

A SIMPLIFIED LABORATORY METHOD OF TESTING THE TOXICITY OF FUNGICIDES

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The need of increased food production is greater today than it has ever been. Science is meeting this need by three approaches: (1) giving the plant its optimum growing conditions; (2) improving the germ plasm through the science of genetics; and (3) protecting the plant from its enemies by means of protective fungicides and insecticides. Regardless of the perfection of the first two factors a plant cannot obtain its maximum productivity if the last factor is neglected.

For years Bordeaux mixture and lime-sulphur have been the two standard protective fungicides. Recently, however, chemistry has supplied many new complexes designed to improve the protective efficiency of fungicides. The production of a new fungicide requires careful testing before it can be recommended for public use. Testing of fungicides has generally been done in the field on the plants to be protected, but this procedure is costly in both time and materials. The work described in the present paper was undertaken to determine whether a laboratory method could be devised that would permit reliable comparisons to be made as to the relative toxicities of both old and new protective fungicides. The method proposed herein does not claim to eliminate field trials, but it will give some information as to the relative toxicity of a preparation to fungus spores before it is sprayed on the plant to be protected. It is recognized that a method in which glass slides are used is lim-

ited in its scope, since reactions between the fungicide, the leaf, and the fungus cannot be tested, however these reactions can be studied by field tests which should naturally follow.

The possibility of fungicide testing in the laboratory is not new. Numerous methods have been suggested, such as bringing the fungus into contact with the fungicide in suspension or in solution- (Burrill) (Hamilton et al.) or in an agar medium (Palmiter). These methods may have rendered useful information but they do not test the toxic relationship of the spore and a dry deposit of the fungicide such as used in the field. The first investigators to evolve a method on this basis were Reddick and Wallace in 1910. Their method proposed the spraying of slides or cover slips with an atomizer until a visible uniform deposit was attained. Such a spore-fungicide relationship was an improvement over the methods previously mentioned, however its limitation centered around the difficulty of getting sufficient uniformity of deposit to give a uniform toxic response of the spores.

An improvement of the dry-deposit method has been proposed by Montgomery and Moore, East Malling Research Station, in 1938, and by the author in 1939. The researches of both were conducted simultaneously and the fundamental principles embodied in both were based on the idea that comparative results could best be obtained if an accurately measured quantity was uniformly

distributed over a delimited area. The toxicity of a preparation was determined by finding the range between a completely toxic and a non-toxic dilution. From this range of dilutibility a number of factors may be determined regarding a fungicide as: (1) its potency in relation to other fungicides; (2) its potency according to different methods of mixing; (3) the effect of varying pH upon its toxic range; (4) how other compounds as spreaders or insecticides might affect its range and; (5) the adhesiveness of the fungicide after the slides have been exposed to weathering, especially to rain.

The materials required for this method of testing include glass slides on which small circles have been etched, a finely calibrated pipette (a serological pipette or glass tube may be drawn to a fine bore and marked so that the same quantity of the dilution will be delivered each time), bell jars, petri-plate lids, four-inch squares of window glass, cultures of fungus in a sporulating condition, and the top or bottom of a moist chamber.

The circles on the slides were etched in the glass by covering the slides with a coating of paraffin and then stamping out three equidistant circles 15 mm. in diameter with a large cork-borer. These grooves were then filled with hydrofluoric acid (care must be taken to go around the circle with a sharp-pointed object to insure uniform contact of the acid with the glass). After the paraffin was removed the slides were cleaned so they were grease-free, otherwise uniform distribution of the fungicide within the circle would not have been possible. This was done by dropping them into concentrated chromic acid cleaning solution. After thoroughly rinsing in tap water and finally in distilled water

they were wiped dry and placed with the circles down on typing paper. Before turning them over to receive the fungicide any traces of oily film that might remain on these areas was removed by vigorously rubbing the surface on the typing paper. The heat generated by the friction made possible the complete removal of the slightest trace of oily film which might have remained.

By means of a serological pipette (or other pipette with a fine bore) a uniform quantity was delivered within each circle. The amount to be delivered should be determined and must never vary in the slightest if toxic comparisons are to be made. Five hundredths of a milliliter was found to be satisfactory in that it was sufficient to completely cover the circle, while the surface appeared level over the entire circle. Uniformity of depth is important to insure the same toxic response over the entire area. Care must be exercised during the process of measuring the fungicide. Before delivering the measured quantity all droplets adhering to the outside should be removed by pulling the pipette through some soft absorbent paper, such as Kleenex. In addition it is important to guard against any carry-over when changing from one dilution to another or from one fungicide to another. The pipette should be carefully cleaned with hot dilute chromic acid cleaning solution, then thoroughly rinsed before using it with a new fungicidal preparation. When going from one dilution to another with the same type of fungicide, the pipette should be rinsed with distilled water and then filled and emptied several times with the new dilution before delivery is made. After the various dilutions have been delivered and spread to the etched border they are appropriately

marked with a glass marking pencil and spread to dry. The range of dilutions will of course vary depending on the fungicide and the kind of spore being tested.

The next step in this process is in getting a uniform spore distribution over the same area. Fungus spores or conidia should be chosen on the basis of their parasitic importance and their ability to grow and produce abundant spores on culture media. *Venturia inequalis*, *Alternaria solani* and *Monilinia fructicola* have been found to be suitable for these tests. Just as it is important to get a uniform spray distribution, it is also important to get an equally uniform spore distribution. The spores used should be grown on the same kind of culture media and should be the same age when tested. By placing the scrapings from the surface of the mat in distilled water and vigorously stirring the water the spores or conidia are dislodged from the mycelium. By straining the mixture with several thicknesses of cheesecloth a mycelium-free spore suspension can be obtained. To obtain the desired number of spores per unit area the above-mentioned spore suspension is diluted and examined microscopically, until the desired number (within a range, as 10-15 under high power), is obtained. The quantity of spore suspension delivered on each area must be held constant and the spore suspension should be agitated with a stirrer when the quantity is taken out to eliminate any settling factor. Sufficient suspension is taken so that the entire delimited area is covered uniformly.

After the encircled areas are covered with the spore suspension the slides are ready for incubation. This is best accomplished by placing the slides in petri-plate lids which have

sufficient diameter to accommodate two slides (the slides may be placed in the lids before the spore suspension is added, thus eliminating the danger of dropping or spilling the suspension over the edge of the circle). The petri-plate lids are next stacked in a moist chamber the bottom of which is covered with paper toweling and soaked with water. The plates are easily and safely stacked by placing a 4-inch square of window glass between the petri-plate lids. A large bell jar is then set over the stack with its base resting on the soaked paper toweling in the moist chamber. By adding water to the area outside of the bell jar an airtight seal is made, and the spore suspensions within the bell jar are subjected to a saturated atmosphere. These units are incubated in darkness at room temperature or as near 20° C. as possible for 48 hours.

Germination results are determined by microscopic observation from each of the three areas on the slide, thus checking in triplicate. Toxicity may be recorded according to the following arbitrary standards: (0) normal germination, similar to the check plates in each bell jar, no visible evidence of toxicity; (1) any visible evidence of toxicity, such as a shortening of the germ tube or contortion of it, decreased percent of germination; (2) if only a few very short germ tubes, merely protuberances existed it was classed very toxic; (3) the concentration which permitted no germination was considered the toxic limit.

If greenhouse facilities permit, further testing of the fungicide may be carried on to determine its phytocidal effect on the foliage. Preparations may appear promising from the standpoint of its toxicity to the spore, but may also be very toxic or otherwise unsafe to the foliage of

the host plant. Theoretically, a fungicide should first protect the foliage, should be adhesive to the leaf surface, and should be compatible with supplementary insecticides. For these preliminary studies apple, cherry or peach seedlings lend themselves readily. The fungicide may be applied at varying concentrations

and subjected to varying environmental conditions such as varying the temperature and moisture content of the air or mineral nutrients. Indoor tests such as described should not be considered conclusive, but may furnish a clue which can be investigated further by field tests.