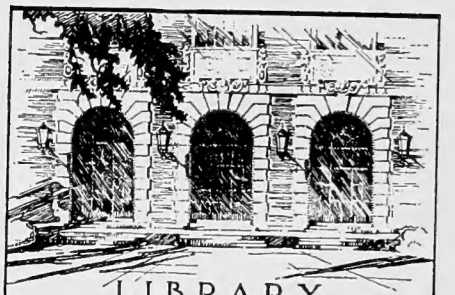

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The Value of IN VITRO FUNGICIDE TESTS

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The Value of IN VITRO FUNGICIDE TESTS

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Because chemists now seem able to synthesize an unlimited number of compounds, we can expect that many fungicides developed in the future will prove to be better than those we are using at the present time. Appropriate methods must be employed to select the better fungicides from among the mass of candidate materials.

Separating the successful and unsuccessful fungicides is an expensive procedure. Wellman (1967) estimated industry's cost of research on one new fungicide to be $3 million. Since it becomes increasingly difficult to find a fungicide that is more successful than one just introduced, these costs can be expected to increase. With this vast expense involved, research agencies must develop and use techniques and methods that are both efficient and reliable.

The three traditional steps in the search for an effective new fungicide are laboratory evaluation, greenhouse evaluation, and field evaluation. With the proper tests and the proper interpretation of the test results, this still seems to be the only practical system. Opinions vary among investigators as to the importance of each of the steps. This paper will review the test methods used to seek and evaluate fungicides in the laboratory, and specifically the in vitro tests. It will be further limited to those methods used to develop protective foliar fungicides. The specialized methods used to develop eradicative, systemic, and soil fungicides are not included.

The value of in vitro tests to appraise fungicides in the laboratory is determined by the ease, speed, and simplicity of test manipulations and the reliability and usability of test results. Alternative in vivo methods are time consuming, which prohibits extensive use of greenhouse and field techniques in the initial evaluation of chemicals. In vivo tests are essential, however, in determining how effectively chemicals control plant diseases.

The literature concerning in vitro testing of fungicides has been reviewed by several investigators (Horsfall 1945*a*, 1945*b*, 1956; McCallan 1947, 1959), most recently by Torgeson (1967). In this paper I wish to emphasize the variety of in vitro techniques that can be used as the basis for selecting fungicides for greenhouse and field studies. Many of these in vitro techniques have additional value, for in recent years they have been extensively used in studies seeking the toxic principles of fungicide groups and the mode of action of chemical structures on plant pathogens.

The significance of fungicide tests in an artificial rather than a natural situation is occasionally questioned. Marsh (1936) and Howard (1939) compared spore germination on leaves and on glass slides and found comparable results. Miller (1943) compared the retention of copper on leaves and on pyralin plates and found laboratory methods to be fairly accurate.

FUNGICIDE-FUNGUS INTERACTIONS

In vitro tests are used to measure and rank the fungitoxicity of fungicides, to measure fungal specificity, and to measure fungicide deposition, redistribution, tenacity, persistence, stability, and volatility.

Chemicals toxic to fungi have been observed to affect the fungi in various ways. The fungus mycelium may cease growing, change metabolic processes, or be killed. The fungus spores may fail to swell before germinating, fail to germinate, plasmolyze, or be killed. Each of these reactions is measurable. Each has been used in the past to determine fungicide toxicity in vitro. Standard methods of determining toxicity have not been universally accepted.

Fungitoxicity

While we accept the general definition of a fungicide as "any substance that destroys fungi or inhibits the growth of the spores or hyphae," we should restrict the use of the adjective *fungicidal* to the conditions that kill fungi and the term *fungistatic* to those conditions that prevent additional growth or sporulation of fungi without killing them (McCallan & Wellman 1942). The term *fungitoxic* has come into use in those situations where detrimental morphological or physiological fungal changes occur but no effort is made to determine whether or not the fungi are killed by the treatment.

Many techniques have been developed during the last 40 years that attempt to measure the fungitoxicity of chemicals or compare the fungitoxic values of chemicals. These techniques are usually bioassays involving a chemical and a test fungus. The use of a fungus introduces biological variability and reduces

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the significance of each measurement of toxicity. Many of the techniques now used have been developed with the express purpose of reducing biological variability.

Testing With Spore Germination.—Early tests of Reddick & Wallace (1910) and McCallan and coworkers (McCallan 1930; McCallan, *et al.* 1941*b*; McCallan & Wilcoxon 1938, 1939; Wilcoxon & McCallan 1939) and Horsfall, *et al.* (1940) were spore germination tests involving the deposition of chemicals on glass slides, evaporation to dryness, and the addition of a drop of water containing spores of the test fungus. The American Phytopathological Society (1943) published instructions for this technique.

One problem encountered with this technique was the variation in size of the water drops. Montgomery & Moore (1938) attempted to solve this by drawing a circle on the glass slide with a diamond pencil, Young (1944) etched a circle with hydrofluoric acid, and Miller (1949) drew a circle with a wax pencil. Others have used depression slides (Barratt & Horsfall 1947, Kirby & Frick 1953, and Prusova 1962), small petri dishes (Tamura 1954), microbeakers (Shafer 1952 and Spencer 1962), or raised cover slips on slides (Peterson 1941).

Another problem was that of obtaining an accurate determination of the amount of toxic material on the slide. Elaborate laboratory sprayers have been built to uniformly deposit the chemical. An alternate solution by McCallan & Wilcoxon (1938) and Peterson (1941) involved mixing the toxicant with the spore suspension directly and not previously forming a dry deposit on the slide. Procedures for this test tube dilution technique were described by the American Phytopathological Society (1947).

Although germinating spores on glass slides are easily examined microscopically, glass is not the ideal substrate for spore germination. Marsh (1936) observed that fungus spores germinated better on glass slides that had been coated with cellulose. Transparent materials other than glass have been successfully used. McIntosh (1961) and Spencer (1962) used transparent plastic polymers. Neely & Himelick (1966) germinated spores on nonwaterproof cellophane discs placed on filter paper pads previously saturated with the toxic solution or suspension. The cellophane discs were transferred to slides for microscopic determination of fungistatic activity or transferred to agar for determination of fungicidal activity.

Many fungus spores do not germinate well in water drops. Davies, *et al.* (1948) found that shaking the culture greatly improved the uniformity of spore germination. Darby (1960) described a shaker method with a 4-hour germination period. Gattani (1954) observed that spores germinate better on agar than in water drops. He mixed the fungicide with warm agar, allowed the agar to solidify, seeded the agar with spores, and counted the germinating spores by placing the petri dish under a microscope. Heyns, *et al.* (1965) placed seeded, toxicant-agar preparations on microscope slides.

Rapid tests involving swelling of spores prior to spore germination also have been used to measure fungitoxicity. Mandels & Darby (1953) described a 3-hour test with the failure of spores to swell as the measurable character. Koopmans (1959) based his results on the loss of turgidity of powdery mildew spores in toxic solutions.

Macroscopic Tests.—Counting germinating spores has always been a tedious, fatiguing task. In vitro tests utilizing macroscopic observations of fungus colony growth have often been used. Lee & Martin (1927) and Wellman & Heald (1940) placed fungus spores in known aqueous concentrations of the toxicant for varying lengths of time before transferring them to bouillon or agar. Their tests are based on reduction in number of fungus colonies formed.

The most commonly used macroscopic tests involve germination of spores on toxicant-agar preparations in petri plates with colony counts being made or the absence of colonies noted (Palmiter & Keitt 1937). With the roll culture technique of Manten, *et al.* (1950), the toxicant-agar layer is formed along the sides of round bottles rather than in petri dishes. A closely related method involves placing known quantities of toxicant at specific spots on seeded agar plates. The fungicide diffuses through the agar. The diameter of the clear area where spore germination was inhibited is measured. The fungicide may be placed in holes in the agar (Mildner, *et al.* 1963) or added with saturated filter paper pads (Thornberry 1950 and Leben & Keitt 1950) or string (Kuhfuss 1957).

In vitro techniques measuring toxicity of chemicals to fungus hyphae are often useful, especially with fungi that fail to sporulate or sporulate poorly in the laboratory. A common procedure is to incorporate toxic materials into agar and then seed with uniform mycelial colonies in agar blocks (Carpenter 1942), on filter paper pads (Sharvelle & Pelletier 1956), or on agar-coated cover slips (Bomar 1962). The reduction in fungus growth is then measured. Fungus cultures on agar plates also have been dusted or sprayed with toxic materials (Henry & Wagner 1940), submerged beneath toxic materials (Grosser & Friedrich 1947), or placed near toxic materials (Moreau & Moreau 1959), after which the resulting inhibition of growth was measured.

The fungus mycelium growing in toxic solutions has been linearly measured (Mason & Powell 1947) and weighed (Le Tourneau & Buer 1961). Forsberg (1949) placed a fungus-infested string in toxic dilutions for varying periods of time, then transferred the string to agar to determine fungicidal activity.

Mandels & Siu (1950) describe fungitoxicity tests

with a manometric system where the growth of the organism is measured by the amount of oxygen absorbed. McCallan, *et al.* (1954) and McCallan & Miller (1957) felt that measurement of oxygen consumption was not a particularly useful means of determining fungitoxicity.

Comparisons of Methods of Testing.—There have been relatively few published reports in which the methods for determining fungitoxicity have been compared. In observations of techniques involving spore germination, Frick (1964) found the test tube dilution test less variable than the dried deposit-slide germination test. Gottlieb (1945) found the seeded, toxicant-agar method and the test tube dilution method equally sensitive. Manten, *et al.* (1950) found the roll culture method more useful than the seeded toxicant-agar, dried deposit-slide germination, and gravimetric methods. Himelick & Neely (1965) found the cellophane transfer technique more sensitive than the seeded agar-toxicant spot method. Walker (1955) compared spore germination, spore respiration, and mycelia respiration techniques and found that, in general, the three methods gave the same ranking of fungicides.

Measurements.—In establishing the value of fungitoxicity tests we must not only know the method of testing but also the method of measurement and the means of expressing the resulting data. In bioassays this is a difficult task. McCallan and his co-workers have found that the amounts of fungicides absorbed by spores of different fungal species vary greatly and suggest that possibly fungitoxicity should not be based on the concentration of the external solution or suspension but on the weight of toxicant actually absorbed by the fungus (McCallan & Miller 1958; Miller, *et al.* 1953).

One problem encountered with the dried deposit-slide germination technique was whether to express the results in terms of weight of toxicant per unit area of slide or weight of toxicant per unit volume of water. Since the amount of toxicant in solution was not readily known, the results were usually expressed in weight per unit area.

A second problem in giving the results with spore germination tests was how to express the percentage of germination at different fungicide concentrations. It could be shown graphically with ease using a dosage-response curve, but it was difficult to express verbally. McCallan & Wilcoxon (1938) used the term LD50 for that toxic concentration at which there was a reduction of 50 percent in spore germination. Later they used logarithmic paper to estimate the LD50 (Wilcoxon & McCallan 1939), more appropriately called the ED50 (McCallan 1948).

Interpretations of the role of the dosage-response curve in the evaluation of fungicides and the increasingly elaborate statistical methods that accompanied use of the dosage-response curve were the subjects for numerous research papers from 1940 to 1960 (Dimond, *et al.* 1941; Horsfall 1956; Litchfield & Wilcoxon 1949; McCallan, *et al.* 1959). In many reports written during this period the results were expressed in a statistical language unreadable by many plant pathologists. Kundert (1956) commented on the exaggerated application of statistics in the assay of fungicides. Many researchers are now reporting results as the lowest external concentration that completely inhibits spore germination or mycelial growth expressed in parts per million or its equivalent (Guillemat & Lambert 1960; Pianka, *et al.* 1966; Luijten & van der Kerk 1961; Neely & Himelick 1966; Nisikado, *et al.* 1951; Pluijgers & Kaars Sijpesteijn 1966).

Fungal Selectivity

Although phycomycetes, powdery mildew fungi, other ascomycetes, and basidiomycetes are not equally sensitive to fungicides and although certain fungi are sensitive or resistant to specific fungicides (Horsfall 1951; Horsfall & Lukens 1966; Wellman & McCallan 1943), most fungicides now in commercial use have a broad spectrum of activity against fungal species. The test organism commonly used in the laboratory will often rank candidate fungicides in the same order as a specific disease-causing organism. McCallan, *et al.* (1941*a*), in a spore germination test with 6 fungi and 20 compounds, reported the fungi similarly sensitive. Neely & Himelick (1966), in a test with 7 fungi and 24 compounds, in general confirmed these results. Casarini & Pucci (1957) in a test with 6 fungi and 6 compounds felt the resulting differences between fungi sufficiently variable to recommend that the same fungus be used in in vitro trials as in field trials whenever possible.

Deposition, Redistribution, Tenacity, Stability

To be successful a fungicide must prove itself on a natural surface and in a natural environment. This involves the physical and chemical factors of deposition, redistribution, tenacity, and stability (Burchfield 1960, 1967). Modifications of the basic fungitoxicity methods have been developed with the aim of learning as much as possible about these additional factors in the laboratory.

Redistribution of a fungicide is often essential for control of a disease because plant growth, poor tenacity, or poor coverage may leave the fungicide particles widely distributed. Powell (1961) measures the ability of fungicides to redistribute by placing a known fungicide concentration in one depression of a glass slide and a spore suspension in the second de-

pression, making a water bridge between the two with filter paper, and measuring the decrease in spore germination.

The tenacity of fungicide deposists on glass slides was measured by Heuberger (1940) by dipping and swishing the slides several times in water, allowing them to dry, and seeding with a spore suspension. Chapman, *et al.* (1950) suspended the slides in circulating water and removed them at varying intervals of time before drying and seeding. Kovacs (1961) applied the fungicides on polyethylene discs, sprayed them with artificial rain, and placed the discs on seeded agar.

The stability of fungicide deposits on glass slides was measured by Barratt (1946) by subjecting them to high humidity and seeding with spore suspensions. Serra (1964) exposed polyethylene discs dusted with fungicides to ultraviolet light for varying periods of time, then transferred the discs to seeded agar. Ciferri, *et al.* (1961) found that zineb frequently increased in fungitoxicity after 2 to 6 years storage although chemical tests showed a decrease in the amount of "active ingredient."

Predicting Field Performance

In vitro fungitoxicity studies are often used as a means of predicting the field performance of candidate fungicides. The literature relating to predicting field performance from laboratory tests has been reviewed by Ciferri (1952), Rich, *et al.* (1953), McCallan (1959), Block (1959), and Torgeson (1967).

Horsfall and his co-workers were among the first to state that the protective value of fungicides could be predicted on the basis of laboratory results. Horsfall, *et al.* (1941) felt that fungitoxicity and tenacity were the two fundamental components of protective value. Keil, *et al.* (1952) called attention to the necessity of chemical stability. Waggoner, *et al.* (1952) later stated that a statistical prediction of field performance could be made on the basis of toxicity, tenacity, and stability of fungicides.

The relationships between laboratory and field results are not always clear cut. A hesitancy to predict field results from laboratory tests often arises in conversations or personal correspondence, but is infrequently found in the literature. Martin (1942), however, stated that to expect a simple correlation between fungitoxicity alone and field performance is too ambitious. Smith & Read (1961), in studies with the cucumber powdery mildew fungus, found a great variance between laboratory and field results.

Yet, in vitro bioassays often correlate well with field trials. Holloman & Young (1951) reported that laboratory assay was a reliable criterion for field performance for control of Botrytis leaf spot on gladioli. Klomparens & Vaughn (1952) reported field and laboratory trials very consistent for control of Helminthosporium on bent grass. Brook (1957) found that laboratory and greenhouse experiments rated fungicides in almost the same order for control of Botrytis gray mold on tomatoes. Monroe (1963), in studies on bean powdery mildew and bean rust, stated that in vitro and in vivo methods generally ranked the fungicides in the same order. Misato (1963), in studies on rice blast, found the in vitro methods measuring inhibition of mycelial growth and spore formation to be satisfactorily correlated with field results.

Although a prediction based on in vitro tests may prove erroneous on a particular host with a particular set of environmental conditions in the field, the success or failure of the fungicide can often be explained with confidence if a sufficiently varied group of in vitro tests have been performed. Most researchers seeking protective fungicides at least agree with the statement that in vitro testing will result in the selection of chemicals worthy of greenhouse and field testing.

FUNGICIDE-FUNGUS-HOST INTERACTIONS

In vitro tests in the laboratory will not in the foreseeable future replace greenhouse and field trials (Hamilton 1959 and McCallan 1966). An effort is being made, however, to introduce a plant or plant part into in vitro tests so that the reactions of fungicide, fungus, and plant are present. Leben (1949) sprayed leaf discs with an antibiotic, then transferred the leaf discs to seeded agar to determine if the material remained fungitoxic. Leben & Keitt (1949) applied artificial rain to fungicide-treated leaf discs to see if the material was tenacious. Chaves (1954) evaluated the effects of fungicide stickers in much the same manner. Adams, *et al.* (1951) devised a leaf punch so that numerous leaf samples with uniform areas could be easily obtained for use in fungicide bioassays.

The ultimate step in in vitro testing is not simply a bioassay using a plant part, but the prevention of the disease itself. Schmidt (1951) treated the primary leaves of celery with fungicides, inoculated with *Septoria apii*, and obtained results on protective fungicides within 2 weeks. The laboratory fungicide trials of Hislop & Park (1962), using detached, treated, and inoculated pods of *Theobroma cacao*, correlated well with field tests for control of *Phytophthora palmivora*. Horn (1964) described a similar detached cucumber leaf method for screening fungicides for control of cucumber anthracnose. Niemann & Dekker (1966) described a method of evaluating compounds for control of powdery mildew of cucumber by floating leaf discs treated with fungicides on water, inoculating, and observing disease symptoms.

CONCLUSIONS

In vitro fungicide tests are extensively used in the initial evaluation of potentially valuable fungicides. In recent years these tests have provided valuable information on the toxicity of various chemical structures and their modes of action against fungi.

The methods of testing to determine the fungitoxicity of chemicals are extremely varied and are constantly changing. This probably means that an acceptable method that can be universally used as a standard procedure is yet to be developed. The procedures for fungicide testing should be quick, easy, and simple and the results should be reliable, reproducible, and usable.

In vitro fungicide tests are used not only to determine fungitoxicity of test chemicals but also range and degree of susceptibility of fungi, deposition and redistribution of fungicides, stability to resist chemical change, tenacity to resist rainfall and wind, and persistence to resist other degrading elements. The results from these in vitro tests often will explain why a fungicide performed well or poorly in the field.

When a varied group of in vitro tests are performed in the laboratory, the candidate fungicides most likely to give satisfactory protection to plants will be selected for continued greenhouse and field testing. Not all of the complex chemical and environmental conditions of disease development can be reproduced either in the laboratory or the greenhouse. The introduction of plants or plant parts into in vitro tests does more closely approach natural conditions, and the use of these techniques will probably increase in the future.

At present there are no practical substitutes for in vitro tests for the initial screening of candidate fungicides. Thus these tests are invaluable as one of a series of steps required in the evaluation of fungicides.

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