

EXPERIMENTS ON THE CULTIVATION OF THE ACTIVE AGENT OF MOSAIC DISEASE IN TOBACCO AND TOMATO PLANTS.

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(Received for publication, October 23, 1924.)

An examination of the extensive literature which has accumulated since the first description¹ of mosaic disease in tobacco reveals the fact that the nature of the inciting agent has not yet been definitely determined.

At present, several views are held concerning it. Beijerinck² supposes it to be what he has termed a *contagium vivum fluidum*; that is to say, a living material without form which is capable of propagating itself. Some later workers believe the agent to have the nature of an enzyme or perhaps to be some other substance capable of causing disturbance. Mayer,¹ Allard,³ and others maintain that it is a bacterium or filterable virus hitherto non-cultivable. Since the work in 1892 of Iwanowski⁴ all investigators of the incitant of the disease, irrespective of their beliefs in one or the other of these views, have agreed that it is filterable.

The experiments here reported⁵ show, we believe, that the incitant of mosaic disease of tobacco and tomatoes is a living, multiplying body, capable of propagation through many generations in an artificial medium.

¹ Mayer, A., *Landwissensch. Versuchs-Station*, 1886, xxxii, 450.

² Beijerinck, M. W., *Centr. Bakt., 2. Abt.*, 1899, v, 27.

³ Allard, H. A., *U. S. Dept. Agric., Bull.* 40, 1914, 1; *Phytopathology*, 1923, xiii, 555.

⁴ Iwanowski, D., *Centr. Bakt., Beihefte Botan.*, 1893, iii, 266; see also *Z. Pflanzenkrankh.*, 1903, xiii, 1.

⁵ The writer acknowledges his indebtedness to Dr. David T. Smith, with whom the work was begun, for his cordial and helpful cooperation.

Material.

In 1908, Clinton⁶ proved, by cross-inoculation tests, the identity of mosaic disease of tobacco (*Nicotiana tabacum*) with that of tomato (*Lycopersicum esculentum*). Later Allard^{3,7} demonstrated that almost all of the nightshade family (Solanaceæ) are susceptible to the affection. The solanaceous plants, in addition to tobacco and tomato, thus far known to be involved are of the following genera: *Petunia*, *Physalis*, *Datura*, *Hyoscyamus*, *Solanum* (*Solanum nigrum* and *Solanum carolinense*), and *Capsicum*. The observations of Melhus⁸ and Gardner and Kendrick⁹ indicate that the disease as occurring in these plants is identical with that of the potato (*Solanum tuberosum*).

There were available for our studies two strains of mosaic disease among the Solanaceæ: one propagated in tomato, the other in tobacco plants. Tomato cuttings were obtained from Dr. L. O. Kunkel of the Boyce Thompson Institute for Plant Research; a tobacco seedling was given us by Dr. Charles E. Simon of the Johns Hopkins University.¹⁰ From the tobacco parent, the disease was transmitted to healthy tomato plants and from the tomato to tobacco. The parents and plants to which the disease was transmitted constituted our first stock of mosaic material. It was also determined as a preliminary that the active agent in this material was readily filterable through Berkefeld filters, sizes V and N. The subsequent experiments were all made on tomatoes, of which there were available an unlimited number of normal seedlings and full grown plants. These were growing vigorously under greenhouse conditions, at a temperature of 28–30°C.

The signs of the mosaic disease in the tobacco and tomato plants of the initial stock and in those to which the affection was transferred experimentally, in the manner which will be outlined further on, were identical with those described by other investigators, notably by Allard.³ According to the latter the affected plant shows (a) partial or complete chlorosis; (b) curling of leaves; (c) dwarfing and distortion of the leaves; (d) blistered or savoyed appearance of leaves; (e)

⁶ Clinton, G. P., *Connecticut Agric. Exp. Station, Biennial Rep.*, 1907–08, 857.

⁷ Allard, H. A., *Phytopathology*, 1916, vi, 328.

⁸ Melhus, I. E., Mosaic studies; abstracted in *Phytopathology*, 1922, xii, 42.

⁹ Gardner, M. W., and Kendrick, J. B., *Purdue Univ. Agric. Exp. Station, Bull.* 261, 1922, 1.

¹⁰ We are grateful to Dr. Kunkel and Dr. Simon for their generous aid.

mottling of them with different shades of green; (*f*) dwarfing of the entire plant; (*g*) dwarfing and distortion of blossoms and, in tobacco, blotched or bleached corollas; (*h*) mosaic sucker growths; and finally (*i*) death of the tissues. The incubation period is stated by Mayer¹ to be 10 to 12 days and by Duggar and Armstrong,¹¹ 10 to 14 days. But as Allard,³ Johnson,¹² and others have pointed out, the incubation period is shortened to 5 to 7 days and the disease is especially active in vigorous plants growing at 28–30°C. Conversely, the onset of the first symptoms may be delayed to 3 weeks or even longer in individuals growing slowly because of a lower temperature.

Methods.

A number of media were tentatively employed in the first attempts at cultivation of the active agent of mosaic. The tests resulted in failure, which is scarcely surprising in view of the fact that the media failed to support an active growth of ordinary plant bacteria. When, finally, a medium had been found which favored the luxuriant growth of at least twenty different species of these bacteria, and closely approached the hydrogen ion concentration of extracts of normal or diseased leaves and stems (pH = 5.3 to 6.0), we were encouraged to proceed. The medium was prepared as follows:

Preparation of Medium.—All glassware was chemically cleansed and washed. 80 gm. of carefully selected fresh young stems, leaves, and shoots from tomato plants which were shown by experiments to be free of the disease and as the later control observations showed, must have been free from it, were minced with scissors and ground by hand in a large mortar to a soft pulp. This was mixed thoroughly with 250 cc. of sterile distilled water having a pH of 5.3 to 5.4. The entire mixture was centrifuged at a high speed (1,500 to 2,000 R.P.M.) for 1 hour. The resultant clear, greenish, supernatant fluid was passed through a sterile Berkefeld filter, size N, and the filtrate refiltered through another similar candle previously unused. The double filtration was performed to insure sterility of the product. The entire process was carried out under strict aseptic conditions. The clear, yellow or amber-colored filtrate was tested for its hydrogen ion concentration with the Sørensen scale and if the reading was between 5.3 and 6.0, it was retained; if not, it was discarded, for no artificial adjustment by adding acid or

¹¹ Duggar, B. M., and Armstrong, J. K., *Ann. Missouri Bot. Garden*, 1923, x, 191.

¹² Johnson, J., *Phytopathology*, 1921, xi, 446; 1922, xii, 438.

alkali was resorted to. Finally, the filtrate was kept for 7 days at 28–30°C. If no evidence of contamination existed, if no precipitation of the contained albumins and globulins was visible, and if the crystal clearness was maintained, it was regarded as suitable for use.

Materials for Culture.—Aqueous extracts of the affected tobacco or tomato leaves and stems prepared in the manner just described were filtered through sterile, new, V Berkefeld candles. These filtrates constituted the materials for the earlier cultures. They gave successful results, but a more satisfactory method to obtain active mosaic material was devised later. A stout tomato stem or a large tobacco leaf was selected and cut from the plant with a razor. The cut end was sterilized by searing in a flame, and a sterile capillary pipette, connected with a small rubber bulb, was inserted into the stem or into the midrib of the petiole of the leaf, in the direction of the long axis. It was found that the needed amount of liquid containing the active agent could in this way be aspirated directly into the pipette.

Cultural Procedures.—A number of tubes measuring 15 by 100 mm. and containing 3 to 5 cc. of the medium were inoculated with 0.1 to 0.2 cc. of the filtrate or with 0.01 cc. of the plant liquid and placed in a dark cabinet in the greenhouse, at a temperature of 28–30°C. After 7 to 10 days, subplants were made by adding 0.1 to 0.2 cc. of this fluid to fresh tubes of the medium, and so on indefinitely. Tests showed that the active agent survived in the original tubes for at least 33 days. In each experiment, uninoculated tubes were included, not only for the better discrimination of changes consequent on the inoculation, but also as controls to initial contamination.

Plant Inoculation.—Young and vigorous tomato plants were employed in the attempts to induce mosaic disease with the culture material. It is generally agreed that the signs of the disease may be masked in slowly growing individuals and that the lesions are more noticeable on young shoots. The usual procedure was as follows: Three leaves on different stems were scratched, as in scarifying the skin of man for a vaccination, and the test material was rubbed in. In addition, if the plant had had prior cuttings, the substance was injected into its stems through the cut ends. A plant received not more than 0.1 cc. of the test fluid in all. Those inoculated with the same material were isolated together in glass cages having close meshed gauze flaps. So too were the stock diseased growths.

Cultural Results.

The medium into which the mosaic filtrate or fluid had been introduced showed as a rule, after 7 to 10 days, or more, a faint, uniform, translucent, almost imperceptible haze. In some instances, no changes could be made out by inspection with the naked eye on comparison with the controls. Stained specimens, however, revealed more granular material than in the latter. Nevertheless, by the available tinc-

torial methods, by dark-field examinations, and by supravital and unstained preparations studied with the ordinary microscope, we failed to differentiate formed elements as distinct from granules or precipitate, which latter were to be found in the uninoculated medium as well.

To determine whether the agent of mosaic disease had multiplied, recourse was had to the inoculation of plants. Since the agent is known to be extremely active, even in high dilutions, careful attention was given to the possibility that a mere transfer of the original active material from tube to tube might be responsible for the results.

Effect of Dilution on the Activity of Mosaic Materials.—Allard¹³ concluded that the agent inducing mosaic disease, when diluted 1 part in 1,000 of water, is quite as effective in producing the affection as the original undiluted active agent. At dilutions of 1 part in 10,000 of water, the disease is not produced so often and is not so severe. At greater dilutions it is not likely to occur. Doolittle¹⁴ also found that the upper limit of dilution of cucumber mosaic "virus" at which activity is still present is 1:10,000.

For the following experiments on the effect of dilution upon the active agent, plant tissues affected with mosaic were pulped in a mortar, water was added, and filtration carried out through cotton. Of six tomato plants receiving a 1:1,000 dilution of the fluid thus obtained, all showed signs of mosaic disease within 20 days. With 1:5,000 dilution, four of six were affected within the same time; with 1:10,000 dilution, two of six showed the disease within this period. On the other hand, a 1:1,000,000 dilution induced the affection in only one of twenty-two plants, and this after 22 days, while a 1:10,000,000 dilution failed to induce the disease in any of the thirty plants inoculated. The results were the same, whether the inoculum was derived from tomato or tobacco plants. The findings support the conclusions of other investigators and indicate, furthermore, that no interpretation can be made regarding the power of a subplant in artificial media to induce mosaic disease, unless the original inoculum

¹³ Allard, H. A., *J. Agric. Research*, 1914-15, iii, 295.

¹⁴ Doolittle, S. P., *U. S. Dept. Agric., Bull.* 879, 1920, 1.

in this subplant is diluted at least 1:1,000,000. Under our cultural conditions this point was reached by the fourth subplant, in which latter the original inoculum was present in an estimated dilution of 1:10,000,000.

Results of Cultivation Experiments.—Seventeen separate attempts were made to culture the active agent of mosaic disease in the filtrate medium already described. Of these, five were made with materials derived from the original stock mosaic tobacco plants, three with those from tomato plants to which the disease was transferred from the tobacco, and nine with those from the original stock of tomato growths.

If multiplication of the active agent be indicated by the capacity of the fourth and more remote subplants to induce mosaic disease in normal plants, then all these attempts must be considered as successful but one. The fourth subplants in four experiments and the fifth in three others caused typical mosaic disease in plants previously normal. Further work with them was then discontinued because of the appearance of contaminating microorganisms in the cultural fluids. The seventh, ninth, and tenth subplants induced the affection in three, three, and one tests respectively. More remote subplants in these series were not made. In yet another case the eleventh, and in still another the twelfth, subplant induced typical mosaic disease after inoculation into normal tomato tissues.

In all, 258 plants were inoculated with the cultural fluids. Of these, 37 of 39 which received material of the fourth and fifth subplants developed the disease, as did 40 out of 42 receiving that of the sixth to eighth subplants and 19 of 24 inoculated from the ninth to twelfth subplants. The remaining individuals were inoculated with the first to third subplants and all became affected.

The incubation period in general varied but slightly from that required by the original, undiluted active agent, namely about 10 days, save in the case of the sixth and seventh subplants, when it appeared earlier. With these the first signs were noted in some of the inoculated plants after 6 days. In the case of the fourth, fifth, and eighth subplants, the earliest effects appeared after 9 days; in that of the ninth, after 11 days; in the cases of the tenth and eleventh subplants after 10 days; and after 15 days in that of the twelfth.

Once the experimental disease gained a foothold, there was no difference in the severity of the affection following inoculation of the early and remote subplants from that induced by the original, undiluted active agent itself. All the characteristic signs mentioned at the outset of this paper were present.

We have already stated that the fourth subplant represented a dilution of the original inoculum of 1:10,000,000. The more remote subplants contained, of course, the initial inoculated material in increasingly great dilutions; for example, the seventh contained it in a dilution of $1:3 \times 10^{-10}$; the tenth, in $1:1 \times 10^{-13}$; and the twelfth, in $1:4 \times 10^{-16}$.

Experiments on Refiltration of Subplant Materials.—The material of the first to fourth mosaic subplants was filtered through tested Berkefeld V and N filters after 7 to 10 days at 28–30°C. The filtrate without further incubation regularly induced typical mosaic disease in normal plants. But with the material of the fifth subplant, the filtrates were noted to be much less effective. Of five separate tests only one gave positive results. And with the sixth subplant, two trials ended in failure, although in each instance the activity of the unfiltered inoculated medium remained unchanged.

Unlimited Transmission of the Experimental Disease.—The experimental mosaic disease induced by the culture fluids could be transmitted from plant to plant in unlimited series both by direct application of the liquid of the affected plant to normal tomato leaves and by inoculation of subplants of the liquid in the artificial medium. An illustration of the latter sort was afforded by the following, one of several similar tests.

A fourth subplant, derived from a filtrate of a suspension of the stock tobacco mosaic tissues, caused the experimental disease in each of three tomato plants inoculated. The liquid from one of the latter individuals was cultured, and consecutive subplants in the artificial medium were made until the twelfth was obtained. The fourth, sixth, seventh, tenth, and twelfth subplants, the material of which had been inoculated into normal tomato tissues, all induced the disease.

Control Experiments.—50 plants were inoculated, after the manner employed in inducing the experimental disease, with distilled water, with fresh uninoculated medium, and with the latter incubated for

7 to 10 days at 28–30°C. None of these showed mosaic disease after observation for 2 weeks or more. In addition, none of over 1,000 seedlings and full grown tomato plants comprising the normal stock and growing in the same greenhouse exhibited the affection.

SUMMARY AND CONCLUSION.

The results of the attempts here described to cultivate the incitant of mosaic disease of tobacco and tomato plants give good reason to suppose that this has been accomplished. The agent in the tissues of affected plants induces the disease not infrequently in a dilution of 1:10,000 but only very rarely at a higher dilution. During its transference from tube to tube of the special medium used in the present study it underwent a dilution far beyond these effective limits—in one experiment to 4×10^{-16} . Nevertheless, the material of the remote subplants proved effective in inducing the disease, which appeared as rapidly and in as active a form as if the undiluted inoculum had been employed. Material derived from plants in which mosaic developed as result of inoculation with the culture fluid induced the disease in yet other plants, and from these again the agent could be propagated *in vitro*, or transfers to other plants could be made.

In the course of the experiments a significant fact was noted; namely, that the agent present in remote subplants which can induce the disease was not readily filterable. The nature of the change thus indicated remains to be determined. No formed elements could at any time be distinguished in the medium.

The conclusion seems justified that the incitant of mosaic disease of tobacco and tomatoes is a living microbic body which can be cultivated in an artificial medium.