EVALUATION OF A BREEDING MATERIAL FOR RESISTANCE TO VERTICILLIUM DAHLIAE USING MARKER PHYTIMMUNITY ENZYMES

DSc. Kurbonov A.Y.[1],prof.Avtonomov V.A.[1],prof. Axunov A.A.[2], DSc.HashimovaN.[2]
[1]*Cotton breeding, seed production and agrotechnologies research institute,Tashkent, Uzbekistan*
[2]*Institute of Bioorganic Chemistry Academy of Sciences of Uzbekistan*
e-mail:kurbonov.abrorjon@mail.ru

Keywords: cotton, variety, enzyme, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, hybrid, resistance, Verticillium wilt.

In the world's cotton-growing countries, cotton is cultivated on the territory of 89 countries, on a total area of more than 30 million hectares, from which more than 22.4 million tons of cotton fiber are obtained. Today, there are problems in the production of high-quality cotton fiber yield. One of these problems is pathogens that cause significant damage to cotton production, with losses in the world amounting to 12-15%. In the world, considerable attention is paid to the study and control of the pathogen *Verticillium dahliae Klebahn*, which affects cotton.

The problem of breeding wilt-resistant varieties of cotton is complicated by the search for new methods and donors of resistance to the pathogen. It is necessary to improve the method of selection of parent pairs during hybridization and qualitative assessment of interspecific hybrids at the early stages of the breeding process in order to increase the efficiency of breeding, speed up the process of introducing new varieties of cotton into production.The selection must be carried out on the basis of physiology and biochemistry of signs of resistance of the initial breeding material. The initial stage of selection should be based on test signs of resistance, which are associated with the catalytic activity of some enzymes involved in the formation of phytoimmunity against fungal infections (peroxidase, phenylalanine ammonialyase, polyphenol oxidase).The greatest interest of researchers is attracted by protective mechanisms, including the processes of lignification of cell walls and the biosynthesis of phenolic phytoalexins. These mechanisms simultaneously create a mechanical and chemical barrier to the penetration of fungal structures into the cell, preventing the spread of the pathogen.

Peroxidase, polyphenol oxidase and phenylalanine ammonia lyase are directly involved in the biosynthesis of lignin and phytoalexins. [1]

Glucansynthetase, peroxidase, polyphenol oxidase, which play a practical role in the creation of new varieties of cotton.

Polyphenol oxidase is involved in the oxidation of polyphenols to quinones, which have antimicrobial activity and lignification of cell walls during microbial invasion. A number of studies show that polyphenol oxidase is involved in protective and hypersensitivity reactions that induce systemic resistance of plants to fungi [7].Peroxidase is an enzyme of the oxidoreductase group. It takes an active part in the oxidation of phenols, suberization and lignification of plant cell walls in response to infection by phytopathogens. [2] Such a

resistance mechanism is associated with the induction of peroxidase activity. [6, 8, 11] Most studies of plant – pathogen interactions show that the accumulation of lignin and phenolic compounds correlates with plant resistance to disease [9, 10].Phenylalanine ammonia lyase is a key enzyme in the mechanism of biosynthesis of phenylpraponoid compounds, the presence of which is shown in plants infected with the pathogen. Previously it is established that different plant species increases the activity of the phenylalanine ammonia lyase under biotic and abiotic stresses, including fungal infection.

The object of the study is cotton hybrids of the species *G.hirsutum* L., created on the basis of complex interline hybridization L-101, L-102, L-103, L-104, L-105, L-106, L-107, L-108.

Research Methods. The research used modern methods of bioorganic chemistry and traditional breeding . The determination of chitin-specific peroxidase was carried out according to the method of N.R. Hashimova. Statistical processing of the material — in accordance with the method of O. Yu. Rebrova, included testing the hypothesis that the tabular data corresponded to the normal distribution law using the AtteStat data analysis program, v.10.9.6, which works as an add-in to the Microsoft Excel-2007 program, with using the modules "Normality Check" and "Emission Processing". Then, the average value and standard deviation σ were calculated using the "Descriptive Statistics" module. In calculating these indicators, parametric criteria were used. Variation-statistical processing of the research results was carried out using the methods of O. Rebrova and B. Dospekhov.

Results.

The first step in the work was the determination of the activity of enzymes, which should be used as markers. The following enzymes were selected for research: oxidoreductase (peroxidase), polyphenol oxidase, phenylalanine ammonia lyase. In the above enzymes, optimal conditions for determining activity were studied, activity in cotton seedlings and responsiveness to the most important stressors were established and methods of identification of the best selection-significant families of different generations are worked out.

In the reserch were studied five hybrids (48 families) of the fifth generation, in the second stage five hybrids (21 families) of the sixth generation, in the third stage six hybrids of the eleventh generation, that are of interest from a breeding point of view.

As a result of experimental work, a change in the enzymatic activity of seedlings of cotton seeds of various origins was established. The regularities of the biochemical adaptation of cotton hybrids to growing conditions were analyzed and generalized, and the relationship of the above forms of enzymes with various conditions of cotton growing was studied.A method for using enzymatic activity as a marker of the resistance of cotton samples to *V.dahliae* is proposed. Cotton families have been identified that should be used in breeding aimed at creating new resistant varieties for *V.dahliae*.

The activity values of the above enzymes in the parent forms determine the resistance in hybrids and breeding material. Using the developed test system allows the selection of fork-resistant breeding material at all stages of selection. Therefore, the use of biochemical approaches allows us to determine its perspective in the laboratory.

In the evaluation wilt resistance, biochemical tests using marker enzymes of phytoimmunity - chitin-specific peroxidase - were used. As a stress load, the isolation of pathogen *V.dahliae* isolated from a naturally infected background under the field conditions of CBSPARI was used by the isolate.

Table 1.

Assessment of the resistance of families of various hybrid combinations of cotton to the pathogen *V. dahliae*

№	№ family	Enzyme activity u/mg protein					
		Peroxidase		Polyphenol oxidase		Phenylalanine ammonia lyase	
		control	experience	control	experience	control	experience
1	2	3	4	5	6	7	8
F_5[F_4(Л-105×Л-106)×Л-105]							
1	2605	490,86	2345,75	1115,51	1200,10	130,00	257,13
2	2622	1178,99	2407,60	567,50	1186,1	177,40	405,36
3	2631	3207,40	891,60	489,70	101,20	19,10	5,71
4	2656	3761,36	1492,50	3814,2	211,1	15,42	11,56
5	2684	5241,32	147,98	1228,9	284,3	20,60	13,90
6	2726	4222,83	1210,32	918,1	187,1	22,34	16,71
7	2747	137,70	153,00	237,44	232,4	52,95	63,14
8	2755	138,80	123,77	623,85	169,5	14,17	12,03
9	2754	126,82	134,33	220,64	910,5	20,00	139,4
10	2767	97,35	92,49	94,17	65,18	19,68	16,00
11	2768	104,85	100,71	217,78	130,20	0,00	0,00
12	2782	158,81	145,42	214,45	240,72	0,00	06,00
F_5[F_4(Л-101×Л-108)×Л-102]							
13	2901	156,57	48,43	156,07	109,78	20,68	20,00
14	2904	158,1	113,98	556,70	923,50	101,2	408,0
15	2902	141,16	140,53	284,05	204,20	0,00	13,46
16	2916	153,09	121,40	278,08	108,91	11,88	12,4
17	2920	161,41	152,45	110,64	95,04	0,00	0,00
18	2922	115,8	110,96	400,25	63,2	0,00	0,00
F_5[F_4(Л-105×Л-106)×Л-106]							
19	2923	181,93	181,18	69,08	130,00	0,83	0,75
20	2925	115,98	117,30	60,76	78,05	5,07	6,32
21	2928	122,23	120,84	63,52	70,32	8,12	2,60
22	2939	110,80	111,51	36,57	38,57	7,04	6,77

continuation of table 1.

1	2	3	4	5	6	7	8
23	2943	122,26	119,55	259,38	67,60	0,00	10,05
24	2944	129,07	213,53	626,24	909,12	23,00	96,34
F_5[F_4(Л-101×Л-105)×Л-106]							
25	2887	146,04	274,58	356,0	1094,5	83,00	277,50
26	2886	139,74	251,56	341,0	1015,7	57,01	138,75
27	2827	133,01	241,71	187,0	1033,4	70,50	117,7
F_5[F_4(Л-105×Л-108)×Л-104]							
28	2749	132,30	253,86	191,0	264,25	192,3	365,0
29	2775	142,53	130,77	183,75	134,01	6,30	6,00
30	2779	142,60	251,14	167,00	285,54	79,40	276,80
31	2780	128,84	238,08	101,00	263,12	95,00	208,05
32	2785	132,88	130,98	106,20	106,06	6,62	6,30
33	2788	120,65	149,04	164,80	196,02	154,30	272,0
34	2784	130,02	116,17	125,10	100,00	5,60	5,29
35	2790	125,44	118,62	1065,0	72,14	148,6	104,0
36	2799	123,09	118,69	165,60	181,70	17,55	14,44
37	2737	128,04	113,65	148,30	105,30	7,61	0,00
38	2733	132,52	110,74	98,62	94,00	6,60	6,12
39	2714	146,39	111,16	126,74	122,20	2,83	2,33
F_5[F_4(Л-103×Л-106)×Л-102]							
40	2836	139,62	115,4	164,10	128,30	2,80	3,63
41	2839	173,12	168,23	101,60	101,50	20,30	14,00
42	2834	244,25	344,57	176,31	297,90	33,61	197,14
43	2831	189,01	525,57	232,14	444,80	21,30	155,00
44	2847	183,31	107,17	104,19	119,41	0,30	0,00
45	2874	114,43	108,40	101,10	98,90	0,00	0,00
46	2849	106,38	101,79	107,80	105,63	6,15	6,69
47	2884	166,73	325,54	160,75	308,60	65,00	120,00
48	2880	164,05	161,22	150,00	76,9	1,80	1,70

During the experiment was used the method of accelerated determination of the wilt resistance of cotton. F_5-F_{11} hybrid families were tested to assess the molecular genetic diversity of the source, hybrid and breeding material when creating new varieties using biochemical criteria for disease resistance.

As can be seen in table 1, the level of peroxidase enzyme activity in the F_5 hybrid [F_4 (L-105 × L-106) × L-105] (family 2605, 2622, 2754) in the presence of *V.dahliae* fungus increases many times.

That the level of activity of the enzyme polyphenol oxidase in seedlings of the hybrid F_5[F_4(L-105×L-106)×L-105] - family numbers 2605, 2622, 2754; F_5[F_4(L-101×L-108)×L-102] - family numbers 2904; F_5[F_4(L-105×L-106)L-106] - family numbers 2944; F_5[F_4(L-101×L-105)×L-106] - family numbers 2887, 2886, 2827; F_5[F_4(L-105×L-108)×L-104] – family numbers 2749, 2779, 2780, 2788; F_5 [F_4 (L-103×L-106)×L-102] - family numbers 2834, 2831, 2884, in experimental versions in the presence of the fungus *V.dahliae* increases many times.

Table 2.

Peroxidase activity in seedlings of the studied families, complex hybrids of the sixth generation cotton (n = 3; M ± m)

№	Hybrid combinations	№ family	Protein content μg / g dry weight (Lowry)	Peroxidase activity (control) unit / mg protein	Peroxidase activity (experience) unit / mg protein
1	F_6 [F_4-Л-105 x Л-106] x Л-105	2	0,32	427,8±1.2	350,3±3.0
2	F_6 [F_4-Л-105 x Л-106] x Л-105	4	0,27	428,9±1.8	380,1±2.5
3	F_6 [F_4-Л-105 x Л-106] x Л-105	5	0,45	435,6±2.1	375,5±1.2
4	F_6 [F_4-Л-105 x Л-106] x Л-105	7	0,41	429,5±3.4	301,7±1.0
5	F_6 [F_4-Л-105 x Л-106] x Л-105	8	0,33	423,9±1.9	398,6±1.7
6	F_6 [F_4-Л-105 x Л-106] x Л-105	9	0,29	425,2±1.4	369,2±1.4
7	F_6 [F_4-Л-101 x Л-108] x Л-102	10	0,52	311,5±2.4	362,9±1.7
8	F_6 [F_4-Л-101 x Л-108] x Л-102	16	0,61	332,6±1.9	347,5±1.8
9	F_6 [F_4-Л-101 x Л-108] x Л-102	20	0,55	361,6±1.1	369,2±2.8
10	F_6 [F_4-Л-101 x Л-108] x Л-102	26	0,48	338,1±2.0	359,8±2.7
11	F_6 [F_4-Л-101 x Л-108] x Л-102	27	0,54	369,8±1.3	395,9±2.6
12	F_6 [F_4-Л-101 x Л-105] x Л-106	46	0,6	216,8±2.0	567,3±1.0
13	F_6 [F_4-Л-105 x Л-106] x Л-106	32	0,40	223,8±1.6	270,9±2.4
14	F_6 [F_4-Л-105 x Л-106] x Л-106	35	0,61	217,4±1.5	272,4±1.4
15	F_6 [F_4-Л-105 x Л-106] x Л-106	38	0,34	238,6±1.0	268,9±1.6
16	F_6 [F_4-Л-105 x Л-106] x Л-106	42	0,54	230,9±1.9	275,4±1.8
17	F_6 [F_4-Л-103 x Л-106] x Л-102	50	0,55	225,4±2.3	276,3±1.2
18	F_6 [F_4-Л-103 x Л-106] x Л-102	52	0,61	224,8±2.5	280,1±1.8
19	F_6 [F_4-Л-103 x Л-106] x Л-102	53	0,58	221,2±1.5	270,9±1.4
20	F_6 [F_4-Л-103 x Л-106] x Л-102	58	0,54	227,3±1.5	285,4±2.8
21	F_6 [F_4-Л-103 x Л-106] x Л-102	61	0,62	219,9±1.3	269,9±2.0

That the level of activity of the enzyme phenylalanine ammonia lyase in seedlings of the hybrid F_5[F_4(L-105×L-106)×L-105] - family numbers 2605, 2622, 2754; F_5[F_4(L-101×L-108)×L-102] - family numbers 2904; F_5[F_4(L-105×L-106) x L-106] - family numbers 2944; F_5[F_4(L-101×L-105)×L-106] - family numbers 2887, 2886, 2827; F_5[F_4(L-105×L-108)×L-104] – family numbers 2749, 2779, 2780, 2788; F_5 [F_4 (L-103×L-106)×L-102]- family numbers 2834, 2831, 2884, in experimental versions in the presence of the fungus *V.dahliae* increases many times. (tab.1).

Table 2 shows the most resistant families of F_6hybridsto*V.dahliae*. As a result of the conducted biochemical research in laboratory conditions, it was found that families isolated from a complex interline hybrid combination of F_6[F_4-L-101 x L-105] x L-106 are resistant to *V.dahliae*.The activity of chitin-specific peroxidase in selected families exceeds the activity by more than 2 times compared with the control, which indicates that the selected families are most resistant to damage.

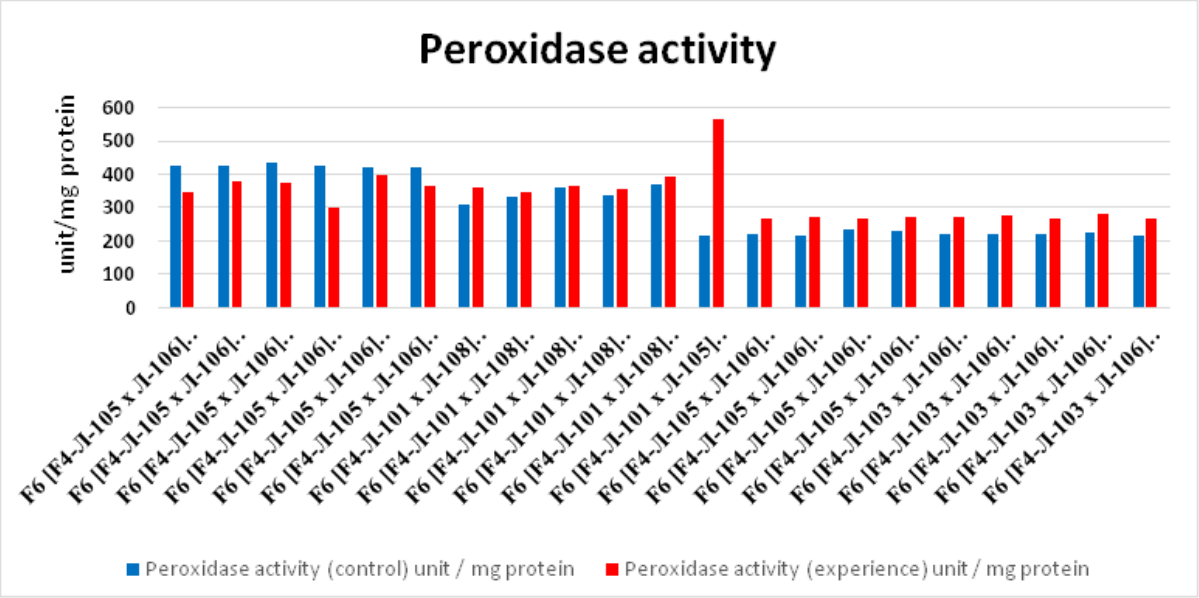

Fig. 1. The activity of peroxidase in seedlings of the investigated samples of cotton.

At the third stage, the study of peroxidase activity in cotton hybrids showed that when interacting with a phytopathogen in all experimental variants, with the exception of Bukhara-6, S-6524 varieties and in the hybrid F_{11}[F_6 (L-101 x L-106) x L- 105], F_{11} [F_6 (L-105 x L-106) x L-105], F_7 [L-175/276 x Namangan-102] the enzyme activity decreased 1.7–1.88 times from the control level (Fig. 2).

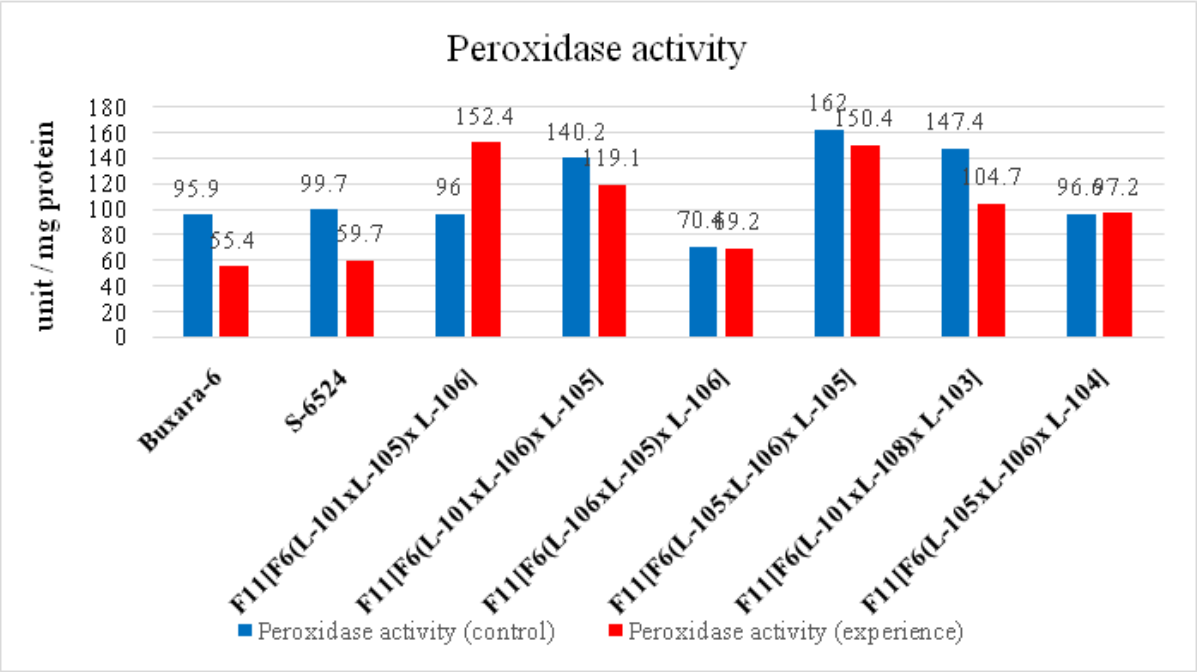

Figure 2 - Peroxidase activity in the roots of 7-day-old seedlings of various cotton hybrids after 48 hours of exposure with conidia of the fungus *V.dahliae*.

In hybrid F_{11} [F_6 (L-101 x L-105) x L-106], peroxidase activity was 1.3-1.6 times higher than its control, which allowed the plant to become involved in the processes that prevent the multiplication of infectious structures fungus, participating in the reactions of strengthening the cell walls when exposed to a pathogen.

A decrease in enzyme activity in other hybrids may be due to inhibition of peroxidase activity due to the production of H_2O_2 in response to the spread of fungal structures in cotton tissues.

It is assumed that resistance to pathogens such as *Verticillium*, depends primarily on the mechanical isolation of the pathogen during the determining phases of colonization and reaction inside and outside the vascular system [13].Lignin forms a structural barrier that limits the spread of pathogenic fungi and prevents the diffusion of extracellular enzymes and toxins. Strengthening the cell wall can confer resistance only if lignification occurs quickly and before the penetration of the pathogen hyphae. Therefore, strengthening the cell wall by deposition of lignin and lignin-like polymers, which is preceded by the induction of the peroxidase enzyme responsible for their formation, therefore plays an important role in the protective response of cotton seedlings against *V.dahliae*.

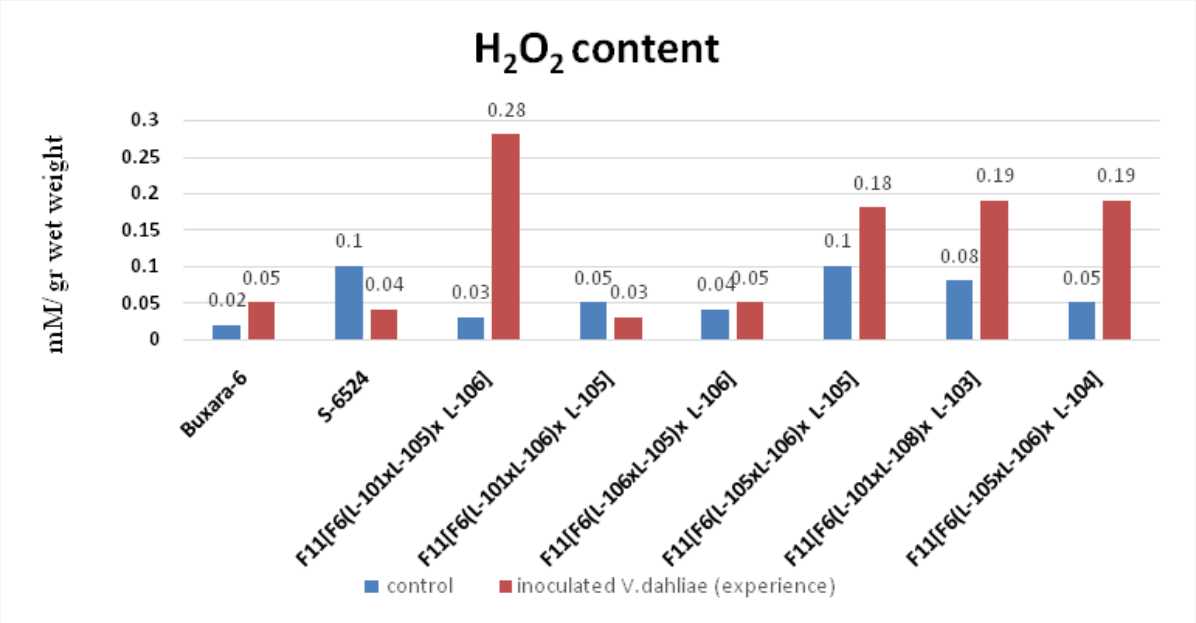

Figure 3 - The content of hydrogen peroxide in the roots of 7-day-old seedlings of various cotton hybrids after 48 hours of exposure with conidia of the fungus *V.dahliae*

An important role in protective reactions is assigned to hydrogen peroxide, which possesses the properties of a signal molecule in low concentrations, and which exhibits a direct biocidal effect in high concentrations [3, 4]. In addition, H_2O_2 with the participation of peroxidases enhances cell wall strengthening by lignification [5].

The earliest protective reactions of plants that form in response to damage by pathogens include the formation of reactive oxygen species, including H_2O_2 [12]. 48 hours after inoculation in plants, the content of H_2O_2 sharply increased (Fig. 3).

Conclusion

As a result of the conducted biochemical research, it was established:

- that at the first stage, among the F_5 hybrids, the following hybrids are of greatest interest from a breeding point of view: F_5[F_4 (L-105 × L-106) × L-105] (families numbers 1, 2, 9), F_5 [F_4(L-101 × L-108) × L-102] (family number 14), F_5[F_4 (L-105 × L-106) × L-106] (family number 24), F_5[F_4 (L-101 × L-105) × L-106] (families numbers 25, 26 and 27), F_5[F_4 (L-105 × L-108) × L-104] (families numbers28, 30, 31, 33 , 35), F_5[F_4 (L-103 × L-106) × L-102] (families numbers 42, 43, 47), as the most resistant to *V.dahliae*.
- at the second stage, among the F_6 hybrids, the most interesting from the breeding point of view is the hybrid F_6[F_4(L-101 × L-105) × L-106], as the most resistant to *V.dahliae*.
- at the third stage of biochemical assessment, only promising cotton families selected for resistance to *V.dahliae* are studied, which allows the breeder to select breeding significant families.As a result of a biochemical reserch in laboratory conditions, it was established that a

hybrid $F_{11}[F_6$(L-101 x L-105) x L-106] was selected from the presented material resistant to *V.dahliae*.

Using the results of biochemical assessment to create new breeding material and new varieties resistant to *V.dahliae,* optimizes and accelerates the work of the breeder.

References

[1] Allashov B., Ibragimov Sh., Ibragimov P.Sh. The study of the effectiveness of crossbreeding according to economically valuable traits in the selection of medium fiber cotton. // The state of cotton breeding and seed production and development prospects. // Materials of the International Practical Conference - Tashkent. - 2006. - P. 49-50.

[2] Akhunov A.A., Golubenko Z., Hashimova N.R., Mustakimova E.Ch., Vshivkov S.O. The role of chitin-specific peroxidases in the resistance of cotton to wilt // Chemistry of Natural Compounds. - 2008. - No. 4. - P. 394–396.

[3] Almagro L., Gomes Ros L.V., Belchi-Navarro S., Bru R.,Ros Barcelo A., Pedreno M.A.// J. Exp. Botany. 2009.- V. 60. - №. 2. – P.377–390.

[4] Cosio C., Dunand C.// J. Exp. Botany. 2009. - V. 60.- P.391–408.

[5] Curtis M.D., Rae A.L., Rusu A.G., Harrison S.J., Manners J.M.// Mol. Plant-Microbe Interactions. 1997. - V.10. - P.326–338.

[6] Goncharova E.A. Strategy for diagnosing and predicting the resistance of agricultural plants to weather and climate anomalies. // Agricultural biology. - 2011. - No. 1. - P. 24–31.

[7] Gechev T., Gadiev I., Van Breusegem F. et al. Hydrogen peroxide protect tobacco from oxidative stress by inducing a set of antioxidant enzymes // Cell. Mol. Life Sci. 2002. V.59.- P.708-714.

[8] Hayrullin RM, Yusupova ZR, Maksimov IV. Protective reactions of wheat when infected with fungal pathogens. 1. The interaction of anionic wheat peroxidases with chitin, chitosan and teliospores Tilletiacries. // Plant Physiology 2000. Vol. 47, No. 1, P. 108–113.

[9] Hayrullin P.M., Yarullina L.G., Troshina NB, Akhmetova I.E. Activation by chitoligosaccharides of oxidation of orthophenylenediamine by wheat seedlings in the presence of oxalic acid // Biochemistry. - 2001. - T. 66. - No. 6. - P. 354–358.

[10] Hashimova N.R., Akhunov A.A., Pshenichnov E.A., Avtonomov V.A., Amanturdiev Sh.B. Patent "Method for determining the resistance of cotton varieties to wilt", Application No. IAP 20110549. Registered on January 20, 2015.

[11] Mittler R.// Trends Plant Scie. 2002. - V. 7. -№ 9. -P.405–410.

[12] Maksimov I.V., Cherepanova E.A., Khayrullin R.M. "Chitin-specific" peroxidases in plants. // Biochemistry. - 2003. - T. 68. - Vol. 1. - P. 133–138.

[13] Nicholson, R. L. and Hammerschmidt R. // Annu.Rev. Phytopathol.1992.- V.30.- P. 369.