 hours. After these treatments seeds were postwashed for 6 hours with running tap water (Doll and Sandfaer,1969).

In field experiment for each treatment 300 seeds were used. Totally 6000 seeds including control and treated with EMS (1200 seeds for were kept as control and 4800 seeds were treated with EMS) were used. All the seeds were planted in the field in rows spaced 50 cm apart with 10 cm distance between plants.

Results and Discussion

In Tables 1 and 2 effect of pre soaking time and various EMS concentration on percentage of germination and on seedling height are presented , respectively.

As it is seen from Table 1, presoaking time and mutagen doses on the germination percentage were determined to be significant at 1% level. However, as shown in Table 1 and Figure1, the average percentage of germination increased with increasing pre-soaking time.

The Germination percentage did not decrease in the TAEK A-3 variety compared to the control, when pre-soaking time was “0”hours in 0,100 M EMS dose, when pre-soaking time is “6” hours in all EMS doses, when pre-soaking time “18” hours in all EMS doses except 0,050 M EMS doses. Likewise, in TAEK C-10 soybean variety depending on EMS doses, on different pre-soaking time there was non significant effect on germination percentage (Anonymous, 1977, Sağel, 1994).

According to the Table 2 values of presoaking time, seedling height and mutagen dose were determined to be statistically significant at the 1% level between cultivars. When pre-soaking time was “0” and “6” hours there was no significant difference in seedling height but it was determined seedling height at “18” hours of pre-soaking increased.

As shown in Figure 2, statistically significant differences at 1% level were apparent in both soybean varieties for seedling height, parallel to increase with increasing EMS concentration, as compared to control .

According to the values in Table 3 and Figure3, results of the field experiment, as compared with control for TAEK A-3 number of plants did noot change when presoaking was “0” hours and at 0.025, 0.050 and 0.075 M EMS concentration.Whereas at 1.00 M EMS treatment the number of plant was reduced. On the other hand for TAEK A-3 as compared with control, plant height, number of pods and seed yield were also reduced at 0.025, 0.050 and 0.075 M EMS.

As compared with control for TAEK C-10 soybean variety when presoaking time was “0” hour , number of plant was also reduced with 0.075 and 0.100 M EMS treatments, whereas plant height was not affected and number of pods were reduced only at 0.100 M EMS concentration. On the other hand seed yield was decreased as the concetration of EMS increased (Constantin, *et al.*, 1976)

Although number of survived plants was less than TAEK C 10 for TAEK A-3. Because of the increase in branching and increased area per plant the seed yield was more in TAEK A3 soybean variety.

When presoaking time was 6 hours, as compared with control for TAEK- A3, number of plants was decreased at 0.050 and 0.100 M EMS concentrations. Whereas plant height, No. of pods and seed yield were decreased from 0.050 M EMS concenration.

For TAEK C-10 soybean variety as compared with control when presoaking time was “6” hour, No. of plant did not change , but plant height and No. of pods were decreased at 0.100 M EMS. Seed yield was also decreased at 0.075 M and 0.100 M EMS treatment.

When presoaking time was “0” hours, for TAEK-A3 soybean variety, effect on seed yield, No. of pods and plant height physiological effect was not much , whereas when presoaking time was 6 hours, as compared with control for TAEK- A3, these traits were decreased at 0.050 M EMS concentrations.

When presoaking time was “0” hours, as compared with control for TAEK- C10, yield was decreased. from EMS concentrations.

When presoaking time was 6 hours, as compared with control for TAEK- C-10,yield was decreased from 0.075 M EMS concentrations

Chemical mutagens, especially EMS have high mutagenic effect on seeds but main criteria is to find maximum physiological damage.

The result of the greenhouse experiment revealed that in TAEK A-3 and TAEK C-10 soybean varieties seedling height decreased with increasing mutagen dose, but preasoaking time did not have any significant effect. In both soybean varieties the average percentage of germination increased with increasing pre-soaking time (Zakri, *et al.*, 1982)

As evident from results of the field experiment, with 6 hours presoaking, physiological damage decreases in M_1 generation after EMS treatment.

Table 1. Influence of presoaking time and EMS concentration on germination percentages of seeds in TAEK A-3 ve TAEK C-10 mutant soybean varieties

EMS Doses (M)	Varieties	Germination percentages of seeds		
		Presoaking time		
		"0" hours	"6" hours	"18" hours
0 (Control)	TAEK A-3	37,0 (74,0)	43,0 (86,0)	38,3 (76,6)
	TAEK C-10	39,3 (78,6)	39,3 (78,6)	42,7 (85,4)
0,025	TAEK A-3	49,3 (98,6)	34,7 (69,4)	46,0 (92,0)
	TAEK C-10	36,0 (72,0)	49,7 (99,4)	44,0 (88,0)
0,050	TAEK A-3	40,0 (80,0)	31,3 (62,6)	33,7 (67,4)
	TAEK C-10	46,3 (92,6)	39,3 (78,6)	46,3 (92,6)
0,075	TAEK A-3	43,3 (86,6)	41,3 (82.6)	41,3 (82,6)
	TAEK C-10	31,3 (62,6)	48,3 (96,6)	45,0 (90,0)
0,100	TAEK A-3	35,3 (70,6)	41,3 (82,6)	49,0 (98,0)
	TAEK C-10	49,7 (99,4)	48,3 (96,6)	43,7 (87,4)
		40,8 (81,6) C	41,7 (83,4) B	42,9 (85,8)A

Figure 1. Influence of presoaking time on germination percentages of seeds in TAEK A-3 ve TAEK C-10 mutant soybean varieties

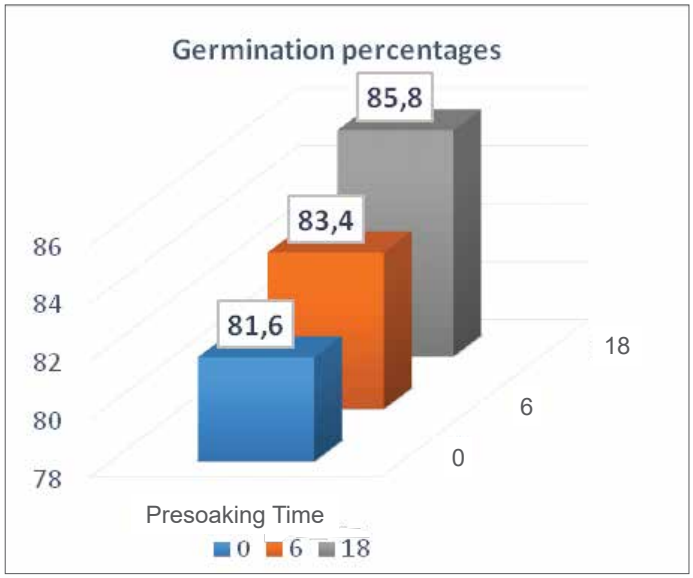


Table 2. Influence of presoaking time and EMS concentration on seedling height of seeds in TAEK A-3 and TAEK C-10 mutant soybean varieties

EMS Doses (M)	Varieties	Seedling height (cm)						EMS Doses (M)	
		Presoaking time (hours)							
		"0" hours		"6" hours		"18" hours			
0 (Control)	TAEK A-3	9,35	(100,0)	9,75	(100,0)	9,83	(100,0)	10,03 A	(100,0)
	TAEK C-10	10,25	(100,0)	10,48	(100,0)	10,52	(100,0)		
0,025	TAEK A-3	8,55	(91,5)	8,77	(89,9)	10,45	(106,3)	9,58 B	(95,5)
	TAEK C-10	10,22	(99,7)	10,41	(99,3)	9,01	(85,6)		
0,050	TAEK A-3	8,30	(88,8)	8,04	(82,4)	9,04	(92,0)	8,79 C	(87,6)
	TAEK C-10	9,65	(94,1)	9,30	(88,7)	8,41	(79,9)		
0,075	TAEK A-3	7,85	(83,9)	7,73	(79,3)	9,79	(99,6)	8,67 D	(86,4)
	TAEK C-10	8,98	(87,6)	9,28	(88,5)	8,37	(79,6)		
0,100	TAEK A-3	7,53	(80,5)	6,29	(64,5)	8,43	(85,7)	7,89 E	(78,7)
	TAEK C-10	8,57	(83,6)	8,80	(84,0)	7,73	(73,5)		
		8,93 B	(97,3)	8,88 B	(96,8)	9,17 A	(100,0)		

Figure 2. Influence of presoaking time on germination percentages of seeds in TAEK A-3 and TAEK C-10 mutant soybean varieties

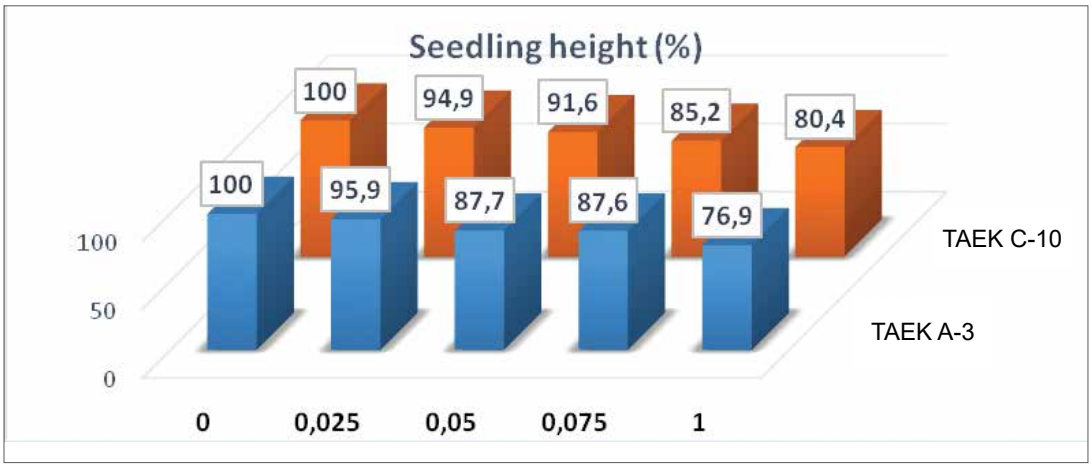
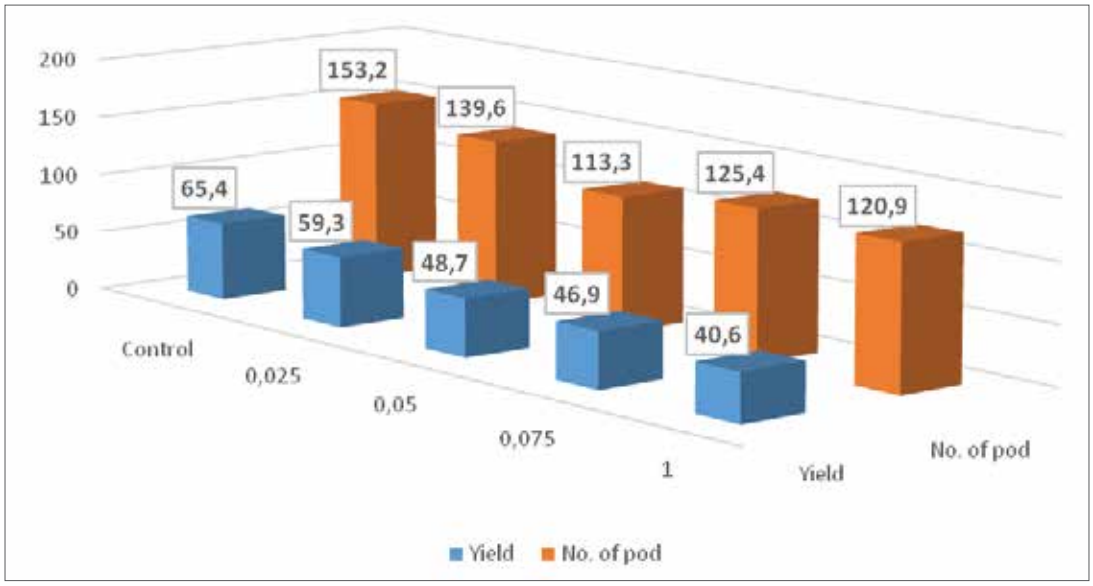


Table 3. Effect of presoaking time and chemical mutagen (EMS) on average No. of plants, plant height, No. of pods and seed yield.

EMS Doses (M)	Varieties	No. of plant			Plant height (cm)		No. of pod (pod/plant)			Yield (g/plant)		
		Presoaking time (hours)										
		0	6	Ort	0	6	0	6	Ort	0	6	
Control 1	TAEK A-3	22	17	42,8 C	66,23	62,12	212,55	220,21	153,2 A	93,79	86,87	65,4 A
	TAEK C-10	78	54		67,09	62,96	88,80	91,16		41,56	39,22	
0,025	TAEK A-3	31	24	56,3 A	61,52	63,63	144,68	230,55	139,6 B	63,45	95,18	59,3 B
	TAEK C-10	92	78		66,42	64,90	91,46	91,72		40,94	37,60	
0,050	TAEK A-3	36	13	48,0 B	55,64	60,38	97,97	150,29	113,3 E	43,99	61,22	48,7 C
	TAEK C-10	78	65		66,28	67,45	103,60	101,49		40,36	42,18	
0,075	TAEK A-3	26	28	48,8 B	58,46	55,14	153,96	158,92	125,4 C	66,62	57,81	46,9 D
	TAEK C-10	77	64		67,01	65,59	93,04	95,55		38,02	32,26	
1,00	TAEK A-3	10	12	35,0 D	66,20	53,00	228,10	101,00	120,9 D	77,56	34,82	40,6 E
	TAEK C-10	59	59		67,53	60,44	85,85	68,53		30,89	19,08	
		50,9 A	41,4 B							53,7 A	50,6 B	
Varieties	TAEK A-3		21,8 B					169,8 A			68,1 A	
	TAEK C-10		70,4 A					91,1 B			36,2 B	

Figure 3. Effect of chemical mutagen (EMS) on average No. of pods and seed yield.



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Research Article

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Genetic Variation in Drought Linked Morpho-physiological Characters and Microsatellite DNA Loci in Rice (*Oryza sativa* L.)

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ABSTRACT

Evaluation for drought tolerance and SSR (microsatellite) markers based molecular polymorphism were investigated in F_6 plant population raised via single seed descent method from a cross between drought tolerant *japonica* rice variety Azucena and drought sensitive premium traditional Basmati rice variety Taraori Basmati HBC 19. A total of 50 F_6 plants were evaluated individually for drought tolerance on 1-9 scale on the basis of agronomic characteristics, root and shoot traits, relative water content and visual observations; the average score ranged between 1to 8.3. Fourteen plants each in the category of drought tolerant and drought sensitive were selected from F_6 population for SSR marker analysis using 30 SSR markers covering all the chromosomes. The 28 Azucena × HBC19 F_6 plants had an allele from either of the two parental lines (homozygous condition) or alleles from both the parental rice varieties (heterozygous condition). Frequency of HBC19 specific alleles was higher in comparison to Azucena in selected drought tolerant and drought sensitive Azucena x HBC19 F_6 plants, which may be indicative of segregation distortion. At ten SSR loci new/recombinant alleles were obtained which indicate the active recombination between genomes of two rice varieties. Cluster tree analysis and principal component analysis demonstrate high level of diversity between Azucena and HBC19 with the clustering of 28 Azucena × HBC19 F_6 plants with HBC19.

Keywords: Genetic diversity; drought stress; microsatellite; *Oryza sativa*; root traits; recombinant inbred lines

Introduction

Rice (*Oryza sativa* L.) is a staple food for almost half of the world's population and it is grown in tropical, subtropical and temperate regions of the world. More than 90% of the world's rice is grown and consumed in Asia, where rice is cultivated on 135 million ha with an annual production of 516 million tonnes. In India, area under low land rice is about 14.4 million hactares which accounts to 32.4 percent of the total rice crop area in the country. Yields of rainfed lowland rice are drastically reduced by drought due to unpredictable, insufficient and uneven rainfall during the growing period. Further, upland rice which accounts for 13% of the total area is always prone to drought during a part of the growing season. In developing countries like India, rainfall is the main source of water available to crops and irrigation facilities are often lacking, so the problem of water stress is more acute in these countries. Thus, emphasis has been given to alleviate this problem in recent years.

Under drought conditions, the performance of crops may be improved by number of morphological, physiological and phenological characters (Hemamalini *et al.,* 2000). Several scientists have suggested adaptive mechanisms of plants in response to water stress (Fukai and Cooper, 1995; Nguyen *et al.,* 1997). Root system is one

of the most significant components of drought tolerance. Nguyen *et al.,* (1997) reported that traits such as root thickness, depth of rooting, and deep root to shoot ratio have been found to be associated with this mechanism. Maximum root depth and dry weight of roots below 30 cm were good indicators of drought resistance in rice (Ahmadi, 1983). Desirable root characteristics could be useful in selecting rice genotypes for drought resistance breeding. However, phenotypic selection for most root traits is challenging and labor intensive. Molecular marker technology is a powerful tool to overcome these drawbacks. It has been successfully utilized for molecular dissection of complex agronomical traits, marker assisted breeding and in linkage mapping (for review see Flowers *et al.,* 2000). Molecular marker technology can significantly enhance the efficiency and accuracy of breeding process. A number of genes have been mapped which include genes/QTLs for several agronomically important traits such as yield, quality and resistance against abiotic stresses including salinity and water stress (Forster *et al.,* 2000; Zhang *et al.,* 1999). Among abiotic stresses maximum progress has been made towards the salinity tolerance and there have been only a few studies to map QTLs for drought tolerance (Babu *et al.,* 2003). Several types of marker such as RFLP, RAPD and AFLP, microsatellites (SSRs) have been used for drought tolerance in rice (Hemamalini *et al.,* 2000). However, PCR based markers such as AFLPs and microsatellites have revealed a great potential in the analysis of genetic diversity, gene tagging and genome mapping studies because they are very informative, technically simple, require less time, and need small amounts of DNA. Microsatellites are tandemly repeated short sequences of DNA with repeat unit of less than 6 bp in length. They exhibit high level of polymorphisms and have been successfully applied in the study of genetic diversity in wheat (Plaschke *et al.,* 1995), barley (Saghai-Maroof *et al.,* 1984) and rice (Xiao *et al.,* 1996). Rice grain yield under drought conditions may be improved with the help of marker-assisted breeding approaches due to the availability of genome wide molecular markers, inexpensive genotyping platforms and sequence information of rice genome.

In this paper, we report the genetic evaluation and microsatellite marker analysis of F_6 advance population derived from a cross between a drought tolerant *japonica* rice variety (Azucena) and Taraori Basmati and its application in linkage mapping for drought tolerance and Basmati rice breeding.

Material and Methods

Plant Materials

A population of 211 Azucena x HBC19 F_6 plants was raised through single seed descent method of which 50 were used for drought tolerance analysis. Azucena is a drought tolerant *japonica* rice variety and HBC19 (Taraori Basmati) is a commercially important traditional Basmati variety, which is quite sensitive to drought.

Evaluation for drought tolerance

The dehusked F_5 plant seeds along with parental genotypes were germinated in large size pots in the green house of the CCS Haryana Agricultural University, Hisar. Two sets of 50 Azucena x HBC19 F_6 lines were taken for recording observations. Each set contained four plants per line. Water stress was given to one set of plants by with-holding water at 60 days after sowing while the other set comprised plants under control conditions and these plants were regularly irrigated. Observations consisted of plant height (PH) in cm, tiller number (TN), grain yield (GY) in g/plant, thousand grain weight (TGW) in g, maximum root length (MRL) in cm, shoot and root fresh weight (SFW, RFW) in g, shoot and root dry weight (SDW, RDW) in g, root:-shoot ratio (RSR), harvest index (HI), relative water content (RWC), leaf drying(LD) and recovery of water stressed plants (RWSP). RWC of youngest expanded leaf was calculated as suggested by Weatherly (1950). Drought tolerant index (DTI) was then calculated for agronomic characteristics (PH, TN, GY, TGW), shoot and root trait (SFW, RFW, SDW, RDW, MRL, RSR), HI and RWC (Ribaut *et al.,* 1997) and on the basis of DTI all the F_6 plants were individually grouped under 1,3,5,7 and 9 score categories for drought tolerance. Further, grouping of these F_6 plants was done on the basis of visual symptoms of leaf drying and recovery on a 1-9 scale as per IRRI's standard evaluation system, where lower score stated for tolerant and higher scale for sensitive (Gregorio *et al.,* 1997). Average scores were calculated for each of the F_6 plants and data was used for the selection of drought tolerant and drought sensitive surviving plants.

DNA isolation and microsatellite DNA loci amplification

Genomic DNA was extracted from leaf samples using modified CTAB method (Saghai-Maroof *et al.,* 1984) from parents and fourteen F_6 plants each selected for the both extremes i.e. most drought sensitive and most drought tolerant plants. Thirty microsatellite primer pairs (Table 1, Research Genetics, Inc.) were used to amplify microsatellite DNA loci using genomic DNAs as templates. PCR reaction was conducted in a volume of 20 µl containing 50 ng template DNA, 1X Taq DNA polymerase buffer, 100 µM of each of four dNTPS, 0.4 µM each primer, 1.2 mM $MgCl_2$ and 1 unit Taq DNA polymerase (Perkin Elmer). The

PCR amplifications were performed on a PTC100 (MJ Research) thermal cycler under the following conditions- a hot start at 95°C for five minutes; followed by 35 amplification cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes. Amplification products were resolved on 4% polyacrylamide gels using aluminium backed sequencing system model # 535 (Owl Scientific, Inc., USA) with silver staining.

Molecular weights of electromorphs were estimated using 10 bp DNA ladder from Gibco BRL, Md.

Data Analysis

The band patterns were scored for each microsatellite primer pair in each rice genotype. Presence and absence of each band in each rice genotype was coded as 1 and 0, respectively. The 0/1 matrix was used to calculate similarity genetic distance using simqual sub-program of NTYSYS-pc program (Rohlf, 1990). The resultant distance matrix was employed to construct dendrograms by the cluster tree analysis sub-program of NTYSYS-pc.

Results

Evaluation of Azucena x HBC19 F_6 population for drought tolerance

It has been suggested that traits, particularly RFW, RDW, RSFW, RSDW, MRL, RWC and visual symptoms (LD and RWSP) are more important for drought resistance in rice. In this study, some shoot traits such as PH, TN, TGW, GY, SFW and SDW were also recorded as summarized in Table 2. Significant variation in all the investigated traits indicated the presence of high genetic diversity among of Azucena x HBC19 F_6 genotypes. Mean drought tolerant index (DTI) which is the average of DTI values calculated on the basis of agronomic characteristics, shoot and root traits and RWC ranged from 42.1% (F_6 genotype no. 9) to 90.6% (Azucena). Regarding MRL, thirteen F_6 genotypes were observed to have higher DTI than Azucena. All the 52 genotypes (Azucena, HBC19 and 50 F_6 genotypes) were further scored for drought tolerance. Mean score values were calculated on the basis of scores given to DTI values and visual symptoms and it was found to be varied between 1 to 8.3. Out of 50 F_6 genotypes, two genotypes (genotype no. 14 and 46) were as tolerant as Azucena (mean score value -1.6). Genotype 48 was highly susceptible to drought conditions. Maximum numbers of plants (20 plants) were found to be moderately tolerant with mean score values of 4-5, followed by 12 plants in tolerant category with mean score values in the range of 3-4.

Microsatellite Marker Analysis

Microsatellite (SSR) DNA fingerprint database was generated for 28 selected plants (14 drought tolerant and 14 drought sensitive plants) from a population of Azucena × HBC19 F_6 lines using 30 SSR markers covering all the 12 chromosomes. The 28 Azucena × HBC19 F_6 plants had an allele from either of the two parental lines (homozygous condition) or alleles from both the parental rice varieties (heterozygous condition). Silver stained gels displaying allelic polymorphism among selected F_6 plants for SSR markers RM 332 and RM 247 have been shown in Fig 1a, b. Number of of F_6 plants with parental alleles in heterozygous condition varied from 1 (RM 170, RM 21, RM 232, RM 218, RM 332, RM 316, RM 24, and RM 247) to maximum of 6 (RM 169 and RM 180). 27 of 28 selected F_6 plants amplified HBC19 specific alleles at RM 207 locus, while 14 F_6 plants showed Azucena specific alleles with RM 18. In some cases, new (rare) alleles were also observed in combination with a parental allele or in the homozygous state. 10 (RM 304, RM 171, RM 241, RM 335, RM 180, RM 22, RM 332, RM 247, RM 204 and RM 310) of 30 SSR markers amplified rare (new) alleles, which were different to those present in two parental rice varieties. Number of F_6 plants with rare allele(s) varied from 1 (RM 304) to 10 (RM 22 and RM 332). At 5 SSR loci (RM 304, RM 241, RM 180, RM 247 and RM 204) rare alleles were present alone, while for rest of SSR loci rare allele was present alone as well as an allele from either of the parents.

The frequency distribution of Azucena and HBC19 specific alleles in 28 selected plants is shown in the Figure 2). Plant number 5 showed maximum number of Azucena specific alleles with Azucena alleles present at 9/30 loci in homozygous condition while the maximum number of Azucena alleles (sum of homozygous and heterozygous state) were observed at as many as 11 of 30 loci in plant number 5 and 6. While plant no. 15 and 25 had as many as 26 HBC19 specific alleles (sum of homozygous and heterozygous state), the plant no. 8 and 25 had maximum no. (25 alleles) HBC19 specific alleles in homozygous condition. All 28 F_6 plants had higher number. (>15 alleles) of HBC19 specific alleles.

SSR allelic database for 28 Azucena x HBC19 F_6 plants and the two parental rice varieties was used for generating similarity matrices data (Table 3) and UPGMA tree cluster/PCA analysis. The similarity coefficient ranged from 0.39 to 0.86 and dendrogram resolved 28 F_6 plants and their parents into two groups (Fig. 3). Group 1 was further divided into two subgroups. Subgroups- II had plant numbers 14 and 23.

Subgroup– I had HBC19 and rest of F_6 plants. Group 2 had a lone parent plant Azucena which merged with group 1 at similarity coefficient of 0.37. The groups identified by PCA were very similar to those linked by cluster analysis (Fig.4).

Discussion

Molecular marker technologies have revolutionized the genetic analysis of crop plants and its application has been suggested for the molecular dissection of complex physiological traits such as drought tolerance (Steele *et al.*, 2013; Sehgal et al., 2012). Using DNA markers, comprehensive molecular marker/ linkage maps have been developed in variety of crops. However, a mapping population such as recombinant inbred lines (RILs), double haploid lines (DHLs) and backcross/ F_2/ F_3 families is a prerequisite for the development of most of the maps. The main objective of the present study was to develop the mapping population, F_6 lines and RILs, to increase the efficiency of QTLs mapping for drought tolerance. F_6 lines were derived from the cross between Azucena (drought tolerant *japonica* rice variety with good root growth) and HBC19 (drought sensitive *indica* rice variety with poor root growth). Drought tolerant and drought sensitive plants were selected on the basis of agronomic characteristics (plant height, number of productive tillers per plant, 1000 grain weight and single plant yield), shoot and root related traits (root length, root weight, shoot weight and root: shoot weight ratio), relative water content, harvest index and visual symptoms like leaf drying and recovery from drought. Ingran *et al*., (1990) reported that among the selection indices used to screen rice, visual scoring of stressed plants was the best method of scoring for drought resistance. DeDatta *et al*., (1988) used visual scoring method to evaluate rice germplasm during the vegetative stage. Malabuyoc *et al*., (1985) reported that drought recovery ability is more important than drought tolerance. Various parameters used to assess the drought tolerance clearly showed tremendous variation for drought tolerance in Azucena x HBC19 F_6 population.

This was evident from the variation in the overall mean score of individual F_6 line (1-8.3) calculated on the basis of score given to each parameter. Yogameenakshi *et al.,* (2003) evaluated rice varieties for drought tolerance on the basis of yield and drought tolerant traits viz., days to 50 per cent flowering, plant height, number of productive tillers per plant, panicle length, 100 grain weight, proline content, relative water content, root length, dry root weight, root: shoot weight ratio, harvest index and single plant yield. Kanbar *et al.,* (2004) also evaluated transgresant backcrosses of rice for drought resistance on the basis of root morphological traits. Most of F_6 plants were moderately drought tolerant, followed by 12 plants in tolerant category. Two plants were as tolerant as parental drought tolerant rice variety Azucena. These studies indicate that it should be feasible to improve the drought tolerance by developing new elite combinations of genes/QTLs from different sources by marker-assisted selection in plant breeding programs.

SSR markers have been preferably employed for DNA fingerprinting and varietal identification (Olufowote *et al.,* 1997; Bligh *et al.,* 1999), linkage mapping and marker-assisted selection (Guvvala *et al.,* 2013; Joseph *et al.,* 2004;), assessment of genetic diversity and phylogenetic relationships (Jain *et al.,* 2004), detection of cases of adulteration (Bligh, 2000) in *Oryza* species. In this study, a total of 30 polymorphic SSR markers were tested on 28 selected F_6 plants comprising of 14 drought tolerant and 14 drought sensitive plants. The two parental rice varieties, Azucena and HBC19 had a similarity coefficient of 0.21, which indicates that two parents are considerably genetically divergent. Evaluation of population of Azucena x HBC19 F_6 plants derived through single seed descent method, showed considerable variation for drought tolerance. Selected 28 F_6 plants had alleles from either or both the parental rice varieties, Azucena and HBC19. Most of the selected F_6 plants (24 plants) had both the parental alleles at one or more (up to 5) of the 30 SSR loci. Frequency of HBC19 specific alleles was higher in comparison to Azucena in selected drought tolerant and drought sensitive Azucena x HBC19 F_6 plants, which may be indicative of segregation distortion. However, it is difficult to be conclusive since only limited number of markers/F_6 plants were analyzed for SSR diversity. Segregation distortion has been frequently reported in wide crosses of rice (Maekawa and Kita, 1985). A number of genetic markers have been found to show segregation distortion in wide crosses. Many instances of segregation distortion have been reported through studies of isozymes (Wu *et al*., 1988; Guiderdoni *et al*., 1989) and RFLP alleles (McCouch *et al*., 1988; Saito *et al*., 1991). The genetic basis of the segregation distortion may be the abortion of male or female gametes or selective fertilization of particular gametic genotypes. Lin *et al*., (1992) studied segregation distortion via male gametes in hybrids between *indica* and *japonica* or wide-compatibility varieties of rice (*Oryza sativa* L.). Notably several new/rare alleles also appeared in selected F_6 plants, which were entirely different from those present in parental rice genotypes. The origin of these rare alleles may be another interesting area to

work on. Occurrence of such new or rare (recombinant) alleles may have resulted from crossing over. Some of the microsatellite loci are hot spots because here mutations occur up to 100 times more frequently than the normal mutation rate, a hotspot is a center of high activity within a larger area of low activity, a hot spot can be a position on the DNA where mutations occur with an unusual high frequency or a position on the DNA where recombination occur with an unusual high frequency. Brar *et al*., (1996) also detected some non-parental bands for some of the RFLP markers during their studies on the molecular characterization of introgression of genes for brown plant hopper and bacterial blight resistance, which have been transferred from wild *Oryza* species to cultivated rice.

However, both morpho-physiological traits and SSR markers provided independent, yet different estimates of genetic variation among F_6 rice plants. However, both markers were proficient at distinguishing the genotypes. It was evident from the present study that the genetic relationships estimated from SSR-based markers enhanced the resolution of diversity and thus provided an improved representation of variability. Analysis of genetic diversity suggested differentiation that is more ecotypic. Appropriate parents with regard to drought – resistance components (e.g. root traits, RWC) may be selected using such estimates of diversity at morpho-physiological and DNA levels so as to develop a population for mapping QTLs of interest. Research can be pursued to look for marker association with important genes/traits/QTLs using appropriate population.

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Table 1. A brief description of microsatellite markers used for SSR analysis

Marker	Clone no.	Map position	Repeat type & length	Forward primer	Reverse primer	Size range (bp)
RM 1	GA12	8	$(GA)_{18}$	GGAAAGAATGATCTTTTCATCC	CTACCATCAAAACCAATGTTC	77-113
RM 17	GA56	12	$(GA)_{21}$	TGCCCTGTTATTTTCTTCTCTC	AACACAGCAGGTACGCGC	168-184
RM 18	GA97	7	$(GA)_4AA(GA)(AG)_{16}$	TTCCCTCTCATGAGCTCCAT	GAGTGCCTGGCGCTGTAC	151-163
RM 21	GA275	11	$(GA)_{21}$	ACAGTATTCGGTAGGCAGG	GCTCCATGAGGGTGGTAGAG	133-164
RM 22	GA580	3	$(GA)_{22}$	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	187-197
RM 24	GA5	1	$(GA)_{29}$	GAAGTGTGATCACTGTAACC	TACAGTGGACGGCGAAGTCG	152-198
RM 31	GA257	5	$(GA)_{15}$	GATCACGATCCACTGGAGCT	AAGTCCATTACTCTCCTCCC	141-153
RM 38	GA334	8	$(GA)_{16}$	ACGAGCTCTCGATCAGCCTA	TCGGTCTCCATGTCCCAC	250-260
RM 42	GA376	8	$(GA)_{26}$	ATCCTACCGCTGACCATGAG	TTTGGTCTACGTGGCGTACA	159-165
RM48	GA479	2	$(GA)_{17}$	TGTCCCACTGCTTTCAAGC	CGAGAATGAGGGACAAATAAC	199-219
RM169	OSM69	5	$(GA)_{12}$	TGGCTGGCTCCGTGGGTAGCTG	TCCCGTTGGCCGTTCATCCCTCC	164-194
RM170	D15716	6	$(CCT)_7$	TCGCGCTTCTTCCTCGTCGACG	CCCGCTTGCAGAGGAAGCAGCC	106-119
RM171	OSM71	10	$(GATG)_5$	AACGCGAGGACACGTACTTC	ACGAGATACGTACGCCTTTG	318-343
RM174	D48756	2	$(AGG)_7 (GA)_{10}$	AGCGACGCCAAGACAAGTCGGG	TCCACGTCGATCGACACGACGG	207-222
RM180	M2	7	$(ATT)_{10}$	CTACATCGGCTTAGGTGTAGCAACACG	ACTTGCTCTACTTGTGGTGAGGGACTG	107-204

Continuing table 1

Marker	Clone no.	Map position	Repeat type & length	Forward primer	Reverse primer	Size range (bp)
RM204	CT19	6	$(CT)_{25}$	GTGACTGACTTGGTCATAGGG	GCTAGCCATGCTCTCGTACC	146-174
RM207	CT41	2	$(GA)_{25}$	CCATTCGTGAGAAGATCTGA	CACCTCATCCTCGTAACGCC	123-142
RM218	CT125	3	$(TC)_{24}ACT(GT)_{11}$	TGGTCAAACCAAGGTCCTTC	GACATACATTCTACCCCCGG	125-153
RM232	CT339	3	$(GA)_{24}$	CCGGTATCCTTCGATATTGC	CCGACTTTTCCTCCTGACG	143-166
RM235	CT368	12	$(GA)_{24}$	AGAAGCTAGGGCTAACGAAC	TCACCTGGTGGAAAATGAG	90-132
RM241	CT404	4	$(GA)_{31}$	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTC	104-149
RM247	CT462	12	$(GA)_{16}$	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	127-178
RM252	CT206	4	$(GA)_{19}$	TTCGCTGATCCCGAGAACG	ATGACTTGATCCCGAGAACG	184-267
RM257	CT522	9	$(GA)_{24}$	CAGTTCCGAGCAAGAGTACTC	GGATCGGACGTGGCATATG	104-192
RM259	CT550	1	$(GA)_{17}$	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGCGCCCATGT	152-174
RM304	GT25	10	$(GT)_2(AT)_{10}(GT)_{33}$	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG	138-175
RM310	GT137	8	$(GT)_{19}$	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC	85-120
RM316	GT264	9	$(GT)_8(TG)_9(TTTG)_4(TG)_4$	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	150-290
RM332	CTT38	11	$(CTT)5\text{-}12\text{-}(CTT)_{14}$	GCGAGGCGAAGGTGAAG	CATGAGTGATCTCACTCACCC	162-183
RM335	CTT50	4	$(CTT)_{25}$	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG	104-155

Table 2. Overall mean score of F_6 rice plants for drought tolerance

	Drought Tolerant Index (DTI)																	
Genotypes	**PHw**	**TN**	**TGW**	**GY**	**SFW**	**SDW**	**RFW**	**RDW**	**RSFW**	**RSDW**	**MRL**	**HI**	**RWC**	**Mean DTI**	**Score on the basis of mean DTI**	**LD Score**	**RWSP Score**	**Mean Score**
Azucena	79.3	82.4	79.9	89.6	93	77	95	83.2	97.7	108.2	106	112	75.1	90.6	1	3	1	1.6
HBC19	82.6	41.2	67.1	50.2	36	56	50.4	62.3	154	106.5	71.2	87	62.1	71.3	5	9	9	7.6
1*	93.8	66.7	78	59.8	91	80	34.1	45.2	41.7	53.4	94.5	98.5	70.4	69.7	7	7	5	6.3
2	76.2	54.5	88.7	54.2	65	37	62.6	41.4	99	125.2	88.8	128	72.6	76.3	3	7	3	4.3
3	79.5	75	69.9	70.9	90	76	64.8	91.7	68.9	118.7	86.4	88.7	59.2	75.5	3	7	3	4.3
4*	73	66.7	89.1	49.8	74	70	93.9	81.9	132	117.8	97.2	67.6	53.8	70.7	5	7	5	5.6
5	88.2	63.6	93.4	68.1	92	61	76.4	54	81.3	72.7	111	111	78.2	80.8	1	5	5	3.6
6	75.9	61.5	77	72.6	84	76	43.8	86.3	65.9	110.5	123	95.5	48.3	71.9	5	5	3	4.3
7	83.2	66.7	77.3	61.1	70	47	91.2	85.5	114	182.6	70.9	118	60.5	71.7	5	5	5	5
8*	64.5	68.8	75.9	56.6	84	73	67.4	63.7	75.4	76.5	125	76.3	83.6	76.1	3	3	3	3
9	46.1	57.1	78	50.2	44	55	86.4	85	196	166.2	70.7	86	79.1	42.1	9	1	1	3.6
10	68.2	62.5	81.2	25.7	58	62	82.7	65.1	112	117.8	79.6	42.6	35.8	72.7	5	5	5	5
11*	76.1	50	75.7	39.8	77	51	46.9	64.6	69.6	126.9	61.3	77.3	71.8	68.3	7	5	5	5.6
12	77.9	57.1	82.6	73.5	86	70	91.6	74.3	94.2	131.5	50.6	98.8	61.1	73.9	5	3	3	3.6

Continuing table 2

Genotypes	Drought Tolerant Index (DTI)																	
	PHw	TN	TGW	GY	SFW	SDW	RFW	RDW	RSFW	RSDW	MRL	HI	RWC	Mean DTI	Score on the basis of mean DTI	LD Score	RWSP Score	Mean Score
13*	86.9	71.4	87.9	70.9	94	70	30.3	72	54	93.4	94.7	90	63.7	73.7	5	3	1	3
14*	70	66.7	77.4	50.9	64	44	88.3	79.2	98.4	236.3	75.8	103	62.9	85.9	1	3	1	1.6
15*	65.2	37.5	85.6	6.1	40	31	91.4	54	169	168.3	48	16.4	61.9	61.2	9	7	3	6.3
16	81.7	50	77.6	71.8	94	64	21.5	32.9	35	53.1	99.4	131	63.2	74.7	5	5	3	4.3
17	73.7	71.4	64.6	76.6	76	81	96.4	67.9	131	83.2	104	104	68.8	84.4	1	7	5	4.3
18	90.7	60	88.1	65	45	52	51.8	39.4	134	87.1	86.5	130	83.1	86.7	1	7	5	4.3
19	93.6	76.5	84.5	70.9	83	81	90.3	82.9	97.9	96.2	138	87	81.3	75.7	3	3	3	3
20	73	63.6	54	68.4	79	64	86	66.4	89	132.1	122	101	67.9	81.9	1	7	3	3.6
21*	86.7	72.7	70.7	79.3	84	80	97.3	80	108	111.4	117	111	90.1	82.7	1	3	3	2.3
22*	74	69.2	87.9	81.5	91	52	84	87.5	97.1	133.9	119	117	76.9	76.7	3	7	5	5
23	53.8	58.8	87.1	42.4	57	73	94.7	90.2	171	125.5	87.4	52.6	77.6	82.3	1	7	5	4.3
24	64.1	55.6	65.5	48.4	45	61	76.2	79.6	153	132.4	140	76.3	76.7	60.1	9	3	1	4.3
25	83.9	66.7	71.4	55.1	43	53	35.8	43.9	134	94.6	116	109	49.4	77.7	3	5	3	3.6
26*	73.1	71.4	80	58.7	42	43	96.8	44	114	95.4	94.4	129	58.3	76.9	3	7	5	5

Continuing table 2

Drought Tolerant Index (DTI)																		
Genotypes	**PHw**	**TN**	**TGW**	**GY**	**SFW**	**SDW**	**RFW**	**RDW**	**RSFW**	**RSDW**	**MRL**	**HI**	**RWC**	**Mean DTI**	**Score on the basis of mean DTI**	**LD Score**	**RWSP Score**	**Mean Score**
27	93.6	50	78.7	50.5	73	63	76.7	82.2	104	123.4	102	73	85.4	89.6	1	7	5	4.3
28*	68.7	63.6	75	36.8	41	37	43.3	53.9	129	152.8	61.6	111	53.3	78.7	3	7	5	5
29	88.4	44.4	76.4	47.7	88	73	67.7	72.8	96.7	99.6	113	72.1	63	77	3	5	3	3.6
30*	63.9	57.1	78.2	79.3	76	67	77.8	79	127	122.7	139	113	72.5	59.9	9	7	5	7
31*	56.1	60	83.4	57.1	45	63	92.5	81.6	210	155.7	102	96	47.9	79.7	3	7	5	5
32*	73.2	70	85.6	46.3	80	71	48.6	44.3	41.1	53.4	106	73.5	73.5	66.6	7	7	1	5
33*	89.1	44.4	68.4	76.6	87	56	45.5	22.9	63.9	46.1	68	167	74.7	85.1	1	5	3	3
34*	91.5	44.4	72.4	55.9	59	61	91.9	60.8	128	98.1	90.5	100	80.7	80.7	1	5	5	3.6
35*	79.2	33.3	77.6	43.4	59	59	20.2	53.7	42.5	105.1	95.4	74.3	66.9	62.3	9	3	3	5
36*	68.2	30.4	77.3	23	28	26	13.5	17.1	77.8	67.8	76	102	72.9	64.2	9	5	3	5.6
37	52.2	22.2	78.3	31.6	27	25	11.7	8.6	46.3	34.5	55.7	163	66.2	81.7	1	7	3	3.6
38	92.6	40	87.1	68.6	76	50	56.6	48.9	90.9	109.3	103	141	62.5	78.8	3	5	3	3.6
39*	81.7	54.5	68.1	54.7	81	78	98.7	86.1	111	91.1	75.2	68.3	71.4	77.7	3	7	5	5
40*	88.9	50	87.7	72.3	88	57	83	47.7	79.9	104.5	88.6	143	67.2	82.7	1	3	3	2.3

Continuing table 2

	Drought Tolerant Index (DTI)																	
Genotypes	**PHw**	**TN**	**TGW**	**GY**	**SFW**	**SDW**	**RFW**	**RDW**	**RSFW**	**RSDW**	**MRL**	**HI**	**RWC**	**Mean DTI**	**Score on the basis of mean DTI**	**LD Score**	**RWSP Score**	**Mean Score**
41*	83.9	42.9	81.8	61.2	38	30	19.5	33.7	63.5	139.8	44	197	75.7	70	7	1	1	3
42*	59.1	66.7	69.4	51.7	38	39	10	49	58.6	112	30.2	127	46.7	55.1	9	1	1	3.6
43*	52.2	60	81.5	66.5	35	25	22.6	54	85.5	219.3	60.6	244	64.4	83.7	1	3	3	2.3
44	61.7	66.7	74.3	51.9	42	49	25.6	53.4	67.1	110.8	77.2	98.6	72.4	65.3	7	3	3	4.3
45	61.7	42.9	73.2	48	61	55	84.4	84.3	115	173.8	94.2	81.1	75.1	57.7	9	3	3	5
46*	67.9	42.9	70.7	77.9	48	56	95.1	82.5	170	138.8	48.5	127	65	84.7	1	1	1	1
47*	66.4	57.1	64.6	61.3	68	44	82.4	100	128	246.8	60.5	124	69.9	90.2	1	3	1	1.6
48*	55.5	42.9	64.6	59.7	37	37	105	69.7	197	195.9	91	136	82.6	51.5	9	9	7	8.3
49*	82.5	76.9	80.4	87.3	79	71	115	80.1	114	95.4	151	116	59	85.7	1	5	5	3.6
50*	78.9	66.7	90.9	77.3	83	63	98.9	80.5	107	127.3	84.4	115	84.5	88.9	1	5	5	3.6

* Plants selected for SSR analysis

Plant height (PH) in cm, ttiller number (TN), grain yield (GY) in g/plant, thousand grain weight (TGW) in g, maximum root length (MRL) in cm, shoot & root fresh weight (SFW, RFW) in g, shoot & root dry weight (SDW, RDW) in g, root:shoot ratio (RSR), harvest index (HI), relative water content (RWC), leaf drying(LD) and recovery of water stressed plants (RWSP).

Figure 1a. A silver stained gel showing allelic polymorphism among selected F_6 plants of cross Azucena × HBC19 and parental lines at RM 332 locus. L represent the 10 base pair ladder, lanes 1-30 represents drought tolerant F_6 plants (1-14), drought sensitive F_6 plants (15-28), Azucena (29) and HBC19 (30).

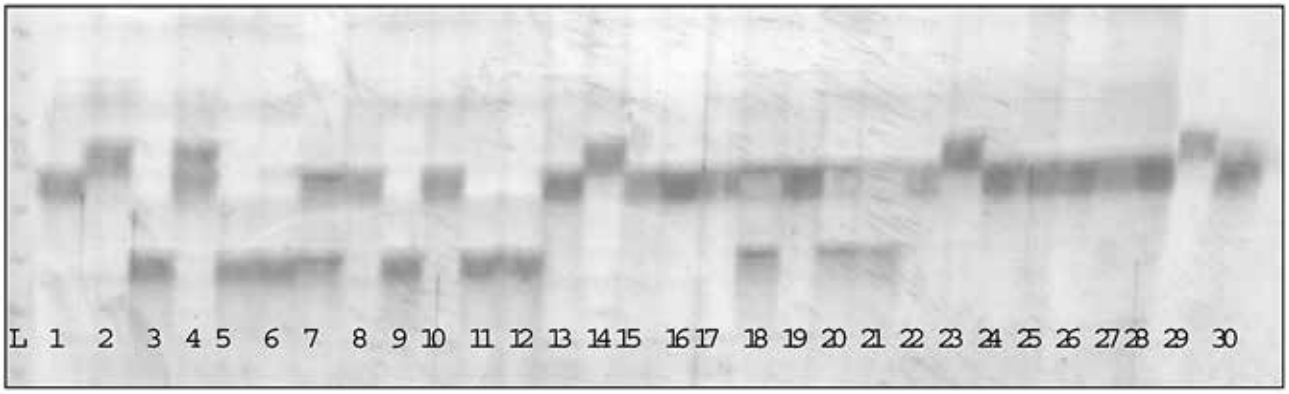

Figure 1b. A silver stained gel showing allelic polymorphism among selected F_6 plants of cross Azucena × HBC19 and parental lines at RM 247 locus. L represent the 10 base pair ladder, lanes 1-30 represents drought tolerant F_6 plants (1-14), drought sensitive F_6 plants (15-28), Azucena (29) and HBC19 (30).

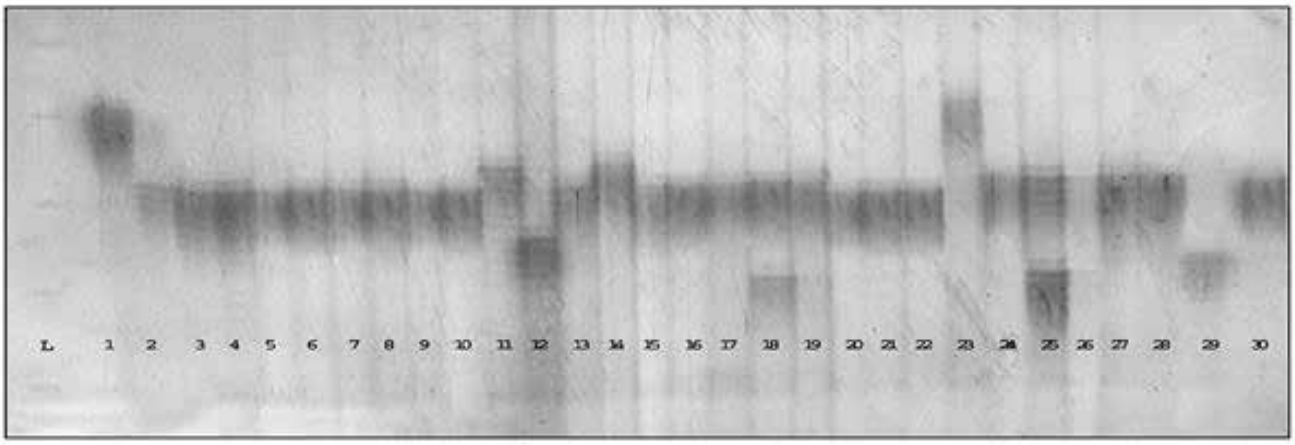

Figure 2. Distribution of Azucena and HBC19 specific alleles in 28 selected F_6 plants of Azucena x HBC19

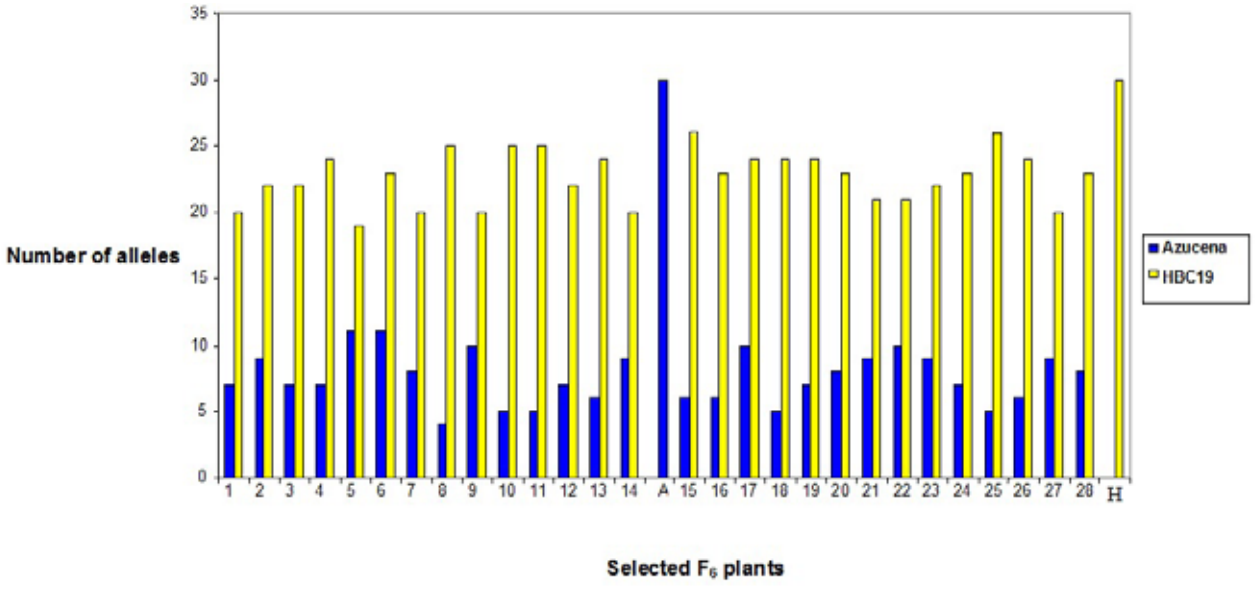

Figure 3. Dendrogram of selected 28 Azucena x HBC19 F_6 plants based on SSR diversity at 30 loci.

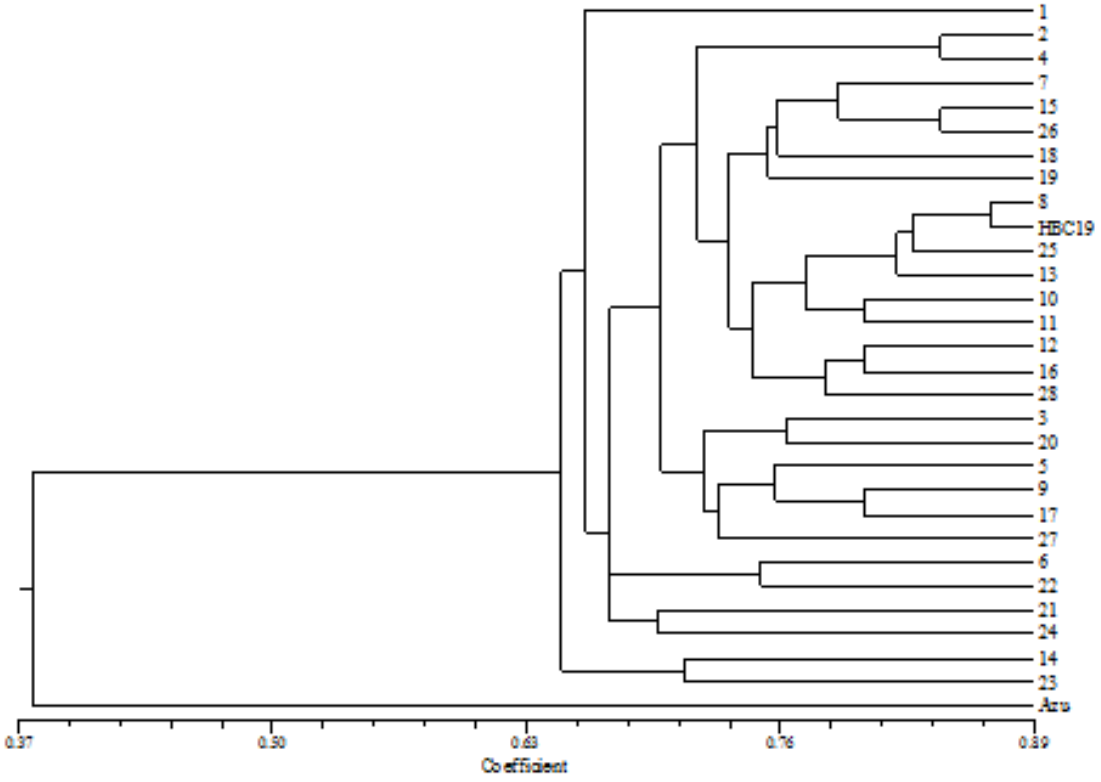

Figure 4. Three dimensional PCA scaling of selected Azucena x HBC19 F_6 plants using SSR diversity data at 30 loci.

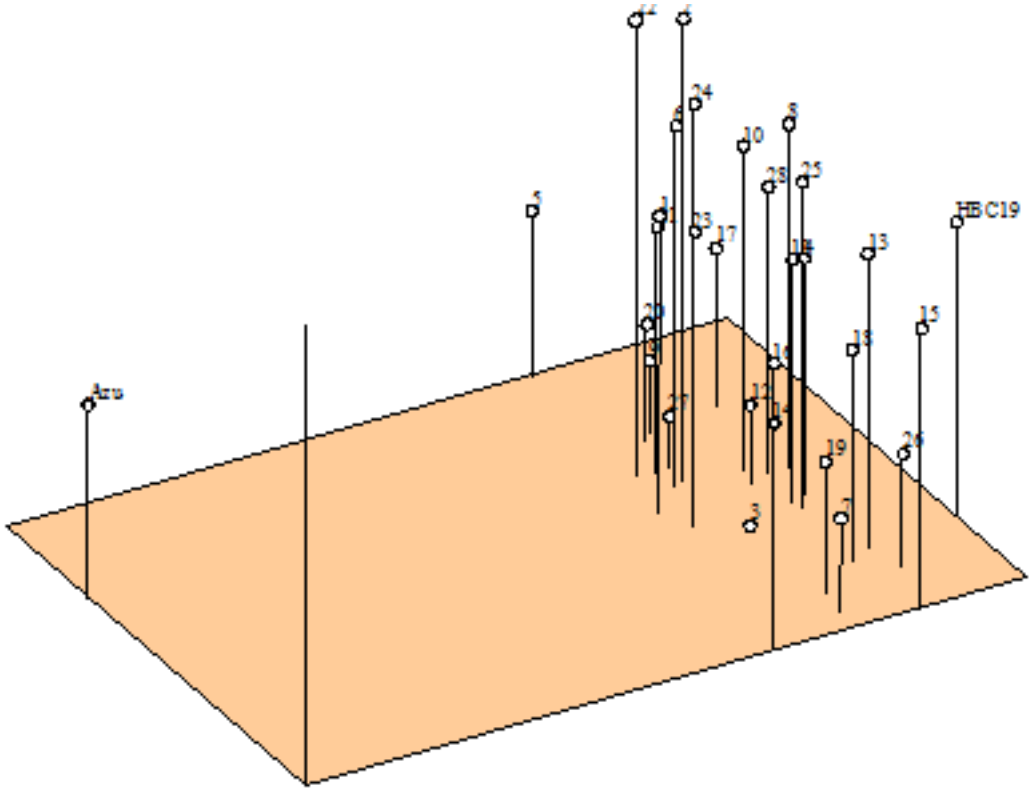

Table 3. Similarity matrix data of 28 Azucena x HBC19 F_6 plants and parental rice varieties using the allelic database at 30 SSR loci

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Azu	HBC19	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1.00																													
2	0.68	1.00																												
3	0.67	0.62	1.00																											
4	0.71	0.84	0.75	1.00																										
5	0.59	0.67	0.68	0.67	1.00																									
6	0.61	0.71	0.67	0.66	0.70	1.00																								
7	0.59	0.57	0.74	0.67	0.61	0.70	1.00																							
8	0.72	0.70	0.63	0.70	0.76	0.70	0.66	1.00																						
9	0.61	0.66	0.72	0.68	0.72	0.66	0.64	0.72	1.00																					
10	0.70	0.67	0.61	0.72	0.71	0.70	0.66	0.84	0.67	1.00																				
11	0.76	0.71	0.67	0.74	0.67	0.71	0.72	0.78	0.74	0.80	1.00																			
12	0.64	0.64	0.74	0.75	0.71	0.64	0.74	0.68	0.72	0.71	0.75	1.00																		
13	0.72	0.72	0.71	0.78	0.63	0.78	0.79	0.82	0.67	0.76	0.75	0.71	1.00																	
14	0.50	0.61	0.62	0.63	0.57	0.61	0.67	0.67	0.61	0.62	0.66	0.62	0.67	1.00																
15	0.64	0.75	0.74	0.80	0.58	0.72	0.76	0.74	0.62	0.74	0.72	0.68	0.82	0.75	1.00															
Azu	0.38	0.43	0.39	0.38	0.47	0.43	0.42	0.32	0.41	0.34	0.30	0.37	0.37	0.41	0.34	1.00														
HBC19	0.72	0.78	0.76	0.83	0.68	0.75	0.74	0.87	0.75	0.82	0.80	0.76	0.84	0.72	0.87	0.21	1.00													
16	0.68	0.61	0.72	0.74	0.72	0.55	0.75	0.83	0.74	0.75	0.71	0.80	0.75	0.61	0.67	0.36	0.80	1.00												
17	0.67	0.67	0.76	0.75	0.79	0.72	0.66	0.76	0.80	0.74	0.70	0.79	0.74	0.57	0.68	0.39	0.82	0.80	1.00											
18	0.66	0.63	0.70	0.68	0.64	0.63	0.78	0.78	0.71	0.72	0.79	0.75	0.67	0.68	0.75	0.30	0.83	0.76	0.70	1.00										
19	0.59	0.62	0.71	0.72	0.61	0.59	0.71	0.68	0.67	0.63	0.64	0.66	0.71	0.67	0.76	0.37	0.82	0.72	0.71	0.75	1.00									
20	0.67	0.67	0.76	0.75	0.74	0.67	0.66	0.68	0.72	0.71	0.72	0.68	0.71	0.62	0.68	0.42	0.74	0.70	0.71	0.64	0.68	1.00								
21	0.58	0.68	0.64	0.68	0.70	0.66	0.67	0.70	0.71	0.70	0.68	0.67	0.59	0.63	0.67	0.41	0.75	0.68	0.72	0.71	0.67	0.72	1.00							
22	0.59	0.72	0.61	0.67	0.66	0.75	0.63	0.71	0.59	0.71	0.62	0.63	0.68	0.54	0.71	0.45	0.74	0.67	0.68	0.70	0.66	0.71	0.70	1.00						
23	0.55	0.71	0.59	0.68	0.64	0.66	0.64	0.67	0.71	0.62	0.61	0.78	0.70	0.71	0.62	0.41	0.75	0.71	0.72	0.68	0.67	0.62	0.63	0.70	1.00					
24	0.62	0.70	0.58	0.64	0.63	0.62	0.66	0.74	0.62	0.66	0.64	0.63	0.68	0.59	0.63	0.39	0.76	0.72	0.74	0.67	0.68	0.55	0.70	0.71	0.67	1.00				
25	0.71	0.74	0.70	0.79	0.64	0.68	0.64	0.80	0.74	0.72	0.71	0.67	0.80	0.68	0.72	0.36	0.86	0.76	0.78	0.76	0.67	0.64	0.71	0.62	0.71	0.72	1.00			
26	0.67	0.70	0.79	0.80	0.66	0.67	0.82	0.74	0.70	0.74	0.72	0.79	0.82	0.64	0.84	0.34	0.87	0.78	0.79	0.75	0.79	0.66	0.67	0.61	0.67	0.68	0.72	1.00		
27	0.63	0.63	0.67	0.76	0.70	0.66	0.64	0.72	0.76	0.72	0.68	0.70	0.70	0.61	0.70	0.41	0.75	0.74	0.72	0.63	0.70	0.75	0.63	0.57	0.61	0.54	0.66	0.72	1.00	
28	0.70	0.72	0.68	0.80	0.68	0.70	0.66	0.76	0.67	0.76	0.72	0.79	0.71	0.59	0.76	0.34	0.82	0.78	0.76	0.75	0.66	0.66	0.59	0.74	0.70	0.68	0.75	0.74	0.75	1.00

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Additional Sources of Resistance for Southern Corn Leaf Blight in Indian Maize Germplasm

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ABSTRACT

Seventy nine maize inbred lines were screened under artificial epiphytotic condition at two locations *viz*, Nagenahalli and Varanasi for continuously for 2 years to identify the additional sources of resistance for 'Southern Corn Leaf Blight (SCLB). *The Southern Corn Leaf Blight* caused by *Bipolaris maydis* is also known as 'Maydis Leaf Blight'. The present study has helped in the identification of 26 resistant inbred lines, 25 moderately resistant, 16 susceptible and 12 highly susceptible maize genotypes. Ten lines viz. V53, V 178, V 190, V 336, V 340, V 341, V 345, V 348, CM 104 and CM 145 by scoring below 1.5 disease score showed high level of resistance, whereas inbred lines *viz*., V 49, CM 126, CM 127, CM 202 and CM 212 showed high level of susceptibility as they scored above 3.5 disease score across the environment. It was also observed that average disease incidence was high in Nagenahalli than Varanasi thus indicating that isolates of *Bipolaris maydis* were more virulent at Nagenahalli than Varanasi.

Keywords: Southern corn leaf blight, *Bipolaris maydis*, *Zea mays*, resistance, susceptible.

Introduction

Maize is prone to a number of biotic stresses like, foliar diseases, ear rot and stalk rot caused by fungi and bacteria, under favorable environmental conditions. These pathogens are capable of causing severe losses and deteriorate the quality of the produce. Maize breeding requires vigorous field and greenhouse testing to determine the kind and level of resistance to different diseases. Southern Corn Leaf Blight is one of the most important maize diseases and caused by the fungus *Bipolaris maydis* Shoemaker, Teleomorph *Cochliobolus heterostrophus* (Drechs.). It is also commonly known as *'Maydis Leaf Blight'* and crops affected by this disease are Corn (*Zea mays*), Sorghum and Teosinte. This disease has great significance in the history of agriculture because of its epidemic propositions in 1970 in US and subsequent devastation of most of the corn crop that year. It tends to be limited by temperature and climate to the warmer part of the US (Hooker *et al.*, 1970) spore production is influenced by temperature (Warren, 1975). Infected tissue is extensively covered with spots and chlorosis rendering them non productive. It is found to have a higher saprophytic ability (Blanco and Nelson, 1972) and hence high primary inoculums level is likely to be found in areas with high disease occurrence. In South East Asia it is reported to cause heavy losses in Pakistan, India, Nepal, Kampuchea, Philippines, Indonesia, Vietnam and China. 'Southern Corn Leaf Blight' is serious disease in India particularly in J & K, Himachal Pradesh, Sikkim, Meghalaya, Punjab, Haryana, Rajasthan, Delhi, UP, Bihar, MP, Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu. The disease is prevalent in warm humid temperate to tropical region, where the temperature ranges from 20-30^{0}C during cropping period. Resistance to this disease is polygenically governed and most of improved cultivars at BHU, Varanasi have a moderate degree of resistance. Ideal maize breeding programme with high

level of SCLB resistance requires to be supported by additional new sources of resistance at regular intervals. The objective of present study is to identify additional sources of resistance for *Southern Corn Leaf Blight* particularly to support breeding programme of Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Material and Methods

The present study was carried out at Agriculture Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and Zonal Agriculture Research Station, V.C. Farm, Mandya (Nagenahalli), Karnataka during 2010 & 2011. In this experiment 79 entries were included for screening of *'Southern Corn Leaf Blight'* under artificial epiphytotic field condition as well as in glass house conditions. The 79 entries were evaluated in 3-row plot of 3.0 m x 1.8 m each and a local susceptible variety Dhiari Local was planted at regular interval as infector row in RBD with two replications. Further, it was planted in glass house condition with five plants of each of 79 genotypes in pots during *Kharif* (summer season) 2010 & 2011. The data analyzed is the average of the 4 trials in 2 locations for 2 years. The inoculation was applied as suggested by (Meena Shekhar and Sangit Kumar 2012) after inoculation the disease symptoms developed within 1-2 weeks of inoculums and by the time of flowering disease were severe in the infector rows. Disease Score were taken as per (Payak and Sharma 1985) and disease scoring was done as per symptoms mentioned below:

1.0 - Very slight to slight infection, one or two to few scattered lesions on lower leaves.

2.0 - Light infection, moderate number of lesions on lower leaves only

3.0 - Moderate infection, abundant lesions are on lower leaves, few on middle leaves.

4.0 - Heavy infection, lesions are abundant on lower and middle leaves, extending to upper leaves.

5.0 - Very heavy infection, lesions abundant on almost all leaves plants prematurely dry or killed by the disease.

Results and Discussion

A total of 79 maize genotypes were screened to identify additional sources of resistance for *'Southern Corn Leaf Blight'* (Fig. 1).

The results of screening against '*Southern Corn Leaf Blight*' have been presented in Table 1.

The screening led to the identification of 26 sources of resistance *viz.,* V53, V 178, V 190, V 336, V 340, V 341, V 345, V 348, CM 104, CM 145, HKI-586, HUZM-81-1, HUZM-60, CML-192, HKI 1105, HUZM-211-1, HKI-PC-8, CML-150, HKI 193, CML-161, HUZM-53, HKI 323, HKI-164-4-(1-3)-2, HKI-1352-5-8-9, CML-172, HUZM-36. The lines such as CM 104, CM 145 and V 341 have earlier been reported as resistance (Ali and Yan 2012, Durrishahwar *et al* 2008.) where as rest of lines are being reported as resistance for the first time. The maize inbred such as CM-145, V-336 and V-338, was resistance in both the environment and inbred such as NAI-219-J, V-335, V-351, HUZM-121, CM-212, V-25 and CML-395, was susceptible in both the environment. These are the valuable material as it expressed resistance across the environment. Similar screening have been done for Southern Corn of Blight by Ali and Yan (2012) and Durrishahwar *et al*., (2008) where as many studies have been carried out in past for other diseases like *Northern Corn Leaf Blight* (Adipala *et al*., 1993, Muriithi and Mutinda 2001, Singh *et al*., 2004, Chandrashekara *et al*., 2012) and *Grey leaf spot disease* (Maroof *et al*., 1993). Based on susceptible and resistant reactions of maize genotypes, an attempt was made to identify the resistant gene pool sources for the disease. It was observed that Vivekananda Population, CIMMYT Population 31 and Indian public sector genotypes were a very good source for breeding resistant cultivars for SCLB (Table 2).

It may be mentioned here that the Indian public sector material majority coming from Directorate of Maize Research, New Delhi or its network have been generated out of CIMMYT and US, materials. Thus, the CIMMYT and US have contributed tremendously for the development of resistant cultivars for '*Southern Corn Leaf Blight'* in Indian Maize Breeding Programme.

Acknowledgements

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Figure 1. Plant susceptible to MLB

Table 1. Screening of maize inbreds for identification of Additional Sources of '*Maydis Leaf Blight*' at Naganahalli and Varanasi

Inbred lines
Resistant: V53, V 178, V 190, V 336, V 340, V 341, V 345, V 348, CM 104, CM 145, HKI-586, HUZM-81-1, HUZM-60, CML-192, HKI 1105, HUZM-211-1, HKI-PC-8, CML-150, HKI 193, CML-161, HUZM-53, HKI 323, HKI-164-4-(1-3)-2, HKI-1352-5-8-9, CML-172, HUZM-36
Moderately resistant: V 12, V 13, V 26, V 128, V 241, V 273, V 334, V 335, V 339, V 346, CM 105, CM 118, CM 119, CM 129, CM 141, HUZM-88, HUZM-47, HUZM-97-1-2, CML-451, HKI-287, V-342, CML-140, V-273, HUZM-356, HKI-209
Susceptible: V 17, V 198, V 324, V 350, CM 128, HUZM-457, HUZM-185, HKI-536, V-338, HKI-335, V-386, V-388, HUZM-478, HUZM-509, HUZM-69, CML-152
Highly Susceptible: V 49, CM 126, CM 127, CM 202, CM 212, V-351, V-25, HKI-162, HUZM-80-1, HUZM-121, NAI 219-J, CML-395

Table 2. Reaction of important maize gene pools and populations available at BHU against *Southern Corn Leaf Blight* (*Bipolaris maydis*).

S. No.	**Sources**	**No. of genotypes**			
		Resistant	**Moderate Resistant**	**Susceptible**	**Highly Susceptible**
1	Vivekananda Population	8	12	7	4
2	Indian Public Sector	7	7	3	5
3	CIMMYT Mexico (population-31)	6	2	1	1
4	BHU, Varanasi	5	4	5	2
5	Total	26	25	16	12

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Research Article

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Analysis of Sesame (*Sesamum indicum* L.) Accessions Collected From Different Parts of Turkey Based on Qualitative and Quantitative Traits

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ABSTRACT

An experiment was conducted at the experimental area of Dicle University Faculty of Agriculture, Department of Field Crops in 2014 growing season. A total of 107 diverse sesame accessions collected from Mediterranean, Aegean and Southeastern Anatolia Region of Turkey were sown on 3 May 2014. The experiment was laid out in an augmented experimental design with eleven replications. A total of 25 morphological and agronomical characters of 107 sesame accessions were evaluated following to IPGRI (International Plant Genetic Resources Institute) descriptors of sesame, and the obtained data were evaluated according to their qualitative and quantitative traits. Frequency distribution of qualitative traits and correlation coefficients of quantitative traits were determined. According to the frequency of accessions analyzed, the variability in terms of the qualitative traits examined. Regarding the relationship between quantitative traits, the number of capsules, 1000 seed weight and harvest index were determined significantly affecting the seed yield.

Keywords: Sesame, diversity, accession, correlation, qualitative and quantitative traits

Introduction

Sesame is one of the most important ancient oil seed crops. Though the origin of sesame is not known, archeological remains of sesame dating back to 5500 BC have been found in the Harappa valley in the Indian subcontinent (Bedigian and Harlan, 1986), and it was cultivated and domesticated on the Indian subcontinent during Harrapan and Anatolian eras (Bedigian and Van der Maesen, 2003). Discussion continues about the exact origin of sesame. It is often asserted that sesame has its origin in Africa and spread early through West Asia, China and Japan, which themselves become secondary centers of diversity. With the exception of *Sesamum prostratum* Retz, all the wild *Sesamum* species are found in Africa (Purseglov, 1977).

So far, many countries of the world have sponsored extensive research on breeding methods based on a single and bulk selection of sesame accessions that resulted in the introduction of new varieties. However, sesame breeding studies relative to other crops remains extremely limited, especially in Turkey.

As a result of natural selection for many years , there are numerous varieties and ecotypes of sesame adapted to various ecological conditions of Turkey. But the average seed yield is too low. Low yields are mainly due to absence of non-shattering cultivars suited for mechanical harvest, indeterminate growth, uneven ripening of capsules and biotic and abiotic stresses such as diseases, pests, drought etc. (Venkataramana *et al*., 1999).

Access to a wide range of genetic diversity is critical to the success of any crop breeding programs and the ability to identify genetic variation is indispensable for effective management and use of genetic resources (Varshney *et al*., 2009), and it mainly depends on germplasm characterization (Yogranjan *et al*., 2015).

The above stated constraints to the productivity pose the need for concerted efforts for sesame crop improvement. However, despite, being an ancient but an important oil crop, it is still at an early stage in its breeding history. Characterization of available or existing genetic diversity of crops played a significant role in bringing out a variety with desirable market oriented physio-chemical traits. Information on genetic diversity is noteworthy when working to improve qualitative profile of the crop varieties (Yogranjan *et al*., 2015).

In this context, the aims of the present study were to find out the relationships between the quantitative traits, and to determine varietal difference. Of sesame accessions collected from different regions of Turkey. The study can help breeders better understand the genetic structure of sesame populations which can be used for selection.

Material and Methods

The field experiment was carried out in the experimental area of Field Crops Department, Faculty of Agriculture, Dicle University, Diyarbakır, Turkey (37^0 53^l N and 40^0 16^l E) in 2014. Annual average rainfall in the region is 490 mm, 18%, 44%, 37% and 1% of which falls in autumn, winter, spring and summer, respectively. Water needs of the plant due to lack of rains especially in July-August was met with irrigation water. The soil type was a clay and mid-alkaline. A total of 107 diverse sesame accessions collected from Mediterranean, Aegean and Southeastern Anatolia of Turkey were used as experimental material. The experiment was laid out in an augmented design with 11 replications. Improved cultivars Muganlı 57, Osmanlı 99, and Özberk 82) were repeated as check after every 13 accessions. Each plot had a size of 1.4 x 4 m^2 with two rows (intra-row spacing of 70 cm and inter-row spacing of 10 cm).

Sesame accessions used in the study were examined for qualitative and quantitative traits according to IPGRI (International Plant Genetic Resources Institute) for a total of 25 traits. The frequency distribution of qualitative traits was analyzed. Also, relations between quantitative traits were made through JMP version 11 statistical program according to Jeffers (1967).

Results and Discussion

Morphological descriptors of qualitative traits recorded on 107 sesame accessions are summarized in Table 1.

The frequency distribution of qualitative traits of 107 sesame accessions is summarized in Table 2. Regarding stem hairiness significant differences between accessions were observed, 88 of the total accession being hairless, 18 less hairy and 1 was very hairy (Table 2).

In terms of leaf edges 102 of the accession had flat edge, 3 were the saw-toothed and 2 were observed to be comb-toothed. 103 of accession had straight leaves and 4 of them were less pieced regarding leaf fragmentation.

In terms of leaf hairiness in accession 102 had hairlessness, and 5 had little hairiness. Because of the fact that leaf hairiness has positive effect in terms of preventing water loss, it is a desirable trait in breeding. According to the flower color, variation was seen between accessions, 76 of the accession were light pink and 20 are dark pink-shaded white in and 11 were white. In respect to flower hairiness, 74 of the accession had medium hairiness, 19 were observed to have high hairiness and 14 had very low hairiness. Flower hairiness, as well as preventing the loss of water in flower at high temperatures, it is often preferred because it is considered to have a positive impact on the pollination of flowers. Variability was seen in accessions in terms of stigma length. With 74 of the accessions stigma was observed to be under the anther (short), it was at the anther level in 33 (moderate). Stigma length is an important factor in plant pollination, and if stigma is longer than anther, fertilization may not occur or it may lead to cross fertilization. The fact that stigma is shorter than anther is preferable because it provides a timely fertilization and increases self-fertilization. With respect to the number of flowers in the axil, 94 had only flowering and 13 of accessions were determined to have multiple flowers. With regard to this feature it was determined that, though very little, there was some variability among accessions. The number of flowers in the axil affects fertilization, the number of capsules and efficiency. The accessions were studied in terms of capsule locule number and in 103 of the accession the locule number was 4, while in 4 of the accession the number was determined to be 6. Capsule locule number is important in terms of affecting the number of seeds per capsule and thus affecting the seed yield. In terms of the number of nodes in each capsule variability was observed in the accession, 93 of them were with a single capsule, while 14 had multiple capsules. This feature has a positive effect on seed yield per plant and seed yield per hectare. In terms of capsule form variation was observed in the accession, and in 75 of the accession the capsule had narrow rectangular shape, while 30 were large rectangle type and 2 were observed to have conical shape. In terms of capsule

hairiness in the accession, variation was recorded. 89 of the accession had little hairiness, 8 of them had medium hairiness, 6 of them were hairless, while 4 of them were very hairy. Capsule hairiness is a desired feature as it increases tolerance against plant diseases, pests and drought. Variation was seen in the accession in terms of capsule-beak shape, and 76 of the accession had long beak, 30 were found to have short beak and 1 had bent beak. In respect to capsule cracking, 96 of the accession was totally cracked, and in 10 of the accession partial cracking was observed, while 1 showed no cracking. Capsule cracking is not preferred because of lack of spillage during harvest for prevention of loss of productivity and compliance with machine harvest. Seed coat color varied from white to various shades of brown grey to black or red. Seed coat color is a feature for determining the direction of the fat and protein content of seeds. Variation in the accession was also determined with respect to Seed coat, and 48 of the accession were light brown, 27 medium brown, 13 dark brown, 12 beiges, 5 olive-black and 2 of them were observed to be matt black (Table 2).

The relations between quantitative traits

Correlation coefficients of quantitative traits of sesame accessions are given in Table 3. A significant and positive relationship was found between the first capsule height and plant height, harvest index and number of capsules, capsule width and length of the capsule, 1000 seed weight and seed yield, 1000 seeds weight and harvest index, and grain yield and harvest index. As for the relation between the first capsule height and the number of capsules, and the first capsule height and harvest index it was found significant but negative. Our results agreed with similar positive correlation observed by, Uzun and Cagirgan (2001) and Sumathi *et al*., (2007).

Principal component analysis(PCA) revealed that 25% or more of the total variation, might be explained by the first two or three-axis. PCA offers reliable cluster analysis (Mohammadi and Prassana, 2003). The number of seeds per capsule contributed highest towards the divergence followed by number of capsules per plant (Sudhakar *et al*., 2006). Seed yield, number of capsules per plant, plant height and 1000 seed weight are the important contributing factors (Solanki and Gupta, 2002). There was a positive relationship between number of seeds per capsule, capsule number per plant and seed yield per plant. Studies by Majumdar *et al.,* (1987) and Reddy and Haripriya (1992) also reported a highly significant positive correlation between number of seeds per capsule, number of capsules per plant and seed yield per plant. This study showed a positive relationship between plant height, number of branches and seed production. These results were in agreement with previous observations by Gupta and Gupta (1977) and Pathak and Dixit (1992) who reported a positive relationship between plant height, capsules per plant and seed yield.

Conclusions

When the frequency of genotypes in the accession was analyzed, it was found that there is variability in terms of the criteria discussed. Regarding the relationship between the quantitative traits, the number of capsules, 1000 seed weight and harvest index were determined to significantly affect the seed yield. Clustering methods are based on a phenotypic characterization of traits on accessions from which the core collection is to be selected (Hintum,1995). Varieties with high yield potential can subsequently be combined with improvements of others traits such as oil content, plant height, synchronous flowering, synchronous ripening and resistance to pests. This study is crucial in terms of having more reliable results.

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Table 1. Morphological descriptors of qualitative traits recorded on 107 sesame accessions.

Qualitative traits	
Trait	**Definition**
Stem hairiness	0: Hairless. 3: Little Hairy. 5: Medium Hairy. 7: Shaggy
Basal leaf margin	1: Flat. 2: Sawtooth. 3: Toothed Comb
Lobe incision of basal leaf	1:Flat. 2: Few Pieces. 3: Medium Pieces. 4: Three or More Lobe
Leaf hairiness	0: Hairless. 3: Little hairy. 5: Medium Hairy. 7: Shaggy
Flower color	1: White. 2: White Light Pink Shaded. 3: Dark pink shaded white. 4: Pink. 5: Open Violet. 6:Dark Violet. 7: Purple. 8: Red. 9: Maroon. 99: (Other)
Flower hairiness	0: Hairless. 3: Little Hairy. 5: Medium Hairy. 7: Shaggy
Stigma length	1: Short (stigma below anthers). 2: Medium (stigma anther level). 3: Long (stigma anther above)
Number flower per leaf axil	1: One. 2: More than one
Number of lobe capsule	Capsule On the main stem; 1: Four. 2: Six. 3: Eight. 4: Mix
Number capsule of per axil	1: single Capsule. 2: Very Capsules
Capsule shape	1: conic. 2: Narrow Rectangle. 3: Large Rectangle. 4: Square
Capsule hairiness	0: Hairless. 3: Little hairy. 5: Medium Hairy. 7: Shaggy
Type of capsule beak	1: Short. 2: Long. 3: Inclined. 4: Separate. 99: Other
Capsule dehiscence at ripening	Maturation period; 1: No Cracking. 2: Partly Cracking. 3: Fully Cracking
Seed coat color	1: White. 2: Cream. 3: Dark Cream 4: Light Brown. 5: Medium Brown. 6: dark Brown. 7: Brick Red. 8: Bronze. 9: Olive color Black. 10: Grey. 11: Matte Black. 12: Bright Black. 99: Other

Table 2. The frequency distribution of qualitative traits of 107 sesame accessions.

Qualitative traits	Index	Number	Frequency (%)
Stem hairiness	0	88	0.82
	3	18	0.17
	5	1	0.01
Basal leaf margin	1	102	0.95
	2	3	0.03
	3	2	0.02
Lobe incision of basal leaf	1	103	0.96
	2	4	0.04
Leaf hairiness	0	102	0.95
	3	5	0.05
Flower color	1	11	0.10
	2	76	0.71
	3	20	0.19
Flower hairiness	3	14	0.13
	5	74	0.69
	7	19	0.18
Stigma length	1	74	0.69
	2	33	0.31
Number of flowers per leaf axil	1	94	0.88
	2	13	0.12
Number of carpels per capsule	1	103	0.96
	2	4	0.04
Number capsules per leaf axil	1	93	0.87
	2	14	0.13
Capsule shape	1	2	0.02
	2	75	0.70
	3	30	0.28
Capsule hairiness	0	6	0.06
	3	89	0.83
	5	8	0.07
	7	4	0.04
Type of capsule beak	1	30	0.28
	2	76	0.71
	3	1	0.01
Capsule dehiscence at ripening	1	1	0.01
	2	10	0.09
	3	96	0.89
Seed coat color	3	12	0.11
	4	48	0.45
	5	27	0.25
	6	13	0.12
	9	5	0.05
	11	2	0.02

Table 3. Correlation coefficients among 10 quantitative traits in sesame accessions.

	PH	FCH	NPB	NC	CL	CW	SN	1000 SW	HI
FCH	**0.6746**								
NPB	-0.1142	0.0285							
NC	-0.2779	**-0.3260**	-0.0495						
CL	0.1489	0.0419	-0.1008	0.0981					
CW	0.2212	0.1782	-0.0349	0.0427	**0.3811**				
SN	-0.0388	-0.0956	0.0026	0.0826	-0.1016	-0.1194			
1000 SW	0.0888	-0.1554	-0.0637	0.1569	0.0933	-0.0011	-0.0014		
HI	-0.0022	**-0.2620**	-0.0765	**0.3541**	0.1990	-0.0689	0.0862	**0.5471**	
SY	0.2040	-0.1489	0.2105	0.1198	0.2176	0.0382	-0.0118	**0.3270**	**0.471**

PH: Plant Height, **FCH:** First Capsule Height, **NPB:** Number of Primary Branches, **NC:** Number of Capsules, **CL:** Capsule Length, **CW:** Capsule Width, **SN:** Seed of Number, 1000 **SW:** 1000 Seed Weight, **HI:** Harvest Index, **SY:** Seed yield

* significant at $P < 0.05$, ** - significant at $P < 0.01$. For quantitative traits, Pearson pairwise correlation coefficients were calculated.

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Review Article

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Helicoverpa Resistant Chickpea Plants: From Bt Toxins to Plant-Mediated RNAi

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ABSTRACT

Helicoverpa armigera, the pod borer is a major constraint to global chickpea production. Genetic improvement of chickpea for insect resistance by traditional methods has been hampered by narrow genetic diversity in the elite gene pool. *Bacillus thuringiensis* (Bt) chickpea plants expressing Bt genes as well as pyramids also have been developed already and many are in field trials. But, already available Bt crops like cotton have increased the insect resistance to transgenic plants in *H. armigera.* Although Bt chickpeas have yet to be commercialized, but the sustainability of Btcrops is vulnerable to the insect resistance in *Helicoverpa*. The next generation approach for crop protection against *Helicoverpa* is to knock down the crucial physiology-related genes of insect pests using transgenic plants, which is called Plant-mediated RNAinterference (RNAi). Common small interfering RNAs (siRNAs) for the target genes of *H. armigera,* designed *in silico* could be used to study the lethal effect of down-regulating crucial target genes in chickpea. This review describes the progress of developing resistance to *H. armigera* in chickpea using Bt toxin genes and the future prospects of using plant-mediated RNAi for *H. armigera* resistance. The plant-mediated RNAi approach holds great promise for future development but further studies will be required to optimize RNAi-based strategies for chickpea protection against *H. armigera* using integrated pest management strategies.

Keywords: Chickpea, Bt toxin, Plant-mediated RNAi, siRNA, *Helicoverpa*.

Introduction

Chickpea (*Cicer arietinum* L.), a self-pollinating diploid and world's second most widely grown annual legume crop. Chickpea production is of prime importance to world food security and in diversifying the cereal-based cropping system, owing to its capacity for symbiotic nitrogen fixation (Jukanti *et al.*, 2012). Chickpea is also a good and cheap source of protein for people in developing countries (Gaur *et al.*, 2012). Globally, chickpea is grown in an area of 13.6 mha; producing 13.1 mt with an average yield of about 0.96-ton ha^{-1} (FAOSTAT, 2013). India is the largest chickpea growing country; with 9.6 mha of chickpea grown area and producing 8.8 mt chickpeas with an average yield of about 0.92ton ha^{-1} (FAOSTAT, 2013). There is growing interest in chickpea consumption and increased global demand but chickpea production has increased slowly at an annual rate of 1.3% in the past 20 years (Rao *et al.*, 2010).

The most intractable impediments to global chickpea production are *Helicoverpa*, aphids, bruchids, weeds, drought, salinity, and low methionine content in the seeds (Acharjee & Sharma, 2013a). Gram pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is the most devastating insect pest to chickpea production, which causes severe pod damage and yield failure (ICRISAT, 1992; Yadav *et al.*, 2006). The pod borer is widely distributed throughout the world and has facultative diapauses, which enables them to survive adverse weather conditions.

The larvae feed directly on the pod, causing seed abortion and damage, thereby having the potential to cause major crop losses (Giri *et al*., 1998) (Fig 1). However, there is still no strong resistance which has been identified for *H. armigera* in chickpea cultivars. Therefore, there is urgent need of transgenic chickpea resistant to *H. armigera* to boost production and productivity (Acharjee & Sharma, 2013b). This review will focus on the progress and current status for developing resistance to *H. armigera* in chickpea using Bt toxin genes and the future prospects of using molecular tools like RNAi for plant mediated insect resistance in chickpea.

Conventional approaches for Helicoverpa resistance in chickpea

The conventional breeding approaches and chemical control measures have been useful to create improved chickpea varieties diseases resistance like *Ascochyta* and *Fusarium* but are limited to a certain extent only for insect pests (Acharjee and Sarmah, 2013a). Chemical pesticides are commonly used to control pod borers in chickpea, but unfortunately, extensive and indiscriminate use has resulted in the development of resistance, environmental degradation (Armes *et al.*, 1992). The use of microbial pathogens and biopesticides such as Bt-products have shown some potential to control *H. armigera*, but the high production costs make them uncompetitive compared with the synthetic insecticides (Romeis *et al.,* 2004). Although, the wild relatives, *C. judiacum*, *C. bijugum* and *C. pinnatifidum* have significant levels of resistance to *H. armigera* (Sharma *et al.*, 2005), but these wild relatives are post-zygotic cross incompatible with the cultivated chickpea germplasms (Mallikarjuna, 2001). So, the genetic improvement for insect resistance has been hampered by the limited genomic resources and the narrow genetic diversity in the gene pool of chickpea have hampered breeding for protection (Varshney *et al.*, 2010; Acharjee & Sharma, 2013b). The biotechnological interventions like genetic transformation are likely to improve *H. armigera* resistance in chickpea (Acharjee and Sarmah, 2013a).

Approaches to the generation of transgenic crops using modern genetic transformation technology to incorporate insect resistance have proven suitable for many cultivated crops. *H. armigera* can be effectively controlled by using δ-endotoxin from Bt in transgenic plants, which is very well demonstrated in widely cultivated Bt crops like cotton (James, 2014). The Crystal Insecticidal Protein (CIP) toxins expressed in plants interacts with the mid-gut epithelium receptors and causes an ionic imbalance to break the mid-gut cells and insect death (Schnepf *et al.*,1998; Bravo *et al.*, 2007). The Bt transgenic plants provide a relatively long lasting and seed borne solution for the management of Lepidopteran pests (Tabashnik *et al.*, 2003). Genetic transformation with δ-endotoxin genes from the bacterium *Bacillus thuringiensis* Berliner have been deployed as a means to enhance crop resistance to the insect in several crops for pest management (Sharma *et al*., 2002; James, 2014). The Bt toxins are toxic to lepidopteran pests and non-toxic to humans and animals, which makes Bt crops are one of the most successful plant transgenic technology (BANR, 2000; Cohen *et al.*, 2000). The Bt crops which were commercialized since 1996, have revolutionized the insect pest management strategies and been widely accepted by small and resource-poor farmers and have achieved significant success economically and ecologically in the world (Zhu *et al*., 2012). The area under Bt crops has increased significantly and contributed to more sustainable crop production systems (James, 2014).

Genetic transformation: Bt chickpeas

Genetic improvement by molecular breeding is limited in chickpeas due to their sexually incompatible gene pool of wild relatives (Acharjee and Sarmah, 2013a). Genetic transformation to develop transgenic chickpea expressing toxin genes for various versions of Bt insecticidal genes has been carried out and found to confer resistance to pod borers in the laboratory bioassays (Devi *et al.*, 2011; Acharjee and Sarmah, 2013b). Commercial Bt chickpea lines with resistance to *Helicoverpa* are under development (Sanyal *et al.* 2005; Acharjee *et al.,* 2010; Mehrotra *et al.*, 2011;Asharani *et al.*, 2011; Khatodia *et al*., 2014; Ganguly *et al.,* 2014) which have not yet been released. The first report of successful genetic transformation of chickpea using Bt *cry1Ac* gene came in 1997 (Kar *et al.*, 1997) and thereafter, various research groups initiated genetic transformation of chickpea using *cry1Ac* gene and reported generation of transgenic Bt chickpeas (Sanyal *et al.*, 2005; Indurker *et al.*, 2007; Biradar *et. al.*, 2009) (Table 1). Neelima *et al.*,(2008) presents a non-tissue culture-based *in planta* transformation strategy to generate transgenic plants in chickpea with *cry1AcF* gene using *Agrobacterium*-infected young seedlings. Acharjee *et al.*, (2010) used *cry2Aa* gene to facilitate pyramiding with existing *cry1Ac* chickpea lines. The pyramided transgenic chickpea lines exhibited high levels of *Cry2Aa* and *Cry1Ac* protein and conferred high (98-100%) levels of mortality to *Helicoverpa* larvae in the insect bioassays (Acharjee *et al.*, 2010).

Mehrotra *et al.*, (2011) also generated pyramided *cry1Ac* and *cry1Ab* genes in chickpea. A new synthetic construct *cry1X* was also used forinsect resistance chickpea using *in planta* transformation (Asharani *et al.*, 2011). Ganguly *et al*., (2014) used fused *cry1Ab/Ac* construct to develop different transgenic lines of chickpea expressing constitutively and pod specifically for resistance against *Helicoverpa.* Khatodia *et al*., (2014a & 2014b) developed Bt chickpea plants carrying *cry1Aa3* and *cry1Ac* gene using direct seed *Agrobacterium*-mediated transformation which works without the involvement of any tissue culture procedure and does not require the complex steps for selection of the transgenic events.

Gene pyramiding by incorporating two or more genes may be a more efficient way of enhancing and broadening insect resistance of plants (Li *et al*., 2015). One of the major concerns regarding the development of the transgenic plant is need of expressing high dose of Bt toxin, which can sustain the insect resistance. But the transgenic chickpea lines that showed appreciable levels of expression of Bt toxin were found to exhibit phenotypic abnormalities and these abnormalities ranged from extreme retardation in the growth of the plant to no flowering, and no setting of seeds (Rawat *et al.*, 2010; Acharjee *et al.,* 2010; Khatodia *et al*., 2014). Such observations in chickpea plants have been probably overlooked earlier; however phenotypic and developmental abnormalities with the *cry1Ac* gene have been reported in tobacco (Rocher *et al.*, 1998; Barton *et al.*,1987). A significant reduction in the growth rate and seed production in chickpea lines expressing high levels of Bt toxin when compared to the parental line (Acharjee *et al.*, 2010; Khatodia *et al*., 2014). The high level of Bt toxin protein was causing growth reduction in chickpea. Although, the reasons for this detrimental effect of Bt toxin need to be analyzed.

Field-evolved resistance to Bt crops

The commercialization of transgenic Bt chickpeas containing a single Bt transgene may not give adequate yield advantage, as *H. armigera* is evolved with increased resistance.The widespread use of Bt toxins has prompted concerns that insects might someday become resistant to this important treatment, which can reduce the effectiveness of Bt transgenic crops (Tabashnik *et al*., 2013). Resistance is a genetic change in the insect pest that allows it to avoid harm from Bt toxins. Although the high and consistent levels of toxin production in the Bt plants make them much less favorable for the development of resistance. The laboratory populations of *Cry1A*-resistant Diamond Black Moth have been shown to be able to survive on high levels of Cry1Ac toxin (Tabashnik, 2003). There were no cases of insects developing resistance to Bt transgenic plants in the field initially. The frequency of resistant alleles has increased substantially because of failure to provide adequate refuges of non-Bt cotton and that there is field-evolved *Bt* toxin resistance in bollworm (Tabashnik, 2008). Intensive cultivation of Bt crops has increased field evolved pest resistance to transgenic plants in *H. armigera* in India, China, and Pakistan (Tabashnik *et al*., 2009; Alvi *et al*., 2012; Zhang *et al*., 2013). The field-evolved resistance in *H. armigera* has reduced the efficacy of Bt crops for pest resistance (Tabashnik *et al*., 2013). So, the transgenic crops expressing pyramided two or more Bt toxins to combat the same insect pest have been widely used now to delay the evolution of pest resistance (Carrière *et al*., 2015). But field-evolved resistance and cross-resistance in transgenic plants expressing two different types of Bt toxins has been discovered (Gassmann *et al*., 2014). The insect survival on currently used pyramids is often higher for both susceptible insects and insects resistant to one of the toxins in the pyramid (Carrière *et al*., 2015). The increased resistance to Bt plants suggests that the current approaches for managing Bt resistance should be replaced by new integrated pest management strategies in order to develop the sustainable resistance.

Plant-mediated RNAi for Helicoverpa resistance in chickpea

The insect resistant transgenic Bt plants have been successful to reduce yield loss and pesticide utilization in the past three decades. The potential of using plant-mediated RNAi induced by double-stranded RNAs targeting pest genes came up as a new strategy against coleopteran and lepidopteran pests resistance in crops (Zhu *et al*., 2012). Therefore, down-regulating the crucial physiology-related genes by using specific double-stranded RNAs to induce RNAi in insects, is a key in pest control, which is paving the way for next generation of insect-resistant transgenic crops (Price and Gatehouse, 2008; Huvenne and Smagghe, 2010). The concept of plant-mediated RNAi was first introduced by silencing a cotton bollworm P450 monooxygenase gene, which impairs larval tolerance of gossypol in *H. armigera* (Mao *et al*., 2007). Insect P450 monooxygenase, *CYP6AE14* play a central role in adaptation to plant defense compounds and in developing insecticide resistance (Mao *et al*., 2007). Mao *et al*., (2007) developed transgenic tobacco and Arabidopsis plants expressing double stranded RNA (dsRNA) directed against a detoxification enzyme *CYP6AE14*, which increased the sensitivity to

gossypol leading to mortality. A report of generation and analysis of *CYP6AE14*, dsRNA-expressing cotton plants by Mao *et al*., (2011) showed drastically retarded growth of bollworm larvae and less damage to the transgenic plants. The deleterious effects of RNAi will magnify if multiple genes involved in the P450 complex were targeted (Mao *et al*., 2011). Another target gene for the cotton bollworm RNAi is *CYP6B6*, which is expressed in the fat baby and midgut of the lepidopteran pest, lead to reduced resistance to pyrethroids and other toxic substances (Zhang *et al*., 2013). *HaHR3*, a molt-regulating transcription factor gene, of cotton bollworm has been used as the target gene for plant-mediated RNAi in transgenic tobacco plants resulting in developmental deformity and larval lethality (Xiong *et al*., 2013).

The plant-mediated RNAi technology often results in a mild enhancement of insect resistance (Price and Gatehouse, 2008). Two key steps of plant-mediated insect RNAi are the production of effective forms of dsRNAs in plants and spreading of these silencing molecules into gut cells of insect (Mao *et al.*, 2013). The first barrier to the food components is a midgut peritrophic matrix (PM) layer that prevents large molecules and toxins from entering into midgut cells (Hegedus *et al*., 2009). The plant cysteine proteases could increase the PM permeability and used to improve the plant-mediated RNAi against herbivorous insects (Mao *et al.*, 2013). Expression of dsRNA and protease in the plant provides a better protection as ingestion-mediated RNAi effect against herbivorous insects (Mao *et al.*, 2013).

The nucleotide variations of the dsRNA of target genes in different ecotypes of the target pest, necessitate selection of a highly conserved, off-target, minimized sequence for effective gene silencing using plant-mediated RNAi. The potential insecticidal siRNAs designed *in silico* for *H. armigera* control could be used for crop resistance by synthesizing a plant and delivering a dsRNA (Choudhary and Sahi, 2011). Asokan *et al*., (2012) designed an off-target minimized region for dsRNA synthesis and *in silico* analyzed the nucleotide variationsto design common siRNAs that could be further utilized for downstream applications for *H. armigera*. The effect of diet delivered various concentrations of dsRNA in silencing genes of *H. armigera* revealed that multiple applications of dsRNA resulted in early and persistent silencing of genes (Asokan *et al*., 2013). The chymotrypsin and jhamt were shown to be suitable candidate genes that could be utilized for RNAi-mediated management of *H. armigera* (Asokan *et al*., 2014). Although, the lethal or highly detrimental effects of down-regulating the crucial target genes of *H. armigera* by plant mediated RNAi for resistance in chickpea is yet to be studied. But the accelerated emergence of Bt resistance in *H. armigera* requires plant-mediated RNAi for pod borer resistance in chickpea, which is an alternative tool paving the way for next generation of insect-resistant transgenic crops (Gordon and Waterhouse, 2007).

Future prospects

The sustainability of Bt transgenic crops is already threatened by the accelerated emergence of insect resistance in *Helicoverpa* and Bt chickpea plants have yet to be commercialized. The plant-mediated RNAi have been demonstrated in cotton and tobacco plants using the dsRNA for the target genes (Mao *et al.*, 2007; Xiong *et al*., 2013; Mao *et al.*, 2013; Zhang *et al*., 2013). Therefore, the plant-mediated RNAi using the *in silico* designed siRNA targeting the insect genes may prove to be a very good approach for chickpea plants protection to pod borer. Common high potential insecticidal siRNAs for the target genes of *H. armigera,* designed *in silico* by analyzing the nucleotide variations of the dsRNA of target genes in different populations of the target pest are available for implementation as a pest management strategy in chickpea (Asokan *et al*., 2012). Moreover, the siRNA also reduces the biosafety concerns, being absent in higher eukaryotes, having low off target similarity (Asokan *et al*., 2012). Further, the high expression of Bt toxins in the plants will directly put a great load on the protein production machinery which will ultimately affect the quality and quantity of the Bt crops in terms of growth and development (Rawat *et al.*, 2010; Acharjee *et al.*, 2010; Khatodia *et al*., 2014). Instead, the expression of the dsRNA in the plants to combat the insect will not cause any load on the protein production machinery, which is in particular very important point for chickpea, which is the good and cheap source of proteins with high protein content in seeds.

We propose that the lethal or highly detrimental effect of down-regulating crucial target genes like CytP450 (involved in detoxification of allelo chemicals), *HaHR3* (molt-regulating transcription factor gene) and chymotripsin (involved in digestion of proteins) of *H. armigera* by plant mediated RNAi for resistance in transgenic chickpeas could be studied in future. Transgenic chickpea plants expressing dsRNA will provide the insight of detrimental effects of down-regulating crucial target genes of *H. armigera* by plant mediated RNAi for resistance in chickpea. This strategy could be taken to further advancement for field evaluation and utility in integrated insect pest management.

Conclusions

Genetic improvements of chickpea for *H. armigera* resistance by molecular breeding approaches are limited due to their sexually incompatible gene pool and insufficient to meet up the challenges of the present agricultural state (Varshney *et al*., 2010; Acharjee and Sharma, 2013a). The commercialization of transgenic Bt chickpeas containing a single Bt gene for *H. armigera* resistance may not give adequate yield advantage. This review documents that transgenic chickpeas generated with combinations of suitable genes and approaches like Bt and RNAi is required for protection from *H. armigera* damage in chickpea. The evidence suggests that transgenic plants expressing dsRNA targeting insect-associated genes are able to improve pest resistance. The plant-mediated RNAi approach allows a wide range of potential targets for suppression of gene expression in the insects and holds great promise for future development. So, feeding *H. armigera* with chickpea expressing dsRNA to trigger RNAi could find applications in field control of this insect pest. There is a need for further studies to optimize plant-mediated RNAi for chickpea protection against *H. armigera*. The integrated pest management strategies would require the use of, not only novel Bt transgenics, Plant-mediated RNAi, but also the modern biotechnological tools like targeted CRISPR/Cas-mediated plant genome editing for chickpea protection against the *H. armigera* (Khatodia *et al.,* 2016).

Figure 1. Typical symptoms of *Helicverpa armigera* infestation on chickpea plants. Showing the different stages of larval feeding on leaves and pods, which causes damage and seed abortion respectively, thereby causing major crop losses.

Table 1. The list of the various chickpea transformation made for insect resistance using Bt toxins against *Helicoverpa* pest.

Chickpea Type	Transformation Method	Explants Used	Bt Toxins	Promotors	Selectable Markers	Bt toxin Expression	Stable Integration	Lab Bioassay Mortality	Ref.
ICCV-1, ICCV-6	Biolistic Gene delivery	shoot apex	*Cry1Ac*	CaMV35S	*nptII*	0.004–0.0045%	Yes	72.6% wt reduction	Kar *et al*., 1997
C 235, BG 256, Pusa 362, Pusa 372	*Agrobacterium*	cotyledonary nodes	*Cry1Ac*	CaMV35S	*nptII*	14.5 to 23.5 ng/mg	Yes	>80% mortality	Sanyal *et al*., 2005
ICCC37, PG-12	Biolistic Gene delivery	Epicotyl	*Cry1Ac*	CaMV35S	*nptII*	6 to 20 ng/mg	Yes	13.3-56.6% survival	Indurkar *et al*., 2007
	In planta *Agrobacterium*	apical meristem	*cry1AcF*	CaMV35S	*nptII*	2.06-9.70 µg/g	No	-	Neelima *et al*., 2008
Semsen, ICCV 89314	*Agrobacterium*	embryonic axis	*Cry2Aa*	ats1A	*nptII*	-	Yes	20-98% mortality	Acharjee *et al*., 2010
KAK-2	In planta *Agrobacterium*	embryo axes	*Cry1X*	CaMV35S	*nptII*	0.257-10.77 µg/g	No	49.6% mortality	Asharani *et al*., 2011
P-362	*Agrobacterium*	embryonic axis	*Cry1Ac*	CaMV35S	*nptII*	116 ng/mg	Yes	100% mortality	Mehrotra *et al*., 2011
P-362	*Agrobacterium*	embryonic axis	*Cry1Ab* and *Cry1Ac*	CaMV35S and Pcec	*nptII*	5–40 ng/mg	Yes	86-100% mortality	Mehrotra *et al*., 2011b
C-235	In planta *Agrobacterium*	Seeds	*Cry1Aa3*	CaMV35S	*nptII*	0.091-0.154 µg/g	Yes	55-77% wt reduction	Khatodia *et al*., 2014a & 2014b
C-235, HC-1	In planta *Agrobacterium*	Seeds	*Cry1Ac*	CaMV35S	*nptII*	0.106-0.364 µg/g	Yes	48-75% wt reduction	Khatodia *et al*., 2014 a & 2014b
DCP 92-3	*Agrobacterium*	embryonic axis	Fused *cry1Ab/Ac*	soybean P*msg* and rice *actin1*	*hpt*	4–19 ng/mg	Yes	67-100% mortality	Ganguly *et al*., 2014

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Molecular Characterization of Some Triticale Cultivars in Turkey

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ABSTRACT

Triticale has gained importance as an alternative crop to solve the nutritional problems of the rapidly increasing world population. Triticale gains the yield potential of durum wheat and adaptation of rye to cold, drought and marginal soil conditions in itself. It is also known that triticale is resistant to many diseases and pests. In this study, it was aimed to determine the genetic characterization of some spring and facultative triticale varieties registered in Turkey with the ISSR-PCR technique which is one of the molecular marker methods. Polymorphic and monomorphic band images were formed from 16 primers as a result of analysis of 20 primers in 5 registered triticale isolates, and 71.1% polymorphism was obtained. Four primers did not show any amplification in the triticale cultivars. Triticale cultivars were mainly divided into three groups. Alperbey and Tacettinbey which were in the first group, differed by 28% and Mehmetbey and Ayşehanım in the second group differed by 37%. In addition, EgeYıldızı in the third group differed 48% from Alperbey, 45% from Tacettinbey, 49% from Mehmetbey and 38% from Aysehanım. The genetic distance values between cultivars of triticale ranged from 0.509 to 0.712. These results obtained from ISSR-PCR method will provide significant contributions to the triticale breeding programs.

Keywords: Triticale, molecular characterization, polymerase chain reaction, PCR, ISSR , Inter-Simple Sequence Repeat

Introduction

Cereals have the most acreage and production among the cultivated plants in the world. Cereals provide approximately 50% of the annual protein consumed by humans and animals (Çölkesen, 1994). Cereals and their products have an important place in human nutrition in the world. Wheat, corn, rice, barley, and rye are the most produced cereals in the world. The food supply and nutrition has been the most important issue throughout history for humanity.

Increasing the productivity in agriculture is obligatory because agricultural land and the natural sources are limited although the world population is increasing. For this reason, the scientists have looked for the solution to develop new plants, species and varieties which can survive under extreme conditions and can give high yield even under stress conditions. The first important research for this aim was made in Scotland in 1875 to obtain a cereal which can be grown in different geographical and climatic conditions, can withstand cold, drought and acidic soils, has also the yield and quality potential of wheat. Triticale *(X. Triticosecale* Wittmack) was developed as a result of these researches (Müntzing, 1979).

Triticale which possesses the yield potential of durum wheat and adaptation of rye to cold, drought and marginal soil conditions, has gained importance as an alternative crop to solve the nutritional problems

of the rapidly increasing world population. . It is also known that triticale is resistant to many diseases and pests (Varghese *et al*., 1996). New varieties have been continued to develop for improving the properties of triticale. Since the genetic basis of amphidiploid triticale does not have a very wide variation, sometimes varieties developed rely on a similar genetic basis, which limits genetic advance. Molecular markers are one of the most reliable methods used in the characterization of genotypes and varieties in recent years.

Genetic diversity is the main source for breeding programs. Conducting the breeding studies as based on a broad genetic basis will ensure maintaining genetic diversity during to long-term as well as adaptation to changeable environmental conditions (Dirik H.,1997). In addition, increasing the genetic diversity is important to develop cultivars resistant to plant diseases and pests (Hajjar *et al*., 2008). However, it is essential to choose the most effective method to determine genetic diversity. For this purpose, DNA markers can be used safely, especially in plant breeding and gene mapping studies (Reddy *et al*., 2002).Identification of the DNA markers belonging to plant species and creating genomic maps of these plants will facilitate to develop new varieties in short time, will save time by shorten the breeding period and will provide reliability and convenience in the selection process (Michelmore *et al*., 1991). In recent years, specific sequence differences between two or more individuals could be identified with molecular marker techniques, Besides, developing a detailed genetic map of marker linked to a trait under study is an important tool for increasing the breeding efficiency. Therefore, genome maps of many crops which have agricultural importance have been established (O'Brien, 1993).In this study, genetic differences among spring triticale varieties registered in Turkey were determined by ISSR-PCR method. Important cultivar based on genetic variation were identified for further breeding programme.

Material and Methods

DNA Isolation

In this study, five spring and/or facultative triticale (X. *Triticosecale* Wittmack) cultivars (Alperbey, Aysehanim, EgeYildizi, Mehmetbey, Tacettinbey) were used to determine the genetic distance/ similarity. Twenty-five seeds of each of the Triticale cultivars were germinated by placing seeds in 9 mm petri dishes lined with filter papers and kept in growth cabin (Binder KBW 400). The germinated triticale seeds were planted in pots which were maintained in controlled greenhouses. Then, plants were harvested after having 3-4 tillers and DNA isolation were done according to modified Doyle and Doyle (1987) method by using the samples obtained from young leaves.

Firstly, the amount of isolated DNA was determined by spectrophotometer (Thermo Scientific Nanodrop 2000c) since equal amounts of DNA from each sample would be used in the PCR reaction. Then, the DNA samples were diluted with TE (Tris-EDTA) buffer according to amount of DNA calculated from the OD results for PCR analysis of ISSR primers.

ISSR Amplification

ISSR protocol developed by Zietkiewicz *et al.,* (1994) was used for the amplification of DNA obtained from Triticale cultivars. The volume was kept similar in each probe as 25 microliter amplification reaction solution; 75 mM Tris-HCl, pH=8.8, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 100 mM dATP, 100 mM dTTP, 100 mM dGTP, 100 mM dCTP, 0.2 mM primer, 1.0 unit Taq DNA polymerase and 10 ng DNA. The binding temperature was adjusted (50-55 °C) according to the Tm temperatures of the primers. Generally, temperatures above 2-3°C of the Tm of the primers gave favourable results in terms of amplification. Then, the primers were incubated at 70-75°C for amplification with Taq polymerase (Palumbi *et al*., 1991). Temperature and cycles adjusted in that: Preliminary denaturation during 2 min at 94 ºC, denaturation for 36 cycles during 1 min at 94 ºC, adhesion of primers to DNA during 1 min at varying Tm according to primers, primer elongation during 2 min at 72 ºC and last primer elongation during 10 min at 72 ºC. The reactions were performed eppendorf tubes using Eppendorf Mastercycler Pro S PCR System.

PCR products were analyzed on a 2% agarose gel prepared in 0.5 X TBE (Tris / Borate / EDTA), in an electrophoresis (ThermoMidicell Promo) tank containing 0.5 X TBE buffer, at 100 V during 2-3 hours. After electrophoresis, the DNA was stained with ethidium bromide during 20 minutes in order to visualize and evaluate the DNA. Emerging DNA bands examined under UV light (VilberLourmat) and then they were photographed on a Kodak Gel Logic 200 Imaging System.

Evaluation of the Bands

PCR results were evaluated taking into account the gel electrophoresis views of the ISSR-DNA bands formed. ISSR amplification products were evaluated as having (1) or absent (0), and the obtained data were analyzed in IBM SPSS 20 (SPSS) statistical package program. The genetic similarity index was calculated according to Jaccard. (Jaccard , 1912)

Based on the genetic distance matrix, a dendrogram was constructed using the Unweighted Pair Group Method (UPGMA).

Results and Discussion

Twenty different primers which have 17-18 base pair lengths were used in the study. Sixteen from them exhibited polymorphic bands and the other four primers were showed monomorphic band formation. Out of total ISSR-PCR 145 bands , 103 were polymorphic resulting from 16 primers (Table 1). When the bands were examined assigned to individual primers; it was seen clearly that Primer UBC 821 gave the most polymorphic band, Primer UBC 813 gave the least polymorphic band, primers UBC 813, UBC 826, UBC 827 gave the most monomorphic bands, and the primer UBC 821 gave the least monomorphic band (Table 1).

Triticale cultivars were mainly clustered in three groups. Alperbey and Tacettinbey took place in the first group, Mehmetbey and Aysehanım in the second group and EgeYıldızı in the third group respectively (Figure 1).

The genetic distance values between triticale cultivars ranged from 0,509 to 0,712. The highest genetic similarity was observed between Alperbey and Tacettinbey and followed them Mehmetbey and Ayşehanım with 0.627 (Table 2). The lowest genetic similarity was observed between Mehmetbey and EgeYıldızı while Ayşehanım in the same group as Mehmetbey were found more similar with EgeYıldızı (Table 2)

Conclusion

This study was concerned with assessing genetic distances/similarities among five Triticale cultivars using ISSR markers and genetic information related to triticale cultivars based on genetic similarities gained from Cluster analyzes. Totally 145 bands gained from 16 primers formed 103 polymorphic and 42 monomorphic bands. Maximum of polymorphic bands were formed by primer UBC 808 while most of monomorphic bands were formed by primers UBC 813, UBC826, UBC 827. From the three groups obtained, the first group contained Alperbey and Tacettinbey, the second group contained Aysehanım and Mehmetbey and the last group contained only EgeYıldızı. Alperbey and Tacettinbey were the most similar cultivars to each other genetically.

This study is important in terms of revealing DNA isolation method and PCR conditions that can be used in triticale. The information gained from this study may be used in further similar research. The ISSR technique can be used effectively in the determination of DNA polymorphism in the triticale and genetic characterization.

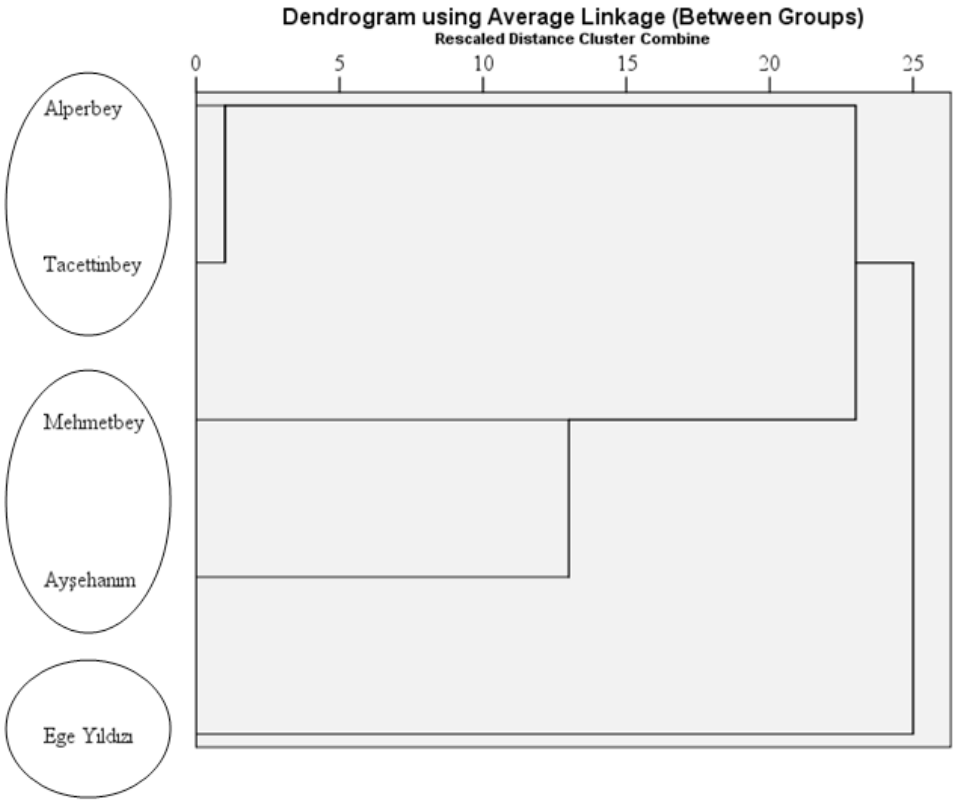
Table 1. Amount of total bands received from amplification of ISSR primers, number of polymorphic bands and polymorphism rate (%)

Primer Name	Number of Polymorphic Bands	Total Number of Bands	Polymorphism Rate (%)
UBC 808	14	16	87,5
UBC 809	-	-	-
UBC 810	4	8	50,0
UBC 812	6	9	66,7
UBC 813	4	9	44,5
UBC 818	4	6	66,7
UBC 820	4	**6**	66,7
UBC 821	8	8	100,0
UBC 822	-	-	-
UBC 824	3	5	60,0
UBC 826	3	8	37,5
UBC 827	5	10	50,0
UBC 829	-	-	-
UBC 830	10	13	77,0
UBC 834	11	13	84,7
UBC 835	5	7	71,5
UBC 840	11	12	91,7
UBC 843	-	-	-
UBC 844	5	6	83,4
UBC 847	6	9	66,7
TOTAL	103	145	71,1

Table 2. Similarity coefficient obtained from cluster analyses according to Jaccard.

	2:Tacettinbey	3:Mehmetbey	4:Ayşehanım	5:Ege Yıldızı
1:Alperbey	,712	,540	,523	,516
2:Tacettinbey		,610	,592	,549
3:Mehmetbey			,627	,509
4:Ayşehanım				,619

Figure 1. Dendograms of some spring and facultative triticale cultivars using ISSR Markers



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Regeneration of *Ruta graveolens* Transgenic Plants

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ABSTRACT

Here we report the construction of transgenic *Ruta graveolens* L. plants. The plants were obtained *via* direct shoot regeneration from leaves and petioles on Murashige and Skoog (MS) solidified medium supplemented with 6-benzyl-aminopurine (1.0 mg L^{-1}) and α-naphthylacetic acid (0,05 mg L^{-1}) in case of *A. tumefaciens*-mediated transformation as well as *via* direct shoot regeneration from rue "hairy" root culture on hormone free half-strength MS medium in case of *A. rhizogenes*-mediated transformation. Frequency of "hairy" root formation was up to 30%. All transgenic root lines were able to form shoots. Using of *A. tumefaciens* allowed to obtain transgenic rue plants with high frequency up to 100%. Indolil butyric acid addition to the medium in concentration 0.5 mg L^{-1} enabled transgenic plant rooting. The shoots regenerated from rue "hairy" root culture were more sensitive to the growth regulator addition and rooted in 2 weeks. Rooting of the shoots obtained after *A. tumefaciens*-mediated transformation was observed only in 6 weeks under these cultivation conditions.

Keywords: *Ruta graveolens* L., *A. tumefaciens, A. rhizogenes*, genetic transformation, plant regeneration, "hairy" root culture.

Introduction

Ruta graveolens L. is a medical plant of Rutaceae family. Numerous compounds of medical properties are known to be synthesized in rue plants. Rutin, alkaloids, essential oils and other chemicals were found in its aerial parts and roots (Orlita *et al*., 2008; Ekiert *et al*., 2005; França Orlanda *et al*., 2015). Rue extracts are characterized by antimicrobial (Ivanova *et al*., 2005; França Orlanda *et al*., 2015), antifungal (Meepagala *et al*., 2005), analgesic (Cunha *et al*., 2015) activity. The antioxidant and anti-inflammatory properties of *Ruta graveolens* L. were analysed earlier (Raghav *et al*., 2006). The study of its anti-tumor activity demonstrated that rue extracts were cytotoxic for lymphoma and carcinoma cells (Preethi *et al*., 2006). Ethanolic rue extracts were not toxic but caused the death of skin melanoma cells (Ghosh *et al*., 2015). So, *R. graveolens* plants could be used as a natural source for medical compounds production and may be used in pharmaceutical industry.

Biotechnological methods of plant improvement include construction of the transgenic plants of numerous species characterized by high level accumulation of valuable natural compounds (Sharafi *et al*., 2013; Zhang *et al*., 2014; Thiruvengadam *et al*., 2014; Majumdar *et al*., 2012). At the same time transgenic plants are able to synthesize the recombinant proteins of medical properties. So the transgenic plants are the promising source both of natural and recombinant biological active substances.

Transgenic plants construction was started at the end of 20th century. *Agrobacterium tumefaciens* and *A. rhizogenes* bacterial strains were used for this purpose. Plant transformation using *A. tumefaciens* strain resulted in direct (from plantlets) or indirect (from primarily formed callus tissue) shoot formation. Transgenic plants can also be obtained *via* shoot regeneration from "hairy" root culture initiated after *A. rhizogenes* – mediated transformation of plants. So, the shoot regeneration methods must be developed

both in case of *A. rhizogenes* or *A. tumefaciens* – mediated transformation. Different types of explants (leaves, roots, hypocotyls, petioles, stems) can be used for shoot regeneration. Methods based on using of growth regulators allow to obtain the regenerated shoots (Diwan *et al*., 2008; Ahmad *et al*., 2010).

A. tumefaciens-mediated transformation method was optimized for *R. graveolens* transgenic plants obtained *via* direct plant regeneration from hypocotyls (Lièvre *et al*., 2009). Transformation efficiency reached up to 22% in case of using for the transformation pTDE4 plasmid harboring genes encoding neomycin phosphotransferase and beta-glucuronidase. Hypocotyls, callus and shoots of *R. graveolens* were used for genetic transformation experiments but "hairy" root culture establishment appeared successful only after inoculation of hypocotyls with *A. rhizogenes* wild *strain (Sidwa-Gorycka et al., 2009).*

The aim of the present study was to obtain transgenic *R. graveolens* plants. We used *A. tumefaciens* GV3101 strain carrying plasmid *pCB124 (*Luchakivskaya *et al*., 2011*)* harboring target *ifn-α2b* gene coding human interferon-α2b synthesis as well the selective neomycin phosphotransferase II gene. We also used *A. rhizogenes* A4 strain for establishment of "hairy" root culture and obtaining of the regenerated transgenic rue plants.

Material and Methods

Plant material

Leaves and petioles of aseptic *Ruta graveolens* plants cultivated on half-strength Murashige and Skoog medium (1/2MS) (Murashige and Skoog 1962) were used for agrobacterial infection.

Determination of selective concentration of antibiotic kanamycin

To find out the optimal concentration of kanamycin for transformant selection we placed the shoots of *in vitro* rue plants on solidified ½MS medium with different concentrations of kanamycin (0–200 mg/l) ("Kievmedpreparat", Ukraine) and cultivated them in Petri dishes for 4 weeks. The medium with minimal concentration of kanamycin which inhibited growth of green plants was chosen as selective one.

Bacterial strains and plasmid vectors

A. tumefaciens GV3101 nopaline strain with the binary pCB124 vector was used for genetic transformation. The T-DNA of the vector contained the neomycin phosphotransferase selective *nptII* gene under the control of nopaline synthase promoter. The used vector carried the target *ifn-α2b* human interferon gene (pCB124 vector) driven by cauliflower mosaic virus *35S* promoter. We also used *A. rhizogenes* agropine A4 strain for rue genetic transformation and "hairy" root culture initiation.

The bacteria were cultivated overnight at 28°C in liquid LB medium (10 g/l of casein hydrolyzate, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2, 100 mg/l carbenicillin and 50 mg/L rifampicin) on rotary shaker (200 rpm). After overnight cultivation the bacterial cultures were resuspended in liquid MS medium (OD_{600} 0.5–0.6).

Genetic transformation, selection and regeneration of transformants

The petiole and leaf segments were inoculated by the bacterial suspension for 30 min and then transferred onto solidified MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BA), 0.05 mg/L α-naphthylacetic acid (NAA), 30 g/L sucrose, 0.7 % agar for direct shoot regeneration in case of *A. tumefaciens*-mediated transformation. The infected plantlets were transferred on the mentioned medium for 1-4 days period and then were cultivated on the same medium with 600 mg/L cefotaxime (Darnitsa, Ukraine) for bacteria elimination. The plantlets were put on MS medium supplemented with 1.0 mg/L BA, 0.05 mg/L NAA, 600 mg/L cefotaxime and 25 mg/L kanamycin after *A. tumefaciens*-mediated transformation for selection of transgenic plants. Regenerated shoots of 1.5–2 cm long were transferred onto 1/2MS medium supplemented with 25 mg/L kanamycin and 600 mg/L cefotaxime. The obtained shoots were cultivated at 24°C under 16-hour photoperiod.

In case of *A. rhizogenes*-mediated transformation the explants were cultivated on 1/2MS solidified medium for "hairy" root induction. The roots formed on the plantlets were transferred to hormone-free 1/2MS medium with 600 mg/L cefotaxime in 3-8 days. The same medium with the antibiotic in mentioned concentration was used for stimulation of transgenic shoot regeneration on "hairy" root culture.

The rooted plants were transferred into soil and grown in the greenhouse.

Molecular analysis of transgenic plants

Total plant DNA was extracted from leaves of the selected transformed and wild-type plants and also from the "hairy" root culture samples by CTAB-method (hexadecyl trimethyl ammonium bromide). The total agrobacterial DNA was extracted according to Draper *et al*., (1988). We used primers 5'-cctgaatgaactccaggacgaggca-3' and

5'-gctctagatccagagtcccgctcagaag-3' for amplification of 622 bp *nptII* gene fragment; primers 5′-ttgatgctcctggcacag-3′ and 5′-ttctgctctgacaacctc-3′ for amplification of 396 bp *ifn-α2b* gene fragment; 5'-atggatcccaaattgctattccttccacga-3' and 5'-ttaggcttctttcttcaggtttactgcagc-3' for amplification of 780 bp (*rolB*) using PCR method.

The amplification of the gene fragments was carried on under following conditions: *nptII* gene fragment amplification - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) - 3 min at 72 °C; *ifn-α2b* - 3 min at 94 °C - 30 cycles (30 s at 94 °C; 30 s at 60 °C; 30 s at 72 °C) - 3 min at 72 °C; *rolB* - 3 min at 94 °C - 33 cycles (30 s at 94 °C; 30 s at 56 °C; 30 s at 72 °C) - 3 min at 72 °C. All samples were fractionated in 1% agarose gel in TBE buffer.

Results

Determination of kanamycin selective concentration

Green shoots were formed if the medium contained less than 25 mg/l kanamycin. Whitening of shoots was observed in case of higher concentrations of antibiotic. Basing these data, we consider 25 mg/l kanamycin to be the best for selection of rue transformants.

Transgenic shoot regeneration after A. tumefaciens-mediated transformation

Earlier we demonstrated the possibility of high efficient shoot regeneration from leaves, roots, petioles and stems of *in vitro* cultivated *R. graveolens* plants (Matvieieva *et al*., 2015). For transgenic shoot regeneration after *A. tumefaciens*-mediated transformation we used the medium optimized for effective plant regeneration. This medium contained 1.0 mg L^{-1} BA+0.05 mg L^{-1} NAA growth regulators. The regeneration frequency of *R. graveolens* plants was found to be affected not only by media composition but also by type of explants (Matvieieva *et al*., 2015). Cultivation of rue petioles and leaves on the medium mentioned above resulted in shoot regeneration of 100% frequency. We consider petioles to be more preferable explants because of their greater length comparing to the length of internodes of *in vitro* cultivated rue plants (Fig. 1 a). So the petiole and leaf explants were chosen for *A. tumefaciens* inoculation because of their high regenerative capability.

We reported the possibility of direct shoot regeneration after plantlet cocultivation with *A. tumefaciens* bacterial culture. Period of plantlet cultivation on the medium without cefotaxime addition was shown to be an important factor for successful shoot regeneration. At the same time the prolongation of the cultivation period on the medium without antibiotic adding from one to four days resulted in increase of frequency of shoot formation from 61.1+1.9 to 100%. The direct shoot regeneration was observed in 10-14 days after transformation (Fig. 1 b). The presence of *nptII* gene in the constructs enabled the selection of transformants so that green plants were formed on the medium containing 25 mg/l kanamycin. The frequency of transformation (number of explants with regenerated shoots) depended on the type of explants (leaves or petioles respectively).

The shoots 10-15 mm long were transferred onto 1/2MS medium for further growth and rooting. Root formation was observed only after 8 weeks of shoot cultivation on hormone free medium (Fig. 1 c). The rooted plants were further cultivated under greenhouse conditions (Fig.1 d).

"Hairy" root culture induction and shoot regeneration

We analyzed the dependence of root formation frequency on the time of plantlets cultivation on the medium without cefotaxime addition (3-8 days) because rue extract was found to inhibit *A. rhizogenes* growth. No root formation was observed in case of cefotaxime addition to 1/2MS medium in 3-5 days after transformation. Increasing of duration of plantlet cultivation on the medium without antibiotic up to 8 days enabled the agrobacterial gene transfer and "hairy" root culture initiation. Root formation on the plantlets started in 14-16 days after their cocultivation with bacterial suspension (Fig. 2 a, b). The frequency of root formation was up to 30%. The induced root culture was characterized by typical "hairy" root phenotype which is associated with the transfer of T_L fragment with *rol* genes from *A. rhizogenes* T-DNA of the agropine type Ri plasmid (Tepfer 1990) and did not require any external auxines addition to the medium for their growth.

The obtained roots were subcultured every two weeks on the medium supplemented with cefotaxime (Fig. 2 c). Cultivation of *R. graveolens* "hairy" roots on hormone-free 1/2MS medium resulted in direct shoot regeneration in 6 months after the transformation (Fig. 2 d). The shoots were taken from the root culture and then grown on the same medium for rooting. Roots formation was observed in 3 weeks of their cultivation on hormone-free medium. The period required for plant rooting was considerably smaller than the one necessary for root formation in case of *A. tumefaciens*-mediated transformation. We consider this fact as an effect of *A. rhizogenes* genes transfer to the plants.

The PCR analysis proved the presence of *nptII* and *ifn-α2b* genes for 100% of studied plants regenerated after *A. tumefaciens*–mediated transformation and also the presence of *rolB* gene in case of study of DNA of the plants regenerated on the "hairy" roots (Fig. 3).

As we observed the time differences in rooting of the transgenic plants regenerated after *A. rhizogenes*- and *A. tumefaciens*–mediated transformation we studied the effect of Indolilbutiric acid (IBA) growth regulator in order to find out the way to make the period of root formation shorter. Using of 0.5 mg L^{-1} IBA resulted in initiation of root formation in 6 or 2-week period on the shoots regenerated after *A. tumefaciens*-mediated transformation and the ones regenerated on the "hairy" roots respectively. So this regulator can be used to advance the period of *R. graveolens* rooting.

There is very limited information about *R. graveolens* genetic transformation though these plants are known to be susceptible to agrobacterial infection (Porter 1991). *R. graveolens* "hairy" root cultures were established after inoculation of hypocotyls with wild *A. rhizogenes* strain LBA 9402 (Sidwa-Gorycka *et al.,* 2009) in order to study the level of pinnarin, rutacultin, bergapten and other compounds accumulation. It should be noted that transgenic plants were not obtained from the "hairy" root in these experiments.

A. tumefaciens bacteria were used earlier for *R. graveolens* transformation by Lièvre *et al.,* (2009). Authors reported obtaining of transgenic plants after hypocotyl co-cultivatation with *A. tumefaciens* strain C58C1Rif containing plasmid pTDE4 harbouring neomycin phosphotransferase and beta-glucuronidase encoding genes using acetosyringone as virulence inducer. Routine transformation efficiency of *R. graveolens* was 11% in these experiments.

Here we firstly demonstrated the possibility of *R. graveolens* plants initiation after *A. rhizogenes*-mediated transformation. The obtained shoots were characterized with more fast rooting compared with the shoots regenerated after *A. tumefaciens*-mediated transformation.

We also demonstrated an opportunity for direct regeneration of transgenic rue plants carrying human interferon-α2b gene. Optimization of transformational conditions in particular the prolongation of the cultivation period on the medium without antibiotic cefotaxime adding from one to four days resulted in increase of frequency of shoot formation up to 100%. We would like to note that acetosyringone as *Agrobacterium* virulence inducer were not used in out experiments. Lièvre *et al.,* (2009) noted a significant increase in the number of kanamycin-resistant plants by using acetosyringone. However, our research has shown also the possibility of obtaining of transgenic *R. graveolens* plants with high frequency without use of this compound.

Conclusions

Thus the transgenic *R. graveolens* plants can be obtained *via* direct shoot regeneration from leaves and petioles in case of *A. tumefaciens*-mediated transformation as well as after shoot regeneration from rue "hairy" roots in case of using *A. rhizogenes* for transformation. Using of *A. tumefaciens* allows the obtaining of transgenic rue plants with higher frequency up to 100%. Frequency of "hairy" root formation was up to 30% because of antibacterial activity of rue plants against *A. rhizogenes*. Time of explant co-cultivation on the medium without selective antibiotic had an effect on the successful *A. rhizogenes*-mediated transformation and "hairy" root formation on the plantlets. The ability to root of the shoots of rue "hairy" root origin exceeded the same ability of the plants obtained *via A. tumefaciens*-mediated transformation. IBA addition to 1/2MS medium in concentration of 0.5 mg/L resulted in root growth stimulation.

The described transgenic technique could be applied to improve plant characteristics and transfer of foreign genes to rue plants. Moreover, such approach enables the obtaining of transgenic *R. graveolens* plants carrying gene coding synthesis of recombinant medicinal compounds.

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Figure 1. Regeneration of rue shoots after *A. tumefaciens*-mediated transformation: *in vitro* cultivated plants used for transformation (a); green shoot regeneration on the selective medium (b); transgenic plants under *in vitro* conditions (c) and in greenhouse (d)

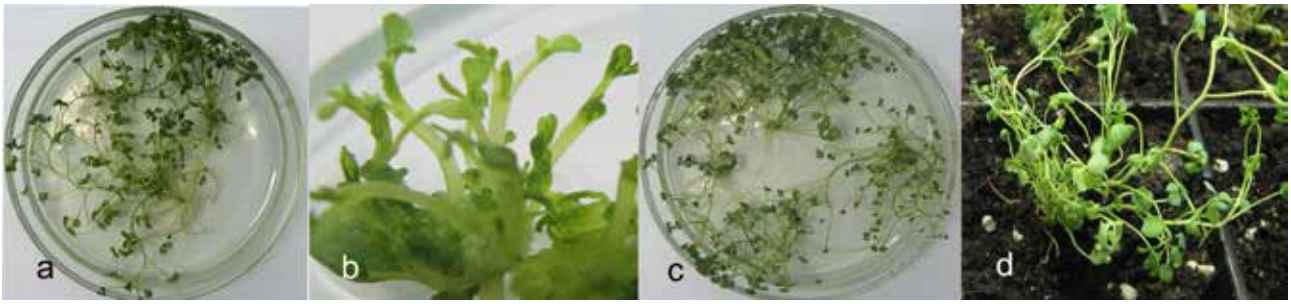
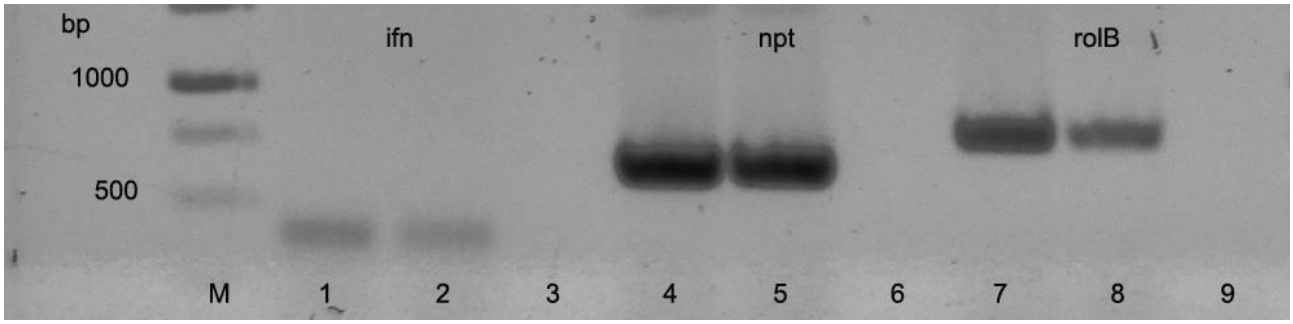


Figure 2. Initiation of *Ruta graveolens* "hairy" root culture and shoot regeneration after *A. rhizogenes*-mediated transformation: root formation on rue petiole (a) and leaf explants (b); "hairy" root culture (c); direct shoot regeneration on hormone free 1/2MS medium (d)

Figure 3. PCR analysis of the rue plants: 1, 2, 4, 5 - DNA of the plants regenerated after *A. tumefaciens*–mediated transformation; 7, 8 - DNA of the plants regenerated on the "hairy" root clones; 3, 6, 9 -negative control (untransformed plant DNA); npt, ifn, rolB - *nptII* (622 bp), *ifn-α*2b (396 bp), and *rolB* (780 bp) genes fragments; M - DNA marker ladder, Fermentas.



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This result was in agreement with result of Sahin and Yildirim (2004).

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Journal article:

Toker C (1998). Adaptation of kabuli chickpeas (Cicerarietinum L.) to the low and high lands in the West Mediterranean region of Turkey. Turk J Field Crop 3:10-15.

Toker C and Canci H (2003). Selection of chickpea (Cicerarietinum L.) genotypes for resistance to ascochyta blight [Ascochytarabiei (Pass.) Labr.], yield and yield criteria. Turk J Agric For27: 277-283.

Toker C, Canci H and Ceylan FO (2006). Estimation of outcrossing rate in chickpea (Cicerarietinum L.) sown in autumn.Euphytica 151: 201-205.

Article by Digital Object Identifier (DOI) number:

Yasar M, Ceylan FO, Ikten C and Toker C (2013). Comparison of expressivity and penetrance of the double podding trait and yield components based on reciprocal crosses of kabuli and desi chickpeas (CicerarietinumL.). Euphyticadoi:10.1007/s001090000086

Book:

Toker C (2014). YemeklikBaklagiller. BISAB, Ankara.

Book chapter:

Toker C, Lluch C, Tejera NA, Serraj R and Siddique KHM(2007) Abiotic stresses. In: Chickpea Breeding and Management,YadavSS, Redden B, Chen W and Sharma B (eds.),CAB Int. Wallingford, pp: 474-496.

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Yasar M (2012). Penetrance and expressivity of double podding characteristic in chickpea (Cicerarietinum L.).Dissertation, Akdeniz University, Antalya.

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Abbreviations

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