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FUNCTIONAL PROPERTIES OF A SERINE PROTEASE INHIBITOR FROM COTTON SEEDS

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It has been shown that dormant cotton seeds contain proteins capable of inhibiting the activity of the proteolytic enzymes of the pathogen Verticillium dahliae *Kleb. An immunochemical method of evaluating the resistance of a cotton plant to this fungus has been developed.*

Interest in specific protein inhibitors from plants acting on microorganism proteases is connected to a considerable degree with the important role that that these proteins may play in the protection of a plant from infection by phytopathogenic microorganisms.

Having developed a scheme for isolating a protease A inhibitor from cotton seeds and determined its physicochemical characteristics, we found that its molecule has a molecular mass of 20 kDa, consists of two subunits, contains no carbohydrates and lipids, and suppresses the proteolytic activity of *Verticillium* by 95% [1]. It appeared of great interest to study the process of the growth of the wilt in the presence of the total inhibiting protein fraction isolated from healthy and wilt-infected cotton seeds. It is known that when the inhibitors are added to a nutrient medium they suppress the germination of the spores and the development of the mycelium [2].

We have isolated a protease A inhibitor by the schenme described previously [1] and also the total inhibiting fractions from healthy and wilt-affected seeds of a cotton plant of the T-1 variety. The germination of the fungus *Verticillium dahliae* was caried out for 24 h in Czapek–Dox medium [3]. Three control flasks without the inhibitor were taken; in the following three flasks the wilt was germinated in the presence of the protease A inhibitor (25, 50, and 75 mg, respectively); then to the next three flasks was added the total inhibiting protein fraction from infected seeds in the same amounts, i.e., 25, 50, and 75 mg, respectively, per 200 ml of the Czapek–Dox medium; and to the following three flasks was added the total inhibiting protein fraction from healthy seeds.

In the control flasks the growth of the fungus was intensive. In the flasks where the germination of the wilt had been conducted in the presence of the total inhibiting fraction from healthy seeds a suppression of growth, as compared with the controls, was observed, but not to the same degree as in the flasks where germination had been conducted in the presence of the protease A inhibitor and of the total inhibiting fraction from infected seeds.

The difference in the degrees of suppression of the growth of the fungus by the total inhibiting fractions from healthy and infected seeds is due to the fact that in infected seeds the level of inhibitors is higher than in healthy seeds. In view of this it was of interest to obtain a quantitative characterization of the levels of the inhibitors in resistant and susceptible culture varieties of cotton seeds.

It is known that immunochemical assay, based on the highly specific interaction of antibodies with the antigen to be determined, is being used successfully in scientific investigations. We have made use of this method of analysis for the quantitative determination of the protease A inhibitor in wilt-resistant and susceptible varieties. We isolated the protease A inhibitor from the wilt-resistant variety 175-F and also the total inhibiting fractions from the varieties T-1, T-6, and S4727 and from eight unknown samples of cotton seeds. Sera were obtained to the protease A inhibitor from variety 175-F and to the total inhibiting fraction from this wilt-resistant variety, with titers of 1/128 and 1/16, respectively.

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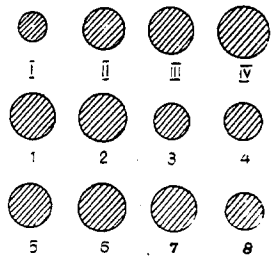

Fig. 1. Scheme of the ring-precipitation reaction [I) S-4727; II) T-6; III) T-1; IV) 175-F; 1-8) the samples under investigation].

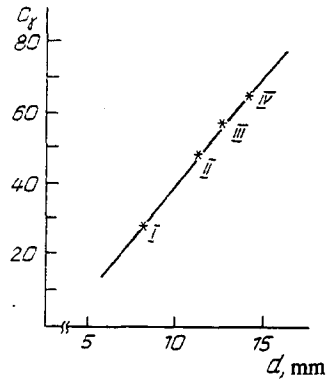

Fig. 2. Graph of the dependence of the diameter of the ring on the concentration of the protease A inhibitor [I) S-4727; II) T-6; III) T-1; IV) 175-F.

The principle of the method of immunoprecipitation is as follows: the protein antigen under investigation, on diffusing from a well in an agar gel containing monospecific antibodies, produces a precipitate in the form of a ring the diameter of which is proportional to the concentration of this antigen [4].

Wells for the antigen were cut out of agar gel with the aid of a punch. Some of the wells (the first four) were charged by a means of a dosing device with a strictly determined volume (20 μl) of a solution of the protein having a known concentration. The remaining wells were charged with the eight extracts of unknown samples. Then the agar plate was placed in a humid chamber. Precipitation rings were formed in the agar gel containing the monospecific antibodies and the diffusing antigen. After a few (4-5) days an equilibrium had become established in the system under observation – i.e., the excess of antigen in the well had disappeared and the diameter of the precipitation ring had ceased to increase. At this stage, a linear relationship exists between the concentration of the antigen and the diameter of the precipitation ring (Fig. 1).

The diameters of the precipitation rings around wells containing diluted standard solutions of the total inhibiting fractions from the seeds of varieties 175-F, T-6, T-1, and S4727 were measured by means of a MD-10 microdensitometer. Then a graph was plotted of the dependence of these magnitudes on the concentration of the solution (Fig. 2). Having measured the diameters of the precipitation rings around the wells with the eight samples under investigation, we used the calibration graph obtained to determine the concentrations of the protein being analyzed. From their inhibitor contents, the eight samples were arranged in the following sequence with respect to resistance: 6 (14.2 mm), 2 (13.6 mm), 5 (13.3 mm), 7 (12.8 mm), 1 (12.6 mm), 8 (10.9 mm), 4 (10.6 mm), 3 (10.0 mm).

An additional confirmation of these results was provided by those obtained in a determination of the diameters of the precipitation rings in the case of the total inhibiting fractions, i.e., when the role of monospecific serum was played by serum to the total inhibiting fraction from variety 175-F. In other words, the resistance sequence is also shown when the total inhibiting fraction is used. If we take the content of the protease A inhibitor from the seeds of variety 175-F as 100%, then for variety T-1 it was 85%, for T-6 73%, and for S4727 37%. For sample 6 it was 100%; 2, 94%; 5, 91%; 7, 86%; 1, 83%;

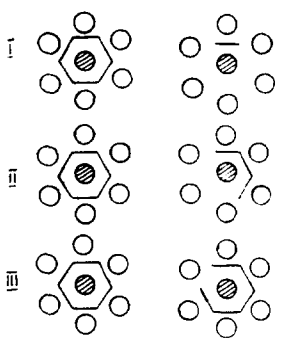

Fig. 3. Titration of the antigen under investigation [I) S-4727; II) T-6; III) T-1].

8, 66%; 4, 61%; and 3, 48%. The error of this quantitative method is 10-15%. Consequently, it is possible to judge the wilt resistance of a variety from its protease A inhibitor content.

In addition to this ring precipitation method, a more sensitive method exists – Feinberg's antibody concentration gradient method [5]. A precipitate is formed as the result of the diffusion of an antigen in a plate of agar gel containing immune serum. The greatest dilution of the antigen at which precipitation still takes place is its titer. In our case, the antigen was the protease A inhibitor. A central well was charged with 20 μl of immune serum, and peripheral wells with a preparation of the antigen under investigation (the protease inhibitor) made up beforehand in the appropriate dilution. A strictly constant volume of solution (20 μl) was added to each well with the aid of a micropipet.

After 24 h in a humid chamber at 37°C, precipitation bands had formed around the peripheral wells in the agar gel (Fig. 3). By recording them, we determined the highest dilution of the antibody at which the precipitation reaction was still observed and took this as the titer of the antigen under investigation. In our case, for the infected seeds the titer for the T-1 variety was 1/1024; for T-6, 1/256; and for S4727, 1/64. This indicates that in the resistant varieties the level of the inhibitor was an order of magnitude higher than in the susceptible ones and confirms yet again the dependence of the resistance of a plant to wilt on the amount of inhibitors synthesized in it at the moment of intrusion of the pathogen.

Thus, enzyme inhibitors, together with phytoalexins – compounds of phenolic or other nature –, may play an important role in the protection of plants from disease. The results of the quantitative method that we have developed can be used to accelerate the selection process for finding varieties resistant to this pathogen. The authors express their gratitude to colleagues in the Institute of Selection and Seed Production of the Uzbek Academy of Agricultual Sciences for providing the seed material.

EXPERIMENTAL

The proteins of the causative agent of verticillium wilt were grown in a liquid mineral medium (Czapek–Dox medium) with the following composition (g): calcium nitrate, 0.5; potassium nitrate, 0.125; magnesium sulfate, 0.25; monopotassium phosphate, 0.5; dextrin, 15; distilled water, to 1 liter. The collected fungus was washed with water, freed from excess moisture, and frozen.

To prepare agar plates containing antibodies, 0.1 M NaCl was added to 3% agar in 0.03 M potassium phosphate buffer solution, pH 8.0, and it was placed in a water bath at 56°C. The appropriate monospecific serum, also heated to 56°C, 8 ml of the heated 3% agar, and 8 ml of heated antiserum were mixed and poured onto a glass plate with dimensions of 8 × 10 cm placed horizontally. After the agar had solidified the plate was placed in a humid chamber. Wells for the antigen with a diameter of 2.4 mm were cut out in the agar gel at a distance of 12 mm from one another in the agar gel with the aid of a suitable punch. The diameters of the precipitation zones were measured after 5 days with the aid of a MD-100 microdensitometer.

REFERENCES

1. L. G. Mezhlum'yan, É. F. Redina, and P. Kh. Yuldashev, Khim. Prir. Soedin., 274 (1994).
2. I. I. Benken, V. V. Mosolov, and N. V. Fedurkina, Mykol. Fitopatol., 10, 198 (1976).
3. B. B.-O. Gromova, E. S. Lantas, and N. N. Guseva, Methodological Instructions: Immunological and Biochemical Methods in Plant Immunology [in Russian], Leningrad (1982), p. 5.
4. E. Bayer, F. Born, and K. H. Reuther, Angew. Chem., 640 (1957).
5. V. G. Feinberg, Int. Arch. Allergy, 129 (1957).
6. J. Shain and A. M. Mayer, Physiol. Plant. **18,** 853 (1965).
7. A. Royer, M. N. Miege, A. Grande, et al., Planta, **119,** 1 (1974).
8. S. Meizel and S. K. Mukerji, Biol. Reprod., **13,** 83 (1975).