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Variety Development and Variety Testing, Variety Registration (Release) and Protection (PVP) Systems in The Eco Countries

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ABSTRACT

The Economic Cooperation Organization (ECO) is an intergovernmental regional organization established in 1985 by Iran, Pakistan and Turkey for the purpose of promoting economic, technical and cultural cooperation among the Member States. In 1992, the Organization was expanded to include seven new members, namely: Afghanistan, Azerbaijan, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan and Uzbekistan. Seed systems and seed trade in ECO region has been already constrained somewhat by regulations and policies that were established when formal seed production were dominated mainly by the public sector. Variety release procedures were designed to meet the needs of public research institutes and seed certification system was mainly focused on public or parastatal seed enterprises. Procedures for variety testing and approval constituted a significant barrier to seed trade and inhibited the spread of new varieties beyond national boundaries. This led to delays in release and often rejection of useful varieties that did not meet the criteria and procedures. A variety released in one country faced long battles to gain release in a second country. Commercial seed trade was also hampered by lack of intellectual property protection for plant varieties and by different procedures for import and export of seed. Under the FAO-ECO launched "Seed Sector Development in Countries of the Economic Cooperation Organization (ECO)", the Regional Seed Agreement and Regional Seed Strategy were developed. In this presentation, variety development; variety testing and registration; variety protection system in the ECO Countries have been evaluated.

Keywords: variety development, variety testing and registration; variety protection, ECO

Introduction

The Economic Cooperation Organization (ECO) is an intergovernmental regional organization established in 1985 by Iran, Pakistan and Turkey for the purpose of promoting economic, technical and cultural cooperation among the Member States. In 1992, the Organization was expanded to include seven new members, namely: Afghanistan, Azerbaijan, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan and Uzbekistan.

Especially in some ECO Countries, only a limited proportion of land has been planted with quality seeds of improved varieties and several factors limited farmer's access to better seed.,

In most of countries, governments have continued to control seed industry, even though public seed production and distribution has usually proven to be an ineffective system of seed supply (Karahan, 2014a).

Considering the above mentioned constraints; the first initiative on harmonization of seed regulations of ECO member countries was FAO-ECO Technical Cooperation Program (TCP) project on "**Strengthening the Seed Supply in the ECO Regio**n" and was launched in 2006. And then, different activities and meetings were realized on this issue.

"The First Workshop on Harmonization of Seed Regulations under the TCP on Strengthening the Seed Supply in ECO Region" was organized by ICARDA, ECO, and FAO was hosted by the Federal Seed Certification and Registration Department (FSCRD) from 17-19 January 2007 in Islamabad, Pakistan. The

objective of the workshop was to review and discuss Variety Release and PVP (Plant Variety Protection) Systems and Phytosanitary / Quarantine Measures in ECO member countries.

The ECO Seed Association (ECOSA) was established in March 2009, in Antalya.

FAO/Turkey Partnership Programme funded project **"Seed Sector Development in Countries of the Economic Cooperation Organization (ECO) (GCP/ INT/ 123/ MUL)"** was built upon the achievements and lessons learnt from a concluded FAO/ECO regional seed project and above mentioned meetings and it was put in effect in May 2013 (Muminjanov, 2013a).

The series of workshops and Seed Trade Conferences under this project were organized (Turner, 2014a).

Under this project, the Regional Seed Agreement and Regional Seed Strategy (Strategy for Coordinated Development of the Seed Sector in Countries of the ECO Region) were discussed and developed at the Final Workshop of the project was held on 4-5 November 2015 in Antalya, Turkey (Turner, 2014c and Karahan, 2014c-d).

In this paper, variety development; variety testing and registration; variety protection system in the ECO Countries have been evaluated.

The Regulatory Framework of the ECO Countries (Karahan, 2014a)

Constraints:

- In some countries, although currently seed legislation is more or less developed and harmonized with many important international acts, however, the implementation of the legal acts by state bodies is unsatisfactory.
- In some countries, seed legislation is not developed and harmonized with many important international acts.
- Regulations are not implemented efficiently and correctly in some countries.
- Constraints in implementation of laws, policies and regulations of seed & plant protection and quarantine are main handicaps.

Recommended Strategies (Karahan and Turner, 2014; Karahan, 2014g; Turner, 2014c):

- ECO countries should take measures to make laws and regulations for easy movement of seeds. Governments should be encouraged for enactment of various seed policy bills into laws in the ECO countries. These laws may also regulate the way in which new crop varieties enter the market or there may be a separate law for plant varieties.
- Rationalization and harmonization of the seed policies, laws, regulations for variety registration and variety protection in order to facilitate the movement varieties with fair and reasonable regulations to promote and develop seed industry in the region.
- The detailed technical content of these regulations is one main area through which regional harmonization can be achieved if the relevant specialists from each country have a consultation forum.
- If regulations place a heavy burden on the agencies responsible for carrying out the technical work then the tasks may not be done to the required standard, or there may be delays.
- Legal provision will be made to encourage private sector participation in Basic Seed production through access to Pre-basic seed of public sector varieties.
- A Seed Strategy-Policy provides an overall guideline for the development of the seed sector with a medium to long-term horizon but it should be revised on a regular basis to take account of the progress made. Therefore the Policy can be regarded as a broad umbrella covering all aspects of the seed sector, while the law(s) and regulations focus on specific elements of the policy that can be enforced.
- It is important that the policy and the law are in harmony with each other and certainly there must be no contradictions because that would cause confusion.

Breeding and Variety Development Activities of the Eco Countries

Constraints:

- There is lack of investment in research and development by the private seed sector in the most of ECO Countries. In some countries only public entities are involved in research programs.
- Especially public research institutions are not attractive for young scientists. Public research system is not properly functioning.
- Most of the varieties are released by public research institutes and hybrids are imported, therefore, still plant variety protection right is not applied.

Recommended Strategies (Karahan and Turner, 2014; Karahan, 2014g; Turner, 2014c):

- The public research has to be dedicated to the real need of the seed market, where there is a lack of good products.

- Access to foreign germplasm, but the recognition of Plant Breeders' Right is necessary.
- Breeding Activities should be done and supported also at private research Institutions.
- To achieve maximum impact, it is essential that improved varieties move quickly from research to farmers and with sufficient information to exploit their full potential in the production system.
- In the major cereal and legume crops, the International Agricultural Research Centers of the GCIAR work in close partnership with national research institutions.
- Closer integration of international and national efforts in plant breeding is strongly advocated, and this should include the sharing of trials information between national systems.
- Especially for hybrid maize and vegetables in which public breeding institutions cannot compete effectively with the large investments made by private companies who concentrate on profitable F1 hybrids. Public breeding programmes in these crops should be critically reviewed to decide if they are sustainable and competitive.
- In addition to national agricultural research institutes, Universities may also engage in plant breeding as part of their research programmes in genetics and agronomy.
- All member countries of the ECO are expected to use the resources of the major cereal and legume crops in gene banks managed by the CGIAR centers and this material is freely available under procedures established by the International Treaty on Plant Genetic Resources for Food and Agriculture (IT-PGRFA) using a Standard Material Transfer Agreement.
- It is therefore important that national gene banks are well-managed to ensure effective conservation of their stocks.

Jan (2014), mentioned in his country report (Report on status of Afghan national seed policy adoption & implementation) that, **in Afghanistan,** Agriculture Research Institute of Afghanistan (ARIA) is a public organization under MAIL with mandate for development & release of improved crop varieties and research on other crop husbandry practices to increase the productivity and resilience of agriculture in Afghanistan, through its 7 zonal and 10 sub stations at different Agro ecological zones spread across the country. ARIA as public department in with collaboration and funding of international research organizations only conducting conventional breeding methods (introduction&selection.

Afghanistan Scientific Academy and Agricultural colleges under ministry of higher education to conduct the research but their achievements are not sensible.

Research activities mainly dominated by crop sector specially cereal crops and only conducting by public departments or NGO's in with collaboration of MAIL, still private sector is not involved, while law and policies allowing them for investment.

In Azerbaijan, the research component of the agricultural innovation system consists of 26 research institutes. Of these, 15 come under the Agrarian Science Center (ASC) of the MoA, six under the Academy of Sciences.

The ASC was established in 1999 and coordinates the research programs and activities carried out by its 15 institutes. Research is carried out on the MoA's 20,000 hectares of land, at its experiment stations, base stations, and subsidiary experiment farms. The dissemination of the institutes' research outputs is coordinated by the MoA's Information Dissemination Unit.

The Agricultural Research Board (ARB) was formed in March 2000 to coordinate the reform of the competitive grants system and knowledge system. The Board is directly responsible to the State Commission for Assistance to Agricultural Private Farm Sector Development.

After 1991, research institutes lost their clients and entered a period of financial difficulty. As a result, they were forced to use their resources to generate income for operations. Typically, experiment stations that belong to the institutes are used to produce, process, and then market seed. With these market-oriented activities, the institutes have developed relations with private seed supply and plant protection companies. They have also maintained close ties with large private farms and state farms. In addition, experts at the experiment stations have, from time to time, developed relations with international organizations within the context of joint projects.

The internal problems of the research institutes are equally serious. Just about everything is inadequate for production of useful research outputs. The institutes need a mandate, qualified personnel, sources of finance, and access to knowledge and information about new technologies. With limited financial resources and too many (and most of the time unqualified) personnel, it is unrealistic to expect new programs to be initiated. In the past, even when financial resources were available, around 70-75 percent was allocated to staff salaries and only around 9 percent to research activities. Yet, there is still some scope for improvement at the individual institute level.

Research structure is undergoing major structural, institutional, and organizational changes.

Institutes need a mandate, qualified personnel, sources of finance, and access to knowledge.

Research priorities need to be established, funding mechanisms developed, and agricultural research organizations reduced in number.

Large farms obtain information on new varieties from experts in institutes and international agencies. The role played by the extension and information unit in the Ministry of Agriculture is negligible.

An up-to-date agricultural knowledge and information system is required to set agricultural research priorities and to develop science and technology strategies (Turner, 2014b).

The Government expects reform of agricultural research to focus on the amount and quality of research work done. Its analysis has identified a need for research institutes in the areas of sericulture, viticulture, and fruit and vegetable farming to be reorganized according to the needs of the country. Finally, given the problems mentioned earlier, the research institutes can use existing resources (experiment stations, breeding farms, land, and farm equipment) more efficiently (Guliyev, 2014).

In Iran, in 1975, the Agricultural and Natural Resources Research Organization was established as a central agency to formulate policies, develop strategies, prioritize and coordinate the activities of agricultural research institutes. The organization played an important role in providing appropriate policies to increase production of agricultural and horticultural crops in the country.

In the 1990s agricultural research organizations have been restructured to improve their performance. In 1993, the Agricultural Research Organization was merged with the Directorates of Agricultural Education and Extension. In 2002, agricultural extension was separated from Agricultural Research, Education and Extension Organization.

In each province, agricultural research centers were also established to coordinate the activities of different branches of each research institute.

The Seed Control and Certification Department of the Seed and Plant Improvement Institute is responsible for the quality of imported seed.

At present the Agricultural Research and Education Organization administers an extensive network of agricultural research institutes working on different crops and agro-ecological regions. These include 12 semi-autonomous agricultural research institutes, which are commodity, multidisciplinary, or farming system oriented and linked to a network of 30 regional or provincial agricultural research centers.

In addition, agricultural research is also carried out by various organizations affiliated to the Ministry of Jahad-eAgriculture and colleges of agricultural sciences.

The Seed and Plant Improvement Institute is the largest agricultural research institute with the main mandate for improvement of strategic agricultural, horticultural and industrial crops. These crops include cereals, food legumes, oilseeds, forages and vegetables. Apart from basic research and crop improvement, SPII continue to play a major role in seed production and seed quality control and certification in the country. SPII has eight departments and at present employs 320 PhD and MSc level professional research staff and more than 400 technical staff (Karahan, 2014a).

In Kazakhstan, emphasis is given to the modern cultivars of staple crops and development/establishment of markets for local varieties is not a national priority.

The "Concept of agriculture development till 2010" has a section on plant growing diversification, which has directed to increase the areas under profitable crops and reception of high-quality, competitive production (MOA RK, 2004). According "Concept" there is a necessity to increase diversity in agricultural systems by the strengthening of breeding, seed-growing, and increasing of the areas under sowings of main crops.

Several scientific bodies affiliated to the state, such as the National Academy of Science, the National Research Institute of Agriculture, the National Research Institute of Fruit Growing and Viticulture, and the National Research Institute of Vegetable and Potato, carry out research on a modest scale. Research on wheat is shared between two ministries: that of agriculture and that of science (under the National Centre of Agricultural Research - NAZAI). The centre is responsible *inter alia* for the production of the elite generations used for certified seeds and intended for the market. Again, because of financial constraints, these seeds are unaffordable by farmers and serve only for experimental purposes.

The centre receives support from and collaborates with CIMMYT, ICARDA, CIP, ICRISAT and IPGRI. The national collections of plant genetic resources are located in various centres of research, botanical gardens or protected areas (Karahan, 2014a).

In Kyrgyzstan, the first experimental breeding stations in Kyrgyzstan (Kyrgyz SSR) appeared in 1925 and 1946 roughly covered all areas of the country, the breeding of sugar beet, corn, tobacco, wheat, fiber crops and medicinal plants , as well as fruit and vegetable crops. First winter wheat varieties were created in the 30s of last century, and corn hybrids in the 40'ies of the last century (Sergey, 2014).

After the country independency, national plant breeding programs significantly have been cut off and only few of them continue on their activities. Government through MoAM support plant breeders' activities for some numbers of priorities crops by giving resources such as field plots for multiplication breeders and super elite seeds.

Two existing units identified by a recent project for crop breeding in republic: the Institute of Land Culture for wheat, barley, maize and vegetable breeding, and Fodder Breeding Institute for pasture and fodder crops. They need changes to make them more autonomous, a management capable of developing realistic business plans, new equipment, training programmes, and adequate financial support.

Kyrgyz Research Institute of Agriculture is a major center for the developing of varieties and hybrids in Kyrgyzstan. Breeders developed more than 100 varieties and hybrids for such crops as winter and spring wheat, winter and spring barley, corn, as well as technical and legumes, for different climatic zones of the republic and in neighboring countries.

Kyrgyz experimental breeding station for sugar beets

Currently breeding work on sugar beet seed production has been stopped, and represented as cooperative, which produces seeds of alfalfa, sugar beets, wheat and barley.

Agricultural cooperative machine-testing station (MIS)

The main activities are breeding seed crops, livestock breeding and dairy production, and forage production. Breeding program was focused on wheat, barley and triticale.

Sugar beet seed production of "MIS" and its hybrids on CMS ones engaged for more than 50 years. Also on the basis of "MIS", there is a single plant in Central Asia for the preparation of sugar beet seeds. Sugar beet seeds are sold in local market and also in Russia, Belarus, Kazakhstan and Turkmenistan. "MIS" produce annually 110-140 tons of cereal seeds for breeding kennels whose seeds are used to grow super-elite and elite.

National Academy of Sciences of the Kyrgyz Republic

National Academy of Sciences (NAS KR - www.nas.aknet.kg) brings together 25 research institutes, including 5 (Forest Institute, Institute of Biotechnology, Botanical Garden, Institute of Soil Biology, Institute of walnut and fruit crops) which activities focused on collecting plant material, research work, plant breeding material of endemic, medicinal, fodder, fruit and flowers crops).

In Pakistan, the National Agricultural Research System (NARS) is the key aspect for bringing scientific advancements in a country. NARS is the single largest agricultural research system and is spread over Federal Research Institutes of the Ministry of National Food Security and Research (NFSR) and Pakistan Atomic Energy Commission (PAEC), Provincial departments, four agricultural universities/agriculture colleges. At the national level, variety development is coordinated by Pakistan Agricultural Research Council (PARC) and Pakistan Central Cotton Committee (PCCC). One of the main functions of PARC as an apex National Agricultural Research Organization is to conduct, promote and co-ordinate R&D activities in NARS. It is also the funding agency of projects of national importance.

Crop variety development is the main domain of public sector organizations in the provinces, and a substantial number of varieties were developed by provincial and federal research institutes through conventional/mutation breeding and using the tools of genetic engineering. At the national level, variety development is coordinated by Pakistan Agricultural Research Council (PARC) and Pakistan Central Cotton Committee (PCCC).

The Pakistan Agriculture Research Council (PARC) administers the largest portion of the Federal part of Agricultural Research. One of the main functions of PARC as an apex National Agricultural Research Organization is to conduct, promote and co-ordinate R&D activities in NARS. Overall, there are 74 Research establishments at Federal level and 106 Research institutes at provincial level. A substantial number of varieties were developed by provincial and federal research institutes through conventional/mutation breeding and using the tools of genetic engineering.

Priority of Government is specifically confined to major crops like wheat, cotton, rice & sugarcane. Vegetable crop variety development remained at low profile and consequently, depending on imported costly hybrid seed.

Pakistan Central Cotton Committee (PCCC) and The Pakistan Atomic Energy Commission (PAEC) use mutation breeding to develop grain legume, rice and cotton varieties.

National Institute of Biotechnology and Genetic Engineering contributes to the release of genetically modified crop varieties.

Some Agricultural Universities and faculties of agriculture are conducting research (Iqbal, 2014).

In Tajikistan, plant breeding in Tajikistan is mainly done by public institutions: Tajik Farming Institute of Tajik Academy of Agricultural Sciences with its regional

branches are dealing with breeding of cotton, cereals, pulses, forage crops etc.

Tajik Horticulture Institute of TAAS with its regional branches, breeding activities of potato, vegetables and fruits are carried out. Beside the TAAS, Institute of Botany, Plant Physiology and Genetics of Tajik Academy of Science and different departments of TAU are also conducting research on plants and basic works on plant breeding. National Centre of Plant Genetic Resources of TAAS owns Gene Bank and is responsible for collection, conservation, characterization and use of plant genetic resource.

Apart from public institutions, during the last decade there have been some farms from private sector showing interest for plant breeding. This trend is especially can be seen in wheat breeding, where private seed farms dealing with breeding are competing with public. The examples are the seed farm of "Chilgazi" in Isfara rayon and the seed farm of Latif Murodov in Hisor rayon (Husenov *et al*., 2014 and Sanginov *et al*., 2014).

Collaborations for wheat breeding improvement with CIMMYT, ICARDA, International Winter Wheat Improvement Program (IWWIP), Oklahoma State University (OSU) and other research programs have been developed. New wheat lines from these programs have annually been received and tested under Tajik climate growing conditions. These collaborations were reason of releasing many varieties (Muminjanov *et al*., 2008).

In Turkey, R&D studies started in 1926 with the establishment of "Wheat Research Stations". Between 1926-1970, studies were concentrated on cultivation techniques such as soil tillage, sowing time, the amount of seed, fertilizer and chemical usage, etc.

In 1963, variety concept gained importance with the adoption of the Variety Registration and Certification Law.

After 1990, the studies on developing new varieties gained momentum and so many varieties developed in numerous species which are grown widely today.

The majority of public research in Turkey is carried out by research institutes and research stations under the General Directorate of Agricultural Research and Policy (TAGEM) affiliated to the Ministry. Public research institutions carry out joint research activities with other research institutions as well as universities and private sector organizations.,

The rights of production of newly developed varieties are transferred to TIGEM and private sector by contract in order to quickly spread the varieties to larger areas. Sunflower and wheat varieties developed by the Institutes are demanded by Chile, Spain, Azerbaijan, Sudan, Turkmenistan, and Iran; Osmancık rice variety is also demanded by Bulgaria, Ukraine, Russia, Macedonia, and Greece.

While private sector previously used to import seed mainly, in recent years as a result of the policies implemented by the Ministry, they have been also producing the hybrid or standard varieties developed by their breeding programs and by the institutes.

Besides MFAL, Tea Research Institute under the General Directorate of Tea Enterprises (ÇAYKUR) in Rize works on tea research, TMO works on poppy, and Sugar Institute under Turkey Sugar Factories Inc. (TURKSEKER) conducts research on agricultural and technological issues of sugar beet.

Some of the authorized private companies can do research and breeding activities, truly.

In Turkmenistan, prior to 1991, most plant genetic resource conservation and breeding activities were coordinated by the Vavilov Institute in St Petersburg, Russia. To date, agricultural research and crop improvement has been conducted by crop specific research institutes established as part of a 'Turkmengrain', within the government diversification policy.

Agricultural Production Services and Scientific Division of the MoA supervise the agricultural research institutions which develop new crop varieties and organize seed multiplication and quality control at scientific experimental centers and specialized seed farms in the regions.

There are three main agricultural research institutes involved in cereal, cotton and vegetable improvement and natural resource (land) management. About 82% of commercially grown varieties come from abroad, and national breeding activities are relatively limited, with the exception of cotton, melons, onions and sorghum.

Plant genetic resource conservation Turkmenistan, in collaboration with ICARDA-CAC, established a gene bank for some agricultural crops. Since 2006, NRICC operates a national gene bank for conservation of genetic resources

The National Research Institute of Cereal Crops (NRICC) is responsible for variety development, crop management and seed production. . It has three main departments: cereal crops, legume crops and seed production.

The Cotton Research Institute (CRI) develops cotton varieties with deciduous properties, and suitable for cultivation in harsh conditions such as drought, high temperatures, and low water use combined, but with high quality fiber and yield. At present, CRI has branch offices or experimental stations in five provinces.

In Uzbekistan, since independence, wheat has become second in importance to cotton. In order to attain food security, wheat area under irrigation increased over the years, currently at 1.3 million ha including rainfed

production. This increase in cultivated area stimulated the use of modern production approaches in the national wheat program (Akhmedov, 2014 and Akhmedov *et al*., 2014).

In the past, many Russian and foreign wheat varieties were introduced to Uzbekistan and were widely grown in large areas. This scenario has changed with release of some newly developed wheat varieties by the national agricultural research programs.

In 2002, the State Scientific Committee was re-established and named as the Center for Science and Technology under the Cabinet of Ministry, Republic of Uzbekistan. The state programs for basic research, science and technology development and innovation are being financed by the Center. Since 1991, each year the Government has allocated US$ 100,000 for wheat breeding program through the Science and Technology Center. This funding has accelerated the wheat breeding program resulting in developing new varieties where 37 winter and spring type bread and durum wheat varieties are currently released and under seed production. In order to further evaluate new breeding lines, more funding support will be critical.

Traditionally, wheat breeding has not been profitable enough to attract private sector interest and investment. But this is changing significantly with the new potential opened up by local institutes and a greater awareness brought about by the International Centers. One thing is certain - those involved in both the breeding and funding side will have a major say in setting the agenda for the future.

The future orientation is towards the development of hard and good quality wheat with ideal plant type.

Yield increase, as well as stabilizing agricultural production, is the priority challenge for the scientists and farmers in Uzbekistan.

The breeding and seed production activities in the Republic are carried out by different public research institutes and Stations (23) under Ministry.

Under MAWR has Uzbek Scientific Production Center for Agriculture, which includes in its membership 23 institutes and experimental stations 17 of which are dealing with the issue of selection and seed of various crops.

In addition to these research institutes in the Academy of Sciences there are several institutions that are engaged in breeding and seed row with / crops.

To disseminate knowledge on breeding and seed MAWR has universities and / institutions:

- Tashkent State Agrarian University,
- Andijan Agricultural Institute,
- Samarkand Agricultural Institute

Variety Testing and Registration (Release)

Constraints:

- Variety release procedures were designed to meet the needs of public research institutes in the most countries.
- Procedures for variety testing and approval constituted a significant barrier to seed trade and inhibited the spread of new varieties beyond national boundaries. This led to delays in release and often rejection of useful varieties that did not meet the criteria and procedures.
- A variety released in one country faced long battles to gain release in a second country.
- Insufficient variety testing and registration infrastructure is the case in the most of ECO Countries
- Variety release and seed certification systems not properly functioning.
- Introduction of new varieties of crops is very slow.

Recommended Strategies:

- For major crops, all countries have an official trials system for evaluating new varieties.
- Support involvement of private seed enterprises in variety development and release process and other research programs.
- Public and private sector should be supported for exporting their developed varieties.
- Facilitating of conducting of DUS/VCU tests
- Private sector will be facilitated to participate in variety testing trials force and DUS examination.
- The public research has to be dedicated to the real need of the seed market, where there is a lack of good products.
- The length of the variety registration period should be reduced to only two seasons or two years. This can greatly improve availability of improved seed varieties and increased private sector participation in the variety registration process.
- Extending the testing period delays the release of promising new material to farmers and reduces the impact of the gains made by plant breeders.
- The use of standardised trials protocols combined with good statistical designs increases the accuracy of data more than large numbers of trials sites.
- The standardization of trials procedures within the region will facilitate the comparison and exchange of information on new varieties.
- The status, membership and functions of this important Committee should be clearly defined in the Seed Law or its Regulations.

- It is important that the committee includes representatives of all the concerned stakeholders and is not controlled by public sector breeders. The NVRC may be designated as a technical sub-committee of the National Seed Board/ Committee, which generally has the final authority for approving the registration and release of varieties.
- Carrying out DUS tests and VCU trials in parallel so that both sets of data can be presented to the National Variety Release Committee at the same time.
- Allowing a provisional or conditional release of a variety if there is some uncertainty about trials data.
- Allowing the pre-release multiplication and certification of promising varieties while still in the testing system so that large-scale multiplication can begin as soon as the variety is officially released/registered.
- Allowing fast-track registration of varieties that are already on the National List of other neighbouring countries with similar agro-ecology. This would be a key step towards regional harmonization and should ultimately lead to the establishment of a Regional Variety List for the major crops, thereby making considerable savings in the resources required for variety testing by each country.
- Establish officially the possibility for an ECO country to use DUS results obtained by the other ECO Countries and may be Russia, Ukraine etc.
- Strict regulation on transgenic (GDO) varieties for registration production and marketing shall be key issue.
- The regional variety catalogue should be established listing varieties that have been released in more than one ECO member country, hence enhancing access to information on the varieties available for farmers on the market in the ECO Region.
- Capacity development, where preparation of new generation of specialists in the seed sector should be a core.

In Afghanistan, at present, there is no independent agency responsible for the variety evaluation and release in Afghanistan. Presently, the DUS and VCU tests are not strictly followed by the variety testing and registration committees and mostly open pollinated and aged varieties are used in crop production chain.

Under FAO/EU Seed Project, ICARDA provided assistance in establishing a variety registration system through DUS testing of existing commercial varieties.

Collection and conservation of local plant genetic materials is also neglected, only national catalogue of wheat varieties is prepared and published.

Introduction of varieties from neighbour or region countries and application of hybrid seed production technology would accelerate the variety development & release process (Kugbei *et al*., 2011).

In Azerbaijan, number of variety testing points has also been decreased, some of them were merged.

The State Service for Registration of Plant Varieties and Seed Control is responsible for variety registration.

In Iran, Seed and Plant Certification and Registration Institute (SPCRI) which has been established in 2004 is the national authority for the seed certification and protection of the new varieties of plant. SPCRI is a subsidiary body of Agriculture Research, Education and Extension (AREEO) of the Ministry of Agriculture.

The Registration & Protection department is responsible for granting PBR and also conducting DUS tests.

The agricultural research institutes conduct variety evaluation in different agricultural research centers (e.g 30 stations for cereals) representing different agro-ecological zones to identify promising lines suitable for major crop growing regions of the country.

The procedures of variety release and registration are similar to procedures practiced elsewhere except that the same institutes that bred the varieties are also responsible for final evaluation of the new varieties. The breeder or the research institute is responsible for variety performance trials. The trials compare the agricultural value of promising varieties with existing commercial varieties and identify those found to be superior in certain agroecological zones.

The Seed and Plant Improvement Institute is responsible for variety performance trials. After the report is reviewed and confirmed by the two Technical Committees and the Commission it will be submitted for approval to the Higher Council for Agriculture Research and Education (HCAREO).

The final report of performance trials is prepared by the breeder and submitted for release and registration to be reviewed at three levels i.e. (1) the multidisciplinary Technical Committee at the department level (e.g. Cereals Research Department of SPII); (2) the Technical Committee at the Institute level (e.g. SPII); and (3) the Research Project Coordinating Commission of the Agricultural Research and Education Organization. After the report is reviewed and confirmed by the two Technical Committees and the Commission it will be submitted for approval to the Higher Council for Agriculture Research and Education (HCAREO). The

HCAREO is composed of representatives of various research institutes, faculty members of agricultural colleges or universities, extension services and the Deputy Minister of Crop Production (Horticulture).

Upon approval of the variety release the certificate of registration is signed by the Minister of Jahad-e-Agriculture and sent to the Agricultural Research and Education Organization.

After the variety is released breeders or the breeding institutes are responsible for variety maintenance and seed production of early generation materials (breeder, prebasic and basic seed) based on the plan of the Crop Technical Committee.

In Kazakhstan, the structure of State Variety Testing Commission includes 12 provincial and 3 regional inspectorates, 4 state stations and 70 variety testing plots, 2 laboratories for assessment of the grain quality. Annually in test for economic utility are about 800-900 varieties and hybrids.

In Kyrgyzstan, the State Center for Variety Testing and Genetic Resources (SCVTGR) is responsible body. In order to preserve the genetic diversity of flora in Kyrgyzstan, as well as for breeding purposes by the Ministry of Agriculture, Water Resources and Food Processing, "Plant Genebank" was established in 2009.

SCVTGR also conducts variety testing and registration of agricultural crops in the state testing plots (SCVTS - 13 around country, excluding Naryn region) and field testing usually takes 2-3 years for yields.

Field methodology includes DUS and VCU tests.

Annual results are officially published once a year in a catalogue "National Variety List" (Muminjanov, 2013).

In Pakistan, the Seed Act (1976) provides a regulatory framework for variety registration and seed quality control.

Federal Seed Certification Agency (FSCA) & National Seed Registration Agency (NSRA) merged together as Federal Seed Certification &Federal Seed Certification & Registration De (FSC&RD) in 1997.

When a breeder selects a candidate variety, simultaneously submits seed sample (Breeder/ Nucleus Seed) of candidate variety along with its tentative "Botanical Description" to two agencies. (1) PARC (all crops except cotton) and PCCC (cotton varieties only) and (2) FSC&RD.

Variety Evaluation Committees (VECs) of PARC & PCCC evaluate candidate varieties in National Uniform Yield Trials (NUYTs) or National Coordinated Varietal Trials (NCVTs) various specific locations in the province/country.

VCU evaluation is at least for two years.

DUS test is two seasons/years with a minimum two replications.

A variety that meets the requirement of VCU and DUS is then accepted for registration and release.

Respective Provincial Seed Council during after Spot Examination and discussion approves variety and recommends its approval from National Seed Council.

After Seed Amendment Bill, the role of private sector companies, seed dealers and seed processing units can be enhanced, creating conditions for making available the pre-basic seed for the production of basic and certified seed in the private sector (Iqbal, 2014; Shah *et al*., 2014 and Karahan 2014b).

In Tajikistan, every new plant variety to enter the production must go through official testing and then be registered. In Tajikistan, "State commission of plant variety testing and variety protection" (SCVT) is the official authority responsible for variety testing and release. SCVT conducts DUS and VCU tests of new varieties.

SCVT, according to the variety testing results and decision of the commission, annually updates the State Register (Catalogue) of commercial and protected plant varieties (Ministry of Agriculture, Tajikistan and Mahkamov, 2014)

In Turkey, Variety Registration and Seed Certification Center (TTSMM) located in Ankara is responsible for the plant variety registration and seed certification in Turkey under MFAL.

After becoming a member of UPOV in 2007, TTSMM has been conducting DUS tests according to the UPOV rules in Turkey.

Registration process is performed in two separate stages with simultaneously conducted VCU and DUS tests by VRSCC. Vegetable, fruit, grapevine, and strawberry species are registered with DUS tests only.

The varieties are registered for a certain period which is 10 years for field crops and vegetables. There is no time limit for fruits, grapevines, and strawberry varieties. The registered varieties are listed in the National Variety List (Mermer and Karahan, 2014).

In Turkmenistan, the State Variety Testing and Seed Certification of the MoA is responsible for variety testing and release. The MoA together with agricultural research institutes approves crop varieties for testing and release.

An application for new crop variety developed by a breeder (or group of breeders) for testing and inclusion in the State Variety Register, should be submitted by the breeders' organization (research institution, company, etc.) and hybrids submitted for state variety testing.

Applications should then be submitted to the State Variety Testing and Seed Certification organization with the required documents.

New varieties developed by research institutions have to undergo three years of examination, compared with the best commercial variety, before submitting for state variety testing.

Since 2004, the State Variety Testing and Seed Inspectorate maintain the list of crop varieties in the State Variety Register, allowed for use in Turkmenistan. Foreign varieties not registered in the State Register are subject to state variety testing before seed can be imported and distributed (Saparmuratov *et al*., 2014).

In Uzbekistan, the regulation for the State Register of Agricultural Crops is governed by Resolution of 1997. The regulation maintains a state register of agricultural crops recommended and released in Uzbekistan. The State Variety Testing Committee (SVTC) is a national agency under MAWR entrusted with responsibility for implementing the variety testing, registration and release system. The SVTC is a legal entity whose functions include the testing new varieties of agricultural crops developed by the breeders before their official release.

The SVTC has proposed a major overhaul of the entire variety release system with changes being implemented since 1991. All stations of SVTC were oriented to test cotton and other industrial crops. Eight additional stations were established in different regions for testing cereals and legume crops.

State Commission conducts testing of varieties and hybrids in two directions: VCU and DUS tests.

Registration is made for the respective region.

Gossortkomissiya published annually the State Register and Bulletin (FAO-SEC and MAWR of Uzbekistan, 2014) (Mukhamedov, 2014).

Plant Variety Protection (PVP)
(Plant Breeders Rights)

Plant Breeders Rights provide a property right so that the breeder of a new variety can obtain income from the use of that variety by others for a certain period.

Constraints:

- Most of the varieties are released by public research institutes and hybrids are imported, therefore, still plant variety protection right is not applied.
- Even some countries are UPOV members and have PVP laws; they do not have enough system to control and they are reluctant to reinforce law provisions.
- The royalty payment system works well in 'mature seed markets' where production and marketing are controlled by a strong regulatory framework. It may be more difficult to implement and enforce when many farmers save their own seed or where the seed supply system is relatively unregulated.
- It should be emphasised that the breeder only benefits from these property rights if there is an effective royalty collection system supported by a reasonable mechanism for enforcement.

Recommended Strategies:

- Access to foreign germplasm, but the recognition of Plant Breeders' Right is necessary.
- Facilitation for membership with UPOV and having PVP law for the variety protection.
- UPOV is able to provide guidance on the preparation of national PVP Laws and ECO countries planning such a law are recommended to contact the UPOV Secretariat at an early stage in this process.
- UPOV members exchange information about the varieties they have protected and this can save on the costs of DUS testing.
- It is recommended that the law on plant variety protection is kept separate from the law on seeds because these are different types of legislation.
- Effective mechanism for collecting royalties on crops.

In Afghanistan, according to the National Seed Policy, although plant variety protection including provisions for Plant Breeders' Rights and Plant Patents are not considered as issues with immediate consequence for the country.

In Azerbaijan, PVP related law is "Selection achievements". Legal document meets international standards and comply with the requirements of UPOV. Relevant law for the UPOV Convention was passed in December 2003 and Azerbaijan joined the International Convention of UPOV in December 2004.

In Iran, country is not a member of UPOV and currently its sui generis system is applicable only for Iran`s residents. The Plant Varieties Registration, Control and Certification of Seeds & Seedlings Act of 2003 has provision on this issue. 18 years variety protection.

Seed and Plant Certification and Registration Institute (SPCRI) conducted a FAO TCP project entitled: Strengthening Capacity on Plant Variety Protection (PVP) which has been implemented during 2007-09 in the Institute.

A book entitled Principles of Plant Variety Protection was also complied by a group of authors in Persian language, funded by the project.

In Kazakhstan, since the Republic of Kazakhstan law on the protection of selective breeding achievements

was enacted in 1999, the system of protection - including plant varieties - has not been noticeably amended. Currently, the issue of Kazakhstan acceding to the International Convention on the Protection of New Varieties of Plants (convention) is discussing.

The Committee and the State Commission for the Testing of New Varieties of Agricultural Plants (commission) are in charge of plant variety protection.

The Kazakh legislation and the convention provide for the temporary legal protection of a new plant variety. The duration of protection complies with convention provisions. Also, a patent's validity can be extended at the patent holder's request.

In Kyrgyzstan, Law of the Kyrgyz Republic "**On Legal Protection of Selection Achievements**" came into force in 1998 and amended in 2003, 2005 and 2006.

The royalty collection system is in place and is expected to be fully operational in the near future. Varieties may be protected by Kyrgyz patent. An agreement to simplify the system for foreign companies to protect their varieties is also in place. Farms are now able to multiply seed under license agreements with the plant breeder or license holder and to pay royalties through the Kyrgyz Patent Office (Islamov *et al*., 2007).

In Pakistan, Seed Act, 1976 which scarcely contains IP protection provisions. This law does not provide protection to the rights of Plant Breeders. The Plant Breeders' Rights Acts, 2015 was passed in Parliament in 2015 that registration for protection will be established under IPO-Pakistan. It provides 20 years protection for the crops and 25 years for trees and wines (Shah, 2014).

In Tajikistan, the Law of the Republic of Tajikistan "On the Plant Variety Protection", #672, December 29, 2010 (Husenov, 2014).

In Turkey, in 2004, the "Protection of Breeder's Rights of New Plant Varieties Law" No. 5042 was accepted implemented effectively. The law has been prepared in full compliance with the EU directives. With the approval of this Law, application was made for UPOV membership and Turkey was approved as the 65th member of UPOV in November 2007.

Implementations under the framework of this law are carried out in accordance with CPVO (Community Plant Variety Office). Data related to breeders' rights are transferred periodically to CPVO, and CPVO central database can be utilized online by Turkey.

In Uzbekistan, the 'Breeding Achievement Act' 29 August 2002 covers intellectual property rights, including procedures for testing and granting protection, breeders' rights and the patent office. In October 2004, Uzbekistan became the 57th member of UPOV.

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In Vitro Tissue Culture Studies in Sunflower (*Helianthus* SPP.)

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ABSTRACT

Sunflower (*Helianthus annuus* L.) is the fourth most important oilseed crop in the world in terms of total yearly production, after soybean, rapeseed, and groundnut. The classical breeding studies made from 1880 until today have been focused on major characters, such as high fat, pests and diseases resistance. The narrow genetic base in sunflower causes a major problem at breeding and selection of desired sunflower lines eg. cytoplasmic male sterility, disease and insect pest resistance, fertility-restoration, agronomic and seed-oil characteristics, drought tolerance, protein content, imidazolinone resistance and fatty acid composition. The growth or maintenance of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro* described as plant tissue culture, is an important tool in both basic and applied researches as well as in commercial application. The first and important step of tissue culture is development of *in vitro* regeneration system. Researchers have successful attempts by various explants, such as immature embryo mature embryo, meristem, anther, hypocotyl, protoplast, cotyledonand embryo sac. Although the specific techniques like, embryo culture, organogenesis, somatic embryogenesis have been succesfully used, anther culture, microspore culture ovule cultureneeds to be improved.

Keywords: sunflower, plant tissue culture, *in vitro* regeneration

Introduction

Sunflower (*Helianthus annuus* L.) is the fourth most important oilseed crop in the world in terms of total yearly production, after soybean, rapeseed, and groundnut. The classical breeding studies started from 1880 until today have been focused on major characters, such as high fat, pests and diseases resistance (Faure *et al*., 2002; Seiler and Gulya, 2004; Vassilevska-Ivanova *et al*., 2014). The conventional breeding studies have resulted in cultivars with improved agronomic characteristics. However, the lack of suitable genetic resources in modern sunflower varieties affects negatively development of new sunflower hybrids possessing high disease resistance and new oil and protein qualities, highy tolerence to stress conditions (drought, salt etc.). New technologies are necessary to broaden the genetic variation of cultivated sunflower. Biotechnology involving tissue culture and genetic engineering might be useful tool to exploit genetic variation (Larkin and Scowcroft, 1981). The main target of tissue culture and molecular techniques is crop improvement.Plant tissue culture is powerful tool for studying basic and applied problems in plant breeding. The growth or maintenance of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro* described as plant tissue culture, is an important tool in both basic and applied researches as well as in commercial application (Thorpe, 1990). Plant cell tissue culture technologies also have considerable potential for genetic improvement of sunflowers, with the generation of somaclonal variant, transgenic and somatic hybrid plants illustrating the potential of these approaches to promote conventional breeding. Molecular techniques facilitate the selection of germplasm for incorporation into sunflower breeding programs.

Application of various tissue culture methods to sunflower crop improvement, throught regeneration via both organogenesis and somatic embryogenesis, interspecific hybridization and embryo culture, haploid production, somaclonal variation, protoplast culture are subjects of the review.

Tissue culture and plant regeneration

The application of biotechnological methods (whole plant regeneration *via* tissue culture and molecular studies) for most sunflower breeding studies to improve the characteristics of sunflower (salinity tolerence, drought and disease resistant, *in vitro* mutagenesis, and somatic embryogenesis eg.) are limited mainly by the difficulty of regenerating plants in a reproducible and efficient way (Flick *et al*., 1983, Phillips, 2004). Sunflower species are highly recalcitrant nature and difficult to regenerate plants especially when they are subjected to *in vitro* conditions (Moghaddasi, 2011; Davey and Jan, 2010; Nichterlein and Horn, 2005; Mezzarobba and Jonard, 1986). Thus, the first and important step of tissue culture is development of *in vitro* regeneration system. Development of a highly regenerable tissue culture system for many *Helianthus* species for crop improvement would be possible with techniques such as embryo rescue, somatic hybridization, somaclonal variation and morphogenesis. Sunflower tissue culture study was started with sunflower tumor cells in 1940's (Hildebrandt *et al*., 1946). It is reported that the sunflower tumor cells continued to proliferate in the absence of *Agrobacterium tumefaciens* (White and Braun, 1941), only root regeneration from tumor cells was obtained but there was no report of shoot regeneration from those tumors. In early, most studies of *Helianthus annuus* L. (*H. annuus* L.) tissue culture were concerned about crown gall cells. In 1954, *H. annuus* with non tumorous callus was obtained from growing stem or hypocotyl segments on simple media with auxin (Henderson, 1954; Kandler, 1952). In the same year, Henrickson (1954) cultured shoot tips of 5 day old *H. annuus* cv. 'Mammoth Russion' on a modified White's medium to obtain whole plant. It is shown that a whole plant without callus formation can grow from a single shoot tip but flowered in culture within 3 months. Rogers *et al*., (1974) successfully established callus from a CMS sunflower line, but this callus induced only roots. The first whole plant regeneration was obtained from callus tissue isolated from the stem pith tip of a 2 month old sunflower plant in medium with 1 mg/l IAA by Sadhu in 1974. Since then many successful attempts on sunflower regeneration protocols have been done by various explants, such as immature embryos (Finer, 1987; Jeannin *et al*., 1995; Dağüstü *et al*., 2010; Encheva *et al*., 2004), mature embryo (Ozyigit *et al*., 2007), meristems (Paterson, 1984), shoot tips and embryonic axes (Paterson, 1984; Malone-Schoneberg *et al*., 1994; Elavazhagan *et al*., 2009), leaves (Greco *et al*., 1984; Lupi *et al*., 1987; Paterson, 1984; Inoka and Dahanayake, 2015), roots and stems (Inoka and Dahanayake, 2015), anthers, ovaries (Badea *et al*., 1989; Mohmand and Quraishi, 1994; Thengane *et al*., 1994; Nurhidayah *et al*., 1996), hypocotyls (Lupi *et al*.,1987; Mohmand and Quraishi, 1994; Müller *et al*.,2001; Sujatha *et al*., 2012; Greco *et al*., 1984), protoplasts (Guilley and Hahne, 1989; Fischer *et al*., 1992; Henn *et al*., 1998; Rákosy-Tican *et al*., 2007), and cotyledons (Fiore *et al*., 1997; Greco *et al*., 1984; Sujatha *et al*., 2012; Ceriani *et al*., 1992), embryo sacs (Popielarska and Przywara, 2003; Popielarska, 2005). Greco *et al*., (1984) also pointed that every part of the seedlings except roots was cabable of regeneration in sunflower. Punia and Bohorova (1992) studied with six wild species of sunflower showed that the effect of genotype, explant and medium were very important on callus induction and plant development. The parallel results were obtained by Dağüstü (2002) that the effect of genotype, age of seedlings and interactions between genotype and light in sunflower genotypes were very important on callus induction and plant regeneration of sunflower genotypes. Plant regeneration parameters have been shown to be under quantitative genetic control in sunflower (Sarrafi *et al*., 1996ab; Delgene *et al*., 1997; Berrios *et al*., 1999a).

Application of tissue culture methods in sunflower breeding

Organogenesis and Somatic Embryogenesis

A variety of techniques for regeneration by organogenesis (Pugliesi *et al*., 1993; Witrzens*et al*., 1988; Power, 1987; Espinasse and Lay, 1989; Chraibi *et al*., 1992; Ceriani *et al*., 1992; Berrios *et al*., 1999b) or somatic embryogenesis (Finer, 1987; Freyssinet and Freyssinet, 1988; Espinasse and Lay, 1989; Pelissier *et al*., 1990; Prado and Berville, 1990; Jeannin and Hahne, 1991; Nestares *et al*., 1996) have been described in sunflower. Sunflower regeneration capacity by organogenesis is highly variable and depends upon genotype, specific media components, the explant type, age of seedling, concentrations of hormones in callus induction medium, lighting conditions and tissue culture methods (Moghaddasi, 2011; Dagüstü, 2002).

Interspecific Hybridization and Embryo Culture

Embryo rescue culture is the growth of an immature embryo under sterile conditions *in vitro* for obtaining a viable plant (Bürün and Gürel, 2001). Embryo culture has been used successfully by plant breeders in solving the problems of seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of symbiotic partner, shortening the breeding cycle, rapid seed viability test, obtaining rare hybrids and homozygous lines, and haploid production (Bhojwani and Razdan, 1996; Chandler and Beard, 1983; Dağüstü *et al*., 2012; Gürel *et al*., 1991ab; Raghavan, 2003; Torresàn *et al*., 1996). Dagustu *et al*., (2010) managed to regenerate fertile plants from sunflower (*H. annuus* L.) *via* immature embryo culture. Embryo culture proved to be an useful tool to overcome post-zygotic hybrid incompatibility in different *Helianthus* spp. and significantly increases the efficiency of distant hybridization (Nenova *et al*., 2014).

The wild *Helianthus* species are of considerable interest as a source of genetic variation for economically important characters such as fatty acid composition, male sterility, fertility restoration, protein content, disease resistance, drought tolerance, salt tolerance, herbicide tolerance, chemical constituents, garden and ornamental sunflower types with novel plant and flower characteristics (Breccia *et al*., 2009; Chandler and Jan, 1985; Christov, 2012; Christov, 2013; Fernandez-Martinez *et al*., 2010; Jan and Chandler, 1985; Kaya, 2015; Mandel *et al*., 2011; Miller and Al-Khatib, 2004; Petcu and Pacureanu, 2011; Sauca and Lazar, 2011; Seiler, 2012; Seiler and Rieseberg, 1997; Seiler *et al*., 2017; Škorić, 2009; Serieys and Christov, 2005; Sukno *et al*., 1999; Tan *et al*., 1992; Tosun and Ozkal, 2000). The use of interspecific hybridization in sunflower has started in 1916, when the Russian scientist Sazyperow produced an interspecific hybrid between *H. annuus* and *H. argophyllus* T. and G. in an attempt to develop sunflower with resistance to rust (Cockerell, 1929). Interspecific hybridization in Russia continued with Galina Pustovoit's research on perennial *Helianthus tuberosus* L. (Škorić, 1988). The first regeneration from interspecific sunflower species was obtained from partial compatible *H. annuus* and perennial tetraploid *H. decapetalusvia* embryo rescue culture by Georgeiva - Todorova *et al*., (1980). With improvement of embryo rescue, 33 interspecific hybrids with an overall success rate of 41%, producing many new hybrid combinations was developed Kräuter *et al*., (1991).In recent years many successful studies have been carried on with embryos arising from interspecific and intergeneric hybrids in sunflower by embryo rescue (Sukno *et al*., 1999; Christov, 2008; Dağüstü *et al*., 2010; Faure *et al*., 2002).At first time, Chandler and Beard (1983) developed a two-step embryo culture procedure that produced 53 interspecific cross combinations without multiple pollinations. Out of 53 interspecific crosses, 21 combinations developed regeneration that had not been accomplished using conventional procedure. Development of chromosome doubling with colchicine greatly accelerated interspecific hybridization in sunflower(Jan, 1988). Today, lines with the high and middle oleic acid content have been successfully transferred to cultivated sunflower genotypes by interspecific hybridization, mutation breeding and gene transfer methods (Sukno *et al*., 1999; Abbas Mohamed 2005). Jambhulkar (1995) developed a rapid embryo-raised plant system for sunflower production from immature embryos, which allows five cycles in 316 days. In the recent study the breeding cycle of sunflower using immature embryo culture was shortened by taking 4 generations in a year (Dagustu *et al*., 2012). Nenova *et al*., (2014) showed succesfull results using embryo culture on interspecific hybridization between cultivated sunflower (*Helianthus annuus* L.) and the perennial species *Helianthus ciliaris.* Four lines 1131/н, 1135/н, 1145/н, 1171/p, 1161/p and 1151/p among the selected possessed complete resistance to the pathogens of downy mildew and broomrape and some lines had higher seed oil content.

Haploid Production

Haploid plants are plants with a gametophytic chromosome number and doubled haploid plants are haploids that have undergone chromosome duplication. Anther and microspore culture for haploid production greatly reduce the time required for development of improved cultivars by providing homozygous doubled haploids within a comparatively short time (Bürün and Gürel, 2001). Several methods have been applied for the production of haploid sunflower plant through gynogenesis (Yang *et al*., 1985; Gelebart and San, 1987), anther culture (Bohorova *et al*., 1985; Gürel *et al*., 1991a; Pugliesi *et al*., 1993; Saji and Sujatha, 1998; Thengane *et al*., 1994; Yang *et al*., 1990; Zhong *et al*., 1995) microspore culture (Gürel *et al*., 1991b; Coumans and Zhong, 1995) and induced parthenogenesis (Todorova *et al*., 1997). Thengane *et al*. (1994) cultured anthers of 4 genotypes and the only one genotype (eg. interspecific hybrids) regenerated plantlets from embryos. Nenova *et al*. (2000) had successes with anthers of some wild species by direct organegenesis. Several publications have described extensive callusing induced from anthers of various interspecific sunflower hybrids cultured *in vitro* (Bohorova *et al*., 1985; Mezzarobba and Jonard, 1986). Optimization of anther culture with

regard to the induction of callus formation and direct embryogenesis was studied with interspecific hybrids of *H. annuus* with *H.tuberosus, H. laetiflorus* and *H.resinosus* by investigating six different induction media and four regeneration media by Nurhidayah *et al*., (1996). Sunflower proved to be very recalcitrant in anther culture and culture response is strongly affected by physical, nutritional, physiological and genetical factors (Gürel *et al*., 1991b; Mezzarobba and Jonard, 1986) and the regeneration rates are very low. Although the results of anther culture of cultivated sunflower has been unsatisafactory so far, whereas the interspecific hybrids have been successfully used in anther culture of sunflower (Alissa *et al*., 1985; Bohorova *et al*., 1985; Mix, 1985; Jonard and Mezzarobba, 1990; Nurhidayah *et al*., 1996).

Isolated microspore culture of cultivated sunflower was assayed by Coumans and Zhong (1995) in order to avoid development of the anther wall and other somatic tissues. It is reported that viability and initial division rate of microspore response was increased and sustained division and microcallus formation were achieved after addition of aminocyclopropane carboxylic acid, an ethylene precursor. The only groups of cells had hairy types of structures developed into calluses.

An alternative method for haploid production in *Helianthus* spp. are induced parthenogenesis. It is an applicable approach for rapid production of doubled haploid lines in sunflower. Todorova *et al*., (1997) used irradiated pollen-induced parthenogenesis obtained the number of agronomically useful DH lines that were fertile and resistant to downy mildew. But the efficiency of the method was depending on the female genotype and pollen donors.

Somaclonal Variation

Genetic variation is revealed in crops and their progenies raised through cell and tissue culture techniques. This is defined as somaclonal variation (Larkin and Scowcroft, 1981). Many types of genetic changes occur in somaclonal variation including alterations in DNA sequence e.g. single gene mutation, transposition, amplification; in gross chromosome structure e.g. duplications, translocations, deletions; in chromosome number e.g. polyploidy or aneuploidy; and in chloroplast or mitochondrial genomes. This types of changes are stable through succeeding generations. However, the variation exposed as a result of a tissue culture cycle can be non-heritable (epigenetic) which would not be transmitted through meiosis and it may be reversible during the life of a plant. Hence it is worthless for sexually propagated plant production. Changes have also been identified that are both heritable and unstable (Karp, 1990). It is believed that somaclonal variants can be enhanced for some characters during culture *in vitro*, including resistance to disease pathotoxins and herbicides and tolerance to environmental or chemical stress. However, at present few cultivars of any agronomically important crops have been produced through the exploitation of somaclonal variation as in sunflower. Pugliesi *et al*., (1991) showed plant regeneration and genetic variability in tissue cultures of sunflower cotyledons. Somaclonal variation and *in vitro* selection can be applied in sunflower in many aspects. Examples of beneficial changes have included higher oil content in seed, higher 1000 seed weight, good combining ability, full resistance to *Phomopsis helianthi*, shorter vegetation period and reduced height (Encheva *et al*., 2003; 2004).

Protoplast Culture

Sunflower protoplasts from various sources (mesophyll, stems, cotyledons anhypocotyls) have been tested for their capacity to divide in culture (Lenee and Chupeau, 1986), only 6% of the initially plated protoplasts reached the stage of calli in a medium containing glutamine or ammonium succinate as sole sources of nitrogen and a reduced amount of naphthalene acetic acid (NAA) (0.1 mg/l).Bohorova *et al*., (1986) isolated and cultured protoplasts of wild and cultivated sunflower and obtained roots and meristematic regions from protoplast derived callus. The scientists managed regeneration of fertile plants from *H. petiolaris* and *H. annuus* protoplasts in 1991 (Burrus *et al*., 1991; Chanabé *et al*., 1991). Simple and efficient method for routine callus formastion from calli protoplasts and for plant regeneration from hypocotyl protoplasts of a sunflower commercial cultivar Girapac SH222 was described by Santos and Caldeira (1998). Plant regeneration from hypocotyl protoplasts of sunflower was also achieved for two cvs.'Florom 328' and 'Turbo' by Rákosy-Tican *et al*., (2007).

In conclusion, sunflower regeneration capacity by organogenesis and somatic embryogenesis are highly variable and depend upon genotype, specific media components, the explant type, age of seedling, concentrations of hormones in callus induction medium, lighting conditions and tissue culture methods. Although much work focused on establishment double haploid production, protoplast culture in sunflower, none of the improvements was efficient and applicabale to a wide range of genotypes in plant breeding programmes. The further researches are necessary to optimize tissue culture protocols for use in sunflower breeding programmes.

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Determination of High Oleic Type and Broomrape Resistant Sunflower Hybrids By DNA Markers

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ABSTRACT

Sunflower is one of the most significant oilseed crops in the world. Sunflower lines with high oleic content have high oxidative stability of its oil. *Orobanche cumana* Wallr., which is a holoparasitic plant infecting the sunflower roots, is one of the limiting factors for sunflower production especially in Eastern Europe and Turkey. Screening for high oleic acid content and broomrape resistant sunflower genotypes by standard methods is time consuming and expensive. Molecular markers associated with high oleic acid trait or broomrape resistance are useful and rapid tool in order to facilitate sunflower breeding program. In this study, two markers were chosen; SSR marker and HO PCR specific fragment for genotyping the 250 sunflower inbred lines for high oleic acid content. According to our results, high oleic acid containing hybrids expressed a specific SSR band at 246 bp, and also HO PCR specific fragment at 870 bp. Also, determination of broomrape resistance for these 250 sunflower inbred lines was done by SCAR and SSR markers. The analyzed SCAR markers (RTS28, RTS29, RTS40 and RTS41) and SSR markers (ORS1036 and ORS1040) were linked to the *Or*5 gene [provides resistance to all five races (A-E)]. According to SCAR and SSR analysis, the studied sunflower inbred lines were resistant to races A-E. These results allowed identification of sunflower hybrids for high oleic acid traits and broomrape resistance by DNA markers.

Keywords: *helianthus annuus*, marker-assisted selection, SCAR, SSR, oleic acid content, *orobanche cumana*

Introduction

Sunflower is one of the most significant oil crops in the world. Sunflower oil contains high level of unsaturated fatty acids (88%); linoleic acid (48-74%), oleic acid (14-40%) and also saturated fatty acids; palmitic acid (4-9%) and stearic acid (1-7%) (Singchai *et al*., 2013; Nagarathna *et al*., 2011). High oleic sunflower production accelerated the consumption for healthy frying oil, and also non-food uses in recent years. Particularly, non-food applications require oleic acid content that is stable and higher than 90% (Vannozzi 2006; Ferfuia *et al*., 2012). Increasing oleic acid content has become one of the significant goals to improve quality of vegetable oil (Lacombe *et al*., 2004). In order to achieve this aim, Sunflower lines and hybrids which have high oleic acid content in their seeds have been obtained by selection programs from HO (High oleic) Pervenets mutant by chemical mutagenesis (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenet population is higher than 65% in comparison to the normal LO varieties which is about 20% (Berville *et al*., 2009). Afterwards, new cultivars with changed fatty acid content were

developed by different researchers (Osorio *et al*. 1995; Velasco *et al*., 2008; Leon *et al*., 2013; Alberio *et al*., 2016; Cvejic *et al*., 2016).

Although the determination of high oleic acid content with analyzing seed oil is easy way, but marker assisted selection studies with tightly linked markers to high oleic trait could speed up breeding process (Dehmer and Friedt 1998). The phenotypic determination (fatty acid analysis) does not allow rapid and early determination of HO genotypes and also cannot provide differentiation of homozygotes from heterozygotes for the mutation. The use of molecular markers has become popular tool for the genetic and breeding studies and it is rapid, cheaper and simple when suitable markers were developed (Varshney *et al*., 2005).Various sunflower lines and hybrids have been studied to distinguish HO genotypes from LO genotypes by different researchers and molecular marker types (Dehmer and Friedt 1998; Schuppert *et al*., 2006; Nagarathna *et al*., 2011; Grandon *et al*., 2012; Singchai *et al*., 2013; Premnath *et al*., 2016; Dimitrijevic *et al*., 2017).

Orobanche cumana Wallr. (broomrape) is a parasitic plant that can lead to advanced losses in yield, in agricultural lands cultivating sunflower, depending on the sunflower varieties and the level of contamination. The numerous and small sized seeds of broomrape causes contamination in sunflower fields quickly. Depending on attack intensity to the field and development stage of sunflower during infection, decrease in sunflower yield could change 5 to 100% (Miladinovic *et al*., 2014). Broomrape is reported by the main sunflower producer countries in the world like Russia, Ukraine, Romania, Bulgaria, Turkey, and Spain, as well as Serbia, Hungary, Moldova, Greece, Tunisia, Israel, Iran, Kazakhstan, China, Mongolia, and Australia (Molinero-Ruiz *et al*., 2009; Pacureanu-Joita *et al*., 2012; Amri *et al*., 2012; Kaya 2014; Miladinovic *et al*., 2014; Marinkovic *et al*., 2014). Eight races (A-H) of *O. cumana* have been reported in Turkey (Kaya 2014).

There are several methods of broomrape control with different level of efficiency (Louarn *et al*., 2016). Sunflower breeders developed *Orobanche*-resistant hybrids from some wild *Helianthus* genus. Wild sunflowers have resistant genes and these genes incorporated into sunflower inbred lines by classical methods. However, broomrape race composition changes rapidly. Breeding resistant sunflower lines to this type of parasitic weed is an urgent and important task for breeders. To accelerate the process of sunflower breeding for resistance to *Orobanche*, collaboration between the breeders from public institutions, universities and private companies should be needed. Since the most reliable method of screening broomrape resistance is the use of molecular markers, various marker types have been used for the selection of resistant genotypes (Imerovski *et al*., 2013; Perez-Vich *et al*., 2013).The objectives of this study were (1) characterization of sunflower hybrids with high oleic acid content and broomrape resistance by DNA markers and (2) test the effectiveness of different marker types for selection of high oleic and broomrape resistant genotypes.

Materials and Methods

For the purpose of screening on high oleic acid and broomrape resistant genotypes, 250 sunflower F_3 individuals were used. Leaves were collected from the field-grown plants, labeled with individual number and stored at -80ºC until further use. i-genomic Plant DNA Extraction Mini Kit was used for DNA isolation from all samples. The quality of DNA was checked by 1% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum St5. Each of the extracted DNA was diluted as 50 ng per µl and was stored at -20 ºC for later uses.

Genotyping of high oleic (HO) and low oleic (LO) sunflower individuals was performed with two primer pairs; SSR (N1-1F/N1-1R) and HO PCR specific fragment (N1-3F/N2-1R) that were chosen from the patent obtained by Berville *et al*., (2009) (Table 1). Amplified PCR products were controlled by 2% agarose gel electrophoresis and visualized by Gel Imaging System Vilber Lourmat Quantum St5 (Figure 1 and Figure 2). SSR fragments were scored in a Beckman Coulter GenomeLab™ GeXP Genetic Analysis System and fragment sizes were calculated by its Software (Figure 3).Genotyping of resistant (R) and susceptible (S) sunflower individuals was performed with four SCAR and two SSR markers (Lu *et al*., 1999; Lu *et al*., 2000; Tang *et al*., 2003; Iuoras *et al*., 2004; Imerovski *et al*., 2012; Imerovski *et al*., 2013) (Table 2). Amplified PCR products were controlled by 2% agarose gel electrophoresis and visualized by Gel Imaging System Vilber Lourmat Quantum ST5 (Figure 4). SSR fragments were scored in a AATI Fragment Analyzer™ and fragment sizes were calculated by PROSize 2.0 Data Analysis Software (Figure 5 and Figure 6).

Results and Discussion

The Pervenets mutation was labelled by the polymorphism of the SSR locus located on the Δ12-desaturase gene intron (Berville *et al*., 2009). Berville *et al.,* (2009) reported that 16 SSR motives (16 TTA repeats) associated with the Pervenets mutation and high oleic genotypes have this type of Δ12HOS allele. In our study, according to DNA fragment analysis for SSR locus 246/246 Homozygous, 243/243 Homozygous and 243/246 Heterozygous genotypes were identified (Figure 3). DNA sequence analysis was carried out to confirm repeat motifs corresponding to HO genotypes and 246/246 Homozygous genotypes were evaluated as HO genotypes. In order to confirm HO sunflower genotypes, all studied individuals were screened with HO PCR specific fragment (Berville *et al*., 2009). The Pervenets mutation was labelled by the 870 bp PCR fragment across the 5' insertion point by HO PCR specific fragment (N1-3F/N2-1R) (Berville *et al*., 2009). The results showed that high oleic containing sunflower individuals (HO genotypes) showed a specific band at about 870 bp length which was absent in low oleic (LO) genotypes (Figure 2). Nagarathna *et al*. (2011) studied around 350 sunflower genotypes including RHA-lines, cms lines, inbreds and germplasm lines to screen high oleic genotypes with HO PCR specific fragment and also the determination of fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) was performed by gas chromatography. Nagarathna *et al*., (2011) reported that the genotypes having a specific band showed high oleic content and GC results supported the marker analysis results. Singchai *et al*., (2013) studied the developed lines those were used as the representative of low and high oleic acid sunflowers for genotyping by screening thirty seven SSR primers including 34 primers of ORS set, 2 primers of HA set and HO PCR specific primer to identify DNA samples from two lines (high and low oleic acid contents). Grandon *et al*., (2012) studied F_2 mapping population that was obtained from a cross between R285 and R023 with 386 SSR markers for genotyping oleic acid trait and they also determined fatty acid composition by GC. They reported that 82 of analyzed marker will be used for genotyping and selection of HO genotypes. Singchai *et al*., (2013) reported that out of the 37 SSR primers screened for polymorphism, 10 SSR primers including HO PCR specific primer generated differentiating bands between the high and low oleic lines. With the 10 SSR markers they studied, Singchai *et al*., (2013) reported that it is possible to identify the genetic markers linked to high oleic acid trait which may be useful for further sunflower breeding program. Dimitrijevic *et al*., (2017) studied parental lines (high oleic and low oleic), F_1 and F_2 individuals with molecular markers for FAD2-1D sequence. They reported that studied 2 markers enabled the discrimination of genotypes, while one was monomorphic.

The analyzed sunflower hybrids were also controlled by molecular markers in order to determine broomrape resistance. The analyzed SCAR markers (RTS28, RTS29, RTS40 and RTS41) and SSR markers (ORS1036 and ORS1040) were linked to the *Or5* gene [provides resistance to all five races (A-E)]. According to SCAR and SSR analysis, the studied sunflower hybrids were resistant to races A-E. Although the studied 250 sunflower hybrids were divided into three groups according to *Orobanche* inoculation result (*Or6*); Resistant, Tolerant and Susceptible, we have not found the studied SCAR or SSR markers associated with *Or6* in the analyzed sunflower individuals. Imerovski *et al*., (2013) carried out study in order to determine broomrape resistance of 20 cultivated sunflower inbred lines using SSR and RAPD markers. They reported that ORS1036 (240 bp) and ORS1114 (246 bp) primer had unique fragments in lines with *Or6* gene. Previously developed markers for *Or* genes were not always effective for identification of *Or6* genes in different germplasm. Further studies should be carried out to validate different marker types for *Or6* in different genetic backgrounds. After MAS analysis, 26 high oleic type and broomrape resistant sunflower hybrids were selected for further breeding studies. As a conclusion PCR analysis with molecular markers enabling to discriminate HO genotypes or broomrape resistance was easy and reliable method. Consequently, these molecular markers may be used in selection programs to identify genotypes carrying the Pervenets mutation and to discriminate broomrape resistant genotypes. However, these markers need validation in different sunflower populations, hybrids or lines in order to confirm their capability to identify high oleic acid contents, and also to investigate presence of *Orobanche* resistant genes.

Acknowledgements

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Table 1. Characteristics of markers used to analyze HO and LO sunflower genotypes

No	Primer type	Primer name	Primer sequences (5' → 3')
1	SSR	N1-1F	TTGGAGTTCGGTTTATTTAT
		N1-1R	TTAGTAAACGAGCCTGAAC
2	HO PCR specific prime	N1-3F	GAGAAGAGGGAGGTGTGAAG
		N2-1R	AGCGGTTATGGTGAGGTCAG

Table 2. Characteristics of markers used to analyze resistant (R) and susceptible (S) sunflower individuals

No	Primer type	Primer name	Primer sequences (5' → 3')
1	SCAR	RTS28-F	AGT AGA CGG GCA AAG CGA AAG GAT
		RTS28-R	AGT AGA CGG GTT GAA TAT GTT GAA
2	SCAR	RTS29-F	GCTTCCCCTTAATGATCCGGAAGA
		RTS29-R	GCTTCCCCTTGGCTAGAAGATGAA
3	SCAR	RTS40-F	TCCACCGAGCTACCAGTTCCGGAG
		RTS40-R	TCCACCGAGCGAGCATATTCCGAG
4	SCAR	RTS41-F	TCGTGTTGCTGATCGGAAAGGAAC
		RTS41-R	TCGTGTTGCTCAACAGTGGAGAAT
5	SSR	ORS1036-F	CCCTTTCACTTCCTATTTTCTATTCA
		ORS1036-R	CTAAGAGGGGTCGGTATGATTTC
6	SSR	ORS1040-F	CTGCTGATCGTTTCTTGGATAGA
		ORS1040-R	TGCTAATCCTTCTAATCAACTTCCAC

Figure 1. Amplified fragments with SSR primer for sunflower individuals

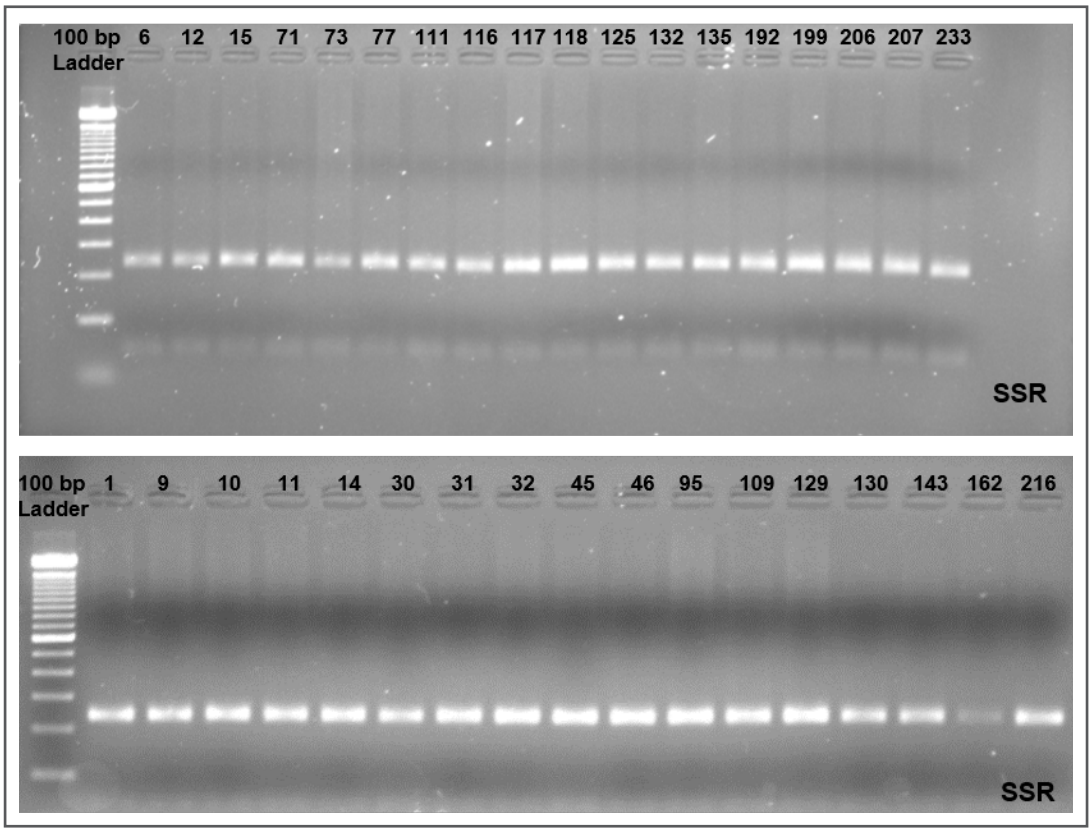


Figure 2. PCR amplification of HO and LO genotypes with HO PCR specific fragment

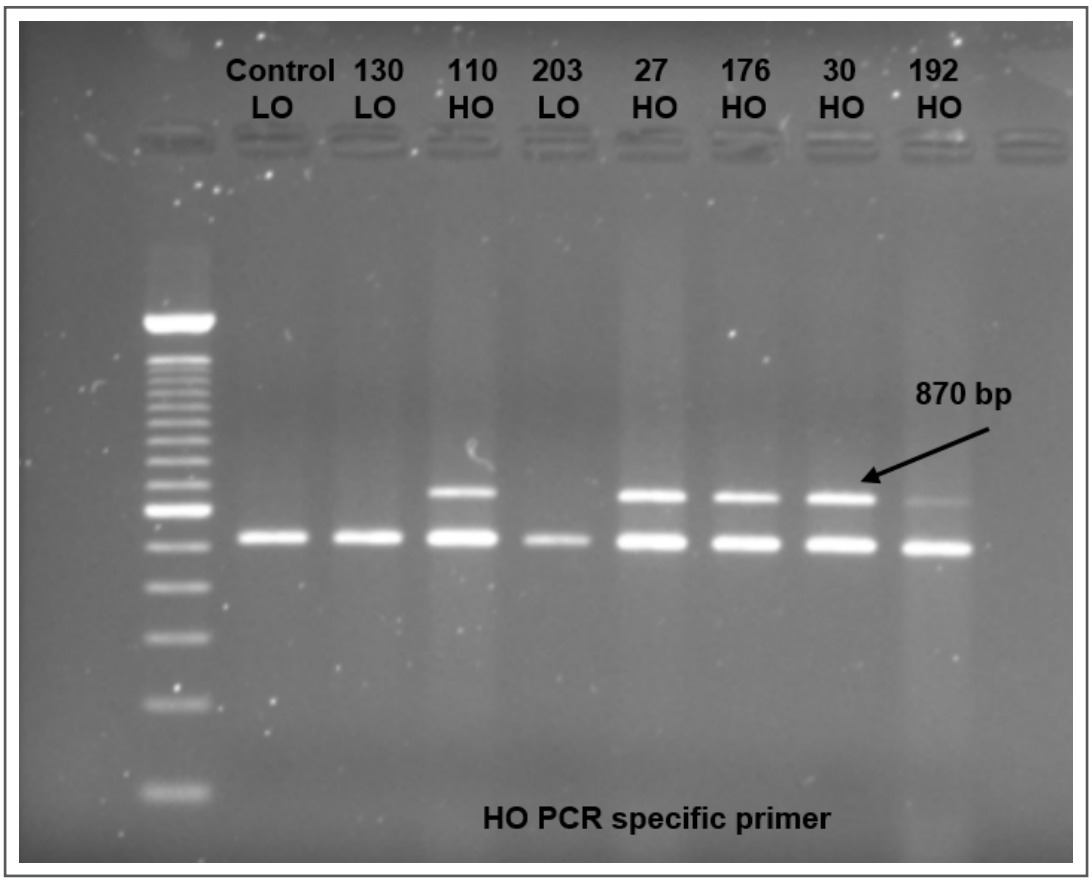


Figure 3. DNA fragment analyses results for SSR (N1-1F/N1-1R) primer

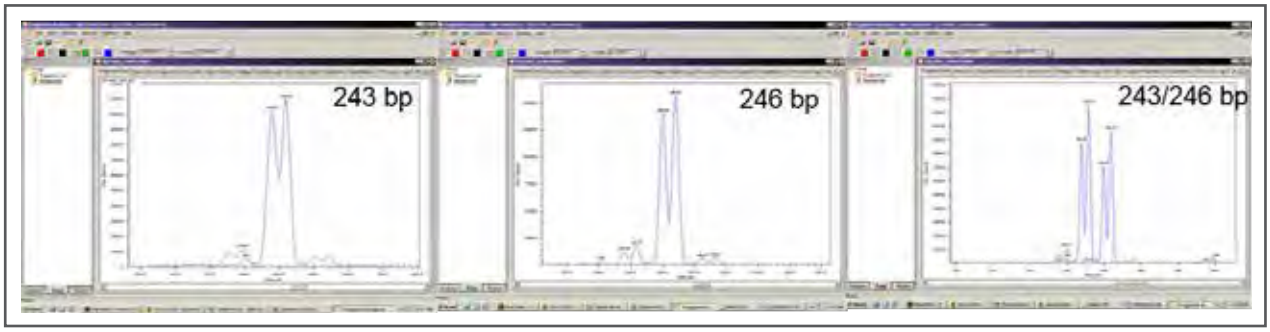


Figure 4. Amplified fragments with SCAR and SSR primers for sunflower individuals

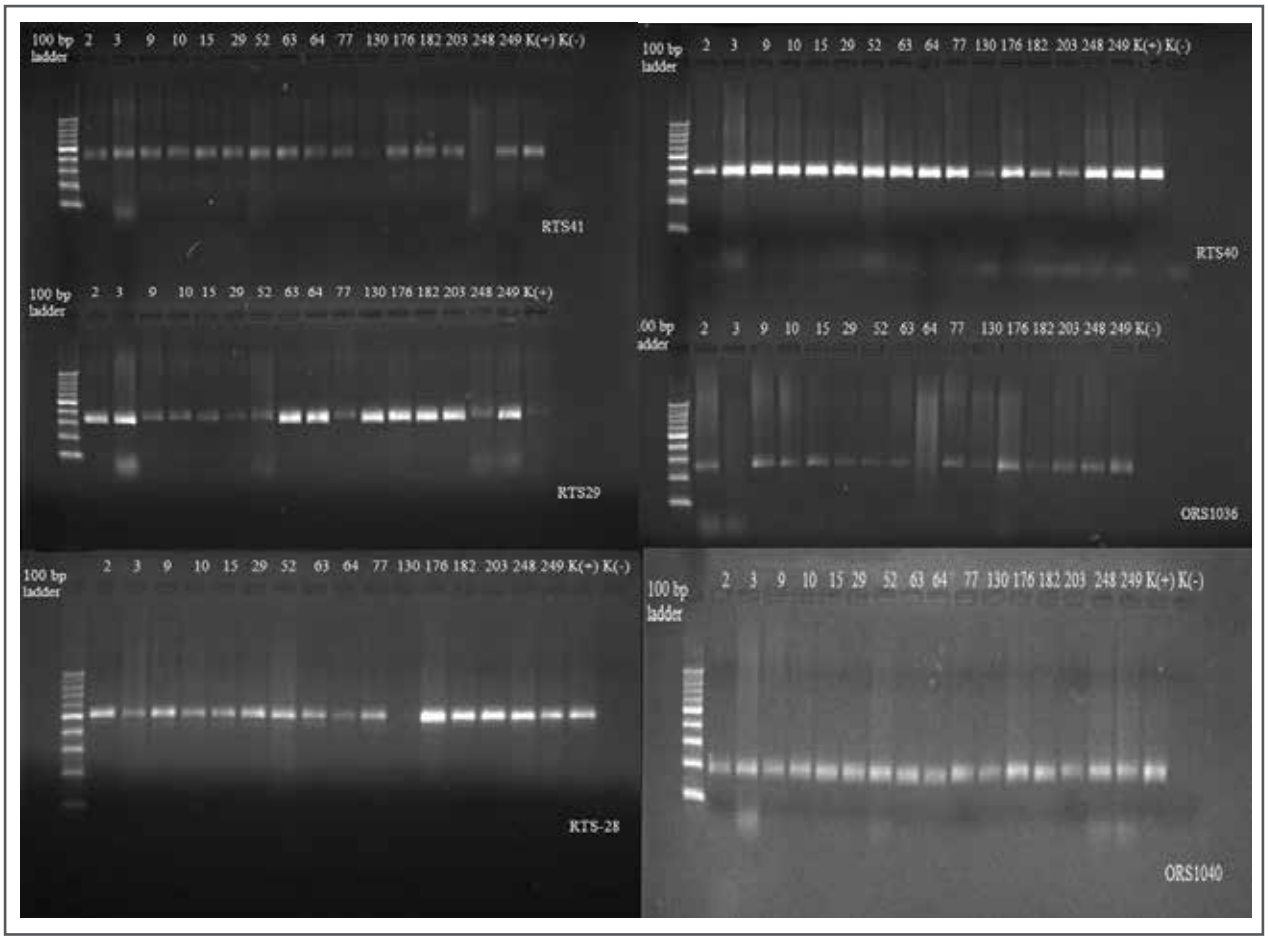


Figure 5. DNA fragment analyses results for ORS1036 primer

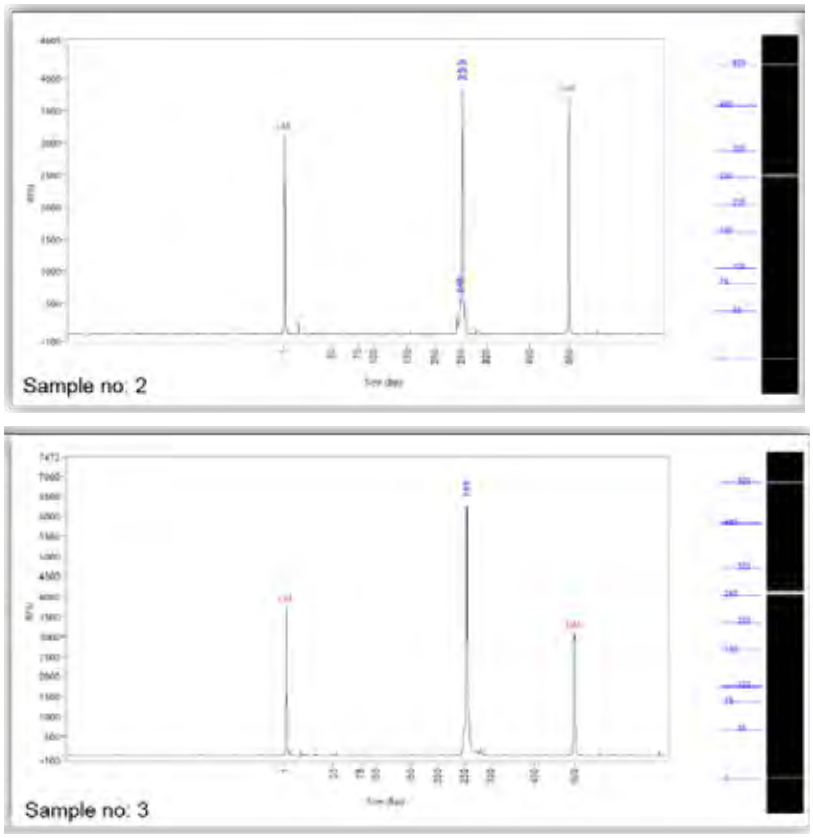
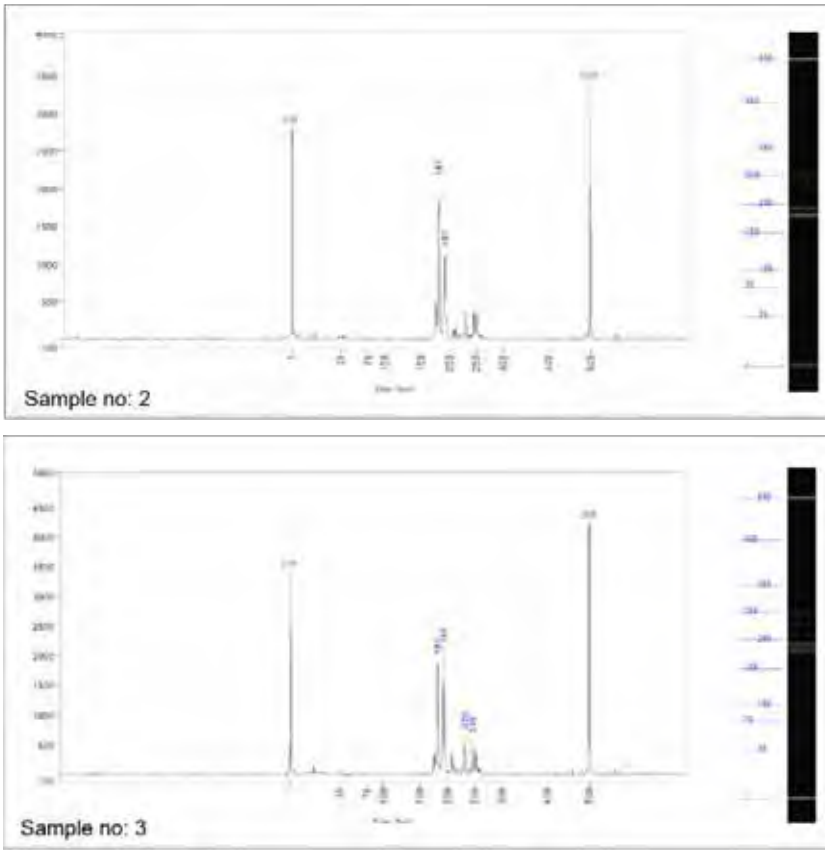


Figure 6. DNA fragment analyses results for ORS1040 primer



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The Determination of Silage Yield and Quality Traits of Candidate Maize Hybrids

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ABSTRACT

This study was conducted to determine some experimental silage yield and quality traits of maize hybrid to be improved by using hybrid breeding. This study was carried out with 15 experimental maize hybrids in 2012 and with 8 pieces in 2013 in Samsun. This study was carried out in the Randomised Complete Block Design(RCBD)with three replications. Genotypes' number of days for 50 % flowering, plant height, leaf/plant ratio, stalk/plant ratio, ear/plant ratio green forage yield and dry matter yield traits were investigated. Inadditon, the traits of silage crude protein, crude cellulose, ADF,NDF,ADL and crude protein yield were investigated. When examined traits were evaluated all together (ripening period, green forage yield, dry matter yield, and silage quality traits), TTM 2011-29, TTM 2011-28, TTM2011-36, TTM 2011-35 ve TTM 2011-7 genotypes, passed the control or involved in the same statistical group, were accepted as the promising varieties and they (TTM 2011-29, TTM 2011-28, TTM2011-36, TTM 2011-35 ve TTM 2011-7) were sent to locations to determine genotype x environment interaction for the purpose of testing within the scope of National Maize Breeding Researches.

Keywords: silage corn, ADF, NDF, maize breeding, candidate hybrid

Introduction

The food stuff demand for increasing population globally is a perceptive threat to food security.woeld over. There is no opportunity to increase present cultivated area in the world, Therefore, increasing crop productivity is important and it can be realized by making the best use of present cultivated areas (Cömertpay 2008).The most important way to increase plant yield is to develop new varieties which can give a highly productive and qualified yield. When it is considered in terms of animal production, this is the animal nutrition with low-cost but qualified feeds and the acquisition of maximum income. Traditionally, the expense of feed for animal production makes up more than half of the business expenses. For this reason, providing cheap feed is so important for business profitability. When cheap and qualified feed comes to, silage is the first thing that comes to mind. Maize is the most common material for silage production (Geren *et al*., 2003). One of the silage feed sources of quality roughage feed for animal breeding operations,matter to meet the demand of animal's living in autumn and winter when pasture, grazeland or feed plants enter the resting period. Maize and sorghum x sudan grass cross come first for silage feed plant production and in recent years plants especially producing high amount of green biomass have been preferred (Kavut *et al*., 2012). Silage maize is the most important source of roughage-succulent feed for ruminants because of its advantages as high energy, easily digestible, and involving the other feed plants mixture. Silage maize which can be cultivated

in very large areas is the most important silage plant cultivated in the world particularly in USA, because of the different reasons such as its capability of producing lots of gren portions from the unit area,its suitability for silage production,its high nutritional value and deliciousness. In our country, silage maize was produced with 18.563.390 ton in 401591ha in 2014 (TÜİK, 2016). The production and cultivation area of silage maize increased approximately 210% in the last ten years (TÜİK, 2016). The main factors of this increases are the increase of the usage of high productive silage maize varieties,involving in the project of feed plant support conducted by Ministry of Food, Agriculture and Livestock, its advanced mechanization,easy storage for the product and the increase of developing silage package industry day by day. In the last 20 years there have been significant increases in improved varieties for hybrid maize breeding. The determinant for these increases is that biotechnology and phytosanitary studies have been integrated into classic breeding methods.

In our country, maize breeding projects have been conducted by Public Research Institution mainly for seed purposes till at the beginning of 2000s, when they initiated silage maize breeding researches as happened in the world in recent years. Dry matter and green forage yield should be high, the period of keeping green colour should be long and it should be easy digestable,hybrids'net energy value should be high in terms of quality yield to choose the best hybrid for silage hybrid maize. Out of 320 registered maize varieties on our country's national list, only 15 have been registered as silage suitable varieties (TTSM 2016). With the increase in silege maize cultivated areas day by day ,the demand for seed is also increasing. This research was carried out to determine the performance of silage maize candidates developed pursuant to Black Sea Region maize breeding studies,considering silage maize yield and quality factors.

Material and Method

This research was conducted in experiment area situated in Black Sea Agriculture Research Institution in Carsamba under first crop conditions in 2012,2013. Inbred lines composed of within maize breeding research Project and materials originated from the abroad were used as study materials. Crosses were made among high ability special combining pure line in 2011 considering genetic proximity-distance and silage potentials (morphological and quality traits). P31Y43, Burak, Samada-07 and Safak varieties were used as control (standard) and 15 single crosses in 2012, and 8 in 2013 were used. The experiment was carried out in Samsun which has rainy and temperate climate. Temperature, precipitation, proportional moisture averages regarding in 2012 and 2013 and long terms averages are given in Table 1. While moisture and temperature values were relatively in each cultivating season (2012,2013) but they differed from long term averages. Average temperatures were measured 1 °C higher than long term averages in both two years. Significant differences were measured for total fall and distribution of fall into months. Approximately, two times more fall was obtained in the first year of the experiment than in the second year. The soil of testing area has clayed-loamy and little alkaline. Total salt and the amount of takable phosphorus were low, but plants were rich in terms of nutrition elements and potassium and lime, but low in terms of organic matter (Table2). The experiment area was cultivated in 14 may, 2012 and 16 may, 2013. Experiments have been carried out for two years under main crop conditions using the randomized block design with three replications. Sowing was made with hands as spreading two seeds in per growing bed and every plot had four lines and plot area was 14 m^{2}. The row to row distance was 70 cm and plant to plant distance within rows was 18 cm. The length of rows was 5m. When the plants reached knee-deep (40-50 cm) in the experiment, the weak one from two plants in the growing bed was thinned. Irrigations were applied with drum irrigation systalk and earthing up was applied with hoeing regularly (Kırtok,1998)

Dressing was made as pure 8 kg phosphorus and 20 kg N/ha totally per decare according to soil analysis. All phosphorised manure and 8 kg/ha of nitrogenous manure were given at the time of sowing as bottom fertilizer, the rest of the nitrogenous fertilizer was given when the plants became 4-6 leafed (V4-V6 phase), reached approximately 40-50 cm. Two lines in the middle were harvested for green forage yield. The harvest was done at the ½ and ⅔ milk lines in other words at the early dough stage. 500 gr sample plant was kept in the oven at the 70 ^{0}C for 48 hours for dry matter ratios. Dry matter yield values were calculated according to dry matter ratios as being weighed when it reached to constant weight. Besides, number of days for 50 % flowering, plant height, leaf/plant ratio,stalk/plant ratio and ear/plant ratio were investigated. Phenological and morphological observations taken during the research were made based on technical order of agricultural values evaluation testings by Ministry of Food, Agriculture and Livestock (Anonymous2010). Genotypes' The silage quality parameters of genotypes were analysed

viz., (ADF, NDF, ADL, rawcellulose, raw protein). These were got done in Blacksea Agricultural Research Institution analyses laboratories in respect of 2012 to determine silage yield. Data obtained from the research were subjected to the varience analysis according to (Düzgünes *et al*., 1987) using Mstat-C software, and multiple comparisons of group averages were made according to Duncan test. Years were evaluated one by one because differences became significant for all observed characters between years according to varience analysis and conclusions which were made regarding the year as factor.

Results

Statistically, differences were found significant at the level of 0.01 between genotypes in terms of the number of flowering days, plant height,first ear height, stalk/plant ratio, leaf/plant ratio, ear/plant ratio, green forage yield and dry matter yield values. Duncan groups are given in Table 3-4 belonging to investigated traits. The average flowering days of genotpes were 74 in 2012, and the earliest flowering was seen in TTM 2011-14 genotype with 69.3 days, and the latest flowering was seen in Burak Standard variety with 77.0 days (Table 3). Genotypes' flowering periods changed between 63.0 and 71.0 days, the earliest flowering was seen in TTM 2011-18 variety candidate, and the latest flowering was seen at Burak standard variety with 71.0 days as similar of the first year (Table 4). The averages of varieties' plant heights changed between 246.7 and 330.0 cm at the first year and it was measured that Burak standard variety had the longest plant height (330.0 cm), and TTM 2011-14 variety candidate had the shortest plant height (246.7 cm) (Table 3). The averages of plant heights were 308.4 cm at the second year and Burak Standard variety had the longest plant height with 351.7 cm and TTM 2011-20 candidate variety had the shortest plant height with 258.3 cm (Table 4). Variety and variety candidates' the first ear heights measured as between 98.3 and 145.0 cm and Burak Standard variety had the longest first ear height, TTM 2011-14 had the lowest first ear height (Table 3). The averages of first ear heights were 129.3 cm in 2013 and Burak Standard variety had the longest one with 161.7 cm and TTM 2011-18 genotype had the shortest one with 105.0 cm (Table 4). Stalk/plant ratios changed between 33.3% and 48.5% in 2012 and the lowest one was taken from TTM 2011-36 candidate variety with 33.3% and the highest one was taken from TTM 2011-14 candidate variety with 48.5% (Table 3). Stalk/plant ratios changed between 36.5% and 43.9% in 2013 and Burak Standard variety had the longest stalk/plant ratio as 43.9% and TTM 2011-36 genotype had the lowest as 36.5% (Table 4).Genotypes' leaf/plant ratios changed between 10.4% and 27.5% at the first year and TTM 2011-7 genotype had the lowest leaf/plant ratio as 10.4% and TTM 2011-26 genotype had the highest leaf/plant ratio as 27.5% (Table 3). Leaf/plant ratios changed between 18.3% and 19.7% at the second year and TTM 2011-9 candidate variety had the highest one as 19.7% and TTM 2011-36 genotype had the lowest one as 18.3% (Table 4). Ear/plant ratios of variety and candidate varieties changed between 36.0% and 52.5% in 2012 and the lowest one determined for TTM 2011-26 as 36.0% and the highest one for TTM 2011-36 as 52.5% (Table 3). Ear/plant ratios changed between 38.8% and 46.0% in 2013 and the highest ear/plant ratio was measured for Burak Standard variety as 38.8% and the lowest for TTM 2011-36 genotype as 46.0% (Table 4). Green forage yields changed between 4614.7 kg/da and 7443.4 kg/da and the highest yield was taken from TTM 2011-29 as 7443.4 kg/ha and the lowest from TTM 2011-14 as 4614.7 kg/da (Table 3). The values of green forage yields were measured between 4616.9 and 6187.8 kg/da in 2013 and the highest green forage yield was measured from TTM 2011-36 candidate variety as 6187.9 kg/da and the lowest from TTM 2011-20 candidate variety as 4616.9 kg/da(Table4). The averages of genotypes' yields changed between 1390 kg/da and 2298 kg/da in terms of dry matter at the first year of the experiment. The highest dry matter yield was determined from TTM 2011-29 as 2298 kg/da and the lowest one from TTM 2011-14 as 1390 kg/da (Table3). The highest dry matter yield was measured for TTM 2011-36 as 2632.1 kg/da at the second year of the experiment and the lowest one for TTM 2011-18 as 1895.7 kg/da (Table 4). Differences between genotypes were found significant statistically in terms of ADF%, raw cellulose%, NDF% and raw protein and differences between ADL% and raw protein (%) ratios were found insignificant statistically. ADF% ratios among varieties involved in the experiment changed between % 21.7-35.0 and the lowest ADF ratio was measured from TTM 2011-36 variety candidate and the highest. ADF% ratios among varieties involved in the experiment changed between% 21.7-35.0 and the lowest ADF ratio was measured from TTM 2011-36 variety candidate and the highest ADF from TTM 2011-20 variety candidate and the average of ADF% was measured as 30.2. The average of genotypes' raw cellulose ratios was measured as 28.0% and the lowest raw cellulose was measured from TTM 2011-18 genotype as 20.6% and the highest ratio from TTM 2011-30 genotype as 36.5%. ALD% ratios of variety and variety candidates changed between 1.2%-

3.1% and raw protein ratios changed between 7.3%-7.9%. NDF% ratios among varieties involved in the experiment changed between 54.2%-67.0% and the lowest NDF ratio was measured from TTM 2011-30 variety candidate and the highest NDF% from TTM 2011-35 variety candidate and the average of the experiment was measured as 59.6. Genotypes' raw protein yields changed between 126.8-171.4kg/da and the highest raw protein yield was obtained from TTM 2011-28 genotype and the lowest yield from TTM 2011-22 genotype (Table 5).

Discussion

High yield, earliness, low seed moisture have composed the basis of maize breeding studies and quality in recent years. Earliness is so important in terms of variety in cultivation period and is the most important criterion for being cultivated crop under main crop or second crop conditions. The great majority of silage maize varieties on the market are stage group temporary varieties. Earliness becomes important because the great majority of silage maize cultivating areas are cultivated as second crop. Genotypes differed from each other in terms of the duration of flowering days considering investigated genotypes and standards flowered later than candidate varieties in both two years. TTM 2011-18 and TTM 2011-36 crosses from variety candidates flowered at the earliest in both two years. (Oner *et al*., 2011)determined that the number of 50% flowering days for varieties was between 58-65 days in their study on the purpose of determining silage maize varieties' some yield and quality traits under Samsun conditions, similarly, (Ozata *et al*., 2012) determined it was between 58-64 days in their study under Samsun conditions, (Erdal *et al*., 2009) determined it was between 60-65 days in their sudy under Antalya conditions, (Sade *et al*., 2005) determined it was between 82-87 days in their study under Konya conditions. When obtained data were investigated, it can be said that genotypes studied with, were close with genotypes in the studies under Samsun and Antalya conditions in terms of flowering day numbers and they were more earlier genotypes than genotypes in the study in Konya. The average plant height was 277.6 cm in 2012 and 308.4 cm in 2013, (Erdal *et al*., 2009) obtained it was 234 cm in the first year and 273 cm in the second year for silage maize varieties in their study under Antalya conditions, (Ozata *et al*., 2012) determined the plant height of silage maize varieties changed between 235-284 cm in their study under Samsun conditions, and (Bolat *et al*., 2011) determined plant height changed between 270-283,3 cm in their study investigated the effect of chemical and microbial fertilizer applications on silage maize yield under Adana conditions. While the first experiment averages of plant height values were in harmony with other studies, the second averages were found higher than other studies. Mostly, plant height arises from variety trait, also is affected from environment conditions. The first ear height was obtained fort he first year average (119.6cm) lower than for the second year average (129.3cm). The first ear height is directly proportional with plant height and generally the height of variety is wanted as between ⅓ and ½ for breeding studies. (Oz *et al*., 2008) stated the first ear height changed between 81-100 cm and the second height changed between 68-111cm, and (Oz *et al*., 2005) the first ear height changed between 109-126 cm at the same conditions. Conclusions were obtained higher than other studies. This difference stalkmed from the differences of genotypes.

The average of experiment was 39.3% at the first year and 39.5% was in the second year in terms of stalk/plant ratios when variety and candidate varieties were investigated.On the basis of variety, the highest stalk/plant ratio was obtained from TTM 2011-14 candidate variety as 48.5% in 2012 and it was obtained from Burak Standard variety as 43.9% in 2013. When leaf/plant ratios investigated, the average of the experiment was 18.5% in 2012 and the average of the second year was 17.9%. The highest leaf/plant ratio was determined for TTM 2011-26 as 27.5% at the first year and for TTM 2011-9 as 19.7% at the second year. When the averages of ear/plant ratios were considered, the highest ratio was obtained from TTM 2011-36 candidate variety as 52.5% at the first year and from TTM 2011-20 genotype as 46.0% at the second year. (Özata *et al*., 2012) have determined that variety and candidate varieties' averages of ear/plant, stalk/plant,and leaf/plant rayios were 40.6% and 41.7% and 17.6% respectively in their study conducted under Samsun conditions. (Oner *et al*., 2011) have stated that leaf/stalk ratios changed between 26% and 43% and ear/plant ratios were changed between 33% and 41% in their study which they investigated quality and yield traits at some silage maize varieties under Samsun contitions in 2010. (Caglar *et al*., 2008) have stated leaf ratio changed between 23.4% and 20.2% and so as to ear ratio between 37.2% and 32.3% and leaf ratio changed between 39.5% and 47.6 at their study conducted under Erzurum conditions. (Geren *et al*., 2003) have stated that leaf, stalk and ear ratios for green forage changed between 34.5% and 42.7% and between 35.9% and 42.1% and between 19.6%-

27.9% respectively at their study conducted under Izmir conditions. (Iptas *et al*., 2002) have stated ear ratio changed between 32.9%-42.0% and so as to stalk ratio between 39.3%-50.1%,and leaf ratio changed between 15.3%-21.2 in their study conducted under Tokat conditions. Obtained conclusions are in harmony with the other studies. Yield (green forage) is an overemphasized selection criterion for silage maize breeding researches as good for maize breeding researches. The average of experiment variety and variety candidates was 5704kg/ha in 2012,it was 532.1 kg/ha in terms of green forage yield in the second year. The highest yield was obtained from TTM 2011-29 genotype as 7443.4kg/ha and the lowest yield from TTM 2011-14 genotype in terms of green forage yield in the first year. 8 variety candidates passed Standard in the first year. The highest yield was obtained from TTM 2011-36 variety candidate as 6187.9 kg/ha and the lowest from TTM 2011-20 genotype as 4616.9 kg/ha in the secnd year. (Ozata *et al*., 2012) determined that the averages of green forage yield changed between 3340.5-6297 kg/ha in their study conducted under Samsun conditions and (Oner *et al*., 2011) determined that they changed between 6075-7391 kg/ha in their study conducted with registered silage varieties in Samsun-Carsamba location. (Erdal *et al*., 2009) stated the average of green forage yields was 6345 kg/ha in 2006 and it was 6504 kg/ha in 2007 in their study under Antalya conditions. (Iptas *et al*., 2002) stated green forage yield changed between 6723-8799 kg/ha averagely at the experiment which they conducted in between 1996-98 under Tokat ecological and main crop conditions. (Akdemir *et al*., 1997) found that green forage yield changed between 4834-6706 kg/ha in the experiment under Bursa conditions. While conclusions were in harmony with the studies conducted in Bursa and Samsun, were lower than the other studies. Dry matter which is one of the yield traits for the production of silage maize is another overemphasized criterion. The average of dry matter yield was determined as 1806 kg/da in the first year of the experiment and as 2278.7 kg/da at the second year. (Ozata *et al*., 2012) stated dry matter yields changed between 1105-1867 kg/da in their study under Samsun conditions and (Erdal *et al*., 2009) stated the average of the first year was 2333 kg/da and the second year of it was 2227 kg/da. (Iptas2002) found dry matter yield changed between 1513.9-2076.6 kg/da in their experiment under the second crop conditions in Tokat. (Oner *et al*., 2011) determined that dry matter yield changed between 1289-2132 kg/da in their experiment conducted with registered silage varieties in Samsun-Carsamba location in 2010, (Akdeniz2004) stated dry matter yield changed between 683-1499 kg/da in the first year and between 767-1723 kg/da in the second year in their two-year study under Van ecological conditions. While obtained data were in harmony with the studies under Samsun and Van conditions and lower than studies under Tokat and Antalya conditions. The content of silage maize, raw protein, raw protein yield, ADF, NDF ratios are also determinants for the energy values of maize silage. In the study, raw protein ratio changed between 6.8-7.7% and raw protein yield changed between 117.3-171.4 kg/da. ADF ratio was averagely 30.2% and changed between 27.1-35.0 and the lowest one obtained from TTM 2011-36 variety candidate and the highest ona from TTM 2011-20 variety candidate. When NDF ratio was investigated it changed between 54.2-67.0 and the lowest one was obtained from TTM 2011-18 cross, the highest one from TTM 2011-35 variety candidate.

Raw cellulose ratio changed between 20.6-36.5% and ADL ratio changed between 1.5-2.0 % (Ozata *et al*., 2012) stated the average of raw protein yield was 6.08%, raw protein yield was 89.3%, ADF ratio was 32.2% and NDF ratio was 53.5% in their study under Samsun conditions. (Erdal *et al*., 2009) determined the average raw protein ratio changed between averagely 7.5% and raw cellulose ratio averagely 20.2% and NDF ratio as 64.0% in thier study under Antalya conditions. (Oner *et al*., 2010) stated ADF, NDF% and raw protein ratio values changed as 31-41%,49-60%, and 3.85-5.85% respectively. (Hutjens1998) determined ADF ratio changed between 21.7-40.7% and NDF ratio between 41.2-70.9 in their study investigated 86 maize varieties' silage traits in 1996 in Illinois, USA. Obtained conclusions are in harmony with the studies. To be high of silage trait is explained with being high of raw protein and being low of ADF, NDF ratios. Generally it is wanted ADF ratio is 30% or lower than it and NDF ratio is between 50-60%

Conclusion

The production and consumption of maize silage have increased commonly due to having high energy value particularly. The average of silage maize (green forage) yield for our country is 4,5ton (TÜIK2013) and higher green forage yield was obtained from all genotypes taken to the experiment. Three traits of plant for silage maize breeding: ripening period, green forage yield and the content of dry matter at harvest are regarded as determinant during selecting. Ripening period, green forage yield and dry matter yield are affected from environment

conditions significantly. Maize plant need total temperature between 2370-3000 °C as well as it differentiates for every plant.Temporal varieties can be cultivated and obtained high yields because Black Sea Region has a mild climate and generally its vegatation period is suitable. Providing, it is desired that varieties give the same yield or close to it in all regions. TTM 2011-29, TTM 2011-8 and TTM 2011-36, TTM 2011-35 and TTM 2011-7 genotypes became prominent crosses in the conclusion of study which was aimed to determine the silage yield and quality traits of silage maize variety candidates. These five variety candidates passed standards in the registration experiments in terms of dry matter yield in both two years or took part in the same statistical group. It is decided TTM2011-28, TTM2011-29 ve TTM2011-36 TM 2011-35 ve TTM 2011-7 variety candidates (for the purpose of being experienced multiple locations) will be involved in Territorial Maize Researches Silage Maize experiment to be evaluated better before varieties are given to registration and to be seen genotype x environment interaction.

Table 1. The 2012-2013 year and for many years some corn during the growing season meteorological data of Samsun *

AYLAR	Mean of Temperature (°C)			Relative humidity (%)			Total rainfall (mm)		
	Many years	2012	2013	Many years	2012	2013	Many years	2012	2013
April	11.1	13.3	12.7	79.5	74.4	76.5	58.3	10.4	64.2
May	15.3	17.5	18.7	80.6	82.3	77.4	50.6	34.4	8.9
June	20.0	21.9	21.6	76.3	76.4	73.0	47.9	24.4	49.7
July	23.1	24.0	23.2	73.4	77.1	72.7	31.3	96.0	43.6
August	23.2	23.0	23.6	73.7	78	76.4	50.9	179.6	26.5
September	19.8	20.1	18.7	74.7	80.4	75.9	87.4	113	44.9
Mean	18.8	20.0	19.8	76.3	78.1	75.3	-	-	-
Total	-	-	-	-	-	-	326.4	457.8	237.8

* (Samsun Regional Directorate of Meteorology, 2012 ve 2013)

Table 2. Some properties of study area*

Parameter	2012	2013	
Soil texture	66.0	68.0	Clay Loam
pH	7.86	7.60	Slightly alkaline
P_2O_5 (kg da^{-1})	2.52	2.50	Very Low
K_2O (kg da^{-1})	94.0	92.0	High.....
Organic Matter (%)	1.76	1.70	Low.....
$CaCO_3$ (%)	6.76	7.50	Medium
EC (%)	0.054	0.061	Nonsaline

* (Samsun, Blacksea Agricultural Researche Institute, Soil Department Laboratory, Analyze Number:362)

Table 3. Some yield and yield characteristics of the silage maize genotypes, 2012

Genotypes	Tasseling	Plant height	First Ear height	Stalk/ Plant ratio	Leaf/ Plant ratio	Ear/plant ratio	Silage yield (kg/da)	Dry matter yield (kg/da)
TTM2011-29	73.0 fgh	303.3 ab	126.7 a-d	40.7 cde	17.8 c-f	41.5 d-g	7443.4 a	2.298 a
TTM2011-28	76.3 ab	295.0 bcd	123.3 a-e	40.1 def	18.1 c-f	41.8 d-h	6722.9 ab	2.247 a
P31Y43 (st)	72.7 gh	296.7 bc	130.0 a-d	35.3 ıjk	22.2 bc	42.5 def	6044.8 bc	1.880 b-e
Burak (st)	77.0 a	330.0 a	145.0 a	42.6 bc	17.9 c-f	39.5 gh	5987.1 bcd	1923 b
TTM2011-20	73.0 fgh	255.0 e	110.0 def	41.8 bcd	15.4 ef	42.7 de	5963.8 cde	1.987 b
TTM2011-35	75.0 bcd	270.0 cde	131.7 a-d	38.6 fgh	15.0 efg	46.4 bc	5928.4 cde	1.905 bc
TTM2011-7	74.7 cde	256.7 e	120.0 b-f	44.3 b	10.4 g	45.4 c	5906.3 cde	1.973 b
TTM2011-36	73.3 e-h	263.3 e	113.3 c-f	33.3 k	14.2 fg	52.5 a	5862.4 cde	1.704 d-g
TTM2011-18	72.0 h	265.0 e	110.0 def	41.7 cde	16.5 def	41.9 d-g	5773.9 cde	1.848 b-e
TTM2011-9	73.3 e-h	268.3 de	120.0 b-f	34.2 jk	23.4 ab	42.4 def	5721.0 c-e	1.917 bc
Samada-07(st)	76.0 abc	296.7 bc	136.6 ab	39.0 fg	17.0 def	44.0 cd	5553.8 c-f	1.941 b
TTM2011-10	74.7 cde	248.3 e	110.0 def	39.3 efg	18.8 b-f	41.9 d-g	5543.4 c-f	1.841 b
Şafak(st)	76.3 ab	310.0 ab	135.0 abc	37.3 ghı	23.3 ab	39.3 h	5538.9 c-g	1.953 b
TTM2011-26	74.3 def	256.7 e	115.0 b-f	36.4 hıj	27.5 a	36.0 ı	5507.6 def	1.689 efg
TTM2011-30	76.0 abc	313.3 ab	131.7 a-d	40.6 c-f	19.3 b-e	40.0 fgh	5422.4 def	1.901 bcd
TTM2011-3	73.3 e-h	260.0 e	103.3 ef	39.5 d-g	16.3 def	44.2 cd	5180.1 ef	1.722 c-g
TTM2011-12	72.3 h	273.3 cde	98.4 f	38.7 fgh	20.8 bcd	40.5 e.h	4884.3 g	1.644 fg
TTM2011-22	74.0 d-g	266.7 de	115.0 b-f	34.3 jk	17.2 def	48.5 b	4778.4 g	1.613 g
TTM2011-14	69.3 ı	246.7 e	98.3 f	48.5 a	19.7 b-e	31.8 j	4614.7 g	1.390 h
Means	74.0	277.6	119.6	39.3	18.5	42.3	5704.0	1806.0
CV(%)	1.2	6.1	11.1	3.7	8.7	3.6	6.3	7.0
LSD (0.05)	1.6	27.7	21.8	2.5	4.9	2.5	594.0	196.0
P	**	**	**	**	**	**	**	**

Table 4. Some yield and yield properties belong to silage maize genotypes, 2013

Genotypes	Tasseling	Plant height	First Ear height	Stalk/ Plant ratio	Leaf/ Plant ratio	Ear/plant ratio	Silage yield (kg/da)	Dry matter yield (kg/da)
TTM2011-36	65.3 b	303.3 ab	136.7 bc	36.5 g	19.3 ab	44.2 abc	6187.9 a	2632.1 a
Burak(st)	71.0 a	351.7 a	161.7 a	43.9 a	17.2 efg	38.8 g	5918.4 ab	2522.7 ab
P31Y43 (st)	66.0 b	310.0 bc	128.3 cde	39.3 cde	17.4 def	43.4 bcd	5886.8 ab	2490.6 ab
TTM2011-35	70.0 a	315.0 bc	133.3 cde	38.5 def	18.6 bc	42.8 cde	5646.7 ab	2374.2 abc
TTM2011-7	69.7 a	311.7 bc	120.0 def	36.7 fg	18.3 c	45.0 ab	5274.2 abc	2286.6 abc
TTM2011-29	70.3 a	315.0 bc	126.7 cde	41.8 b	16.7 fgh	41.4 ef	5269.0 abc	2428.7 ab
Şafak(st)	70.0 a	321.7 a	148.3 ab	40.9 bc	16.5 gh	42.6 cde	5268.1 abc	2199.2 abc
TTM2011-9	69.0 a	312.7 ab	128.3 cde	40.1 bcd	19.7 a	40.2 fg	5245.5 bc	2185.1 abc
TTM2011-28	69.7 a	301.7 bc	121.7 cde	40.4 bcd	17.9 cde	41.7 def	5116.2 bc	2171.6 abc
Samada-07 (st)	70.0 a	313.3 bc	125.0 cde	38.9 de	18.6 c	42.5 cde	4797.8 bc	2079.7 abc
TTM2011-18	63.0 c	286.7 cd	105.0 f	39.3 cde	18.0 cd	42.8 cde	4626.0 c	1895.7 c
TTM2011-20	66.0 b	258.3 d	116.7 ef	37.6 efg	16.4 h	46.0 a	4616.9 c	2078.4 bc
Means	68.3	308.4	129.3	39.5	17.9	42.6	5321.1	2278.7
CV(%)	1.9	5.9	7.2	2.9	2.4	2.6	10.6	11.7
LSD (0.05)	2.2	30.1	15.6	1.9	0.8	1.9	928.7	516.3
P	**	**	**	**	**	**	**	**

Table 5. Some guality belong to silage maize genotypes, 2012

Genotypes	ADF %		Crude cellulose (%)		ADL (%)	NDF (%)		Crude protein (%)	Crude Protein yield (kg/da)	
TTM2011-20	35.0	a	31.5	bc	1.9	62.4	bcd	7.2	143.3	bc
TTM2011-3	34.4	ab	34.2	ab	1.8	61.2	cde	7.0	120.5	e
TTM2011-30	31.9	abc	36.5	a	1.9	60.4	c-f	7.6	143.9	bc
Samada-07 (st)	31.8	a-d	31.0	bcd	1.7	61.2	cde	7.2	148.2	b
TTM2011-22	31.2	a-d	31.2	bc	1.6	66.7	ab	7.9	126.8	e
TTM2011-7	30.8	b-e	23.0	gh	1.5	59.2	d-g	7.4	146.0	bc
TTM2011-12	30.6	b-e	27.6	c-g	2.2	55.8	gh	6.8	117.3	e
TTM2011-28	30.5	b-e	26.3	efg	2.2	63.8	abc	7.6	171.4	a
Burak (st)	30.4	b-e	26.4	d-g	3.1	56.1	fgh	7.5	136.7	b-e
Şafak (st)	30.0	cde	25.3	e-h	1.2	54.4	h	7.0	149.0	b
TTM2011-14	29.6	cde	28.3	cdef	1.7	60.3	c-f	7.2	106.1	f
TTM2011-35	29.5	cde	29.3	cde	1.7	67.0	a	7.3	139.0	b-e
TTM2011-10	29.3	cde	28.8	cde	1.6	60.4	c-f	7.5	138.7	b-e
TTM2011-9	29.1	cde	23.9	fgh	1.9	56.1	fgh	7.5	143.8	bc
TTM2011-26	28.7	cde	32.1	abc	1.5	59.1	d-g	7.6	127.8	de
TTM2011-18	28.6	cde	20.6	h	1.3	54.2	h	7.4	141.8	bcd
TTM2011-29	28.3	cde	28.9	cde	2.0	58.1	d-h	7.7	177.0	a
P31Y43 (st)	27.8	de	24.8	e-h	1.2	57.3	e-h	7.2	144.7	bc
TTM2011-36	27.1	e	21.4	h	1.2	58.5	d-h	7.7	131.2	cde
Means	30.2		28.0		1.7	59.6		7.4	139.6	
CV (%)	8.10		7.55		0.40	4.54		2.36	6.38	
LSD (0.05)	4.0		4.6		-	4.6		-	14.8	
P	*		*		-	**		-	*	

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A Study on Usability of National Registered Durum Wheat Cultivars for Synthetic Bread Wheat Production

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ABSTRACT

The present study was conducted in Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary, from 7th September 2015 to 31st July 2016, and aimed to evaluate the crossabilities of 6 *Triticum durum* (Ç-1252, Kızıltan, Altıntaş, Dumlupınar Yelken and Kunduru) with 6 *Aegilops tauschii* (MVGB605, MVGB1323, MVGB589, CIMMYT224, CIMMYT372 and CIMMYT458). *Triticum durum* and *Aegilops tauschii* parents had been successfully grown, and the desired number of spikes had been emasculated and pollinated (total 171 spikes). Total 34 combinations were made and 164 embryos were obtained from 19 combinations among them. There were no embryos in 15 combinations. Number of maximum embryo rescue was obtained from Yelken/MVGB589 combination (24 embryos). It was followed by Kızıltan/MVGB589, Ç-1252/MVGB589 and Ç-1252/2T combinations (16, 13 and 11 embryos, respectively). 70 germinated plants were obtained from 164 embryos in B-5 medium (in petri dishes). 94 embryos were not germinated. A total of 29 germinated embryos died in two B-5 medium (petri dish and tube). Some older and younger tillers died after colchicine treatment. However, we had new shooting tillers. According to results, the best females were Yelken and Ç-1252 among *Triticum durum*s, while the best male MVGB589, CIMMYT224 and MVGB1323 among *Aegilops tauschii*s, respectively.

Keywords: hybrid, synthetic, wheat, *Ae. tauschii,* crossability

Introduction

Wheat has an important place in Turkey's national economy, and new varieties resistant to both biotic and abiotic stresses will play an important role in ensuring that wheat preserves its current status and importance (Shah *et al*., 1987; Cox *et al*., 1994; Mujeep-Kazi and Hettel, 1995; Mujeep-Kazi *et al*., 2008). In this context, it is important to consider the varieties and benefits which synthetic wheat will provide (Thompson and Zwart, 2008). Many countries all over the world have already begun to use synthetic wheats in production and breeding programs, and interest in synthetic wheats is gradually growing (Mujeep-Kazi *et al*., 2008).

For Turkey, the utilization of synthetic varieties in hybridization programs is of considerable importance. After being first hybridized, it may take up to 18 years for a new registered variety to be used in the production stages in farmers' fields. For this reason, it is important to use synthetic bread wheat varieties for developing new varieties for Turkey that have wide adaptability. Durum wheat, which is used as the source for synthetic bread wheat varieties, already represents a variety with significant adaptability. In this context, the current project plans to produce synthetic bread wheat varieties by utilizing local durum wheat genetic stocks.

Synthetic bread wheats obtained through interspecies have several disadvantages compared to cultivated bread wheats. However, extensive hybridization studies on culture wheats may reduce these disadvantages, or change them into advantages (Villareal *et al*., 1994; Lage *et al*., 2004; Mujeeb–Kazi *et al*., 2008). Compared to culture wheat, synthetic wheats are more resistant and/or hardy against biotic and abiotic environmental conditions. For example, Mujeep-Kazi and Hettel (1995) previously tested the salt resistance as well as the *Helminthosporium sativum, Fusarium graminearum,* and *Tilletia indica* resistance of various synthetic varieties obtained through the hybridization of durum wheats and *T. tauschii.* Based on the obtained results, they determined that the synthetic varieties were all comparatively more resistant to salt and these diseases than their parents. Synthetic hexaploids have 2n=3x=21 chromosomes. After chromosome doubling is performed with the chemical substance colchicine, the seeds generally have 42 chromosomes. However, hypoploids with 41 chromosomes and hyperploids with 43 chromosomes may also be encountered among the obtained synthetic varieties (Mujeep-Kazi and Hettel, 1995). This generally stems from differences in chromosome folding periods following colchicine application (Sears, 1941; Kihara, 1924; Sears, 1944; McFadden and Sears, 1946; Sears, 1956).

The most difficult stage of synthetic bread wheat production is the procedure used for embryo regeneration and chromosome doubling. For this reason, this project is necessary to observe the latest development in embryo culture and chromosome doubling methods at the Agricultural Institute of the Centre for Agricultural Research of the Hungarian Academy of Sciences, and for further increasing our knowledge on these procedures. Using materials from Turkey, the project will enrich our knowledge regarding synthetic wheat production based on the latest international developments and advances in this field. Owing to this project, we will be able to add information regarding the most recent advances in double haploid wheat production in Hungary to the current body of knowledge, and also initiate synthetic wheat production studies that will serve to broaden the genetic basis available in Turkey for breeding studies-which is particularly important at this time when the effects of global warming are being increasingly felt in Turkey.

Materials and Methods

1. Experimental parental materials and Planting

This study was started on 7th September in 2015. The experiments were primarily performed by using six durum wheat varieties (*Triticum durum*) originating from Turkey. These varieties were the Kunduru-1149, Altıntaş-95, Yelken-2000, Kızıltan-91, Ç-1252, and Dumlupınar (Table 1). Durum wheats were sown 8 seeds per variety in jiffy pod. All of durum wheats were sown at 7 different times with seven-day intervals. 8 *Aegilops tauschii*accessions were sown in jiffy pod as father (all of tauschii in 4th and 3 tauschii were replicated in 5th week) (Table 2; Figure 1). It would be ensured that the heading date of durum wheat varieties and the flowering date of *Aegilops tauschii* materials overlap.

2. Emasculation and pollination

3 *Aegilops tauschii* materials determined by the International Maize and Wheat Improvement Center (CIMMYT), Mexico, and 1 *Aegilops tauschii* determined by the gene bank of the Centre for Agricultural Research of the Hungarian Academy of Sciences (MVGB) were used in crossing. Because 5 and 8 *Aegilops tauschii* accessions from CIMMYT have not had enough germinations. We did not use them in the crossing. We want to produce seeds from those *Aegilops tauschii* genotypes. 2 and 3 *Aegilops tauschii* accessions from the MVGB being too late for heading compared to durum wheat.

The heading date was 50% visible of first spike in leaf sheath and the flowering date was visible of the first anthers in spike. There were big differences among durum wheats and among *Aegilops tauschii* materials about heading and flowering. Durum wheats were used as female plants and *Aegilops tauschii* materials were used as male plants (William, *et al*., 1993; Mujeep–Kazi and Hettel, 1995). The crossing with pollen from the male plant was performed using the “twirl method” (Quisenberry, 1967).

2.4-D treatment is important for hybrid seed development (Koba *et al*., 1991). 2.4-D solution was prepared (0.05 g + 2 ml Ethanol + 100 ml bidistilled water) and had been successfully applied by injection into middle of upper internode of spike as soon as after pollination. After 2.4-D solution application, injection hole was covered with vaseline.

In a total 171 crosses were made (Table 3). In all of crosses, 6 durum wheat varieties were used as female and 6 *Aegilops tauschii* were used as male except 5T and 8T. 2D/4T and 4D/4T combinations were not made because of insufficient pollen. 5D/3T has had number of maximum cross (15) compared to other combinations.

3. Sterilization of seeds, embryo rescue, plant regeneration and vernalization

Embryo rescue studies have just been started at the end of February. The spikes and seeds were sterilized by following procedure;

The spikes were put in a bottle. A few drop of dishwashing liquid was added on the spikes. The bottle was filled up with water and shaken well, until the water starts to foam. The spikes were rinsed well with water (=3x).

%70 ethanol was added to the spikes and shaken for 10 min.

They were rinsed with sterile water (=4x).

In sterile box/laminar box:

The spikes in 5% Hypo (sodium hypochlorite) solution for 5 min were sterilized.

They were rinsed with sterile water (=3x).

0.1% $HgCl_2$ solution to the spikes was added, 3 min.

They were rinsed with sterile water (=4x).

Spikes of combination were harvested for spike and grain sterilization between 20 and 24 days after every crossing, (Figure 2). In the first term report, spike and grain sterilization were explained. Embryo rescue operations were started in 16 February 2016 and continued until to 29 May 2016. Embryos from all of hybrid seeds were rescued under microscope in sterilization-cabin (Sharma, 1999). B5 Agar Medium was prepared for regeneration of hybrid embryos and its growing.

Not only there was no endosperm or endosperm abnormality in hybrid grains (Kinoshita, 2007) but also there were no embryos in the majority of hybrid grains (Fujii and Toriyama, 2008) in spite of normal grain appearance (Figure 3). Clark and Wall (1996) reported that genomic relationship of two species was important for crossing. Therefore, crossabilities of species can have some barriers and difficulties. In interspecific hybridization, sometimes there are no hybrid grains or hybrid grains are smaller compared to normal grains (Stebbins, 1958; Linskens, 1972) (Figure 3). Mature seeds cannot be obtained from ovary culture or hybrid embryo because of abortion caused by the mismatch between embryo and endosperm development. Ovule and embryo culture methods are suitable (Bajaj *et al*., 1986). Embryo rescue operation is difficult because of smaller embryos. For this reason, 2.4-D hormone application is recommended for interspecific hybridization because of larger grain and embryo formation (Polgari *et al*., 2014) (Figure 3). Larger grains were created by 2.4-D hormone application.

Rescued embryos were put in B-5 medium in petrie dish (Gamborg *et al*., 1968). Maximum 5 embryos were put in every petrie dish. Petrie dishes were kept under dark and 20^0C condition for embryo germination during 10-13 days. After that, germinated embryos were put under light (14-16 hours per day) for the formation of chlorophyll to start the photosynthesis activity during 2 weeks (Figure 4A).

Haploid plants with sufficient root and shoot length were transferred to tube because of the limited space in petrie dishes (Figure 4B). One haploid plant was only planted in one tube. Tubes were kept under controlled condition (24^0C and 14-16 hours) during 1-3 weeks depending on root growth (Figure 4C). Haploid plants in tube were exposed to vernalization during 6 weeks (at $+2^0$C, and under 12 hours light and 12 hours dark) (Figure 4D). We had contaminations in some petrie dishes and tubes (Figure 5A and 5B). 12 haploid plants died due to contamination. Contaminated plants were 1D/3T (2 plants in 1 petrie dish), 2D/3T (4 plants in 1 petrie dish), 3D/7T (5 plant in 1 petrie dish) and 5D/3T (1 plant in 1 tube) combinations.

4. Transfer to soil

After vernalization, tubes (with plants) were taken to adaptation room (and under 14 hours light and 10 hours dark at 24^0C) during 1 week. After adaptation, plants were removed from tubes and the B-5 medium was washed off from roots thoroughly under tap water, and were transferred to pots (Figure 6A and B)). Planted pots were watered and covered by nylon to prevent moisture loss (Figure 6C). Nylons were removed from every pots after 1 week. Climate conditions per day of phytotron were illuminated during 12 hours at 15^0C and 65% humidity and darkened during 12 hours at 10^0C and 70% humidity. When younger plants are transferred from B-5 medium to soil, they are susceptible to lack of nutrient in the soil. Because of this reason, pots in phytotron were fertilized 2 times per week (on Monday and Thursday) with nutrient special solutions.

5. Colchicine treatment

In colchicine treatment, the following steps were performed;

The haploid plants one by one with sticker and pencil were labeled (Figure 7A),

The whole plants (with roots) were removed from pot,

Soil was washed off from roots thoroughly and bottom of The end of bottom of roots was cut 4-5 cm long (Figure 7B-C),

The plants were put into water (into a beaker) and stored them on 15°C until colchicine treatment,

0,04% colchicine ($C_{22}H_{25}NO_6$) solution was prepared:

To prepare 1000 ml solution, dissolve 0,4 g colchicine (Sigma) in a few drop of dimethyl-sulfoxide (Sigma-Aldrich), mix well and make volume up to 1000 ml (sterile water (MilliQ) or bidistilled water). It was tired at moderate speed.

The plants were put into the colchicine solution from 4.00 p.m. to 7.30 a.m. on 15°C (Figure 8A),

After colchicine treatment, the roots were washed thoroughly (minimum 2 hours) under flowing tap water, not too cold water (Figure 8B),

Plants were again planted into soil and leaves were cut 10 cm from upper level (Figure 9A and B),

The colchicine treated plants were put in phytotron under first condition (Figure 9C).

After colchicine treatment, some deformation and drying were observed on leaves and leaf sheaths of older and younger tillers (Figure 10A and B). Some older and younger tillers were also died by colchicine effect. These were an expected situation because colchicine was normally a very strong poison. New tillers shooted after colchicine treatment (Figure 11).

6. Calculation

Embryo Development Ability, Embryo Germination Ability, Vegetative Growth Ability in Tube and Soil Adaptation Ability for studied traits were calculated using the following formulas (modified from Yuan *et al*., 2016);

Embryo Development Ability
(EDA) = (RE/TF)*100
Embryo Germination Ability
(EGA) = (GE/RE)*100
Vegetative Growth Ability in Tube
(VGA) = (TP/RE)*100
Soil Adaptation Ability
(SAA) = (PS/PT)*100
Where;
TF = *Total Flowers*
RE = *Rescued Embryos*
GE = *Germinated Embryos in Petri-dish Medium*
PT = *Plants in Tube*
PS = *Plants in Soil*

Results

In this study, 171 spikes were pollinated in 34 combinations (a total of 5049 flowers) (Table 3). 164 rescued embryos (RE) were obtained from these crosses (Table 4). 5D female *Triticum durum* genotype had 48 embryos with 29.3% ratio among 164 RE. Because it had higher number of RE compared to other *Triticum durum* wheats (15). 6D female genotype had lowest RE value (9) with 5.5% ratio. According to germinated embryos in petri-dish (GE), GE ratios of 6D, 4D and 3D female genotypes were decreased (1.4%, 7.1% and 8.6%, respectively) compared to 5D, 2D and 1D (40%, 21.4% and 21.4%, respectively). These results were nearly same values of their offsprings in tube (PT). 5D and 1D genotypes among all female durum wheats had the best ratios of plants in soil (PS) trait (46.3% and 26.8, respectively) according to RE ratio (29.3% and 18.3%, respectively). 3T genotype was used maximum number of crosses (67 with 40.9% ratio) as male among all of *Aegilops tauschii*s. Others were nearly same each other's. Maximum GE ratio was observed in 3T male genotype (61.4), while minimum GE ratio was observed in 6T and 1T male genotypes (4.3% and 5.7%, respectively). PT ratios of *Aegilops tauscii*s did not nearly changed compared to GE. 3T, 4T and 2T genotypes among all male *Aegilops tauscii*s had the best ratios of PS trait (53.7%, 14.6 and 12.2, respectively) according to RE ratio (40.9%, 11% and 11.6%, respectively).

3.2% embryos were only obtained from 5049 pollinated flowers. Embryo development ability (EDA) of 5D/6T combination was the highest ratio (9.2%), but all of them were not interestingly germinated (Table 5). Maximum pollinated spikes were in 5D/3T combination (15 spikes) and, as expected, maximum number of embryos was obtained from this combination (24 embryos) (Table 6). 5D/3T combination had not only the highest EDA value (6.3%) but also the highest embryo germination ability (EGA) and vegetative growth ability in tube (VGA) (79.2% and 79.2%, respectively). 2D/3T combination had good values for EDA and EGA (5.3% and 56.3%, respectively). But its value was decreased in VGA (18.8%) and also it had zero (0%) in soil adaptation ability (SAA) value. The results of VGA values for each combination at the stage of colchicine treatment were more clear and important. After B-5 medium conditions, the best combinations about adaptation to soil were 1D/2T, 1D/3T, 1D/6T, 2D/1T 3D/T4 and 6D/4T (100%).

According to results, the best females were 5D and 1D among *Triticum durum*s, while the best female 3T, 4T and 2T among *Aegilops tauschii*s, respectively.

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Table 1. Code, species, accession and origin of experimental materials

Code	Species	Accession	Origin
1D	*T. durum*	Ç-1252	TURKEY
2D	*T. durum*	KIZILTAN	TURKEY
3D	*T. durum*	ALTINTAS	TURKEY
4D	*T. durum*	DUMLUPINAR	TURKEY
5D	*T. durum*	YELKEN	TURKEY
6D	*T. durum*	KUNDURU	TURKEY
1T	*Ae. tauschii*	MVGB605	HUNGARY
2T	*Ae. tauschii*	MVGB1323	HUNGARY
3T	*Ae. tauschii*	MVGB589	HUNGARY
4T	*Ae. tauschii*	CIMMYT.224	CIMMYT
5T**	*Ae. tauschii*	CIMMYT.369	CIMMYT
6T	*Ae. tauschii*	CIMMYT.372	CIMMYT
7T	*Ae. tauschii*	CIMMYT.458	CIMMYT
8T**	*Ae. tauschii*	CIMMYT.895	CIMMYT

** *Few germinated plant (1 plant for 5T and 2 plants for 8T)*

Table 2. Sowing date, start to vernalization date and transfer to greenhouse of experimental plants and their plant numbers (in 2015).

Weeks	Sowing date	Start of vernalization	Transfer to greenhouse	Planted seeds per variety or access	Total
1	07. Sept.	16. Sept.	04. Nov.	6 durum varieties (8 seeds/var)	48
2	14. Sept.	25. Sept.	11. Nov.	6 durum varieties (8 seeds/var)	48
3	21. Sept.	02. Oct.	18. Nov.	6 durum varieties (8 seeds/var)	48
4	28. Sept.	12. Oct.	25. Nov.	6 durum varieties (8 seeds/var), 5 *Ae.tauschii*, CIMMYT (∑55 seeds), 3 *Ae. tauschii*, MVGB (10 seeds/acc)	123
5	05. Oct.	19. Oct.	2. Dec.	6 durum variety (8 seeds/var), 3 *Ae. tauschii*, MVGB (10 seeds/acc)	78
6	12. Oct.	22. Oct.	9. Dec.	6 durum variety (8 seeds/var)	48
Total pots (There was one plant per pot)					**393**

CIMMYT: Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Center)

MVGB: Martonvaser Gene Bank of Agricultural Institute of the Centre for Agricultural Research of the Hungarian Academy of Sciences

Table 3. Numbers of emasculated and pollinated spikes, total flowers and flowers per spike in durum wheat

Cross	Number of		
	Emasculated and Pollinated Spikes (EPS)*	Total Flowers (TF)	Flowers per Spike (TF/EPS)
1D/1T	2	59	29.5
1D/2T	5	161	32.2
1D/3T	8	236	29.5
1D/4T	2	61	30.5
1D/6T	4	133	33.3
1D/7T	2	59	29.5
2D/1T	6	183	30.5
2D/2T	5	140	28.0
2D/3T	11	301	27.4
2D/4T	-	-	-
2D/6T	6	174	29.0
2D/7T	4	121	30.3
3D/1T	5	133	26.6
3D/2T	4	114	28.5
3D/3T	5	154	30.8
3D/4T	4	116	29.0
3D/6T	3	92	30.7
3D/7T	5	136	27.2
4D/1T	6	150	25.0
4D/2T	4	126	31.5
4D/3T	7	187	26.7
4D/4T	-	-	-
4D/6T	3	82	27.3
4D/7T	5	134	26.8
5D/1T	4	130	32.5
5D/2T	6	166	27.7
5D/3T	15	384	25.6
5D/4T	8	222	27.8
5D/6T	3	87	29.0
5D/7T	4	112	28.0
6D/1T	5	174	34.8
6D/2T	4	138	34.5
6D/3T	7	257	36.7
6D/4T	5	188	37.6
6D/6T	3	102	34.0
6D/7T	1	37	37.0
Total	**171**	**5049**	**-**
Mean	**5**	**-**	**30.1**

* *We had 84 pollinated spikes at the end of the first term and we have made more 87 pollination in the first days of the second term*

Table 4. Number and ratio of rescued embryos (RE), germinated embryos in petri-dish medium (GE), plants in tube (PT) and plants in soil (PS) of *Triticum durum* and *Aegilops tauschii genotypes* in all of crosses

		RE		GE		PT		PS	
	Genotype	**Total**	**%**	**Total**	**%**	**Total**	**%**	**Total**	**%**
Triticum durum	1D	30	18.3	15	21.4	11	19.6	11	26.8
	2D	31	18.9	15	21.4	9	16.1	5	12.2
	3D	20	12.2	6	8.6	4	7.1	3	7.3
	4D	26	15.9	5	7.1	4	7.1	2	4.9
	5D	48	29.3	28	40.0	27	48.2	19	46.3
	6D	9	5.5	1	1.4	1	1.8	1	2.4
	Total	**164**	**100**	**70**	**100**	**56**	**100**	**41**	**100**
Aegilops tauschii	1T	1T	17	10.4	4	5.7	4	7.1	4
	2T	2T	19	11.6	6	8.6	5	8.9	5
	3T	3T	67	40.9	43	61.4	33	58.9	22
	4T	4T	18	11.0	7	10.0	7	12.5	6
	6T	6T	21	12.8	3	4.3	3	5.4	2
	7T	7T	22	13.4	7	10.0	4	7.1	2
	Total	**164**	**100**	**70**	**100**	**56**	**100**	**41**	**100**

Table 5. Number of rescued embryos (RE), germinated embryos in petri-dish medium (GE), plants in tube (PT) and plants in soil (PS) of all combinations

Cross	Number of			
	RE	GE	PT	PS
1D/1T	-	-	-	-
1D/2T	11	6	5	5
1D/3T	13	8	5	5
1D/4T	-	-	-	-
1D/6T	6	1	1	1
1D/7T	-	-	-	-
2D/1T	8	4	4	4
2D/2T	-	-	-	-
2D/3T	16	9	3	-
2D/6T	7	2	2	1
2D/7T				
3D/1T	-	-	-	-
3D/2T	-	-	-	-
3D/3T	7	3	2	1
3D/4T	5	2	2	2
3D/6T	-	-	-	-
3D/7T	8	1	-	-
4D/1T	9	-	-	-
4D/2T	4	-	-	-
4D/3T	7	4	4	2
4D/6T	-	-	-	-
4D/7T	6	1	-	-
5D/1T	-	-	-	-
5D/2T	-	-	-	-
5D/3T	24	19	19	14
5D/4T	8	4	4	3
5D/6T	8	-	-	-
5D/7T	8	5	4	2
6D/1T	-	-	-	-
6D/2T	4	-	-	-
6D/3T	-	-	-	-
6D/4T	5	1	1	1
6D/6T	-	-	-	-
6D/7T	-	-	-	-
Total	**164**	**70**	**56**	**41**
[1]Mean 1	**9**	**5**	**4**	**3**
[2]Mean 2	**5**	**-**	**-**	**-**

[1]Mean 1: average of values; [2]Mean 2: average of all of crosses

Table 6. Calculated values of embryo development ability (EDA), embryo germination ability (EGA), vegetative growth ability in tube (VGA) and soil adaptation ability (SAA) of all combinations

Cross	EDA	EGA	VGA	SAA
1D/1T	0.0	-	-	-
1D/2T	6.8	54.5	45.5	100.0
1D/3T	5.5	61.5	38.5	100.0
1D/4T	0.0	-	-	-
1D/6T	4.5	16.7	16.7	100.0
1D/7T	0.0	-	-	-
2D/1T	4.4	50.0	50.0	100.0
2D/2T	0.0	-	-	-
2D/3T	5.3	56.3	18.8	0.0
2D/6T	4.0	28.6	28.6	50.0
2D/7T	0.0	-	-	-
3D/1T	0.0	-	-	-
3D/2T	0.0	-	-	-
3D/3T	4.5	42.9	28.6	50.0
3D/4T	4.3	40.0	40.0	100.0
3D/6T	0.0	-	-	-
3D/7T	5.9	12.5	0.0	0.0
4D/1T	6.0	0.0	0.0	-
4D/2T	3.2	0.0	0.0	-
4D/3T	3.7	57.1	57.1	50.0
4D/6T	0.0	-	-	-
4D/7T	4.5	16.7	0.0	0.0
5D/1T	0.0	-	-	-
5D/2T	0.0	-	-	-
5D/3T	6.3	79.2	79.2	73.7
5D/4T	3.6	50.0	50.0	75.0
5D/6T	9.2	0.0	0.0	-
5D/7T	7.1	62.5	50.0	50.0
6D/1T	0.0	-	-	-
6D/2T	2.9	0.0	0.0	-
6D/3T	0.0	-	-	-
6D/4T	2.7	20.0	20.0	100.0
6D/6T	0.0	-	-	-
6D/7T	0.0	-	-	-
General	**3.2**	**42.7**	**34.1**	**73.2**

Figure 1. Durum wheats were germinated in jiffy pod (A) and transferred to pots in greenhouse (B)

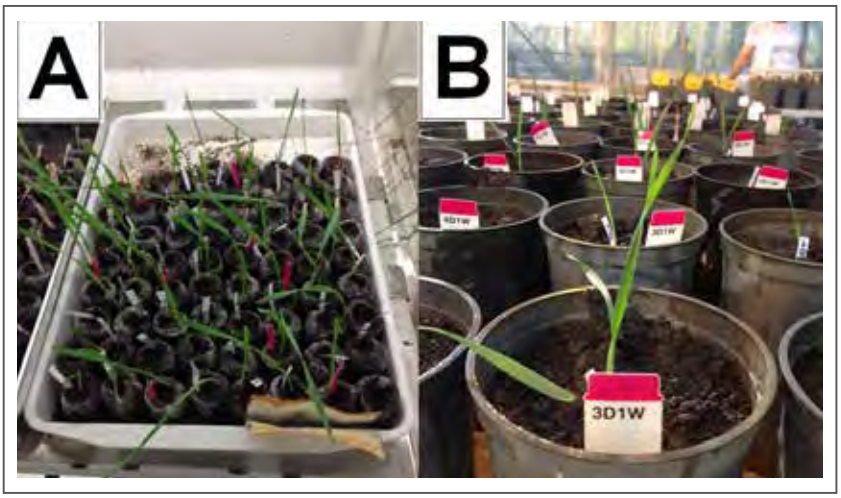

Figure 2. The spikes are ready for sterilization (A) and sterilization processes (B, C)

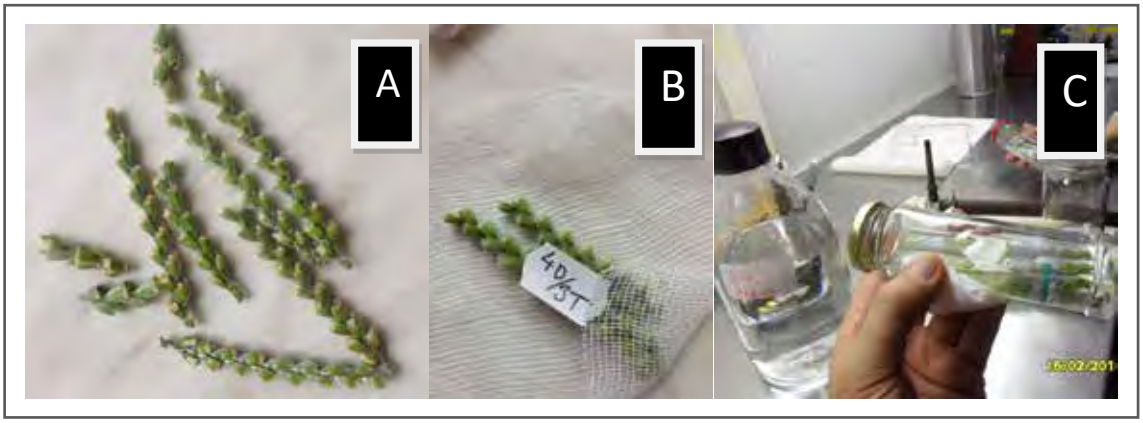

Figure 3. Self-pollinated seeds (on the left in A) in durum wheat; and cross-pollinated durum wheat seeds with male parent *Ae. tauschii* (on the right in A); a successful inter-specific hybridized seed with embryo (C and D); 2.4-D caused normal looking seed (B), but seed had no embryo (C and D)

Figure 4. Germinated inter-specific hybridized seeds in B-5 medium in petrie dish (A), transferring of germinated seeds from petrie dish to tube after three weeks (B), developments of shoot and root of haploid plants in the tubes (C and D)

Figure 5. Some fungal contaminations in B-5 mediums in petrie dish (A) and tube (B)

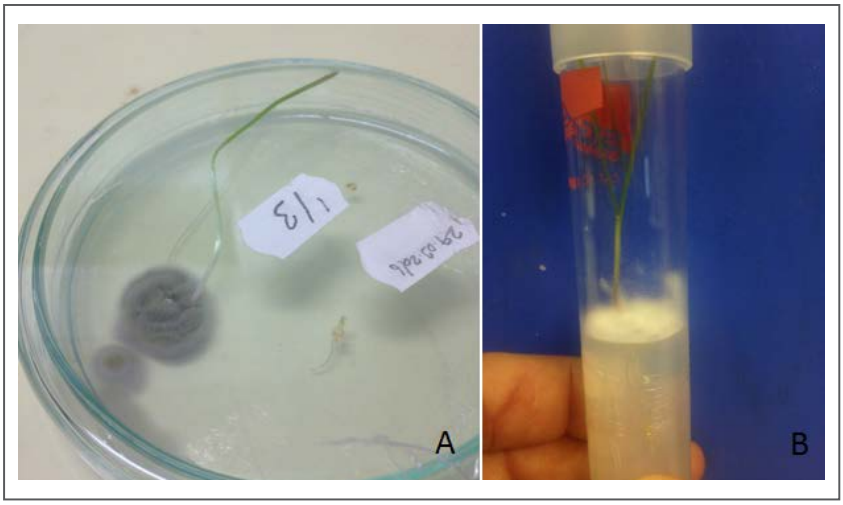

Figure 6. Transfer well formed haploid plants from B-5 medium into soil after vernalization (A and B), covering them with plastic wrap to prevent moisture loss during one week (C)

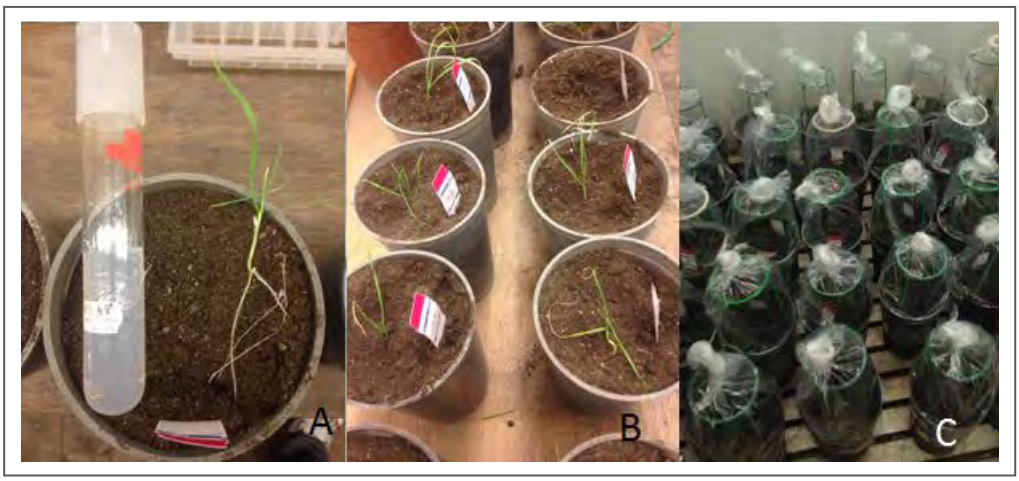

Figure 7. Prepared haploid plants for colchicine treatment (A), washing of roots (B) and cuting of roots (C)

Figure 8. Colchicine treatment to haploid plants (A) and washing of colchicine treated parts of plants under tap water (B)

Figure 9. Cuting of leaves ofcolchicine treated plants (A). Plant transfer into soil (B). Plants in phytotron (C)

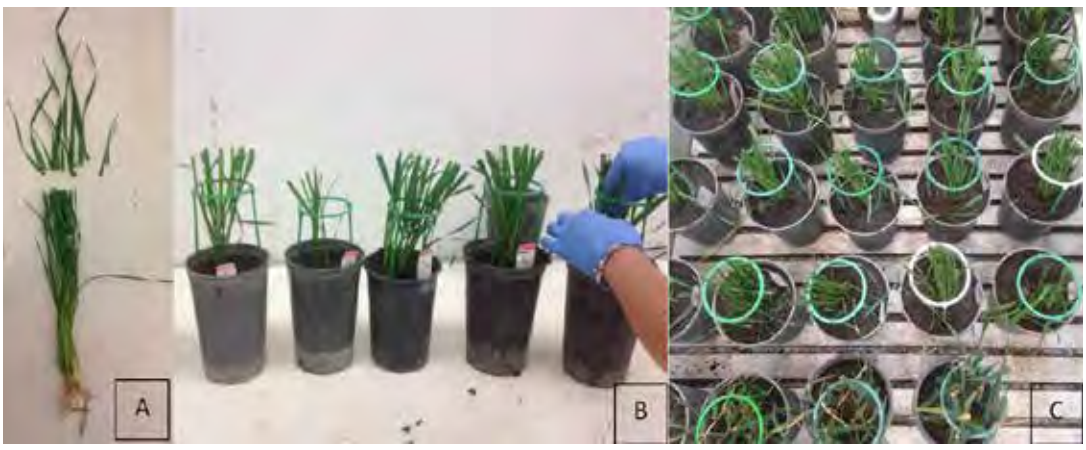

Figure 10. Some deformations and damages on plant after colchicine treatment

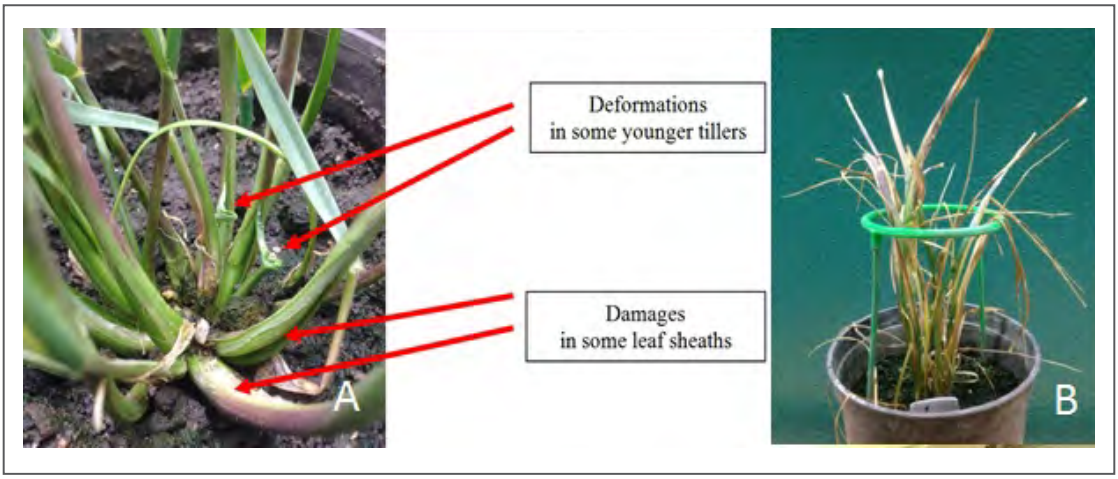

Figure 11. Shooting younger tillers after the death of older tillers

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Development of Turkish Potato Varieties Tolerance to Potato Virus Y and Potato Virus X

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ABSTRACT

Potato is one of the most important crops worldwide. The present study was conducted in Yuksel Seed R&D Center, Antalya. Approximately 120.000 seeds from different genetic backgrounds were obtained and evaluated between 2008 and 2016 for developing new varieties. The aim of the research was to develop superior potato varieties tolerant to PVY and PVX with high agronomic, tuber and quality traits. Potato virus Y (PVY) and Potato virus X (PVX) are among the most important viruses, causing economic crop losses in potato crop in the world. The use of resistance genes is the most effective method to control these viruses. Resistance genes are transferred to new commercial varieties using molecular markers that are tightly linked to resistance genes (Ry_{adg} for PVY, R*x1* and R*x2* for PVX). Potato lines were tested in replicated trials in different potato regions of Turkey (Adana, İzmir, Afyonkarahisar, Niğde, Nevşehir and Kütahya) by 8 years to estimate the genotype x environment interaction and 85 superior lines were selected. At the end of the research 4 early season and 3 main season superior lines were submitted for registration as commercial varieties and 4 of 7 candidate commercial varieties were determined as suitable for French fries and were marketed.

Keywords: breeding, molecular marker, potato, PVY

Introduction

Potato is the fourth most important food crop in the world after maize, rice and wheat. It is planted on 19 million ha with a yield of 382 million tonnes in the world, and it is planted on 128 thousand ha with a yield of 4.1 million tonnes in Turkey (Anonymous 2014). Asia and Europe are the world's major potato producing regions, accounting for more than 80% of world production. The total value of the seed potatoes produced in the EU is estimated at 1 billion Euro. The value of the processed potatoes is estimated to be more than 600 billion Euro.

Potato breeding is based on a phenotypic and genotyping selection scheme that cycle every 6 to 10 years. Potato varieties are developed by crossing hundreds of genotypes in breeding programmes (Gebhardt 2005). An important potato breeding limitation that the breeder has been faced with is the challenge of tetraploid inheritance, imposed by this tetraploid crop (2n=4x=48) which results in complicated genetic segregation (Ross 1986; Matsubayasthi 1991). Conventional breeding for resistance to pests and pathogens involves the identification of resistance sources, which are often found in wild and local genetic sources, the introgression of resistance factors into cultivars by repeated backcrossing to different potato breeding genotypes and phenotypic selection of resistant progeny (Ross 1986; Gebhard *et al*., 2006).

Biological assays for resistance in a greenhouse or field are fundamental, but time and space consuming. Alternatively, DNA based molecular marker-assisted selection can be used without special facilities for respective biological assays and not influenced by different growth stages or growing conditions such as temperature, humidity, water, light intensity, day-length, etc. Marker-assisted selection can identify rapidly and reliably resistant genotypes, becoming an important and practical breeding tool (Babu *et al*., 2004; Xu and Crouch 2008).

Potato crop is commonly attacked by viruses. Potato virus Y (PVY) and Potato virus X (PVX) are most important viruses, causing crop losses in potato crop in the world. The use of resistance genes is the most effective method to control these viruses. Resistant genes are transferred to new commercial varieties using molecular markers that are tightly linked to resistance genes. The objective of this study was to develop new potato varieties with resistance to these viruses (PVY and PVX) for cultivation in the potato growing areas of Turkey.

Material and Methods

Breeding and Selection

Tetraploid F_1 populations including European varieties, exotic cultivars and local genotypes were used as parents for developing new varieties resistant to PVY and PVX in the breeding programme. Approximately 120000 seeds from different genetic backgrounds were obtained and evaluated between 2008 and 2016. After crossing a large population, tens of thousands of F_1 seedlings were grown for visual selection. After a number of years, advanced lines were tested in replicated trials in different locations of Turkey (Adana, İzmir, Afyonkarahisar, Niğde, Nevşehir and Kütahya) to estimate the genotype x environment interaction. Eighty five advanced breeding lines were selected from these F_1 populations for carrying resistance genes and other agronomic and yield traits.

Cultural practices and measured traits

Potato candidate varieties were planted, spaced 30 cm apart in ridges and 70 cm wide, in field conditions. Fertilizer was broadcast at 60/80 kg ha^{-1} N, 40/50 kg ha^{-1} P_2O_5 and 80/100 kg ha^{-1} K_2O in different experiment fields and locations. Weeds were controlled by hand after emergence. Disease control and irrigation was carried out according to practice. The components such as maturity, marketable tuber yield (%), tuber shape, skin and flesh colour, cooking type, dry matter content (%) and starch content (%) and tuber yield per hectare (kg), were measured. Marketable tuber yield were determined as the percentage of bigger than 30mm in early season and 35 mm in medium-late season. Tuber shape: 100 x (tuber long (mm) / tuber width (mm) in 20 tuber in each replication. 112-129: short oval tuber, 130-149: oval tuber, 150-169: long oval tuber, 170-199: long tuber and > 200 very long tuber. Flesh colour were measured in 20 tubers in each replication. For cooking type baked and boiling potato performance were determined. For baked potato performance, 10 clear tubers in each replication 8 short holes with fork were made. Thereafter tubers were baked in oven at 175 °C for two hours. After two hours tuber structure and colour were determined. For water cooking performance; 2 kg tubers were boiled in 2 lt water and tuber structure and colour determined. Dry matter (%) was measured by Zeal potato hydrometer and starch content was determined with a polarimetric procedure (Haase 2003).

Molecular Marker, DNA Isolation and PCR Analysis

Resistant genes in superior lines were verified with molecular markers. Genomic DNA was isolated from young fresh leaves of potato lines using the Wizard Magnetic Kit (Promega) following the manufacturer's instructions. Three primers were used for molecular analysis. The Ry_{adg} gene, resistant to PVY, was identified using the SCAR marker RYSC3 (Kasai *et al*., 2000). Rx1 gene resistance to potato Virus X was screened using the RxSP-S3 and RxSP-A2 primer sets (Ohbayashi *et al*., 2010). For Rx2, the CAPS marker GP21 (AluI) and the marker TG432 (DeJong *et al*., 1997) were tested (Ahmadvand *et al*., 2013). PCR and restriction digestion conditions were as described in literature.

Results and Discussion

In this study, the selected lines were classified as resistant or susceptible to PVY and PVX. The PVY and PVX resistant lines with an acceptable tuber type and shape will continue in the variety development process in subsequent years for the evaluation of agronomic, quality and disease resistance traits and/or used as parental lines in our recurrent selection programme. Eighty five advanced lines were selected from the breeding programme and grown in different potato regions of Turkey. Superior lines were tested in replicated trials in different locations to estimate the genotype x environment interaction. In addition, resistant genes in superior lines were verified with molecular markers. Moreover, Leaf samples taken from potato plants were tested to detect the presence of PVY, PVX, PLRV, PVA, PVS and PVM by DAS-ELISA (Loewe, Sauerlach, Germany).

It is reported that PVY, which is the most common virus in the world and the primary one of the viruses

affecting potato production the most, can cause 10-90% loss of crops in potato depending on the variety (Ramakrishnan *et al*., 2015; Slater 2017). This virus is mechanically transmitted by tuber and aphids. For a campaign, it is necessary to use clean tubers and to fight vectors. Fighting with vectors is mostly done chemically, but does not provide complete protection. The second important virus is PVX, which causes heavier mosaic symptoms if the plants are co-infected with PVY (Gebhard *et al.,* 2006). Therefore, it seems that the best solution is to develop these virus-resistant varieties.

In seven commercial candidate lines, the Ry_{adg}, Rx1 and Rx2 genes were analysed by molecular markers linked to the genes (Table 1). Molecular markers linked to the Ry_{adg}, Rx1 and Rx2 genes produced the expected DNA bands. The SCAR marker RYSC3 produced a 321 bp fragment only in the resistant genotypes bearing the Ry_{adg} gene. The Ry_{adg} marker was present in all commercial candidate lines and produced the expected PCR products. The Rx1 and Rx2 markers were present in four candidate varieties (12-55-07, 12-55-16, 12-68-05 and 22-99-33) and commercial candidates advanced lines (12-55-16, 12-68-05, 12-69-39 and 22-99-33), respectively. In three commercial candidates varieties (12-55-16, 12-68-05and 22-99-33), positive results were acquired from all of the Ry_{adg}, Rx1 and Rx2 markers (Table1).

The study involved 7 commercial candidate varieties taken into the trial during the early season (12-03-85, 12-55-07, 12-55-16 and 12-69-39) and late season (12-68-05, 13-67-25 and 22-99-33). Variety 12-55-16 is a high yielding variety with long-term storage capability, and the resulting big tubers were a nice fresh market type (ware potato) and industry type (French fries) variety candidate in terms of taste and aroma. Variety 12-55-07 had big tubers and was high yielding, and the variety was a nice ware, French fries and Chips variety candidate in terms of quality. Variety 12-69-39 had good fresh quality and was a high yield ware and French variety candidate. The 12-69-39 variety was identified as a candidate variety with a strong plant structure, standard and quality tuber characteristics. Although the 12-69-39 candidate variety exists in the mid-early group among the 7 varieties, it is a variety coming to the forefront in that it is the only kind suitable for breeding during both early and late seasons. Among the 4 varieties tested at early season, the 12-69-39 candidate variety was found to be the one with the highest tolerance to PVY virus and mildew (*Phytophthora infestans*) based on field observations. Variety 12-03-85 has oval shaped tubers and was an early and fresh market variety candidate in terms of quality, aroma and appearance. The 12-03-85 variety also came to the forefront with a homogenous tuber structure and yellow tuber flesh colour (Table 2).

The dry matter contents of 13-67-25 and 12-68-05 candidate varieties were found to be quite high. Particularly the 13-67-25 candidate variety was considered as a variety with high quality, long oval tuber. 22-99-33 candidate variety is a special variety. 22-99-33, which is an early-mid early variety, was regarded as an industrial variety due to the dry matter rate. 22-99-33 candidate variety was also identified as a variety with a quite high starch ratio. 22-99-33 candidate variety has standard, long oval, high-quality tuber properties. As regards to the dormancy, the variety 22-99-33 displays the structure of a variety suitable for industrial production as one with a long dormancy.

Approximately 50 characters including good quality and high yield attributes as well as tolerance to different abiotic and biotic stresses, resistance to diseases and pests are considered in potato breeding thereby making new varietal development in potato very demanding (Gebhardt 2013; Asano and Tamiya 2016). Although, last ten years important advances in molecular breeding provide opportunities for rapid genetic gain (Slater *et al*., 2014), yet the use of molecular approaches requires quantitative genetic analysis of the highly heterozygous breeding populations for development of the complex quality and yield traits with low heritability. Thus, phenotypic selection in potato still remains the common practice in breeding programmes (Gopal, 2015).

In the study, the 120.000 potato true potato seed lines were propagated and selected (2008 to 2016) with an acceptable tuber type and shape continued in the variety development process for subsequent years for the evaluation of agronomic, quality and disease resistance traits and/or used as parental lines in our recurrent selection programme. Superior advanced lines were selected from the breeding programme. Seven of 85 selected lines were submitted for registration as commercial varieties. As a conclusion, in this study seven advanced commercial candidate potato varieties were developed and tested in Turkey's potato growing regions. All commercial candidate potato varieties will be used in large-scale during next 10 years in Turkey and other countries like Morocco, Egypt, Tunisia, Russia, Pakistan, Azerbaijan and other Turkic Republics, South Korea, Bulgaria with high adaptation ability, high yield and good quality characteristics.

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Table 1. The presence of marker and genes advanced potato lines.

Variety	PVY Resistance (Ry-adg)	PVX resistance	
		Rx1	Rx2
12-03-85	+	-	-
12-55-07	+	+	-
12-55-16	+	+	+
12-69-39	+	-	+
12-68-05	+	+	+
13-67-25	+	-	-
22-99-33	+	+	+

+: presence of molecular marker,
-: absence of molecular marker.

Table 2. Properties of candidate potato varieties.

Varieties	Maturity	Marketable yield (%)	Tuber shape	Flesh colour
12-55-16	Early-Mid Early	95-96	Long oval	Light yellow
12-55-07	Early-Mid Early	93-95	Long oval	Light yellow
12-69-39	Mid Early	94-96	Oval	Yellow
12-03-85	Early	90-92	Oval	Light yellow
12-68-05	Mid Early	95-96	Short oval	Light yellow
13-67-25	Early-Mid Early	96-97	Long oval	Yellow
22-99-33	Early-Mid Early	94-95	Long oval	Light yellow
Varieties	**Starch Content (%)**	**Dry matter (%)**	**Cooking Type**	**Consumption**
12-55-16	12.5-14.0	21.0-22.4	Slightly mealy	Ware, French fries
12-55-07	13.0-14.3	23.2-23.9	Slightly mealy	Ware, French fries, Chips
12-69-39	12.0-12.4	21.3-21.4	Firm	Ware, French fries
12-03-85	11.3-11.8	20.5-20.8	Firm	Ware potato
12-68-05	12.2-12.4	19.6-20.4	Firm	Ware potato
13-67-25	10.6-11.2	17.5-18.2	Firm	Ware potato
22-99-33	12.6-15.9	20.4-21.0	Slightly mealy	Ware, French fries

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Organic Tea Production and Tea Breeding in Turkey: Challenges and Possibilities

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ABSTRACT

Tea production can be seen at the zone beginning at the Georgia border of the Black Sea region up to the Fatsa district of Ordu in Turkey. Tea production areas are present foremostly in Rize, followed by Trabzon, Artvin, Giresun and Ordu. These regions are representing the top zone tea production areas in the World. The region beginning from the Georgian border up the district Araklı represents the most suitable and primary high yielding tea plantation areas in Turkey. Tea is the most important income resource of people settled in this region. With the switch of the Hemşin district to organic tea production and with future plans to switch also in Rize gradually to organic tea, in public organic tea production have become important. İn this present work the changeover to organic tea production in Rize, the use of organic fertilizers, possible changes in production and marketing and also general challenges and possibilities are discussed.

Keywords: tea, organic agriculture, breeding, quality

Introduction

Camellia sinensis belonging to family Theaceae, commonly known as tea is a plant species whose leaves and leaf buds are used for tea production. There are 3 types of tea: green, oolong and black. Green and Oolong tea are the most widely consumed beverages in Asian countries and has been familiar in China and Japan for centuries. Oolong tea is semi-fermented during processing, whereas green tea is not fermented and black tea is fully fermented (Rahman *et al*., 2013). Black tea accounts for approximately 72 % of the world's total tea production (Sharangi 2009). In Turkey, generally black tea production is common and a little green tea production is present at the Eastern Black Sea Region.

Agriculture changed direction due to the effect of industrial revolution and green revolution arised in the second half of the 20th century. The aim in the Green Revolution was to increase yield obtained from a unit area to compensate the food need of the human population. The requested yield increase was obtained by intensive application of pesiticides and fertilizers, but it was observed that with time applied pesticides and fertilizers showed a lot of negative effects especially for human health. Besides this environmental problems like the degeneration of the physical structure and nutritive balance of the soil, salinisation and desertification encountered. As a result of all this and other negative developments "Organic Agriculture" arised as an alternative production system (Aksoy 2001).

The purpose of organic agriculture is to rehabilitate the degenerated ecological balance due to the still used conventional agriculture applications, to minimize the agricultural inputs and applications which are responsible for the degeneration of this balance and to use natural products instead of fertilizers, peticides and hormones harmful for human health (Kayahan 2001; Kirazlar 2001).

In Turkey organic farming is developing rapidly due to the increase of demand from foreign countries,

the support of the Ministry of Agriculture, Food and Livestock, the increasing interest of universities, research companies and non governmental institutions, the interest of local farmers and public opinion, the formation of a domestic market etc. (Aksoy and Altındişli 1999; Kenanoğlu and Karahan 2002; Demiryürek *et al*., 2008).

The main cultural application in increasing yield per unit area is fertilization. Materials used as fertilizers can be very different. These are grouped as organic fertilzers and chemical fertilizers. Organic fertilizers are like farm manure of natural, organic character. İn case chemical fertilizers are containing one or more nutrients. Negative effects of the extreme use of chemical fertilizers on human health are detrmined by conducted experiments (Demiryürek 2011).

Tea production are practised in the Eastern Black Sea Region, in a zone beginning from the Georgian border up to the Fatsa district in Ordu. In this region tea production is mainly located in Rize, Ordu, Giresun, Trabzon and (Anonymous2015). If we are considering the tea production areas in the World these regions are located in the top zone. In Asian countries like China, India and Sri Lanka temperature does not falls up to minus degree in tea production areas and tea production is covering the whole year. But in our country where we can have four climates tea plantations are in fallow four six months. The fact that snow falls on Turkish tea plantations bring them an extra important characteristic. Because of this character pesticides are not applied in our tea production areas. This gives Turkish tea compared with teas in the world "the most natural tea" characteristic.

The biggest advantage of Turkish tea sector is that pesticides are not used for its cultivation. The winter conditions decease pests in natural means as it snow in Rize province situated in the north east of Turkey. As a result of that, there is no need to use any pesticides. A small amount of pests have been seen in Rize province actually, but they does not reach the population require to use pesticides. Two important parameters prevent the production of organic tea in general. One of them is the pesticides and the other one is chemical fertilizers. All of the tea, produced in Turkey, will be organic product if organic fertilizers are used instead of chemical ones, as the Turkish tea industry is already not using pesticides. That feature is a great advantage of Turkish tea sector (Saklı 2011).

History of Tea in Turkey

At the start of tea cultivation in Turkey, the primary goal was to meet the domestic demand only. It looked very difficult to introduce a new crop in the area and it was popularly understood that the effort was going to end up in fiasco. However, in a relatively brief space of time, the tea trade and industry have undergone sweeping changes due to the consistence efforts. Today, Turkey holds a significant place among the world's largest tea producers and ranks sixth in world production of tea such that the farmers have no reservations about tea cultivation (Klasra *et al*., 2007).

Tea cultivation was first introduced in Batum (Republic of Georgia), neighboring Eastern Black sea region of Turkey by Russians in the last quarter of 19th century after importing seedlings from China. They had successfully established commercial tea planting here and tea industry had slowly expanded with opening of large acreage of lands for the purpose. Since Russians had successfully introduced tea in Batum, it was felt that tea cultivation must also be introduced in Turkey. Therefore, under the directions of the state, the Department of Agriculture selected Bursa (an important historical city of Ottoman empire surrounded with hills and large number of natural springs and forests), to evaluate the feasibility of tea cultivation by importing seedlings from Japan and China in 1888 (Tekeli 1976). Soon it was discovered that the tea plants needed very specific environmental conditions to produce an economic crop, which led to the identification that tea cultivation was not feasible in Bursa.

Mr. Ali Riza Erten, was assigned the duty to discover feasibility of some other suitable locations with in Turkey for tea cultivation. He made extensive visits to Rize, Artvin, Ardahan (Turkey) and Batum (Georgia) in the Eastern Black Sea region (Kakuzu 1944; Kacar 1986a,b). He made a detailled analysis of the soil and climatic conditions of these areas, to know the feasibility for economic and successful cultivation of crop and found that the ecology of Rize, Artvin and Ardahan was very similar to Batum. He observed tea, orange and bamboo gardens in Batum. He reported economic feasibility of tea cultivation at Rize and (Hatipoğlu 1934 a,b; Arar 1969).

In Turkey, tea cultivation was determined in 1917 around Rize province and cultivation was first started by law in 1924 andthe Tea Research Institute was established. Afterwards, research studies began to be conducted and tea cultivation began on a commercial basis. In 1947, the first plant for processing green tea leaves was opened in Rize. From then on, tea production has been carried out in a microclimate along the Eastern Coast of the Black Sea Region and thus, Turkey takes its place on the upper limit of tea ecologies. Along this region Rize, Ordu, Giresun, Trabzon and Artvin are the provinces in which tea is produced.

In order to supply better service, parallel to the growth in this sector, an economic enterprise, ÇAYKUR (General Directorate of Tea Enterprises) was established in 1971 and was fully authorized as a state monopoly in the tea business. In 1984, with the abolishment of the monopoly in the tea sector, private enterprises were also given the rights of procurement, processing and marketing (Anonymous 2017).

Tea Production in Turkey

Although the tea business in Turkey is a relatively new activity compared with the other producer countries, tea cultivation and the industry have shown very important improvement in a short time. While the production of dried tea was below 25, 000 tons in the 1950's, this figure reached significant quantities in recent years. Today, Turkey holds a significant place among the world's largest producer countries with a share of 3%. According to the Food and Agriculture Organization (FAO) statistics, Turkey ranks 8th place in the world production area of tea after China, India, Sri Lanka, Kenya, Indonesia, Vietnam and Myanmar (Table 1).

Regarding world tea production Turkey ranks at the 6*th* place in the world after China, India, Kenya, Sri Lanka and Vietnam (Table 2).

In Turkey tea production is located in the North-East Black Sea Region. The tea plantations are distributed in the cities Artvin, Rize, Trabzon, Ordu and Giresun (Picture 1).

Table 3 shows the distribution of tea production areas according to related cities. The main tea production area is Rize with 65.96%., followed by Trabzon, Artvin, Giresun and Ordu. Parallelly, the number of tea farmers are following the same ranking.

Why organic tea?

There are various factors influencing the consumer preference to buy Organic Tea. Supplements due to their perception like its health properties and consume safety of organic tea, environment friendly character of organic tea, their hight nutritional value, price of proucts, trust in product certification and availability of products..

Objective of organic tea cultivation is to have an ecologically sustainable plantation, aimed at the conservation of ecology and natural habit without polluting soil, air and water and yet maintaining sustainable tea production. Tea is produced in the absence of synthesized chemicals like pesticides, fungicides, herbicides, growth regulators and concentrated fertilizers. Naturally occurring, mined products and bulky and concentrated organic manures are used for resistant cultivars, regulation of microclimate or by the introduction of biological control agents/the use of biological products, naturally extracted without the use of inorganic solvents.

History of Organic Tea Production

Organic agriculture is practised in almost all countries of the world, and its share of agricultural land and farms is growing everywhere. The market with organic products is growing at a fast rate, not only in Europe, Japan and North America (which are the major markets) but also in many other countries, including many so called developing countries. Lack of state regulations for organic agriculture makes it difficult in many countries to distinguish organic from low-chemical or even non-organic products. Official interest in organic agriculture is emerging in many countries. On an international level FAO is giving in creasing support to organic farming (Williges 2004).

Modern scientific technology encourages the use of chemical pesticides, herbicides, fungicides and chemical fertilizers for the high crops production. It creates the whole agriculture production chemicalization environment and soil become unhealthy, all biodiversity and ecosystem have been sick and whole ecology becomes chemicalized. The modern agriculture technology creates the farming system an organic. Organic cultivation system deals as whole elements of farming i.e. fertilizer, soil management, plant or seeds selection, irrigation, pest and diseases management, biological control method. In that case direct control measures with natural pesticides may be appropriate in organic tea cultivation (Khanal 2012).

Organic agriculture is a production system that sustains the health of soils ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local condition rather than the use of inputs with adverse effects. Organic tea farming is based on four principles that are principle of health, principle of ecology, principle of fairness and principle of care. Organic tea farming promotes and enhances biodiversity, biological cycles, soil biological activity through management practices that resort, maintain and enhance ecological harmony. The organic tea production system is different from non-organic tea production (Acharya 2009).

The organic movement in Sri Lanka started in the 1980s through contact and inspiration of local NGOs with the Philippine organic agriculture move ment. In 1982 a group of local NGO representatives, planters, scientists and environmental officers had drafted a Memorandum of Association to create a movement named Lanka Organic Agriculture Movement (LOAM). This can be seen as the official starting point for the dissemination of organic agriculture in Sri Lanka (Williges 2004).

The past, presence and future Organic Tea Production

Organic products which could not or not enough produced in countries where Organic market is expanding or where demand for organic products is increasing are imported mostly from developed countries. One of this products is tea, a product of high economic value in our region.

In India organic tea farming begun in 1986 with 'Darjeling' and spread to Assam and South India. In India the organic tea farming areas corresponds to 4.000 ha. Tanzania, Japan, Kenya and China started with organic tea in the same period. 1989 organic teas were saled in England.

The tea production area in the world is 2.9 million ha, 4.8 million tonnes tea are produced. But organic tea farming is produced in 5.000 ha and approximately 4-5 tonnes organic tea production exists (Anonymous 2016).

Why Organic Tea Production in the world did not expand enough can be explained as follows:

1. Tea production is located most in the equatorial or near the equatorial region, no winter, battle against diseases and pests are obligatory
2. Besides bacterial and fungicidal diseases more than 100 different insects were dtected in this regions
3. Losses in yield and quality due to diseases and pests specific for tea
4. High residue levels (above the permitted limit)
5. Soil pollution due to intensive chemical inputs
6. Switch to organic farming is a long process
7. High input costs

Organic Tea Production in Turkey

Parallel to the developments in the world ÇAYKUR initiated in 2003 studies to increase organic tea farming in our country. Within the context of organic tea farming Borçka/Artvin and Çamlıhemşin and Hemşin/Rize was choosen as organic tea production areas. İn 2006 ÇAYKUR founded the "Organic Tea Farming Commission" to organize studies regarding organic tea farming and production and to determine a road map for organic tea.

In 2006 "Farmer Briefing Meetings" were organized to inform them about the benefits and contibutions of organic tea farming. In 2007 organic tea farming contract was signed with 135 farmers covering 37,8 ha to initiate the organic tea production project.

As mentioned before Borçka Muratlı, Çamlıhemşin and Hemşin was choosen as organic tea production areas. Between this areas Hemşin was selected for following reasons:

- a old center for population containing historical and natural beauties
- it represents an closed basin
- surrounded totally by mountains and forests,
- suitability of ecological conditions
- high education level, possible easier acceptance of organic tea farming by local farmers
- higher willing of local farmers
- presence of organic honey and egg production in the region; a proportionof local farmers are familiar with organic farming
- supoort local administratives and non governmental institutions
- sufficient technical personal in related managements etc.

After involving the whole Hemşin district in the organic tea farming project; Muratlı/Artvin, the districts of Rize Çamlıhemşin and İkizdere, the Tunca district, Senoz basin in Çayeli district, higher altitudes of the districts Pazar and Ardeşen, a part of the Çağlayan basin in Fındıklı, two settelements in Of/Trabzon, two settlements in Rize center and 1 settlement in Kalkandere was included in organic tea farming.

The selected regions display similar characteristics. These are: are rich in water resources, relatively young tea plantations, upper tea plantation areas, mean yield is low, tea plantations are surrounded by forests, absence of other agricultural practices, rich resources regarding running waters, low settlement and industrialisation, no air, water and soil pollution due to industrialisation, suitability for ecotourism, preservation of their historical tissue and presnece of hot spring and baths.

The agricultural land of the Hemşin district is surrounded by forests. This leads to a closed basin and its ecological conditions are suitable for organic tea farming. Because of this reasons ÇAYKUR declared this region as 'Organic Tea Farming Basin' and all farmers were supported to tend towards organic tea farming.

During Tea Purchase Campaign in 2008 processed in the Taşlıdere Tea Factory and 5.900 kg green tea was produced and saled as 'Zümrüt Yeşil Çay'. After this developments a tea factory was built in the Hemşin district. It was opened in 2009 as "Hemşin Organic Tea Factory".

The tea plant needs higher nitrogen amounts for plant development. With the switch from an inorganic fertilizer containing 25% nitrogen to organic fertilizers with low nitrogen contents increases in yield arised.

ÇAY-KUR has supported from the beginning of the organic tea farming project that all produced organic tea by farmers has to be purchased, no quota or limitations are given, Projenin the price has to be earlier payed compared with conventional tea, 50%

support for organic fertilizer will be given, that farmer commodities has to contact fertilizer companies and that seminaries concerning fertilizer and fertilisation should be given (Anonymous 2016).

Advantages of Turkey regarding Organic Tea Production

Turkey has compared with tea producing countries some advantages. These can be explained as follows:

1- Ecological conditions in this region are suitable for organic tea production
2- The presence of winter prevents damage caused from fungi and insects while tea plants are under the snow cover
3- No yield or quality losses caused by tea specific diseases and pests,
4- No demand for crop protection chemicals
5- No need for chemicals except fertilizers
6- Absence of air, soil and water pollution caused by heavy industry
7- Sufficient technical information and equipment by side of ÇAYKUR
8- The switch to organic production come true in a short time of 3 years
9- Support from local administratives and non governmental institutions because of possible contibution to tourism and regional development
10- Increase in income level of farmers by supports and different price applications
11- Demand for organic tea in the organic market is high
12- The shelf price of organic products are higher compared with conventional products
13- Tea waste obtaied from tea factories can be used as organic fertilizers,
14- The organic tea factory will help to improve the economy of the region.

Organic tea production increased from 378 da in 2007 up to 38.034 da in 2016. Also number of organic tea farmers increased from 135 in 2007 up to 11.786 in 2016 (Table 4).

In Table 5 processed organic black and green tea amounts are given. The amount of bought wet tea, processed black and green tea and in total increased from 2009 up to 2016. There is a remarkable increase in organic tea production in Turkey during the last decade. Organic black tea production increased more compared with organic tea production.

Scientific Work Regarding Tea at the Black Sea Region

In Turkey, most of the tea plantations were established by using seeds; continuous seed ropagation has produced populations with different yield and quality properties, reflecting wide genetic variation. Clonal selection studies were conducted in the Black Sea region and several promising tea clones such as 'Tuglali-10', 'Derepazari-7', and 'Pazar-20' have been identified (Öksüz, 1987). Clones named Muradiye, Gündoğdu, Fener3, Enstitü1, Enstitü2, Hamzabey, Hayrat, Çayeli, Ardeşen, Fındıklı, Pazar and İyidere followed later. Basicly clonal selection work was done by ÇAYKUR in this region.

Molecular characterisation work (Kafkas *et al*., 2009; Beriş *et al*., 2001, 2005, 2016) and the use of plant growth promoting bacteria in organic tea production was conducted by Çakmakçı *et al*., (2012, 2013, 2016).

Organic Fertilizer Studies

Due to the plan of ÇAYKUR expanding the organic tea production area collobaration of ÇAYKUR, Ministry of Agriculture, Food and Livestock begun. The primary aim was to find out the potential of organic fertilizers to be used in tea plantation areas.

A research study was conducted in 2017 using 21 different organic fertilizers and chemical fertilizer. These were compared in a randomized block design with three replications in 8 locations, Çamlı and Pınarlı/Hopa, Fındıklı, Ardeşen, Pazar, Çayeli, Ortapazar and Of/Trabzon. 4 solid, 16 liquid and 1 solid + liquid mix fertilizer were used in this study. Each trial plot was depending on field structure 25-30 m^2 and three replications were used.

In this ongoing Project leaves were collected at possible harvesting times and investigated regarding all components important for tea. Choosen samples will be processed for black tea. Also soil samples were taken before fertilizer application and after every harvest time to determine the changes in soil due to fertilizer application.

Basing on experimental statistics, only after 3 year results it can be possible to recommend any fertilizer for organic tea production in this region.

Further the use of organic fertilizer in an high altitude, in Tunca/Rize was conducted by Prof. Seyis and collegues (unpublished data) to prove the possible yield increase in high altitudes.

Improvement of Tea Quality

In 2016 the Project "Improvement of Black Tea Quality" supported by DOKAP (Doğu Karadeniz Projesi-East Black Sea Project) belonging to the Ministry of Development started. The aim of this study is to characterize the present tea plantations on chemical and molecular level, determination of

tea clones with edible oil quality and multiplication of promising tea clones. Further a mini tea factory will be built for developing a tea trademark for the Recep Tayyip Erdoğan University after research and development focussing on black and green tea quality.

Possible Challenges and Opportunities

The switch to organic tea farming will aslo start some challenges in the production behaviour of local tea producers. Specially, the switch to use of organic fertilizers will allow to restore soil fertility and also water resources in tea production areas. But the switch to organic farming should not be considered only in case of tea, it should be considered as a whole. There are some advantages and disadvantages regarding this issue.

Advantages are:

1- The Hemşin example

The experience in the Hemşin district will help in the switch of other regions to organic tea farming

2- Intensive interest of farmers

The interest of farmers sensitive to the protection of the environment is also an advantage, because they want to live an healthy environment

3- ÇAYKUR

The support of this governmental institution is the biggest advantage.

4- Interest of Ministry of Agriculture, Food and Livestock

This Ministry supports organic agriculture facilities overall in Turkey.

5- Presence of Faculty of Agriculture and Natural Sciences (Recep Tayyip Erdoğan University)

The Faculty of Agriculture and Natural Science is the only Agricultural Faculty in the tea production area. Finished or ongoing research projects in the mentioned faculty and university are highlighting present problems and are giving advices for solutions

6- Farmers experience in tea cultivation

Because tea has a history of nearly 8 years in this region, this experience will help in the switch to organic tea farming

7- Increasing number of educational training courses related with organic tea

The members of Faculty of Agriculture and Natural Sciences are involved in educational training of local farmers regarding organic tea production since 2013. Cooperations with ÇAYKUR and the National Tea Council (Ulusal Çay Konseyi) are ongoing.

Disadvantages:

1- Recommodation for suitable organic fertilizers

Organic tea production is ongoing in Turkey, but there is a lack in recommendation of organic fertilizers. Ongoing studies will hopefully solve this problem

2- Misunderstanding that switch to use of organic fertilizers will decrease obtained yield

Due to wrong applications, there is a misunderstanding that the use of organic fertilizers will led to yield decrease. But in the example Hemşin organic fertilizers are not used and because of corresponding yield losses the gossip arised that yield losses will company organic tea production.

3- Intensive nitrogen fertilization and corresponding problems

During the last 20 years the intensive use of chemical fertilizers has led to soil and environment pollution. In such regions the switch to organic tea production will take time and needs another approach.

Conclusion

Turkish tea, to have the advantage of producing organic tea, is an important opportunity. The increasing importance of healthy consumption today can be treated as a chance for consumers in Europe and in the World as well as Turkish consumers. For this reason, all necessary efforts should be initiated to produce organic tea in tea gardens of Turkey as soon as possible.

The industry must tackle the problem of quality for use advantage of organic tea as needed. The high quality of fresh tea harvested and good quality black tea processed will be given high price in the tea exchange and this will play an important role in the system. Organic tea meeting the high price in the market will eliminate the problem of competition about Turkish tea due to high costs. If organic tea production realized, the factories could pay a higher price to tea farmers and ask them to harvest higher quality fresh tea leaves (Saklı, 2011).

ÇAYKUR is planning to switch to Organic tea production in all tea plantations in Rize in 2018. But the lack of information about the use and kind of organic fertilizers is still present. In near future the farmers has to be educated intensively about the structure of organic tea production, the use of organic fertilizers and they have to be highlighted about future plans of ÇAYKUR and the Ministry of Agriculture, Food and Livestock).

Table 1. Tea production areas in the world

Countries	Tea Area (thousand ha)
China	1984
India	604
Sri Lanka	222
Kenya	203
Indonesia	119
Vietnam	115
Myanmar	83
Turkey	76
Other countries total	382

FAO (2014)

Table 2. Tea production in the world

Country	Yield (tonnes)
China	2.111
India	1.207
Kenia	445
Sri Lanka	338
Vietnam	228
Turkey	227
Iran	119
Endonesia	154
Other countries total	731

FAO (2014)

Pic. 1. Tea production areas at the Black Sea region

Table 3. Tea plantation area and number of farmers

City	Tea Area (da)	%	Number of farmers	%
Rize	574.135	65.96	131.443	61.81
Trabzon	165.982	20.01	51.222	24.08
Artvin	98.433	11.51	20.169	9.48
Giresun	20.844	2.51	9.814	4.61
Ordu	111	0.01	44	0.02
Total	829.505	200	212.692	100

Anonymous (2016)

Fig. 1. Consumer preference buying organic tea (Snehagha and Ramawat, 2016)

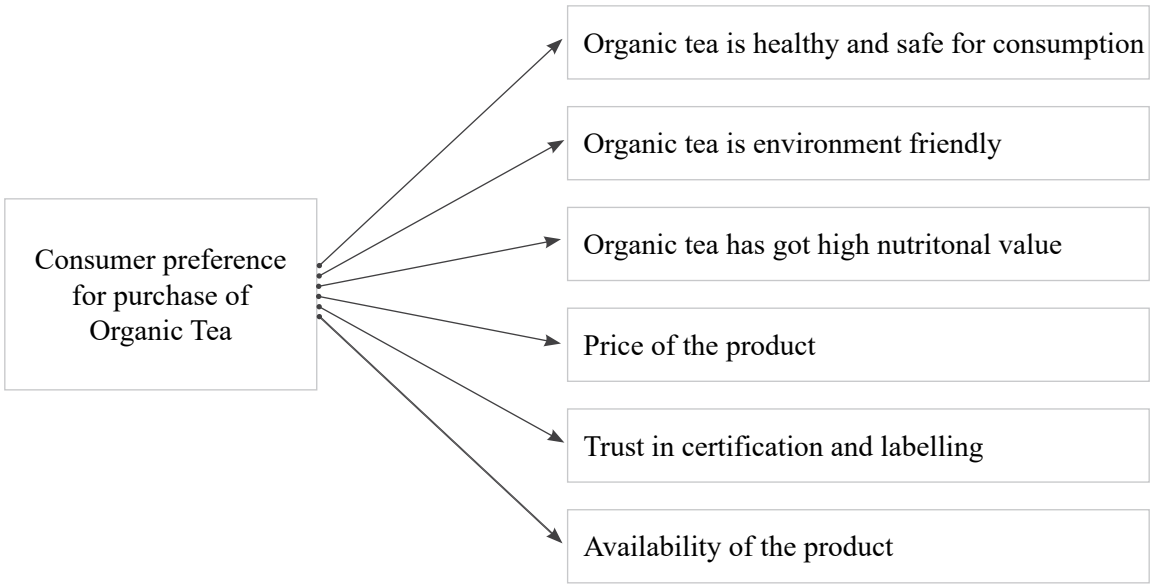

Table 4. Organic tea production areas in Turkey

Years	Numbers of Farmers	Tea area (da)
2007	135	378
2008	400	1.080
2009	1.434	3.558
2010	1.438	3.555
2011	1.448	3.557
2012	3.843	11.298
2013	9.758	28.768
2014	11.155	32.505
2015	11.224	34.665
2016	11.786	38.034

Anonymous (2016)

Table 5. Organic tea leaf production and produced tea amounts (tonnes)

Years	Processed Tea (tonnes)			
	Bought Wet Tea (tonnes)	Black Tea	Green Tea	Total
2009	361	58	3	61
2010	384	152	5	157
2011	1.743	313	13	326
2012	1.724	339	10	349
2013	1.732	353	9	362
2014	1.927	341	26	367
2015	7.381	1.328	21	1.349
2016	22.330	4.449	39	4.488

Anonymous (2016)

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Improving Grain Quality in Pulses: Strategies to Reduce Raffinose Family Oligosaccharides in Seeds

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ABSTRACT

In human diet, pulses are an excellent source of carbohydrates, proteins, dietary fibers, vitamins, minerals and other bioactive compounds. However, the presence of high concentration of raffinose family oligosaccharides (RFO) limits their consumption and acceptance worldwide especially in developed countries. Humans and mono-gastric animals cannot digest RFO but are fermented by large intestinal microflora that produces carbon dioxide, hydrogen and methane causing flatulence and stomach discomfort. Hence, it is imperative to develop strategies to reduce RFO concentration in pulses to promote their consumption in human diet around the world.RFO are sucrosyl galactosidessynthesized during the later stages of seed development. RFO accumulation in seeds is affected by crop species, genotype and growing environment. Genetic strategies have been used to reduce the accumulation of RFO in pulses. Several post-harvest processing methods have also been used to reduce RFO concentration in pulses used for human consumption.

Keywords: oligosaccharides,pulses, raffinose, RFO, stachyose and verbascose

Introduction

Pulse crops, members of the family Fabaceae, are defined by the presence of unusual flowers, podded fruits and their ability to fix nitrogen in their root nodules (de Faria *et al*.1989). The family Fabaceae is further divided into three subfamilies: Papilionoideae, Caesalpinioideae and Mimosoideae (Andrews and Andrews 2017). The three subfamilies show distinct flower characteristics: Papilionoideae has two partially fused petals, two wing petals and a banner like petal; Caesalpinioideae has irregular flowers with no distinct petals; and Mimisoideae is characterized by the presence of spikes. Most pulse crops belong to the subfamily Papilionoideae.

Major pulse crops cultivated for human and animal consumption include field pea (*Pisum sativum* L.), common bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.), broad bean (*Vicia faba* L.), pigeon pea (*Cajanus cajan* L.), cowpea (*Vigna unguiculata* L. Walp.), and lentil (*Lens culinaris* Medik.) (Chibbar *et al*., 2010). Two other legume crops grown primarily for oil production include soybean (*Glycine max* L.) and peanut (*Arachis hypogaea* L.). Pulse crops in general have gained a great significance in crop rotation, due to their nitrogen fixing capability that enriches soil with nitrogen.

Production of pulses has constantly increased during the past decades in Canada that contributed about 35% and 30% to total world production of lentil and beans from 2011-2013, respectively (Table 1). Canada is the largest exporter of lentil/pea and third largest exporter of beans in the world during 2013 (FAOStAT 2016). Nutritionally,pulses have higher amount of protein (20.6-32.2 g/100 g dry weight) compared to cereal grains (10-12

g/100g dry weight), but are also enriched in the essential amino acid lysine that is deficient in cereal grains (Chibbar *et al*., 2010; Shewry and Halford 2002). The major constituents in pulse seeds are carbohydrates contributing 49-68% to total seed weight (Chibbar *et al*., 2010). Carbohydrates can be classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides based on their polymeric structure (Chibbar *et al*., 2010). The total soluble sugars concentration in pulse seeds range from 3 -13 g/100g (Oomah *et al*., 2011). Total soluble sugars in the pulses include monosaccharides (ribose, fructose and glucose), disaccharides (sucrose, maltose, melibiose) and oligosaccharides (raffinose, stachyose, verbascose, ajugose and ciceritol). Among the soluble sugars, concentration of galacto-oligosaccharides or raffinose family oligosaccharidesishighranging from 2.7 to 5.9 g/100g in seeds (Sosulski *et al*., 1982).

RFO or α-galactosides are sucrosyl derivatives characterized by the presence of α(1→6) linkage between the galactose residue and the C-6 of the glucose moiety of sucrose (Gangola *et al*., 2014a).A major limitation to increase human consumption of pulses is the presence ofhigh seed RFO concentration (Gangola *et al*., 2012).Human and monogastric animals lack alpha-galactosidase required to hydrolyze α(1→6)glycosidic linkages, therefore RFO remain undigested in the upper gastrointestinal tract (Gangola *et al*., 2014b). The undigested oligosaccharides are fermented in the lower gut by anaerobic bacteria producing carbon dioxide, hydrogen and methane (Reddy *et al*., 1984). The higher production of these gases causes flatulence that can lead to stomach discomfort, abdominal rumblings, cramps, pain, and diarrhea. RFO in animal diets have also been associated with a reduction in net dietary energy. Adult roosters fed with diets containing 5.3% RFO showed a 20% reduction in net metabolizable energy compared to a diet containing 1% RFO (Coon *et al*., 1990). Diets with high RFO content caused osmotic imbalance (before fermentation by microbial flora) resulting in reduced nutrient absorption and protein utilization (Wiggins 1984; Van Barneveld 1999). In humans, RFO when consumed in low concentrationsmay have potential beneficial effects as prebiotics promoting the growth of beneficial bacteria like bifidobacterium in the large intestine (Guillon and Champ 2002; Martínez-Villaluenga *et al*., 2008b; Roberfroid 1999; Roberfroid *et al*., 1998). In rats, diets rich in RFO showed an increase in bifidobacterial growth and increased immune response (Gulewicz *et al*., 2002). In humans, consumption of soybean α-galactosides increased bifidobacterial and eubacterial growth in the large intestine (Hayakawa *et al*., 1990; Wada *et al*., 1991). RFO also play an important role in plants and participate in several metabolic processes (Obendorf and Górecki 2012; Sengupta *et al*., 2015) such as phloem transport, and defense responsesduring abiotic (Hannah *et al*., 2006; Nishizawa *et al*. 2008) and biotic stresses (Gil *et al*., 2012). RFO are synthesized during later stages of seed development and are postulated to confer desiccation tolerance (Martínez-Villaluenga *et al*., 2008a).

In pulses such as lentil (Tahir *et al*., 2012) and chickpea (Gangola *et al*., 2013), high RFO concentration has been attributed as one of the reasons for reduced consumption of pulses by humans (Gangola *et al*., 2014b).Hence, reduction of RFO concentration might help to promote human consumption of pulses.However, a major consideration is that RFO concentration needs to be reduced to an optimal amount that reduces stomach discomfort while still maintaining adequate amounts required for seed germination and plant growth.

Structures of raffinose family oligosaccharides

RFO are ubiquitous in plant seeds (Blöchl *et al*., 2007).Raffinose is the first member of this oligosaccharides family followed by stachyose, verbascose and ajugose (Figure 1). The first RFO was found and purified in chickpea hence named as "Ciceritol" (Quemener and Brillouet 1983). The RFO nomenclature is as follows:

Raffinose [*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Stachyose [*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Verbascose [*α*-D-galactopyranosyl-(1→6) -*α*-D-galactopyranosyl-(1→6)-*α* - D- galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D-fructofuranoside]

Ajugose [*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D- galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Ciceritol [*α*- D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→2)-1D-4-O-methyl-chiro-inositol]

Biosynthetic pathway of raffinose family oligosaccharide

Raffinose family oligosaccharides are formed by α-(1→6) galactoside linkages between the linear chain galactosyl residues and the glucose moiety of sucrose (Avigad and Dey 1997) (Figure 1). The three galactosyl donors involved in RFO biosynthesis are: UDP-D-galactose, galactinol and RFO. The biosynthesis of RFO is initiated by galactinol synthase (EC 2.4.1.123) which catalyzes the transfer of a galactosyl residue from UDP-D-galactose to *myo*-inositol to synthesize galactinol (Figure2). Raffinose synthase (EC 2.4.1.82) catalyzes the synthesis of raffinose by the transfer of a galactosyl residue from galactinol to sucrose. Stachyose synthase (EC 2.4.1.67) catalyzes the synthesis of stachyose by the transfer of a galactosyl residue from galactinol to raffinose. The enzyme verbascose synthase (VS) catalyzes the synthesis of verbascose by addition of a galactosyl residue from galactinol to stachyose. The main enzymes involved in the RFO pathway, galactinol synthase (GS or*GolS*), raffinose synthase (RS) and stachyose synthase (StS) have been isolated from some plants and the gene sequences coding for these enzymes have been submitted in NCBI or patented (Allan and Hitz 2000; Oosumi *et al*., 1998). RFO are also synthesized by a galactinol independent biosynthetic pathway. In *Ajuga reptans* the enzyme galactan:galactan galactosyl transferase (GGT), that catalyzes chain elongation by galactosyl transfer between two RFO molecules, was reported (Bachmann *et al*., 1994).

In *Ajuga reptans* two different RFO pools were reported: (i) storage pool - RFO synthesized in mesophyll cells, and (ii) transport pool - RFO synthesized in intermediary cells involved in phloem transport (Bachmann *et al*., 1994). Further compartmentalization studies by purification of vacuoles from mesophyll cells indicated that GGT, stachyose and higher RFO (verbascose) were vacuolar; and GS, StS, *myo*-inositol, galactinol, sucrose and fructose were extra-vacuolar. Raffinose was reported to be distributed in both the vacuole and the cytoplasm (Bachmann and Keller 1995). Stachyose synthesized in the cytoplasm was proposed to be transferred to the vacuole through a stachyose transporter in the tonoplast (Bachmann and Keller 1995). Two allelic variants of *GolS* were isolated in *Ajuga reptans* (Sprenger and Keller 2000). Gene expression, RFO accumulation and GS activity suggested functional differences among the two isoforms. *ArGolS1* was predominantly present in the storage RFO pool in mesophyll cells and *ArGolS2* was predominant in transport RFO pools in intermediary cells (Sprenger and Keller 2000).

A. Galactinol synthase

In Cucurbitaceae leaves, GS was a monomeric polypeptide of 38-43 kDa with 318-348 amino acid residues. GS enzyme activity hadpH optima between 5.6 and 7.5, and it requiredMn^{2+} as a cofactor. The K_m values for UDP-D-galactose and *myo*-inositol ranged from 0.16 – 0.53 mM and 4.0 – 6.5 mM, respectively (Keller and Pharr 1996; Peterbauer *et al*., 2001b). Most of the studies on GS suggest that RFO accumulation is controlled by the concentrations of initial substrates, *myo*-inositol and sucrose, rather than only by galactinol synthase activity (Karner *et al*., 2004).RFO concentration in chickpea seeds also showed a significant positive correlation to initial substrates concentrations (Gangola *et al*., 2013).

The presence of more than a single isoform of *GolS* showing differential expression during abiotic stresses has been reported in several plant species. Three genes coding for GS were characterized in *Arabidopsis thaliana* as, *AtGolS1*, *AtGolS2* and *AtGolS3*. All three recombinant AtGolS1, AtGolS2 and AtGolS3 proteins expressed in *E. coli* showed GS activity. Differential expressions of GS genes were obtained during abiotic stresses, where, *AtGolS1* and *AtGolS2* were induced during drought and salinity stress, but not by cold stress. The third isoform, *AtGolS3* was induced by cold stress but not by drought and salinity stress (Taji *et al*., 2002).

In *Zea mays*, three *GolS* isoforms were isolated, but they showed differential genes expressions. *ZmGolS1* expression was not observed during seed development. Transcript accumulation of *ZmGolS2* was observed towards later stages of seed development and rapid decrease of transcripts was observed at imbibition during seed germination. *ZmGolS3* transcripts were only detected when seed germination was interrupted by desiccation (Zhao *et al*., 2004). In *Coffea arabica* three allelic variants, *CaGolS1*, *CaGolS2* and *CaGolS3* encoding polypeptides with 388, 334, 344 amino acids, also showed differential transcript accumulation under drought, salinity and heat stress conditions. *CaGolS1* showed high expression during stressed and non-stressed conditions, *CaGolS2* was expressed only during severe water deficit and *CaGolS3* was expressed during all experimental stresses but at reduced level than *CaGolS1* (dos Santos *et al*., 2011). In tomato (*Lycopersicon esculentum*), *LeGolS1* transcripts weredetected 35 days after anthesis until seed maturity (60 days after anthesis) (Downie *et al*., 2003).

Two GS isoforms in *Ajuga reptans*, *ArGolS1* and *ArGolS2*were characterized. *ArGolS1*was source-leaf specific and *ArGolS2* participated in RFO transport. Gene expression studies showed that *ArGolS1*

transcripts were found in mesophyll and *ArGolS2* in intermediary cells explaining its role in phloem transport (Sprenger and Keller 2000). Recently, two galactinol synthase isoforms, *LcGolS1* and *LcGolS2*, were reported in lentil (*Lens culinaris* Medik.; Kannan *et al*. 2016). Both the isoforms showed expression during lentil seed development however, *LcGolS2* showed maximum expression at 24 days after flowering (DAF) whereas, *LcGolS1* transcripts accumulated mainly during 26-32 DAF.

B. Raffinose synthase

The purification of raffinose synthase (RS) was first reported by Lehle and Tanner (1973) from *Vicia faba*. The purified RS had a molecular mass of 90 kDa and exhibited a pH optimum between 6.5 and 7.0. In a subsequent study pea (*Pisum sativum*) RS was partially purified, which showed apH optimum of 7.0, and K_m values of 7.3 mM and 22.9 mM for galactinol and sucrose, respectively (Peterbauer *et al*. 2002a). Expression of a *RS* cDNA clone in *Spodoptera frugiperda* Sf21 insect cells, produced recombinant RS with kinetic properties like those of the purified RS (Peterbauer *et al*., 2002a). A *RS* cDNA clone isolated from rice (*Oryza sativa*) was expressed in *E. coli* to produce recombinant RS. The rice recombinant RS also showed maximum activity at pH 7.0 at 45°C (Li *et al*., 2007).

In *Arabidopsis*, five putative RS genes (*AtRS1-5*) or seed imbibition proteins (SIP) were described (Nishizawa *et al*., 2008). Among the five *AtRS* genes described, *AtRS5* showed high sequence similarity to the RS characterized in *Pisum sativum*. Heterologous expression of recombinant *AtRS5* showed RS activity (Egbert *et al*., 2013). Further, raffinose concentration was reduced in seeds of Arabidopsis *AtRS5* mutant. No *AtRS5* expression or activity was detected in leaves in mutant plants under unstressed or stressed conditions (Egbert *et al*., 2013). *RS2*/*ATSIP2*showed sequence similarity to α-galactosidase genes. Recombinant protein of *ATSIP2* expressed in Sf9 insect system showed raffinose specific α-galactosidase activity (Peters *et al*., 2010). RS has been reported as the most unstable enzyme in the RFO biosynthetic pathway (Castillo *et al*., 1990; Peterbauer *et al*., 2002a). Low RFO line (*stc*1 mutant) identified in *Glycine max* was associated with RS2 allele showing low raffinose synthase activity and higher accumulation of galactosyl cyclitols (Dierking and Bilyeu 2008; Hitz *et al*., 2002; Obendorf and Górecki 2012; Sebastian *et al*. 2000). The low RFO soybean genotypes showed good field emergence and yield like wild type.

Stachyose synthase

Stachyose synthase (StS) has been purified from adzuki bean, kidney bean, lentil and pea (Hoch *et al*., 1999; Peterbauer and Richter 1998; Peterbauer *et al*., 2002b; Tanner and Kandler 1968). StS purified from mature lentil seeds had a specific activity of 9.09 pkat/mg protein, a molecular mass of 88.6 kDa and an isoelectric point of 4.8 (Hoch *et al*., 1999). The amino acid sequence of StS (853-868 amino acids) was first obtained from *Vigna angularis* (Peterbauer *et al*., 1999). The molecular weight of StS was 85 to 95 kDa, with a pH optima of 6.5 - 7.0 (Richter *et al*., 2000). StS shows a broad range of substrate specificity which includes inositols and inositol O-methyl ethers. StS from adzuki bean showed no conversion of pinitols, whereas lentil StS catalyzed the synthesis of galactopinitol A and ciceritol, in addition to stachyose synthesis (Hoch *et al*., 1999; Peterbauer *et al*., 2001a). StS purified from adzuki bean and lentil showed no synthesis of verbascose (Hoch *et al*., 1999; Peterbauer and Richter 1998). StS from *Pisum sativum* synthesized both stachyose and verbascose (Peterbauer *et al*., 2002b and 2003)

In *Ajuga reptans,* RFO biosynthesis also occurred through a non-galactinol independent enzyme galactan:galactan galactosyl transferase (GGT) present in leaf vacuoles (Bachmann and Keller 1995; Haab and Keller 2002). GGT amino acid sequence showed high similarity to α-galactosidases and a non-sequence specific vacuolar sorting determinant at the C-terminal (Haab and Keller 2002; Tapernoux-Luthi *et al*., 2007). The presence of GGT activity (neutral pH) was reported in pea seeds with a high verbascose concentration and undetectable activities in a low verbascose pea line (Obendorf and Górecki 2012; Peterbauer *et al*., 2002b and 2003;). Since previous reports suggest that galactinol is exclusively cytoplasmic and stachyose is exclusively vacuolar, higher RFO might be produced by the galactinol independent pathway (Peterbauer *et al*., 2001a).

Interestingly, RFO biosynthetic enzymes have shown a wide range of substrate specificity. Galactinol synthase also catalyzed the synthesis of fagopyritol B1 where D-*chiro*-inositol was the galactosyl acceptor (Lahuta *et al*., 2005; Obendorf and Górecki 2012; Ueda *et al*., 2005). Raffinose synthase synthesized galactosyl ononitol and galactopinitol A from D-ononitol, D-pinitol and O-methyl cyclitols not naturally present in pea (Obendorf and Górecki 2012; Peterbauer *et al*., 2002a). Stachyose synthase from Lentil also synthesized fagopyritol B1 from D-chiroinositol and galactinol. Synthesis of galactopinitol B was also catalyzed by lentil stachyose synthase at a lower rate (Hoch *et al*., 1999).

Variation in RFO concentration among different legume crops

RFO concentration varies widely among different legume crops (Table 2). The concentration and composition of RFO depend on type of crop, growing environment and the genotype (Andersen *et al*., 2005; Gangola *et al*., 2013; Martín-Cabrejas *et al*., 2008; Reddy and Salunkhe 1980; Tahir *et al*., 2011). Reddy and Salunkhe (1980) reported verbascose (34.4 g/kg) as predominant RFO followed by stachyose (8.9 g/kg) and raffinose (trace) in black gram (*Vigna mungo* L. Hepper). Faba bean was reported to contain higher amount of verbascose (27.0 g/kg) while field pea was found to have higher amount of stachyose (27.0 g/kg). Sosulski *et al*. (1982) studied the variation in RFO concentration in eleven legumes and reported stachyose as the major RFO component in chickpea (Gangola *et al*., 2014b) and lentil flours. They also reported verbascose as the predominant RFO in mung bean and fababean. Quemener and Brillouet (1983) detected ciceritol in chickpea (28.0 g/kg dehulled seed), lentil (16.0 g/kg), white lupin (6.5 g/kg), soybean (0.8 g/kg), and bean (in traces). Saini and Knights (1984) studied the variation for total oligosaccharides in desi and kabuli chickpeas (seven varieties of each). They concluded that on average kabuli chickpeas (14.7, 53.0 and 1.2 g/kg of raffinose, stachyose and verbascose, respectively) contained 3.2% higher levels of total oligosaccharides compared to desi types (14.8, 50.6 and 1.5 g/kgof raffinose, stachyose and verbascose, respectively). Gangola *et al*., (2013) also reported a relatively higher concentration of total RFO in kabuli type (21.1 - 53.8 mmol/kg) compared to desi type (15.8 - 53.1 mmol/kg) chickpea genotypes.

In cowpea (*Vigna unguiculata*L. Walp.) and soybean, RFO concentration contributed more than 50% to total soluble sugars (Martín-Cabrejas *et al*., 2008). Cicek *et al*., (2006) studied various soybean seed characteristics including RFO variation using recombinant inbred lines among which stachyose (30 - 60 g/kgseed) was found as the major RFO constituent followed by raffinose (2 - 9 g/kgseed). Comparable results were reported by Kumar *et al.,* (2010) in soybean seeds showing range of 6.4 - 25.3 and 20.9 - 71.0 mmol/kgfor raffinose and stachyose concentrations, respectively.

Andersen *et al*. (2005) studied the compositional variations of α-galactosides in barley and various species of Leguminosae and Brassicaceae. The highest concentration of total RFO was reported in Lupin (91.0 ± 26.0 g/kg seeds), while *Brassica* species contained 14.0 ± 5.0 g RFO/kg of seeds (only raffinose and stachyose). Barley (*Hordeum vulgare* L. cv. Vega) contained 5.0 g raffinose/kg of seeds, which was the sole RFO component. However, Lupin was reported to have 3.0 - 19.0, 23.0 - 86.0 and up to 35.0 g/kg of raffinose, stachyose and verbascose, respectively (Martinez-Villaluenga *et al*., 2008a). Among studied species of Leguminosae and Brassicaceae, ajugose was present exclusively in lupin seeds. *L. albus* and *L. mutabilis* contained the lowest ajugose concentration (2.0 - 5.0 and 2.0 g/kg, respectively) followed by *L. angustifolius* (17.0 - 26.0 g/kg) and, *L. luteus* (6.0 - 46.0 g/kg; Andersen *et al*. 2005; Martinez-Villaluenga *et al*. 2008a).

Vidal-Valverde *et al.,* (1998) observed higher amount of verbascose [22.9 g/kg dry matter (DM)] followed by stachyose (11.0 g/kg DM) and raffinose (2.8 g/kg DM) in fababean. Total α-galactosides concentration of 18 pea varieties varied from 22.6 to 63.4 g/kgDM. Stachyose (10.7 - 26.7 g/kgDM) was found in higher amount than raffinose (4.1 - 10.3 g/kgDM), while verbascose was present in fifteen varieties ranging from 1.7 - 26.7 g/kgDM (Vidal-Valverde *et al*., 2003). Tahir *et al.,* (2011) analyzed eleven lentil cultivars, grown in two different environments, varying for stachyose, raffinose and verbascose concentrations that ranged 22.0 - 25.5, 19.5 - 22.2 and 11.5 - 13.3 g/kgof lentil seed meal, respectively. In another study, Lentil seeds RFO concentrations ranged from 9.22 to 19.68 g/kgfor verbascose and from 23.19 to 27.93 g/kg for raffinose+stachyose (Johnson *et al*., 2013).

The significant impacts of genotype, environment and their interaction on seed RFO concentrationshave been reported in some of the legume crops like soybean (*Glycine max* L. Merr.; Cicek *et al*. 2006; Kumar *et al*., 2010; Jaureguy *et al*., 2011), lentil (*Lens culinaris* Medikus subsp. Culinaris; Tahir *et al*., 2011) and chickpea (*Cicer arietinum* L.; Gangola *et al*., 2013). Consequently, broad sense heritability of RFO traits in legumes has been reported from low to high (0.25 - 0.85) depending on the crop, genotype and environment (Cicek *et al*., 2006; Gangola *et al*., 2013; Tahir *et al*., 2011). The environment influenced variation in RFO concentration suggest their role as antioxidants and phloem-mobile signaling compounds during diverse types of stresses. Therefore, environmental conditions like temperature, rainfall and light intensity influence RFO concentration, *i.e.* more adverse condition would result in higher RFO concentration (ElSayed *et al*., 2014).

Strategies to reduce RFO in seeds

Two main strategies have been employed to reduce RFO concentration in the seeds: (i) Post-harvest processing methods, and (ii) Molecular approaches

A. Procession methods

(i) De-hulling

Dehulling of cowpea (*Vigna unguiculata* L. Walp) seeds caused a significant reduction in RFO

concentration (Onyenekwe *et al*. 2000). However, in *Lens culinaris* varieties, dehulling decreased raffinose but increasedstachyose and verbascose concentrations (Wang *et al*., 2009). This shows that interaction of variety and processing method can led to different results in different crop species.

(ii) Germination

Germination has been found to remove RFOs quite effectively in legumes (Chilomer *et al*., 2010; Gulewicz *et al*., 2014; Khalil and Mansour 1995; Martín-Cabrejas *et al*., 2008; Mubarak 2005; Urbano *et al*., 1995; Vidal-Valverde *et al*., 1998). The decrease in RFO concentration during germination has been attributed to increased activity α-galactosidase which hydrolyses the α(1,6)- linkages, thus increasedthe total soluble sugar content and decreased RFO concentration (Martin-Cabrejas *et al*., 2008).

(iii) Aqueous or alcoholic extraction

Soaking has been commonly used during legume processing which decreased RFO concentration in severalpulses (Aguilera *et al*., 2009; Han and Baik 2006; Martín-Cabrejas *et al*., 2004 and 2006; Onyenekwe *et al*., 2000). Reduction due to hydration depends on differential solubility of individual oligosaccharides and their diffusion rates (Aguilera *et al*., 2009; Shimelis and Rakshit 2007; Upadhyay and Garcia 1988) but activation of enzymes like α-galactosidases upon hydration may also be responsible for reduced RFO concentrations (Aranda *et al*., 2001; Onyenekwe *et al*., 2000; Wang *et al*.,2003). Autolysis during soaking and extraction in the soak water and the cook water also decreased oligosaccharide concentrations in seeds (Wang *et al*., 2009). Ethanol extraction of RFO also increased amino acid usage and availability in soybean meal and resulted in more protein and energy dense product (Glencross *et al*., 2003; Leske and Coon 1999; Leske *et al*., 1995). Though it may be an effective method, ethanol extraction is not economically viable for the large-scale production of low RFO concentration seeds (Hagely 2013).

(iv) Changes in temperature or humidity or pressure treatment

Heat treatment (e.g. boiling, autoclaving, microwave cooking and extrusion at elevated temperature) decreased anti-nutritional RFOs (Alajaji and El-Adawy 2006; Devindra *et al*., 2011;El-Adawy 2002; Frias *et al*., 2011; Jenkins *et al*., 1982; Khalil and Mansour 1995; Vijayakumari *et al*., 2007; Wang *et al*., 2008; Wang *et al*., 2010). It was proposed that the decrease in raffinose and stachyose during cooking was due to thermal hydrolysis and formation of disaccharides and monosaccharides or other compounds from the RFOs (Onigbinde and Akinyele 1983; Wang *et al*., 2008). Industrial process of dehydration also affected the α-galactoside content by inducing changes in the carbohydrate fraction including hydrolysis of α-galactosides (Aguilera *et al*., 2009). However, long cooking time can also cause loss in proteins which could be attributed to partial removal of certain amino acids on heating (Aguilera *et al*., 2009; Rehman and Shah 2005; Wang *et al*., 2010; Youssef *et al*., 1986). Cooking after soaking treatment showed more noticeable decrease in RFO content than soaking alone and depended on the crop type (Aguilera *et al*., 2009; Martín-Cabrejas *et al*., 2006; Sánchez-Mata *et al*., 1999). Blanching has also been tried to reduce RFO concentration in pulses (Wang *et al*., 1997). Autoclaving has been suggested as a better method of processing to reduce RFO concentration in seeds (Vijayakumari *et al*., 2007).

(v) Treatment with microbial or plant α-galactosidase

Alpha-galactosidase is present in plants, microorganisms and animals (Dey and Campillo 1984; Kim *et al*., 2002). Endogenous synthesis of α-galactosidases increases during seed germination and results in lower concentrations of RFO in germinating seeds (McCleary and Matheson 1974). This is the biochemical principle for using germination and fermentation to reduce RFO concentration (Devindra *et al*. 2011; Glencross *et al*., 2003; Granito *et al*., 2002; Ibrahim *et al*., 2002; Torres *et al*., 2006; Vidal-Valverde *et al*., 1998;Yamaguishi *et al*., 2009). These procedures take advantage of the natural role of plant and microbial α-galactosidases. However, the potential microbial contamination of germinated grain legume seeds decreased their shelf life, making them unsuitable for food and feed use (Kadlec *et al*., 2006). While many studies showed positive effects for the enzymatic removal of RFOs (Anisha and Prema 2008; Cao *et al*., 2010; Girigowda *et al*., 2005; Leblanc *et al*., 2004; Veldman *et al*., 1993; Yamaguishi *et al*., 2009), still there are reports where diets supplemented with α-galactosidase showed no improvements in protein digestibility (Brasil *et al*., 2010; Irish *et al*., 1995; Smiricky *et al*., 2002). The potential of this approach is restricted due to poor stability of enzymes or their origin from microbes without generally recognized as safe (GRAS) status (Gote *et al*., 2004; King *et al*., 2002; Viana *et al*., 2007). Use of isolated enzymes is another option but it greatly increases processing costs. Since α-galactosidase is sensitive to pH and heat, and loses its activity rapidly during storage at

room temperature, novel coating treatments have been suggested such as encapsulation of α-galactosidase in chitosannanoparticles that could be developed into a pH-sensitive feed enzyme-releasing system (Liu *et al*., 2011). Though microbial enzymes are more efficient (Falkoski *et al*., 2006) and provide convenience of easy growth and isolation, soybean α-galactosidase may be a better choice because it is more suited for high protein and buffered environment of soybean (Viana *et al*., 2005). Alpha-galactosidase from coconut kernel immobilized to sepharose-4B gel also reduced total flatulence by 53-73%. This was advantageous as no clogging occurred when soy milk was passed through glass columns with enzyme containing gels (Dharamsena and Mathew 2002).

(vi) Irradiation

Irradiation is commonly used to control insect infestation and extend the shelflife of pulses (Machaiah *et al*., 1999), but it can also lower RFO levels by their rapid degradation (Al-Kaisey 2003; Machaiah *et al*., 1999). Gamma radiation along with germination showed distinct legume-specific quantitative changes in RFO concentration without altering their positive sensory attributes (Machaiah and Pednekar 2002; Rao and Vakil 1983). However,such treatment increase the cost as well as energy required for pulses production.

B. Molecular approaches to reduce RFO in seeds

(i) Up-regulation of α-galactosidase and galactosyl cyclitols synthesis

Alpha-galactosidase is a well-known enzyme for RFO break down by hydrolyzing α(1→6) linkage. Overexpression of α-galactosidase from coffee (*Coffea arabica* L.) was used to reduce RFO concentration in peas (Polowick *et al*., 2009). The transgenic pea lines showed up to 40% reduction in raffinose and stachyose concentrations without affecting seed germination rate (96%). Zuo *et al*., (1996) had showed that although much lower oligosaccharide concentrations were present in genetically altered soybean meal; no differences were noted between conventional and low oligosaccharide soybean meal in any of the digestion responses in ileally-cannulated dogs.

Further reductions in the endogenous RFOs could be obtained by use of improved vectors, RNAi or antisense technology and development of homozygous lines (Polowick *et al*., 2009). It would be a better strategy if α–galactosidase could be activated after harvesting to degrade RFO after harvesting. This can be based on the transfer of α-galactosidase from a thermophilic bacterium into grain legumes which can be activated during canning (Griga *et al.,* 2001; Wang *et al.,* 2003).

Frias *et al*., (1999) suggested an alternative strategy to reduce RFO concentration byincreasing the synthesis of related compounds such as the galactosyl cyclitols. This would maintain the protective nature of these compounds but decrease their flatus potential, as the ciceritol was more slowly hydrolyzed by α–galactosidase than the RFO. Ciceritol is present in chickpea and lentil but has not been detected in pea. The key to introduce galactinol cyclitols into pea with an accompanied reduction in the RFO content appears to lie with stachyose synthase, which has a vital role in the synthesis of the galactinol cyclitols and in the synthesis of stachyose (Peterbauer and Richter 2001b). It represents a link between the RFO and galactinol cyclitol pathways (Wang *et al*., 2003).The ratio of D-pinitol and *myo*-inositol influenced the RFO concentration in developing tiny vetch [*Vicia hirsute* (L.) S. F. Gray] seeds (Lahuta *et al*., 2005). Galactosyl pinitols can replace RFOs as reserve carbohydrates for seed germination in *Vicia villosa* (Lahuta and Goszczyńska 2009). It has also been reported that free cyclitols inhibit StS and/or VS activity in developing seeds of *Vicia*species (Lahuta *et al*., 2010). Since accumulation of d-chiro-inositol strongly reduced accumulation of verbascose, the main RFO in pea seeds, transformation of pea with genes encoding d-chiro-inositol synthesizing enzymes has been suggested as a strategy to reduce the accumulation of RFO by inhibiting the synthesis of verbascose (Lahuta and Dzik 2011).

(ii) Down-regulation of key biosynthetic enzyme

Galactinol synthase (GS) is considered as the first committed and key regulating step of RFO biosynthesis influencing carbon partitioning between sucrose and RFO (Nishizawa *et al*., 2008). There has already been a patent regarding genetic manipulation of RFO levels by inhibiting galactinol synthase activity (Kerr *et al*., 1993). Bock *et al*., (2009) used an antisense approach to down-regulate the expression of galactinol synthase in canola (*Brassica napus* L.). Consequently, a decrease in galactinol and stachyose concentrationswas observed in transgenic canola seeds. Out of four main targets (*myo*-inositol concentration, sucrose concentration, galactinol synthase and other biosynthetic enzymes) to regulate RFO biosynthesis,galactinol synthase has been suggested as a potential target to reduce RFO concentration in chickpea seeds (Gangola *et al*., 2016)

(iii) Mapping and Breeding

Genetic manipulation of RFO content by plant breeding can be an effective tool to prevent flatulence

caused by legumes. It is known that there is considerable variation in the raffinose and stachyose content among different varieties of legumes. This variation can be either natural or created through mutagenesis. Transgenics require high energy and time input. Further different regulations make it difficult to release varieties especially for food and feed purposes. In such cases, plant breeding can be a good approach, as used in case of soybean. Methods of germplasm screening as well as chemical mutagenesis have been used to select soybean strains with low RFO or high sucrose (Clarke and Wiseman 2000) and in addition, it also helped in determining the genetic basis of some of the available low RFO traits (Hagely *et al*., 2013). These soybean lines with variant alleles for low RFO not only provided soybean meal that was nutritionally superior to conventional soybean meal (Parsons *et al*., 2000), but also a resource to introgress the low RFO phenotypes into other genetic backgrounds including elite cultivars (Hagely 2013).

The breeding program for soybeanutilized mutants characterized by Hitz *et al*., (2002)[including LR33 having low raffinose, stachyose, *myo*-inositol, and phytic acid (with mutation in MIPS1 gene)] and a soybean plant introduction line, PI 200508, (with lower raffinose and stachyose and increased sucrose) identified by Kerr and Sebastian (1998). This linealso showed decreased raffinose synthase enzyme activity in maturing seeds (Hitz *et al*., 2002). Further studies showed that the line had mutant allele for *RS2* gene (Dierking and Bilyeu 2008). Another independent mutant allele of the *RS2* gene was further identified (Dierking and Bilyeu 2009) and, recurrent selection and plant breeding have led to the development of new soybean lines containing more distinct alterations in sucrose, raffinose, and stachyose contents. Carbohydrate profiles of lines representing a range of characterized RS2 genotypes grown together in one location showed that these profiles were heritable in general and RS2 genotype appeared to be the single largest determinant of carbohydrate profile (Hagley *et al*., 2013). In another recent study, though altered carbohydrate soybeans could produce low RFO phenotype across distinct locations, the carbohydrate profile was found to be affected by the environment (Bilyeu and Wiebold 2016)

In pea also, variant stachyose synthase gene resulted in reduced verbascose content in genotype SD1 (Peterbauer *et al*., 2003). Another recent report from soybean showed the inheritance of high sucrose and low raffinose/stachyose contents in V99-5089 soybean seeds. Due to the strong correlations between sucrose and raffinose ($r = -0.88$), and between sucrose and stachyose ($r = -0.96$), V99-5089- can be a good genotype for use as parent in soybean food-grade improvement programs (Mozzoni *et al*., 2013). Another study identified a 33-bp deletion mutant in the putative *StS* gene (Glyma19g40550) of PI 603176A responsible for ultra-low stachyose content (0.5%) therefore, an indel marker associated with low stachyose content was developed (Qiu *et al*., 2015). Identification of variation both natural and through mutation is necessary for successful pulses improvement programs to reduce RFO concentration in seeds. Anti-nutritional factors in legumes can be efficiently and economically reduced through molecular breeding. Marker-assisted selection has proven a rapid and reliable method for selecting desirable lines for seed quality traits. Recent breakthroughs in genomic sequencing of legumes (Das and Parida 2014), molecular breeding becomes more attractive strategy for RFO reduction in pulses (Hagely 2013). Molecular markers correlating with raffinose family oligosaccharides in soybean are already available, and the availability of markers in other species will also increase with increasing genomic information. Although several quantitative trait loci (QTLs) and associated markers have been identified for sugar content in soybean, it is still necessary to validate these QTLsand confirm associated molecular markers in several genetic backgrounds (Mozzoni *et al.,* 2013).

Concluding remarks

Pulses are environmentally friendly due to their nitrogen fixing capability, which reduces the input costs and enriching the soil with nutrition. Pulses are also very nutritionally diverse grains, and rich source of proteins rich in essential amino acid lysine that is deficient in cereal grains. To completely utilize the nutritional benefits of pulses, more emphasis should be placed on pulse seed quality improvement. Genetic and molecular biological techniques have been used to reduce RFO concentration in some pulses. However, similar strategies can be used to improve the pulse seed quality to increase the protein concentration, improve the amino acid composition and above all enhance protein digestibility so that complete benefit can be realized from pulses consumption in human diet. Pulse carbohydrates also have very good health benefits as pulse starch has higher amylose concentration that cereal grain starch. Research to improve starch concentration and composition in pulses will add to the human health benefits of pulses (Chibbar *et al.,* 2010). In conclusion, pulse improvement should focus both to increase yield as well as improve pulse seed quality to realize the complete benefits of these environmentally friendly grains that have the potential to assure global food and nutritional security.

Table 1. Production of pulses in the world and Canada

Legumes	Years	World		Canada		
		Area harvested	Total production	Area harvested	Total production	Export value
Chickpea (*Cicer arietinum*)	1961-1970	11122839	6671439	NA	NA	NA
	1971-1980	10217041	6511618	NA	NA	NA
	1981-1990	9862896	6674667	NA	NA	NA
	1991-2000	10824941	8050048	52343	72269	7183
	2001-2010	10656554	8750603	124410	149350	44986
	2011-2013	13052911	12155054	67267	140533	68260
Soybean (*Glycine max*)	1961-1970	26548450	34442545	108373	210716	6123
	1971-1980	41172412	66468637	204565	439081	10399
	1981-1990	53306881	95580502	421870	998160	40962
	1991-2000	64109804	135011549	854520	2243850	140805
	2001-2010	90766274	213207790	1190820	2979290	539093
	2011-2013	106664474	259829434	1680333	4843700	1445843
Lentils (*Lens culinaris*)	1961-1970	1688968	969648	NA	NA	NA
	1971-1980	2069018	1233391	10083	6806	NA
	1981-1990	2879647	2113347	104720	115630	27346
	1991-2000	3454660	2818101	373487	482190	115842
	2001-2010	3798903	3485664	714900	938620	418174
	2011-2013	4289906	4693991	985500	1650100	883659
Beans, dry (*Phaseolus vulgaris*)	1961-1970	23656624	11930983	34288	48739	3391
	1971-1980	24016286	13040812	55767	80363	20481
	1981-1990	26224268	15649445	42530	71700	34439
	1991-2000	25239779	16935482	99258	178450	71581
	2001-2010	27378366	20425247	153380	297910	184821
	2011-2013	29656745	23422508	90597	207787	214073
Peas, dry (*Pisum sativum*)	1961-1970	9524463	9824185	25240	32154	1576
	1971-1980	7657213	9096667	34036	55223	8002
	1981-1990	8839051	12620974	126360	215560	34303
	1991-2000	6869300	12350106	690110	1516870	161560
	2001-2010	6300669	10302944	1305530	2677390	457821
	2011-2013	6444392	10378984	1233433	3101900	1099015
Cow peas, dry (*Vigna unguiculata*)	1961-1970	4355361	1117818	NA	NA	NA
	1971-1980	4185682.2	1273051	NA	NA	NA
	1981-1990	4328346	1548741	NA	NA	NA
	1991-2000	8336560	2967803	NA	NA	NA
	2001-2010	10587941	4965438	NA	NA	NA
	2011-2013	11061517	5472601	NA	NA	NA

Units for area harvested, total production and export value are hectare, tonnes and ×1000 US$, respectively.

Table 2. Variation in RFO (including Ciceritol) concentrations among different legume crops

Legume crops	Concentration (g/kg dry matter)*					
	Raffinose	**Stachyose**	**Verbascose**	**Ajugose**	**Ciceritol**	**References**
Chickpea (*Cicer arietinum*)	4.5 – 21.0	17.2 – 61.5	ND - 45.0	ND	~28.0	1, 3, 4, 5, 6, 7, 12, 13, 14, 15
Soybean (*Glycine max*)	6.7 - 11.5	27.5 - 28.5	ND - 3.0	ND	0.5 - 0.8	1, 2, 3, 6, 7
Lupin (*Lupinus albus*, L. *luteus*, L. *angustifolius* and L. *mutabilis*)	3.0 - 19.0	23.0 - 86.0	ND - 35.0	02.0 - 46.0	6.5	1, 3, 6, 7
Cow pea (*Vigna unguiculata*)	4.1	32.2 - 44.4	4.8	ND	0.4	1, 2, 3
Lentil (*Lens culinaris*)	3.1 - 10.0	14.7 - 31.0	4.7 - 31.0	ND	16.0	1, 3, 4, 6, 7
Field pea (*Pisum sativum*)	6.0 - 14.0	17.1 - 27.0	23.0	ND	ND	1, 3, 6, 7, 9, 10
Mung bean (*Vigna radiata*)	2.3	9.5	18.3	ND	ND	1
Fababean (*Vicia faba*)	1.0 - 3.0	6.7 - 15.0	14.5 - 31.0	ND	ND	1, 3, 4, 6, 7, 9, 11
Black gram (*Vigna mungo*)	Traces	8.9	34.4	ND	ND	8
Bean (*Phaseolus vulgaris*)	<0.5 - 25.0	2.0 - 42.0	0.6 - 40.0	ND	Traces	3, 4, 5, 6, 7

[1] Sosulski *et al*. 1982; [2] Martín-Cabrejas *et al*. 2008; [3] Quemener and Brillouet 1983; [4] Dilis and Trichopoulou 2009; [5] Wang *et al*. 2010; [6] Andersen *et al*. 2005; [7] Martinez-Villaluenga *et al*. 2008a; [8] Reddy and Salunkhe 1980; [9] Huynh *et al*. 2008; [10] Vidal-Valverde *et al*. 2003; [11] Vidal-Valverde *et al*. 1998; [12] Saini and Knights 1984; [13] Alajaji and El-Adawy 2006; [14] Frias *et al*. 2000; [15] Jukanti *et al*. 2012
*ND = not detected

Figure 1. Chemical structure of *myo*-inositol, UDP- Galactose, galactinol, raffinose, stachyose, verbascose, ajugose and ciceritol

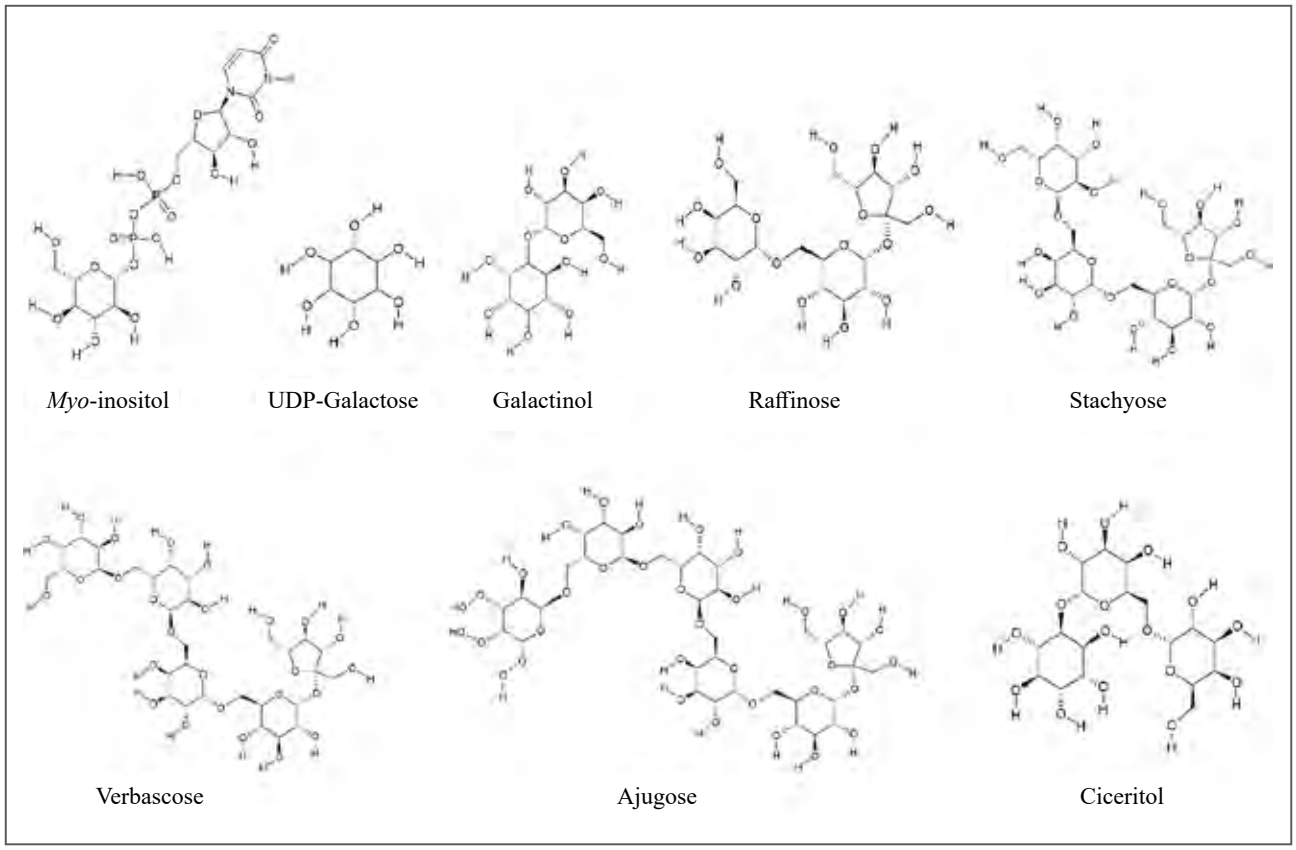
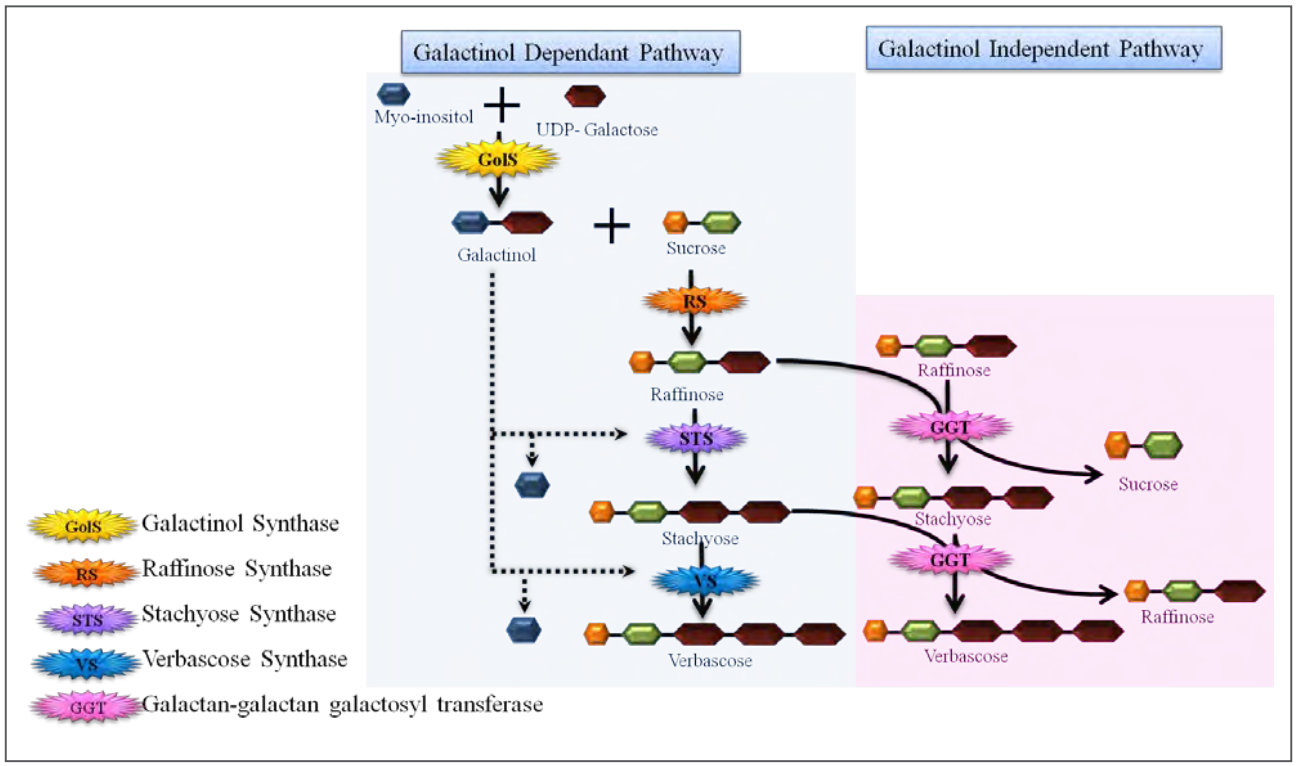


Figure 2. Schematic representation of RFO biosynthetic pathway in plants



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

Similar effect has been widely studied prior to this study (Eser 1991; Bagci et al. 1995; Uzun and Yol 2013).

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References

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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.

Online document:

FAOSTAT J (2013) http://faostat.fao.org/site/567/default.aspx#ancor. Accessed 15 May 2013.

Dissertation (Thesis):

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

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Abbreviations

Abbreviations should be defined at first mention and used consistently.

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