Repeated Germination and Sporulation of Microsclerotia of Verticillium Albo-atrum in Soil

J. D. Farley, S. Wilhelm, and W. C. Snyder

Assistant Research Specialist and Professors of Plant Pathology, University of California, Berkeley 94720, respectively. Present address of senior author: Assistant Professor, Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster 44691.

Supported in part by Cotton Producer's Institute Cooperative Agreement No. 68-98. Published with approval of the Director, Ohio Agricultural Research and Development Center, Wooster, as Journal Article No. 48-70.

Accepted for publication 9 September 1970.

ABSTRACT

Soil, artificially infested with microsclerotia of *Verticillium albo-atrum,* was air-dried and remoistened with sucrose or water nine consecutive times. Microsclerotia germinated and sporulated after each remoistening. Germination percentage and number of germ tubes and conidia formed by each microsclerotial germination decreased with succeeding germinations. Pregerminated microsclerotia were capable of vigorous germination. When they were removed from treated soil and germinated in agar or the rhizosphere of cotton, there was little difference in germination percentage and number of germ tubes produced per germination between microsclerotia which had germinated one, four, or eight times. The propagules responsible for population increases in microsclerotial-infested soil moistened with sucrose solution or water were conidia. Conidia formed by germinated microsclerotia in soil were isolated and germinated in agar and in the rhizosphere of cotton. Phytopathology 61:260-264.

Additional key words: drying and remoistening soil, selective medium.

In field soil, microsclerotia of *Verticillium albo-atrum* Reinke & Berth. are exposed to repeated cycles of soil drying and rewetting and intermittent supplies of nutrients from plant roots and organic matter. Yet almost all studies on microsclerotia have been conducted in soil held at constant moisture levels and where nutrients, if supplied, were added as a single dose at the start of each experiment (4, 5, 6, 7, 12). An exception to this procedure is the work of Menzies & Griebel (9), where microsclerotial-infested soil was subjected to repeated cycles of drying and rewetting. Their data indicated that microsclerotia which had ceased multiplying for several months would again multiply when a food source was provided or when the soil was air-dried and remoistened. An important result of this multiplication was that it weakened the microsclerotium so that it lost its ability to withstand soil drying.

The purpose of this investigation was to study the effects of repeated nutrient amendment and drying and rewetting of soil on microsclerotial germination and multiplication. In addition, the nature of the propagule formed by germinating microsclerotia in soil was studied. Although there is indirect evidence that microsclerotia sporulate in soil, the production of conidia in soil directly from germinating microsclerotia has not been observed (2). An abstract of this study has been published (3).

MATERIALS AND METHODS.—*Soil.*—Soil used in this study was a clay loam containing 30% sand, 36% clay, 34% silt, and 1.5% organic matter. Field capacity (FC) was at pF 2 (30% moisture); and permanent wilting point, pF 4.2 (10% moisture). The soil was from a cotton field in the Central Valley of California in which *Verticillium* wilt has been severe for several years. Soil was sieved and stored at 50% FC in closed plastic containers.

Inoculum.—Soil was infested with microsclerotia from an isolate of *Verticillium albo-atrum* pathogenic to cotton. Microsclerotia were collected from 3-month-old cultures grown on Czapek-Dox agar covered with uncoated cellophane (11). Microsclerotia were scraped off the cellophane, ground in a Waring Blendor with water and fine mesh sand, and washed through a series of graded 60, 120, and 250 mesh sieves. Microsclerotia were collected on a 250-mesh sieve.

Germination and multiplication studies.—The equivalent of 10,000 microsclerotia/g of oven-dried soil was added to air-dried soil. The soil was atomized with water or 0.1% sucrose solution to 50% FC. Microsclerotial germination was measured after 1-day incubation by modification of a technique reported by Papavizas (10). Infested soil (0.2-0.4 g) was added to a test tube containing 2-3 ml water. The test tube was shaken by hand for 30 sec, and after the heavier soil particles had settled (5-10 sec), 1-2 ml of the soil suspension were poured onto water agar. After the suspension dried, cotton blue was added to the agar surface and covered with a No. 1 glass cover slip. Recovery of germinated and nongerminated microsclerotia was nonselective.

Ten days after wetting the soil, the *Verticillium* population was determined by soil dilution plate techniques. A modification of the soil extract agar described by Menzies & Griebel (9) was used as the selective medium. In addition to the use of polygalacturonic acid, suggested by Green & Papavizas (6), pentachloronitrobenzene (PCNB) (10 μg/liter) was added. The addition of PCNB was useful at soil dilutions of 1:100 and 1:1,000, in which PCNB-sensitive fungi often obscured *Verticillium* colonies.

Following population sampling, the soil was quickly air-dried to 3-4% moisture by an electric fan and remoistened to 50% FC with water or 0.1% sucrose solution, depending on the prior treatment; i.e., sucrose treatment followed sucrose, water followed water.

The population was re-estimated immediately after rewetting. Germination was measured again after a 1-day incubation period, and the population re-estimated after 10 days. The soil was repeatedly dried and remoistened at 10-day intervals over a 90-day period. All experiments were repeated twice with two replicates/treatment. One hundred microsclerotia were counted/replication.

Rhizosphere studies.—Three-day-old cotton seedlings (*Gossypium hirsutum* L.) grown in U.C. mix (1) at 27 C were transplanted into *Verticillium* microsclerotial- or conidial-infested soil. After an incubation period of 1-2 days at 25 C, the plants were harvested. Most of the adhering soil particles were removed by gently dipping into water. Soil retained on the roots after dipping (called rhizosphere soil) was removed by vigorous agitation in 2 ml water. This suspension was added to the surface of water agar and stained with cotton blue. Five plants were used/replication; 100 propagules were counted/plant.

Germination of conidia.—Conidia were produced in soil by moistening microsclerotial-infested soil (20,000 propagules/g soil) with 0.1% sucrose solution or water to 50% FC. As a control, washed *Verticillium* conidia from a 7-day-old potato-dextrose agar (PDA) culture were incubated 3 days in soil moistened 4 days previously with 0.1% sucrose or water. Seven days after the addition of amendments, the germinability of PDA conidia and conidia produced by microsclerotia in soil was measured in water agar, cotton rhizosphere, and soil. For measurements of conidial germination in soil, the infested soil was air-dried to 6-8% moisture and remoistened with water. After a 24-hr incubation, conidia were washed from the soil by the method previously described for microsclerotia and placed on the surface of water agar, where they were immediately stained with cotton blue and observed microscopically. Germinability in agar was tested by washing conidia from soil onto water agar amended with 100 ppm streptomycin sulfate. After 12-hr incubation on agar, the soil suspension was stained with cotton blue and covered with a cover slip. Each experiment was repeated twice with two replications/treatment. One hundred conidia were counted/treatment.

RESULTS.—*Germination of microsclerotia.*—Microsclerotial germination occurred on each wetting cycle (Table 1). Germination percentage ranged from 90% on the first germination to 15% on the ninth germination in sucrose-treated soil and from 50% to 7% in nonamended soil. In addition to the decrease in germination percentage, fewer germ tubes were produced; in sucrose-amended soil, for example, 4-6 germ tubes were formed/germination in the first two germinations, whereas after the fifth germination, only 1-3 germ tubes emerged/germination.

Menzies & Griebel (9) suggested that pregerminated microsclerotia may have a reduced ability for growth when contacted by host roots. This possibility was tested in the present study by measuring the germinability of microsclerotia which had previously germinated one, four, or eight times in the rhizosphere of cotton and water agar. Repeated germination in soil only slightly reduced the germinability of microsclerotia in the rhizosphere or water agar (Table 1). For example, microsclerotia germinated 94, 90, and 80% in water agar and 66, 58, and 45% in the rhizosphere after the first, fourth, and eighth germination, respectively, in nonamended soil. Similar results occurred with microsclerotia from sucrose-treated soil.

Multiplication studies.—*Verticillium* populations were measured on the 10th day of each wetting cycle and after the soil had been air-dried in preparation for the next rewetting. At the time of population estimates, all microsclerotial germ tubes had lysed, so that increases were due to propagules produced from germinating microsclerotia.

In sucrose-treated soil, population increases occurred after each germination (Fig. 1). On the basis of the

TABLE 1. Effect of repeated soil drying and rewetting on microsclerotial germination

No. wetting and drying cycles[b]	% Microsclerotial germination[a]					
	Soil[c]		Rhizosphere[d]		Agar[e]	
	Nonamended	Amended	Nonamended soil	Amended soil	Nonamended soil	Amended soil
1	50	90				
2	45	50	66	65	94	92
3	39	65				
4	52	69				
5	50	68	58	54	90	90
6	20	50				
7	25	24				
8	8	17				
9	7	15	45	48	80	76

[a] Each value is the average of two experiments with two replications/treatment. One hundred microsclerotia were counted/replication.

[b] Soil was repeatedly air-dried and moistened up to nine consecutive times. Each cycle was 10 days, with air-drying and moistening on the 10th day.

[c] Soil was moistened with water or 0.1% sucrose solution. Microsclerotial germination was measured 24 hr after each remoistening.

[d] Cotton seedlings were transplanted into soil infested with microsclerotia 10 days after the first, fourth, and eighth cycle. Germination was measured 24 hr after planting. Five plants were used/replication.

[e] Germination on water agar of microsclerotia from soil repeatedly air-dried and remoistened.

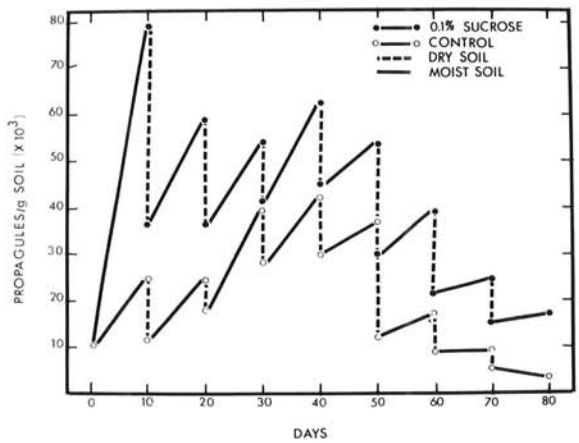

Fig. 1. Repeated sporulation of microsclerotia in soil repeatedly air-dried and moistened with water or sucrose solution. Soil was air-dried to 3-4% moisture and remoistened on the 10th day of each cycle.

number of propagules formed per germinating sclerotium, there were eight produced/sclerotium on the first germination, five/sclerotium on the second, and two/sclerotium on the sixth, seventh, and eighth germination. Results in nonamended soil were similar to sucrose-treated soil, except that population levels were lower and there was no detectable propagule increase in the last two germinations.

The data indicate that microsclerotia are capable of repeated multiplication. With each succeeding multiplication, however, the number of propagules decreased so that after the eighth and ninth germination, few or no propagules were produced.

Production of conidia from germinating microsclerotia.—Attempts were made to determine if population increases were due to conidial production. Microsclerotial-infested soil was treated with 0.1% sucrose. After 9 days' incubation, a soil suspension was poured onto the surface of water agar as previously described. After 12-hr incubation on agar, the soil suspension was stained with cotton blue and observed microscopically under a magnification of ×420. Germinated conidia were observed. Fifty of these germinated conidia were isolated from an unstained plate and placed onto water agar amended with 100 ppm streptomycin sulfate and sterilized barley straw. Ninety-five per cent of these conidia yielded *Verticillium* colonies. No other type of *Verticillium* propagule was observed. In soil treated with 0.1% sucrose and no microsclerotia, this type of conidium was not observed. This experiment was repeated with nonamended soil with similar results.

Conidial germination.—The germinability of conidia produced in soil by microsclerotial sporulation was comparable to conidia produced on PDA and then incubated in soil (Table 2). For example, PDA conidia, incubated in nonamended soil, germinated 95% in water agar, 40% in the rhizosphere, and 6% in nonamended soil, whereas conidia formed in nonamended soil by germinating microsclerotia germinated 80% in agar, 38% in the rhizosphere, and 3% in nonamended soil. Similar results were obtained for the germination of conidia from sucrose-treated soil.

Conidial formation.—Sporulation was observed by using a technique described by Lingappa & Lockwood (8). Twenty g of 0.1% sucrose-amended soil (80% FC) were added to a small petri dish (50 × 15 mm), and a smooth soil surface was made with a steel spatula. A dilute suspension of microsclerotia was added to the soil surface. At daily intervals after the addition of microsclerotia, the germinated propagules were stained in situ with cotton blue, recovered from soil with plastic film, and observed microscopically. Microsclerotia germinated within 18 hr, and began sporulating 2-3 days later (Fig. 2). Conidia were formed in heads at the tip of short condiophores borne at right angles to the germ tube. Frequently, 30-50 conidia were produced/germinated microsclerotium. This is above the average of eight conidia/microsclerotium observed in sucrose-amended soil in the multiplication studies. The inherent artifacts of this direct observation method, i.e., smooth soil surface, continuous water film, and max aeration, might account for the increased conidial production.

DISCUSSION.—Under the conditions of this study, microsclerotia of *Verticillium albo-atrum* were capable of repeated germination and sporulation in soil repeatedly dried and remoistened. With succeeding germinations, however, germination percentage and the number of germ tubes and conidia formed decreased.

TABLE 2. Comparison of the germinability of conidia produced in soil by microsclerotia with conidia produced on potato-dextrose agar (PDA)

Source of conidia	Incubation of PDA conidia and production of sclerotial conidia in	% Conidial germination[a]		
		Water agar	Rhizosphere[b]	Nonamended soil[c]
PDA	Nonamended soil	95	40	6
	Sucrose-amended soil	93	43	5
Sclerotia in soil	Nonamended soil	80	38	3
	Sucrose-amended soil	85	34	2

[a] Each value is the average of three experiments, with two replications/treatment. One hundred conidia were counted/replication.

[b] Cotton seedlings were transplanted into soil infested with conidia. Germination was measured 1 day after planting. Five plants were used/replication.

[c] Soil infested with conidia was air-dried to 6-8% moisture and remoistened with water to 50% field capacity. Germination was measured 24 hr after remoistening.

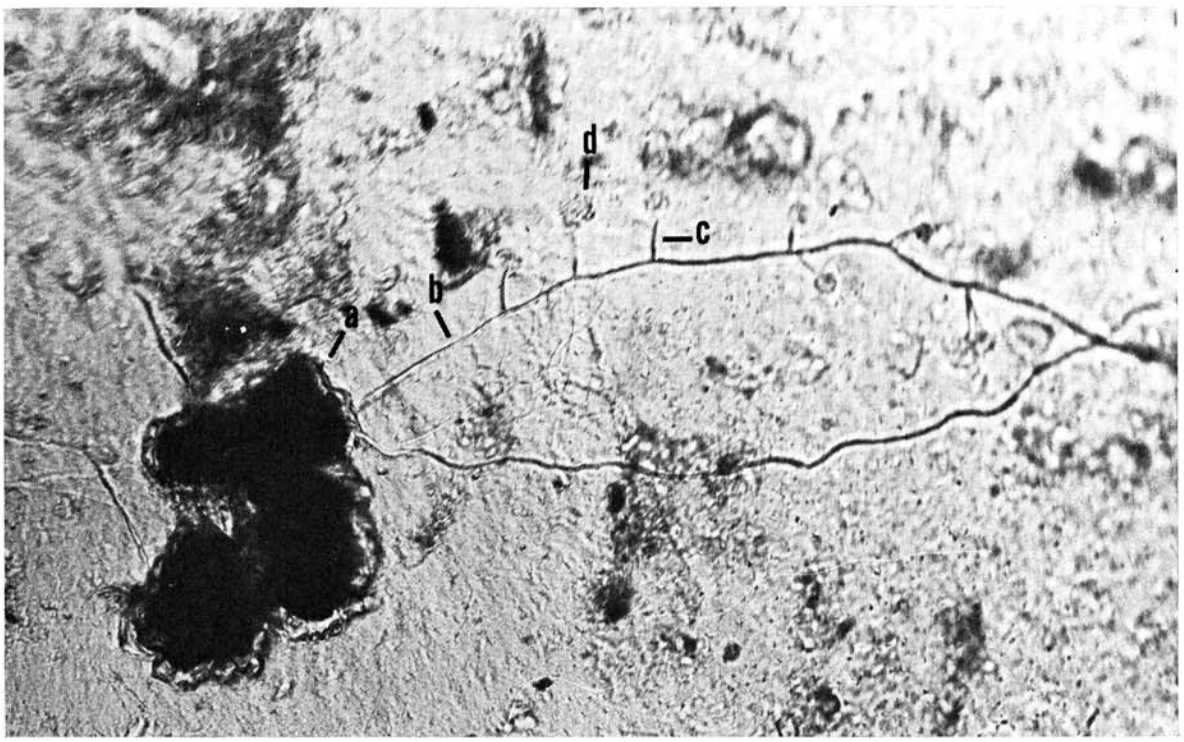

Fig. 2. Sporulation of a microsclerotium on the surface of 0.1% sucrose-amended soil; a = microsclerotium; b = germ tube; c = conidiophore; d = conidial head.

Apparently, microsclerotia were still capable of vigorous germination, for when they were removed from treated soil to agar or the rhizosphere of cotton there was little difference in germination percentage and number of germ tubes produced per germination between microsclerotia which had been pregerminated one, four, or eight times. The decline of germination percentage and number of germ tubes produced in treated soil might be explained in one of two ways: (i) the fungistatic level of the soil was increased by repeated nutrient amendment; or (ii) microsclerotia were more sensitive to soil fungistasis after prolonged incubation and repeated germinations.

The evidence suggests that under field conditions, microsclerotia may germinate and sporulate several times in the vicinity of organic matter or the rhizosphere of nonhost plants and still have the capacity for growth and infection when contacted by host roots. This evidence might partially explain some of the failure of control of *Verticillium* by crop rotation.

Menzies & Griebel (9), Lacy & Horner (7), and Green & Papavizas (6) have reported population increases following microsclerotial germination in soil. Even though the production of conidia in soil from germinating microsclerotia had not been observed, Menzies & Griebel (9) and Lacy & Horner (7) suggested that the increase of propagules was due to sporulation. Recently, Emmatty & Green (2) reported the formation of secondary microsclerotia from germinating microsclerotia, and concluded that these propagules probably accounted for the population increases observed by other workers.

The results of this study implicate sporulation as a primary factor in population increases. Although germination and sporulation was studied up to 10 days after the addition of nutrients, the formation of secondary microsclerotia, as described by Emmatty & Green (2), was not observed. Differences in experimental technique, soils, biotype, and source of microsclerotia might account for the differing results.

LITERATURE CITED

1. Baker, K. F. [ed.] 1957. The U.C. system for producing healthy container-grown plants. Calif. Agr. Exp. Sta. Manual 23. 332 p.
2. Emmatty, D. A., & R. J. Green, Jr. 1969. Fungistasis and the behavior of the microsclerotia of Verticillium albo-atrum in soil. Phytopathology 59: 1590-1595.
3. Farley, J. D. 1969. Repeated germination and sporulation of microsclerotia of Verticillium albo-atrum in soil. Phytopathology 59:1026 (Abstr.).
4. Green, R. J., Jr. 1960. The survival of Verticillium albo-atrum in muck soils. Phytopathology 50:637 (Abstr.).
5. Green, R. J., Jr. 1969. Survival and inoculum potential of conidia and microsclerotia of Verticillium albo-atrum in soil. Phytopathology 59:874-876.
6. Green, R. J., Jr., & G. C. Papavizas. 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of Verticillium albo-atrum. Phytopathology 58:567-570.
7. Lacy, M. L., & C. E. Horner. 1966. Behavior of Verticillium dahliae in the rhizosphere and on roots

of plants susceptible, resistant and immune to wilt. Phytopathology 56:427-430.

8. LINGAPPA, B. T., & J. L. LOCKWOOD. 1963. Direct assay of soils for fungistasis. Phytopathology 53: 529-531.
9. MENZIES, J. D., & G. E. GRIEBEL. 1967. Survival and saprophytic growth of Verticillium dahliae in uncropped soil. Phytopathology 57:703-709.
10. PAPAVIZAS, G. C. 1967. Survival of root-infecting fungi in soil. I. A quantitative propagule assay method of observation. Phytopathology 57:1242-1246.
11. SCHREIBER, L. R. 1961. The soil survival and germination of conidia and microsclerotia of Verticillium albo-atrum Rke. and Berth. Ph.D. Thesis, Purdue Univ. 84 p.
12. SCHREIBER, L. R., & R. J. GREEN, JR. 1962. Comparative survival of mycelium, conidia and microsclerotia of Verticillium albo-atrum in mineral soil. Phytopathology 52:288-289.