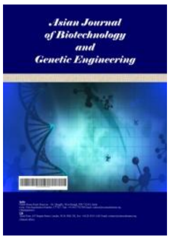

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Genetic Analysis of Drought Resistance in Maize Using Physiological, Morphological and Molecular Markers

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Authors' contributions

This work was carried out in collaboration among all authors. Author KA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Whole studies about the Molecular markers are also carried by author KA. Authors AH and AA managed the physiological and morphological analyses of the study. Author AA managed the literature searches. Authors MA and MB wrote the summary of physiological and morphological markers. All authors read and approved the final manuscript.

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ABSTRACT

Drought is one of the limiting factors to annual maize production worldwide, as every year, about 15 to 20% of maize yield is lost to drought. Improvement in maize drought tolerance has always been one of the main objectives of a plant breeder. Measurement of physiological and morphological characters related to drought tolerance might prove very useful. The main objective of this study is to assess the markers, physiological and morphological traits, as well as molecular markers, which are considered as primary indices of drought tolerance mechanism. Maize, being a C4 crop, has a great mechanism of drought tolerance. Severe water stress may result in hampering photosynthesis, disturbing the overall metabolism, and finally leading to necrosis of the plant.

Similarly, other factors such as membrane stability, Chlorophyll contents, leaf area, delayed

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senescence, and rooting system are the most important markers to identify a crop plant in drought conditions. The final portion of this review discussed molecular markers and DNA marker-assisted selection which have become an amazing option for drought analysis in the maize crop. In this study at the Research Institute of Agrarian Business, about 113 maize lines were selected. With PCR using two Molecular Markers, dhnC397 and rspC1090, the polymorphic SNP sites of dhn1 and rsp41 were determined. As a result of this, using dhnC397, a 164-bp segment was found at the dhn1 site. For a genotype, carrying (CCA AGG) variants, PCR products were digested by using StyII restriction enzyme resulting in 02 bands, 131-bp and 33-bp. A similar approach was used for the identification of rsp41 gene polymorphism. Passing through the HpaII restriction enzyme, resulted in 02 bands, 225-bp and 61-bp. The alleles produced by using PCR and restriction enzymes are associated with drought resistance in maize. In this study, the alleles associated with drought resistance were identified in about 68 lines out of 113 by using these molecular markers. This concept can be used for breeding drought-resistant maize hybrids as well.

Keywords: Maize (Zea mays L.); drought resistance; drought markers; SNP polymorphism.

1. INTRODUCTION

Maize (*Zea mays* L.) in Pakistan is cultivated as multipurpose food and forage crop, mainly by resource poor farmers using marginal land. Maize is currently the leading world cereal both in terms of production and productivity. In Pakistan, it is the third most important cereal after wheat and rice. Plant growth and productivity is adversely affected by nature's wrath in the form of various biotic and abiotic stress factors [1]. Water deficit is one of the major abiotic stresses, which adversely affects crop growth and yield. Drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation due to increase in temperature in nature. Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species. Severe water stress may result in hampering photosynthesis, disturbing the overall metabolism and finally leading to necrosis of the plant. Water stress inhibits cell enlargement more as compared to cell division. Plant growth is reduced by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters. For plant breeders, abiotic stress tolerance is a big challenge because of high genotype x environment interaction, low heritability, and mutagenic nature of abiotic stress responses [1]. This review covers the most beneficial morphological, physiological and molecular markers, breeder can use for drought tolerance in maize crop. No exact figures on yield and economic losses in maize due to drought and salinity are available. They can be assumed extensive and significantly greater for drought than salinity. It is estimated that 20–25% of the global maize area is affected by drought in any given year. Drought in major maize producing countries such as the United States or China routinely affects world maize yields, much more so than for rice or wheat. Most of the total world maize area of 150 million ha is grown under rain fed conditions, and maize is more susceptible to drought than all other cereals except rice. Even though yield fluctuations in the main maize producing countries and more developed economies have the greatest influence on world maize production, impacts of drought on the economies and human well-being in developing economies is likely much greater [2]. In eastern and southern Africa, where maize is the most important staple food for over 300 million people, a close correlation between rainfall and maize yields can be observed, and total maize production can result in close to two-fold variation between two years (FAOSTAT, 2006; 12.5 million ton in 1992; 23.5 million ton in 1993). Drought in these countries can result in wide-spread maize crop failure, affecting the livelihood of millions of people. Between 2003 and 2005 alone, the World Food Program spent USD 1.5 billion to meet food deficiencies due to drought and crop failure in Africa (World Food Program, 200). Maize as a C4 crop efficiently utilizes moisture and sunlight. Depending upon intrinsic factors in the seed and abiotic factors such as temperature and moisture, maize plant covers stages from emergence (VE) to early vegetative 5-leaves (V5) with in 4 to 15 days. It is characterized as the most critic period with fragile roots, growing point of plant below the ground surface. Any type of abiotic stress such as drought stress at this stage causes total crop failure [2]. Drought resistance is considered to be complex mainly when it is viewed from the genomics platform. Most recorded conclusions and complaints about drought resistance being complex arise from studies of gene mining and gene expression, as well as from some molecular mapping attempts of drought resistance. Drought resistance mechanisms generally relate to drought avoidance or drought tolerance. Drought avoidance mechanisms include deep-root systems to acquire soil moisture at lower levels, and plant characteristics to reduce water loss, such as closed stomata, leaf rolling, or waxy substances on the leaf surface. Usually heat and drought tolerance are considered together because high temperatures and drought often accompany each other in the field [3]. Soil moisture gradients from irrigation lines have been used to evaluate strains in different levels of drought stress. Irrigation water supplied will be highest near the irrigation line and will decline outward until a point is reached where no irrigation water is received. Cultivars growing along the gradient are compared for performance. There is a high correlation between tolerance to heat and tolerance to desiccation. Comparative resistance of plant genotypes may be observed by exposure to high temperature, soil drought, or atmospheric drought. Like winter hardiness, plant genotype resistance to heat and drought stress in the field is determined by complex physiological and morphological characteristics and cannot be accurately evaluated by a single laboratory test. Heat and drought stress resistance are quantitative characters with complex inheritance [3]. In this review article some markers are used for genetic analysis. These are physiological, morphological and molecular markers. In addition to utilizing morphological markers for the genetic mapping of the chromosomes, maps may be developed using molecular markers [4]. The molecular markers being utilized include isozymes, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNA (RAPDs). Isozymes are multiple forms of a single enzyme. Chemically, they are complex proteins. Isozymes were the first molecular genetic markers used in plant genetics and breeding. The number and polymorphism level of isozymes are much lower than that of the recently found molecular markers at the DNA level, RFLPs and RAPDs [4].

2. PHYSIOLOGICAL MARKERS

2.1 Membrane Stability

Membrane stability is the measure of drought or salt stress tolerance of a plant. Membrane stability index (MSI) provides the extent of drought tolerance for a particular cultivar in given environment (in vitro). The higher the MSI, the more will be the drought tolerance. Maize (*Zea mays L.)* being C4 crop is very sensitive to drought stress in tropical and sub-tropical regions like Pakistan [5]. Maize plants from different cultivars are grown (in vitro) and drought stress is induced by treatment of Polyethylene Glycol (60% PEG600). Leaves at seedling or any other particular stage are collected to make leaf discs (5g, 1cm diameter), and cellular membranes are observed. Rupturing of membranes shows drought sensitivity, while membrane stability at given laboratory conditions tells about drought tolerance of maize [5]. If a cultivar has high water retention capacity and low membrane injury, it will be drought tolerant. As drought tolerance results because of the accumulation of Glycinebetain (GB) and free proline, the comparison among maize lines in accordance to the accumulation of these osmolytes can help to choose the drought tolerant one [6]. At higher stages of maize plant, water deficiency causes oxygen toxicity. Because of less water availability, plant goes towards avoidance mechanism i.e reduce water loss by closing stomata. As a result of stomata closure. CO_2/O_2 ratio decreases in leaves inhibiting the process of photosynthesis. As a result of which amount of reactive oxygen species such as H_2O_2 and other radicals increases in chloroplast and mitochondria. These highly reactive radicals tend to react with lipids of membranes, resulting in membrane destruction [7]. While some anti-oxidative enzymes and metabolites may reduce membrane damage. Hence, it is concluded that the cultivar with relatively high accumulation of glycinebetain and proline, anti-oxidative enzymes and membrane permeability will be drought tolerant [7].

2.2 Abscisic Acid

In plants having drought stress (DS) the role of plant hormone abscisic acid (ABA) is very important in regulating physiological responses that can lead to adjustment to an unfavorable environment. In regulation of glycinebetaine (GB) metabolism in maize is realated to this hormone certainly on young plant at the seedling stage is not well known yet. The variation in the levels of abscisic acid and sugar in stress response plays a role [8]. Pre-pollination ears, silks, and leaves were analysed for sugars, starch, proline, ABA, ABA-glucose ester, and phaseic acid. The growth is negatively correlated in silk and ears in level of abscisic acid and sugar. Levels of abscisic acid of water stressed plant are meaningfully linked to a certain aldehyde oxidase gene. The disequilibriumin maize which shows that allelic change in these genes causes effect on abscisic acid and carbohydrate metabolism in floral tissue in drought stress. There were directed some hydroponic experiments to examine the modulation role of abscisic acid on plant growth, water relations and glycinebetaine metabolism in the leaves of two maize cultivars, drought tolerant, and drought sensitive, In drought stresses the relative water content of plant (leaves) decreased and also the dry matter of plant decreased but the glycinebetaine increase in both caltivars [8]. In the maize seedlings the contents of abscisic acid and glycinebetaine increased in drought stress, but glycinebetaine accumulated later than abscisic acid's accumulation in drought treatment. These actions were in drought tolerant than in drought sensitive cultivars. Glycinebetaine accumulation enhanced by the application of abscisic acid, production of leaf relative water content and dry matter of shoot in both cultivars [9]. However, the performance of the drought sensitive maize cultivar was better than the other maize cultivar in abscisic acid application.In modulation of betaine aldehyde dehydrogenase, the endogenouse abscisic acid likely connected with the regulation of glycinebetaine metabolism and resulting in specifically in the drought sensitive maize cultivar in regulation water relations and plant growth under drought. Stomata closure is encouraged by the application of the abscisic acid in guard cells by intracellular signalling. Water content decreases, while abscisic acid and electrolyte leakage percentage increased under drought stress. In water stress, the activity of antioxidant enzymes increases by the application of abscisic acid. Abscisic acid's application improved glycinebetaine accumulation, RWC and dry matter production in maize plants [9].

2.3 Chlorophyll Content

Drought stress had a significant effect on chlorophyll content. Chlorophyll content decreased under drought condition. It has been observed that there is 30 to 60 percent decrease in chlorophyll content during drought stress . Change in the climate during recent years, leading to decrease in rainfall causes increase in drought condition which affects yield of plant [10]. Growth of plant are sensitive to abiotic and biotic stress and depend on the photosynthesis. One of major chloroplast component for photosynthesis is chlorophyll. Plants absorb photosynthetically active radiation by chlorophyll. Under drought condition leaf area reduces, resulting in decrease in chlorophyll content which causes decrease in the photosynthesis process. Tolerant lines are either resisted to decrease in chlorophyll content during drought condition or showed minor reduction while susceptible lines showed massive decrease in chlorophyll content [11].

2.4 Proline

The proline is a type of amino acids. It's concentration in plants depends on the water availability. The growing area of maize having less water availability will contain more proline accumulation. The proline concentration is more at root or shoot tips than at the lateral or middle portions of plants [12]. It plays very important role in the maintenance of root elongation even at low water availability. The proline concentration in maize is also important in making maize cold resistant like manitol and abscisic acid concentration. Although the manitol and abscisic acid concentration produced very different effects on proline accumulation and makes the plants more resistant to cold which proline alone could not [13].

3. MORPHOLOGICAL MARKERS

Morphological traits were the first genetic markers used in the scientific studies. Some of these traits are color, shape, and flower which represent the phenotype of the plant. Morphological markers are detectable simply by visual examination. Morphological markers include plant height, presence and absence of awn. They do not require advanced and ultra modern equipments nor preparatory procedures. That is why use of these markers is simple, inexpensive and rapid [14]. Morphological markers are known to be associated with the important agronomic traits such as leaf-tip burning which is associated with the leaf rust resistance gene Lr34 in wheat. There are also some limitation of morphological markers which include maintenance stocks for expressing the various traits, limited number of morphological markers in a specie, and many traits required may have threshold for their expression [14]. According to the recent studies, most of the authors reported that change in the morphology of plant species can be initiated by mutations that effects on the phenotype [15].

3.1 Leaf Area

Correlation of means show that moderate drought stress set off leaf region and maximum leaf area is produce. While during severe drought stress, the area of the leaf is reduced, because of quick leaf senescence. Severe drought stress reduces leaf area, radiation use efficiently and reap the maize altogether by declining the membrane structure, overproduction of ROS and quickened leaf senescence. Cytokinins (CK) plays powerful role in deceleration of leaf senescence. It is applied externally or may be produced internally it retards the leaf senescence by removing free radical involved in leaf senescence and increase leaf area [16]. It has been reported that reduction in leaf area is 15-20% under different condition of drought stress in maize [17]. The inoculation of Arbuscular mycorrhizas (AR)- helps in improving the maize plant to respond efficiently during drought tolerance [17,18].

3.2 Short Stem

Under drought stress growth of cell is retarded and the stem of maize is reduced. It was reported that more dry weight of stem is produced by drought tolerant variety of maize compared to the sensitive one. The diameter of the maize stem had been observed reduced during its vegetative and reproductive growth under drought stress [19]. Drought stress affects the diameter of stem more at vegetative growth time than to reproductive stage. It affects the stem diameter more badly when the condition of drought stress remained prolonged for long time. The earlier studies showed that exogenous application of Gibberellic acid and cytokinin helps to maintain stem diameter during drought stress condition in maize varieties [19]. Drought tolerance involves many biochemical and physiological processes. In this condition, potassium is an important nutrient which helps plants to exhibit its tolerance against drought stress. The deficiency of potassium causes decrease in photosynthesis and other processes involved in consumption of carbon reservoirs. The studies on plants showed that potassium increases stem elongation and also enhances fruit shelf life and root length [20].

3.3 Small Tassels

Maize plant are susceptible to drought stress at flowering and early seed development stage. The tassel emergence was the first observed developmental step can be increased by the moisture deficit treatments. Small tassels allow more light to strike on the leaves as compare to large tassels [21]. Moreover small tassels produce less number of pollen than the large tassels. As pollen decrease associated with small tassels, so if the tassel is too small pollination process may inadequate. The increase in tassels was observed due to an increase in silk delay as measured by the pollen shed to silking interval. As compared to control, mild and severe drought stress treatments increased silk delay by an average of 3- 4 days, respectively [21].

3.4 Erect Leaves

Experiments were performed for the leaf orientation of maize. After these experiments it was concluded that the development of erect leaves in maizeis directly connected with reduced response to hormone , auxin and light in young seedlings of plant in early time of the growth of the plant. Change in maize leaf angle that can be due to any reason, shortage of light and hormones and any other factor alters the three dimensional organization of plant body. If the leaf angle of the plant is narrower then the most upper ear is assumed as compact plant organization [22]. And if the plant's leaf is broader, it is considered as the expanded plant architecture. That is why leaf angle is the most important factor in plant organization. It was being tested to check the possibility that the physiological results of the selection involved the response of auxins and light. Hybrids showed resistance to auxins and light induced responses at the seedling development level, cell level, and at molecular level. Preliminary analysis of mutants showed that ABPs may be involved in the development of leaf angle. It was also being hypothesised that the mutated corn contained erect upright leaves. And these leaves were very helpful against drought resistance, as the leaves are directly involves in the absorbance of light and performance of photosynthesis [22].

3.5 Delayed senescence

When silk emerge in maize plant, the first silk to appear ithe also first to senesce. It was first evident that senescence was due to the loss of turgidity, which was due to loss of water at the base of silk. A description is given about the major changes in the pattern of photosynthate accumulation by the delayed senescence of maize. The major effects noted include high water and chlorophyll contents in leaves at the time of their physiological maturity:- more accumulation of sucrose in stalk during grain filling:- busks and cons with more water: sucrose and protein contents, high protein value in the grains [23]. The high level of sucrose in the stalk at physiological maturity secures the plants from stalk spoiling organisms. The delayed senescence phenotype is controlled by a dominant gene, the same genetic factor which is responsible for the more availability of sucrose in stalk. Different oxidative and antioxidants are also considered as the basis of delayed senesce in maize [24]. In this regard different experiments were performed for checking out the response of these oxidants and antioxidants and then resultantly it was concluded that leaf senesce in early senescence is associated with the higher oxidative stress and decline in antioxidants response towards maturity [24].

3.6 Rooting System

At the time of average growth in maize plants, the roots can utilize up to two hundred cubic feet of soil and can absorb about thirty five to fifty gallons of water. Soil texture is also important in this regard. The roots can expand up to three to four feet on all sides and also penetrate into depth of about five to six feet or up to eight but this is rare. The tillage activity is very important for the growth of the maize roots. The importance of tillage for the roots of maize was reported in a trial of five years [25]. In the trials, two soils were under consideration (loamy silt and Sandy loam soil). The results showed the differences in maize roots growth in the tillage systems. And if the roots are expanded to the area which was not tillage then starter fertilizer should be applied in subsurface especially phosphorus which is immobile nutrient. Three dimensional dynamic system was made for studying the uptake of water and nutrients by the roots of maize plants [25]. From this three dimensional system stimulated by the model, various outputs like root profile and cross section maps which were being compared with the observed value about the roots of maize were being studied and computed. Rooting system and soil water profile vary with different mulching systems in semi-arid areas. Full film mulching on double ridges and furrows increases growth of roots and improves soil texture. Full film mulching is an effective sustainable method for maize production because of the improvement of soil texture, soil profile, increased soil nutrients and soil water contents [25].

4. MOLECULAR MARKERS

Genetic analysis of maize was conducted using by two types of markers that are the basic primers for drought tolerance in maize. One hundred and thirteen maize lines were selected at Research Institute of Agrarian Business for the study. This study was conducted at the Department of Laboratory Studies on qualification expertise of plant varieties (Centre of Certification test) of the Ukrainian Institute for Plant Variety Expertise during period of 2017-18. Samples were allowed for sprouting for 4 to 5 days. DNA was isolated from 100 mg of 05-days sprouts using CTAB and the dissolved in buffer TE **,** the most effective buffer in plant molecular biology with Tris and EDTA components [26]. A sequence of two markers, *dhnC397* and *rspC1090* was used to identify the allelic states of SNP A/G polymorphisms in drought-tolerance genes, *dhn1* and *rsp41* [27]. Polymerase Chain Reaction (PCR) was carried out on the TC-Y amplifier. A reaction mixture of 20 µl containing: 1×DreamTaq^{TM}Greenbuffer, 1 µl DreamTaq^{TM} polymerase, 200 µ M of each Dntp, 30 mg of DNA sample, 0.2 µ M of each primer forward and reverse in reaction mixture according to the following table:

For both the primers, 03 steps or parameters of PCR are setup. For *dhn*C397: step 01_ initial denaturation at 96**°**C for 02 minutes; step 02_ development of reaction products by: denaturation at 94**°**C for 01**,** hybridization of primers at 58°C for 30 sec, elongation at 72°C for 01 minute with number of cycles 30: step 03_ final elongation at 72°C for 02 minutes [28]**.** Similarly, for *rsp*C1090 primer: step 01_ initial denaturation at 95°C for 02 minutes; step_02_ development of reaction products; denaturation at 94°C for 01 minute; hybridization of primer at 58°C for 01 minute; elongation at 72°C for 01 minute with number of cycles 30: step 03_ final elongation at 72°C for 05 minutes [28]. Once the DNA amplification products are obtained using given markers, these were treated with the restriction enzymes *StyII and HpaII.* Electrophoretic separation was carried out in 2% agarose gel for 1.5 hours [29]**.** Polymorphism of two key loci of the dhn1 and rsp41 genes was carried out that are associated with the maize drought tolerance. SNP (single nucleotide polymorphism) of dhn1 was determined by using *dhn*C397. 164 bp amplicon was obtained by the PCR that contained polymorphism (CCAAAG/CCAAGG). This amplicon was then treated with the restriction enzyme *Sty1* that gave out two fragments of amplicons of 131 bp and 33 bp. CCAAAG.

A-type polymorphism is associated with drought resistance. Following figure (Fig.1) shows the electrophoretic separation of fragments after they were treated with the restriction enzyme [29].

Table 1. Description of primers [28]

Primer name	Nucleotide sequence	Restriction enzymes	Hybridization temperature(°C)	Expected size of amplicons(bp)
dhnC397F	Ggaggaagaagggaatca Aggagaagaccaa	*StyII*	58	164
dhnC397R	gccggtggcgtaggagccgc	*StyII*	58	164
rspC1090R	gcgttccattccatgcctgtg	*HpaII*	60	286
rspC1090R	gatccgtttacctggttttc	*HpaII*	60	286

**Description of primers in polymerase chain reaction*
"F – forward primer
"R – Reverse primer

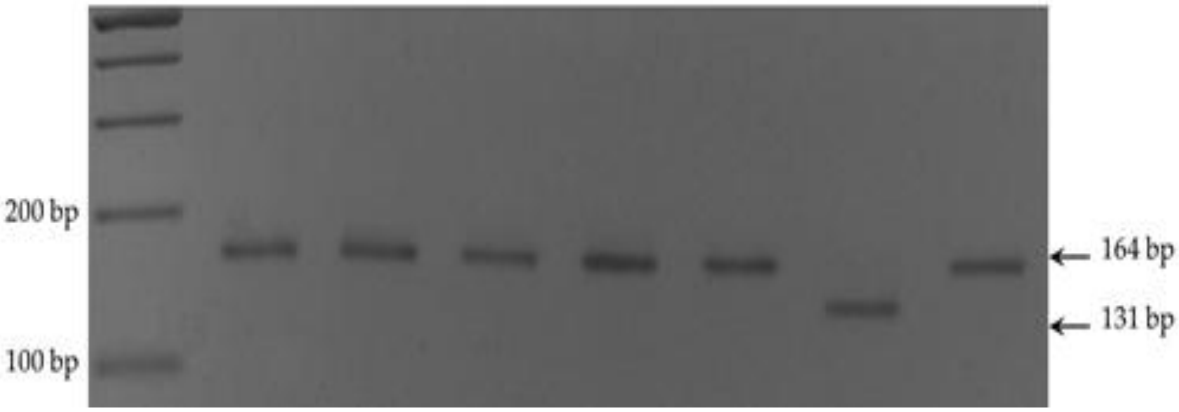

Fig. 1. Electrophoresis of restriction analysis products (primer dhn 397)

Similarly, SNP (single nucleotide polymorphism) of rsp41 was determined by using *rspC1090.* Polymerase Chain Reaction (PCR) gave out the fragment having polymorphism CCAG which characterises the genotype as drought resistance. Following figure (Fig. 2) shows the electrophoretic separation of fragments after they were treated with the restriction enzyme [28].

In this research, the percentage of polymorphisms was obtained. The percentage of genotypes containing single nucleotides polymorphism (SNP)A of the dhn1 gene totalled 88%. In this study, about 22% of studied lines were found very sensitive to drought and for other group of SNP(G) of the rsp41 was about 73%. (Fig.3) [28].

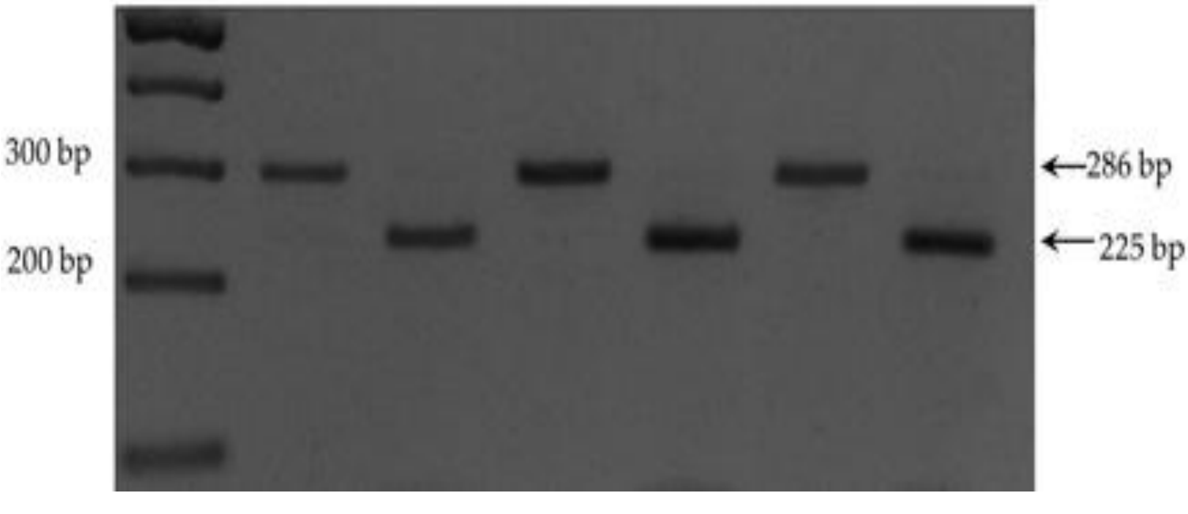

Fig. 2. Electrophoresis of restriction analysis products (primer rsp1090)

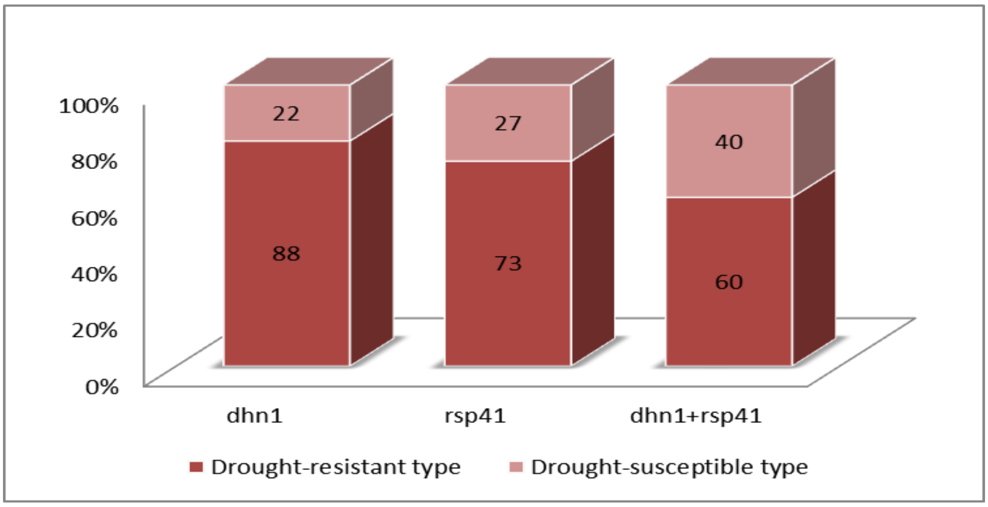

Fig. 3. Distribution of maize lines in accordance to the drought sensitivity

5. CONCLUSION

This review allows to estimate the state of functional factors in regards to maize drought resistance by physiological, morphological and Molecular Markers. Physiological markers are expressed as the results in hampering photosynthesis and thus disturbing the overall plant mechanism due to drought conditions. Similarly, other factors like membrane stability, leaf area and rooting system are also important indices for the identification of maize crop in drought conditions. In view of molecular Markers, we have thus concluded that the favorable genes of dhn1 marker were identified in 100 lines of maize, out of 113. Similarly, in 82 maize lines, genes were successfully identified by using rsp1. So, we can here conclude that about 68 lines of maize have favorable genes by both molecular markers. This fact makes it possible to continue use of these lines for further studies of drought tolerance. This information is also useful in selection process and the other remaining lines can be used as donor for obtaining favorable alleles associated with maize drought tolerance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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