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Research Article

Biotechnology of Accelerated Breeding and Improvement of Cotton Varieties

Shadmanov RK*, Shadmanova AR and Scheffler JA

Institute of Genetics and Plants Experimental Biology, Academy of Sciences of Uzbekistan, Uzbekistan

Abstract

The success of any breeding program depends upon the availability of genetic variability of traits of interest and availability of efficient markers for selection of traits. Within the last 20 years, molecular biology has revolutionized conventional breeding techniques in all areas. Biochemical and molecular techniques have shortened the duration of breeding programmes. In this article are discussed the opportunities of use of biochemical markers (enzymes and proteins) for acceleration of breeding of new and improving of existing cotton varieties on tolerance to different unfavourable environmental factors and with complex of desired traits and varietal purity. We analyzed nineteen (19) local cotton varieties and lines (*Gossypium hirsutum L.*) for this purpose. The indices of oxidoreductases class enzymes and two phosphoprotein markers were developed as objective biochemical markers of earliness, homogeneity, tolerance to Verticillium wilt, drought and salinity.

Keywords: Biochemical markers; Cotton; Isoenzymes; MAS; Micro evolutionary processes; Plant breeding; Proteins; Tolerance; Unfavourable environmental factors

Introduction

Cotton is the major agricultural crop in the Republic of Uzbekistan. There are more than 30 cotton cultivars continually in production, but they all are deficient in one or more agronomic traits. Verticillium wilt resistance, drought, salt tolerance is critical components of a successful cultivar for Uzbekistan and the surrounding countries in the region. That is why it is important to develop early maturing elite lines with Verticillium wilt resistance, tolerant to salinity and drought with high economic characteristics using both the traditional phenotypic selection in combination with biochemical and molecular markers to hasten the selection process, improve the accuracy of identifying desired genotypes and incorporate multiple traits into one cultivar. Using traditional breeding methods the process is long and often requiring 10 to 15 years. Besides, the ecological situation in Central Asia region generates a need for development of cultivated plants varieties with a shorter growing season and maximum adaptability to environmental changes.

In most cases, plants of agricultural crops influenced by various unfavorable factors show stability to these factors, as a result of the adaptation. It happens mainly by means of active adaptations and micro evolutionary processes in populations of plants [1,2].

It should be emphasized that the leading role in the maintenance of intracellular homeostasis and adaptation to stressors is played by enzymes [3]. Thereby the induction of enzymes or elements of their qualitative structure with new properties, or new proteins providing cells protection is an essential factor of survival. It is pointed out that isoenzyme systems possess undesirable for markers ontogenetic, tissue or organoid specificity, variability depending on environmental factors [4]. While absolute advantage of the isozymes is that they are not neutral genome markers [5]. Taking part in different biochemical processes and physiological reactions, they determine organism's adaptation to various environmental factors [6,7]. They have monogenic genetic control, are co-dominant in comparison with some types of DNA markers. In addition, their resolving power is high enough [8].

Using indices of seed enzymes and proteins we have bred cultivars and lines of cotton with such characteristics as earliness, yielding capacity, tolerance to diseases, tolerance to drought and salinity for extremely short period of time.

Materials and Methods

Plant material

Nineteen (19) cotton lines and cultivars of local breeding were used in this study. We analyzed per 100 seeds (genotypes) of each line or cultivar during this research. Today the cotton varieties as C-4727, Namangan-77, 108F, C-6524 suffer from Verticillium wilt and lost their effectiveness. Tashkent-6 and Bukhara-6 cotton varieties are not tolerant to salinity. AH-16 and AH-18 varieties were improved on tolerance to wilt using biochemical markers. Cotton lines and Shodlik varieties are the experimental cotton material received during three reproductions. All the Shodlik cotton cultivars are short-season, with high yield Content. They are also extreme resistant to Verticillium wilt, pests, tolerant to drought and salinity. All the marker indices were based on the analysis of seed material (genotypes). As a result, we received populations possessing traits of interest and absolutely homogenous by morphology (Figure 1).

***Corresponding author:** Shadmanov RK, Institute of Genetics and Plants Experimental Biology, Academy of Sciences of Uzbekistan, Uzbekistan, Tel: +7 (99893) 1849654; E-mail: Rshad@yandex.ru

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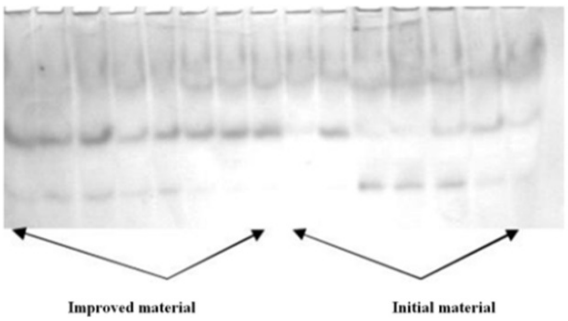

Figure 1: Selection for tolerance to Verticillium wilt on phenoloxidase AN-Bayaut-2 cotton variety.

Selection of adaptive genotypes

Mature cotton seeds have been used for analysis because they are at a fixed stage of development, and contain all the genetic information for the resulting plant. Besides, qualitative indices of enzymes and proteins in seeds are not influenced by environmental factors. Thus, proteins and enzyme systems correlating directly or indirectly with characteristic expression served as markers in our research. A small amount of the seed (genotype) was used for biochemical analysis in order to identify marker enzymes. The rest part of isolated seed was planted in the laboratory conditions and in field afterwards, if the corresponding enzyme or protein marker was found. The methods of electrophoretic analysis of enzymes and proteins were used as biochemical methods of the research [9]. Embryo tissue taken for analysis was ground under cold conditions in a small volume (0.1-0.2 ml) of Tris-HCl buffer (pH 8.6), then incubated for at least 30 minutes in the refrigerator, followed by centrifugation under cold conditions at 15,000 g. The resulting supernatant was used for electrophoretic separation of enzymes or proteins laemmli gel device and a 7.5% polyacrylamide gel (1.5-2 hours at 4 mA/cm). After electrophoresis, gels were stained either with amido black or coomassie blue to visualize the proteins. Determination of enzymatic activity zones (enzyme forms) in gel was carried out using appropriate histochemical methods [10,11].

Phosphoproteins extraction

A protein extract was prepared from one seed or a part of cotton seed, which was ground under cold conditions in a mortar with 10-fold volume of 10% NaCl. 3-4 ml of ethyl ether was added for degreasing and incubated in the refrigerator for 30 minutes, followed by centrifugation at 16-18,000 g for 10-15 minutes. The supernatant was left in thermostat for 1 hour at 80-85°C, followed by centrifugation at 16,000 g for 10 min. The pellet was rinsed carefully in centrifugal tubes with 1 ml of distilled water without sediment detachment. Then the pellet was dissolved in 0.3 ml of distilled water, and left in the refrigerator for at least 1-2 hours. The proteins were detected by electrophoresis in 7.5% polyacrylamide gel at 300 V for 2.5-3 hours. After electrophoresis, gel was stained with 5-7% acetic acid [12].

Germination of selected embryos and generation of seedlings

If the corresponding protein or enzyme marker was found, the seed was germinated and the seedling was allowed to grow either in the greenhouse or the field.

Results and Discussion

Resistance to salinity

Salt-affected soils occupy more than 7% of the earth's land surface, and represent a major limiting factor in crop production. Environmental degradation of the soil due to increasing salinity has been recognized as a major factor limiting crop production in irrigated arid regions including Uzbekistan too. More than 60% of soils in Uzbekistan suffer from salinity. And this problem has worsened with the drying of the Aral Sea basin. Possible mechanisms for resistance to salinity are widely discussed in the literature. Resistance to salinity has been attributed to individual substances such as Ca or the accumulation of a number of compounds as choline, proline, glycine-betaine. However, consistent difference between halophytes and glycophytes in the content or accumulation of similar compounds was not found [13].

We have suggested that the question of tolerance to salinity could be connected with protein structures and their components' metabolism process. Providing one or another configuration in cell elements, these protein structures protect these cell elements against excessive invasion of salt.

While differentiated analysis of seeds proteins we have detected two phosphor proteins in seeds of resistant to salinity cotton plants (Figure 2). In seeds of non-resistant cotton plants these proteins were not detected. Being objective marker traits, these proteins allow selecting genotypes from plants population (Figure 3) and determine level of tolerance to salinity of any cotton cultivar. It's been found out, that two phosphoproteins, typical for tolerant to salinity genotypes, were proteins with relative electrophoretic mobility (Rf)=0.37 and (Rf)=0.56

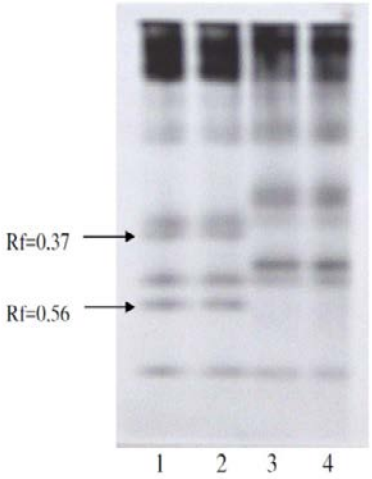

Figure 2: Marker for tolerance to salinity. (Proteins of phosphoproteins fraction).

Note: 1 and 2 - tolerant to salinity biotypes, 3 and 4 - non-tolerant to salinity biotypes

Tolerant to salinity biotypes

The cotton cultivars which are usually sowed in northern regions of the Republic of Uzbekistan suffer from high soil salinity level and

possess low economic characteristics. In this connection, we have carried out selection of genotypes on tolerance to salinity of the recognized in this region variety Khorezm-127 (Figure 4). We sowed the selected genotypes in the same Khorezm region and they showed high tolerance to salinity and led in ripening (7-9 days).

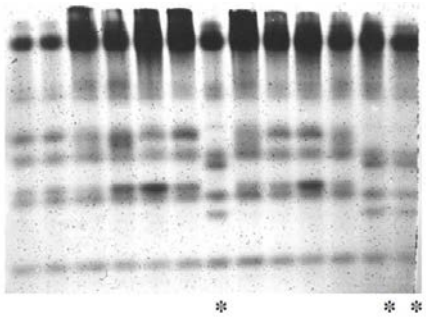

Figure 3: Selection for tolerance to salinity in population of cotton plants Namangan-77 cotton variety.

Figure 4: a) Genotype received after selection from Khorezm-127 variety. b) Initial variety Khorezm-127 (Control check).

Tolerant to salinity cotton line Shodlik-11 was tested for tolerance to drought and salinity under salinity stress in the Republic of Karakalpakstan. According to the results of experiment with a number of dehydrogenase enzymes we found out, that glucose-6-phosphate dehydrogenase enzyme can serve as indirect marker for simultaneous estimation and selection for tolerance to draught and salinity (Figure 5 and Table 1).

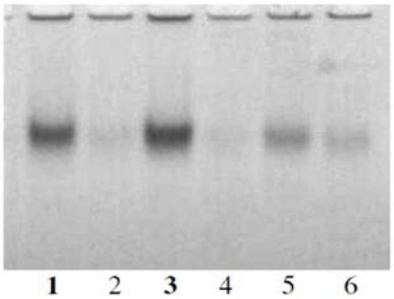

Figure 5: Marker for tolerance to drought and salinity.

(Glucose-6-phosphate dehydrogenase)

1-Shodlik-11; 2-Shodlik-1; 3-Shodlik-9; 4-Tashkent-6; 5-108-F; 6-Bukhara-6.1, 3-tolerant to salinity cultivars.

Cotton Varieties	% of Non Resistant to Drought Plants
C-4534	70.4
Shodlik-11	18.8
C-6524	35

Table 1: Field evaluation for resistance to drought.

According to the field experiments, plants of the experimental, resistant to water deficit Shodlik-11 cotton variety, exceeded non-resistant to water deficit plants in growth and development after selection. And this difference was approximately 15-20%.

Resistance to Verticillium wilt

Verticillium wilt is one of two vascular wilt diseases affecting cotton, the other being Verticillium wilt. Vascular pathogens have the ability to colonize plant roots and penetrate to the vascular tissues, where they are contained and proliferate within the xylem vessels, eventually becoming distributed throughout the plant. Under optimal conditions for infection, the susceptible host is normally killed by a combination of toxic fungal metabolites, accumulated fungal material and host responses to infection, leading to vascular occlusion and moisture deficit.

Uzbekistan is one of the northern most cotton growing areas in the world. The climate in Uzbekistan provides favorable conditions for Verticillium wilt (*Verticillium dahliae*) infection, and yield losses can exceed 40% [14,15]. A role of a polyphenols-polyphenoloxidase system in immunity of plants was well known. Attempts to treat the plants with gossypol to decrease fungal damage have also been unsuccessful. The reason was that high activity of polyphenoloxidase has not been connected to immunity or mitochondria's respiratory system that produces ATP.

Penetration of pathogen in a plant occurs already on the initial stage of germination. Pathogen should pass through epidermal layer cells of the seed coat or young root [14]. In case of presence of active enzymatic systems, oxidizing processes occur intensive in this layer. Products of oxidation possess stronger fungicide activity, in comparison with initial substances and form a powerful chemical barrier [16].

Many researchers observed, but could not explain, mass accumulation of fungi on the surfaces of a root and it's insignificant penetration into plants tissue of plants with low vulnerability to disease and almost complete absence of fungi on roots, but with high content of infection in tissues of susceptible plants. Meeting on the way of penetration cells with high enzymatic activity, the infection eliminates (immune genotypes) or penetrates in vascular system of plant weakened or in insignificant amount. It does not greatly influence the plant's normal developmental processes (tolerant genotypes). We came to conclusion that two non-mitochondrial respiratory pathways, non producing ATP are responsible for resistance to wilt infection. The terminal oxydases of these pathways are phenol- and ascorbatoxidases. Analyzing tissues of plants we have found out that not all genotypes reveal presence of non mitochondrial systems of metabolism. In resistant to wilt genotypes they were always at amount, which provides immunity. It has been shown that phenoloxidase works during initial period of germination of seeds, and ascorbatoxidase-during the period of budding and flowering. The progeny of the genotypes selected according to the signs of these enzymes had a high level of tolerance to pathogens in comparison with initial plants. Thus, from

the experiments, phenoloxidase enzyme can serve as an effective biochemical marker for both the tolerance to wilt infection (*Verticillium dahliae*) (Figure 1 and Figure 6) and for varietal purity evaluation (Figure 7).

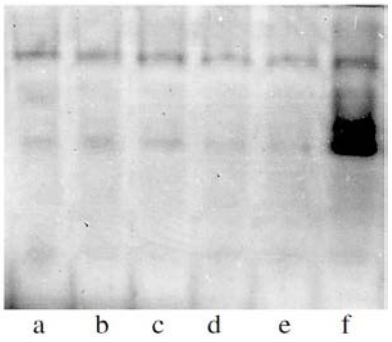

Figure 6: Marker for tolerance to wilt (*Verticillium dahliae*) (Polyphenoloxidase)

Selection of tolerant to Verticillium wilt genotypes from the population of AN-Bayaut-2 cotton cultivar.

A-E - Non-tolerant to Verticillium wilt genotypes

F- Tolerant to Verticillium wilt genotype

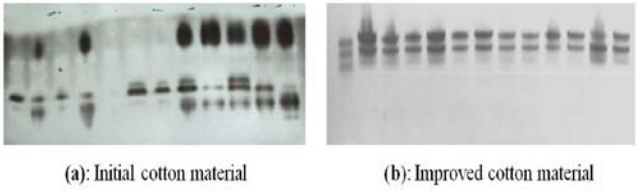

Figure 7: Evaluation of varietal purity (Phenoloxidase).

Heterogeneous population of the AH-16 cotton variety

We have analyzed the genotypes from the population of susceptible to Verticillium wilt AN Bayaut cultivar for tolerance to wilt (*Verticillium dahliae*) during the research (Figure 7). The analysis of the results showed, that the cotton material improved by means of marker-assisted selection with use of biochemical characteristics of enzymes exceed the initial cotton material on tolerance to Verticillium wilt and was homogeneous. According to the field experiments, tolerant to Verticillium wilt plants exceeded non-tolerant. The results meet the table data (Table 2). Both L-2 and Shodlik-9 variety were received from the original susceptible to wilt AN-Bayaut-2 variety, and Line-3 was received from the standard C-4727 variety. According to the table, damage from wilt of the experimental L-2, L-3, Shodlik-9 lines and varieties received, were significantly lower in comparison with initial material.

In order to successfully develop cotton and other crops cultivars, traditional breeding methods and phenotypic screening need to be combined with new approaches and technologies including biochemical and DNA markers assisted Selection to make a breeding process more accurate and efficient. It should be emphasized, that incontrovertible advantage of DNA and protein markers is, that they are closer, then other substances to or are the carrier of hereditary information themselves (DNA) [17].

Cotton lines and cultivars	% of Infected Plants
Namangan-77 (Standard check)	20
AN-Bayaut-2 (Original susceptible cultivar)	62
Shodlik-9	3.5
L-2	3.6
C-4727 (Original susceptible cultivar)	85
L-3	4.6

Table 2: Field evaluation of Verticillium wilt infection

Plants unlike animals due to the fact, that they are tightly bound to the habitats, during long term evolution and changing environmental conditions, had developed a wide range of defense mechanisms. In the process of plants domestication the necessity in some of these defense mechanisms eliminated, but they was preserved in genome [18]. And there are always biotypes with such mechanisms in plants population. But phenotypic selection, especially towards unfavorable environmental factors, is not always efficient to develop these genotypes.

Under adaptation we usually understand a long process in which many generations are involved; however, within a population individual plants can make useful adaptive changes within one generation. Biochemical adaptation takes place on different levels of the metabolism by replacement of amino acids in primary sequence of enzyme, or by change of isoenzymes balance, which cause the proteins polymorphism [19].

The micro evolutionary processes are one of the major factors causing genetic polymorphism of natural populations including cultivars. They lead to occurrence of genetically various individuals in population that finds reflection in distinctions of members of a population by inherited diversified traits: morphological, physiological, biochemical, qualitative and quantitative [20]. But the problem is being able to identify the few useful individuals among the entire population of plants. Often the trait is not visually detectable or varies depending on environmental conditions or presence of a pest. Therefore, selection by the phenotype of plants, especially in relation to pathogens, is not always efficient or is time consuming because of the random factor. Beside for that, it is necessary to take into account that microorganisms also adapt. But, biotypes which have arisen as a result of adaptation, maintain high tolerance to pathogens much longer. The biotechnology, based on identification of such genotypes on marked traits allows very quickly, already at a level of seeds conducting selection for many traits particularly or in complex.

Selection for earliness

Earliness is considered a complex character composed of various temporal and morphological traits. In addition, its expression is influenced by environmental conditions and cultural practices. These factors make selection for earliness difficult. Some of the most effective individual traits that indicate earliness are height of the 1st fruiting branch, timing of the 1st flower and duration of flowering. The heritance of these traits ranges from less than 40% to a maximum of 70%. But often the efficiency of phenotypic selection is low. It such

situations, the use of biochemical markers coupled with field testing could increase the efficiency of selection. On the basis of indices of catalase, as one of key respiratory enzyme, was marked a trait of early ripening (Figure 8).

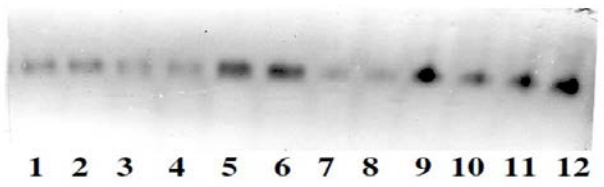

Figure 8: Selection for earliness (catalase).
5, 6, 9, 12-Genotypes selected for earliness.

Together with increasing of tolerance to Verticillium wilt and reduction of vegetative period we have increased weight of one boll and oil content in seeds (Table 3 and 4).

Conclusion

We investigated the mechanisms underlying enzymes and protein fractions correlated with the traits of interest and used biochemical marker technology to identify potentially useful biotypes by screening seeds for desired traits.

Biochemical markers correlating with such important traits as tolerance to drought and salinity, tolerance to wilt, earliness, evaluation of variety' purity and morphological homogeneity were developed and tested with experiments in this research. On their basis we received and tested in field genotypes with complex of above traits. As for resistance to Verticillium wilt, the most simple and effective was electrophoretic quantitative and qualitative assessment of oxidase respiration systems. In particular, regarding tolerance to wilt it was shown that use of isozymes indices of phenoloxidase on seed material was effective for selection of adaptive genotypes.

As for tolerance to salinity we found out that the tolerance was connected to the synthesis of structural proteins and their components, which through their configuration in cells, prevent penetration of the salt. In the course of the study on reserve seed proteins was found out that seed of cotton resistant to high salinity levels always contained two phosphor-proteins that were not present in non- resistant genotypes. Isozymes indices of the glucose-6-phosphate dehydrogenase enzyme were also effective for selection for salinity resistance. According to the results of the experiments with a number of dehydrogenase enzymes we found out, that glucose-6-phosphate dehydrogenase enzyme can serve as indirect marker for simultaneous estimation and selection for two traits, tolerance to drought and salinity.

The research showed that seeds' isozymes analysis allows not only developing genetic predisposition on different traits, but also traits correlation. For example, the study of earliness trait of cotton plants showed that the most essential trait, which could be combined with earliness was tolerance to wilt (*Verticillium dahliae*), because early ripening cultivars are mainly non-tolerant to wilt infection, or faster lose their tolerance during reproduction. According to the study results, catalase enzyme was identified as an indicator for development of early ripening genotypes. And this is not due to coincidence as catalase forms a part of the metabolic respiratory system that defines and controls the level of activity for various metabolic processes.

Thus, the research results showed that it is possible to develop physiologically and Biochemically adapted biotypes in cultivars populations. Using biochemical markers on seed allozymes level, we were able to develop genotypes with tolerance to unfavorable factors and to increase homogeneity and cultivar Population' purity. Such markers based on biological polymers' polymorphism, could be unique phenotypic markers reflecting allelic state of corresponding genes. Their discretization appearing stable on seed level and being environment independent allows high accuracy identification of genotypes, and also allows accelerating breeding process.

Plant Characteristics (Standard)	Namangan-77 (Standard)	AH-16	AH-18	Shodlik-3(9)	III-5	Shodlik-10
Plant height, (centimeter)	108.5±0.4	110.0±0.2	115.0±0.2	105.0±0.2	100.0±0.1	115.0±0.2
Number of bolls	14.0±0.1	20.0±0.2	20.0±0.2	19.0±0.1	17.0±0.09	20.0±0.1
Resistance to wilt disease, % of healthy plants	45.0±0.3	98.5±0.2	96.0±0.2	99.0±0.1	98.0±0.2	98.5±0.1
Yield capacity, Hwt/hectare	30	38	37	40	37.5	36.5
Fiber length, mm	33.0±0.2	34.5±0.19	37.5±0.2	34.5±0.2	34.5±0.1	35.5±0.3
Fiber output, %	37.0±0.17	37.5±0.2	37.6±0.2	38.5±0.23	37.5±0.19	37.0±0.1
Micronair, mic	4.8±0.14	4.2±0.1	4.1±0.2	4.1±0.2	4.4±0.1	4.3±0.1
Oil content in seeds, %	19.5±0.2	22.5±0.3	24.0±0.3	25.5±0.2	23.5±0.2	23.0±0.2

Table 3: Agronomic traits of cotton lines and varieties.

Cotton Lines and Varieties	Days to Maturity	No of Bolls on a Plant	One Boll Weight	Lint %	1000 Seed *Weight (g)*
Namangan-77 (standard check)	108	13.2±0.15	5.3±0.1	38.2±0.1	115±0.3
Bayaut-2 (original susceptible variety)	114	12.1±0.1	5.5±0.09	35.7±0.1	115±0.3
Shodlik 9	108	15.2±0.1	7.4±018	38.7±0.1	141±0.2
Line-2	108	14.0±0.12	7.0±0.1	38.5±0.12	139±0.2
C-4727(Original susceptible variety)	113	13.1±0.2	6.0±0.17	36.2±0.1	125±0.17
Line-3	110	12.5±0.2	6.6±0.2	38.6±0.2	125±0.2

Table 4: Agronomic traits for Uzbekistan field trial.

Acknowledgement

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