Diploidization and Heritable Gene Repression-Derepression as Major Sources for Variability in Morphology, Metabolism, and Pathogenicity of Verticillium Species

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Cooperative investigations of the Plant Sciences Research Division and the Texas Agricultural Experiment Station. Published with approval of the Director as Technical Paper No. 9344.

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The technical assistance of William Bate, Constance Liverman, Barbara Kronke, and Diane Fabian is gratefully acknowledged.

Strains of *Verticillium albo-atrum* Reinke & Berth. that produce microsclerotia (MS) or dark mycelium (DM) are unstable in laboratory cultures (1, 5, 8, 17, 18, 20). Mechanisms proposed to account for such variability can be grouped into three categories: heterokaryosis-monokaryosis (8, 13, 18); gene exchange through the parasexual cycle (7, 9, 10, 11, 12, 13, 15); and gene mutation or loss (13, 18). The parasexual cycle has been studied with ultraviolet-induced, auxotrophic haploid mutants of *V. albo-atrum* which are believed to form unstable heterokaryons (7, 9, 10, 11, 12, 13). The heterokaryons in turn form unstable diploids which through unknown mechanisms yield haploids with new gene combinations (9, 10, 11, 12, 15). Heterokaryotic conidia have not been observed in *V. albo-atrum* (9).

A difficulty in accepting gene exchange through the parasexual cycle as a major cause of heritable variability in *Verticillium* spp. is that unstable wild type isolates are usually haploid and monokaryotic. Nuclear studies with the light microscope (5, 14, 16, 18, 19), electron microscope (2), and on the frequency of auxotrophs among conidia irradiated with ultraviolet (4) indicate a mononucleate haploid state. Roth & Brandt (20) pointed out that hereditary instability exists within homokaryotic MS cultures. Furthermore, the appearance of haploid variants among the conidial population is associated with the production of MS or DM (1, 8, 18, 20, 22), but the various mechanisms proposed for variability do not account for this association. The black cells have been viewed as asexual structures whose presence or absence reflects hereditary changes that occur primarily in conidia and hyphae. Thus, hereditary studies in *Verticillium* have always employed conidial analysis.

As MS of *V. albo-atrum* (6) or *V. tricorpus* Isaac (21) germinate, they often produce conidiophores and conidia before a hyphal colony is formed. Some of the MS cells within aging colonies empty their contents into conidia that are being produced (*unpublished data*). The proportion of conidia that originates from MS increases with colony age. The percentage of haploid variants in the conidial population of MS cultures also increases with age (1, 20). Haploid variants may arise primarily from aged MS or DM within homokaryotic cultures.

The purpose of this report is to show evidence that a haploid-homozygous diploid cycle is involved in forming MS cells, DM cells, and chlamydospores in various species of *Verticillium*; that MS cells of *V. albo-atrum* serve as sites for establishing numerous new heritable patterns of gene repression and derepression within a common haploid genome; and that the resulting haploid variants carry the same gene pool which is translated differently in each variant type.

MATERIALS AND METHODS.—*Isolates.*—The production of haploid variants was studied primarily in two wild type MS isoltes of *V. albo-atrum* from cotton. These are designated as T9 and V44, and were collected at Tulare, Calif., and Lubbock, Tex., respectively. Three additional MS isolates from Texas were also used in more limited studies of haploid variants. The MS progency from five consecutive generations were studied in which each generation represented a different type of haploid variant. Other isolates of *V. albo-atrum* (MS and DM types), *V. tricorpus, V. nigrescens* Pethy., and *V. nubilum* Pethy. from various areas of the world were used in studies of homozygous diploids.

Media.—Potato-carrot-dextrose agar (PCDA) and D medium were used as described previously (22). Complete medium (C) consisted of HV medium (22) modified by the addition of 5 g of Difco yeast extract (Y), 5 g of Difco peptone (P), 20 g of Difco Noble agar, and the adjustment of potassium phosphate buffer to 10 mM at pH 7. The -Y, -P, and minimal (M) media were identical to C except for the omission of Y, P, and Y-P, respectively.

Maintenance and inoculation of cultures.—Cultures were maintained at 24 C on PCDA. Inoculations were made by single hyphal-tip transfers from the advancing fronts of colonies 10 to 14 days old.

Harvest of MS and conidia for dilution plates.—Microsclerotia were removed from cultures 20-42 days old, washed, and air-dried aseptically. They were the only viable propagules present after air-drying. The dried MS were resuspended in distilled water (25 ml) with a Waring Blendor (30 sec). A series of five concentrations of MS was made by

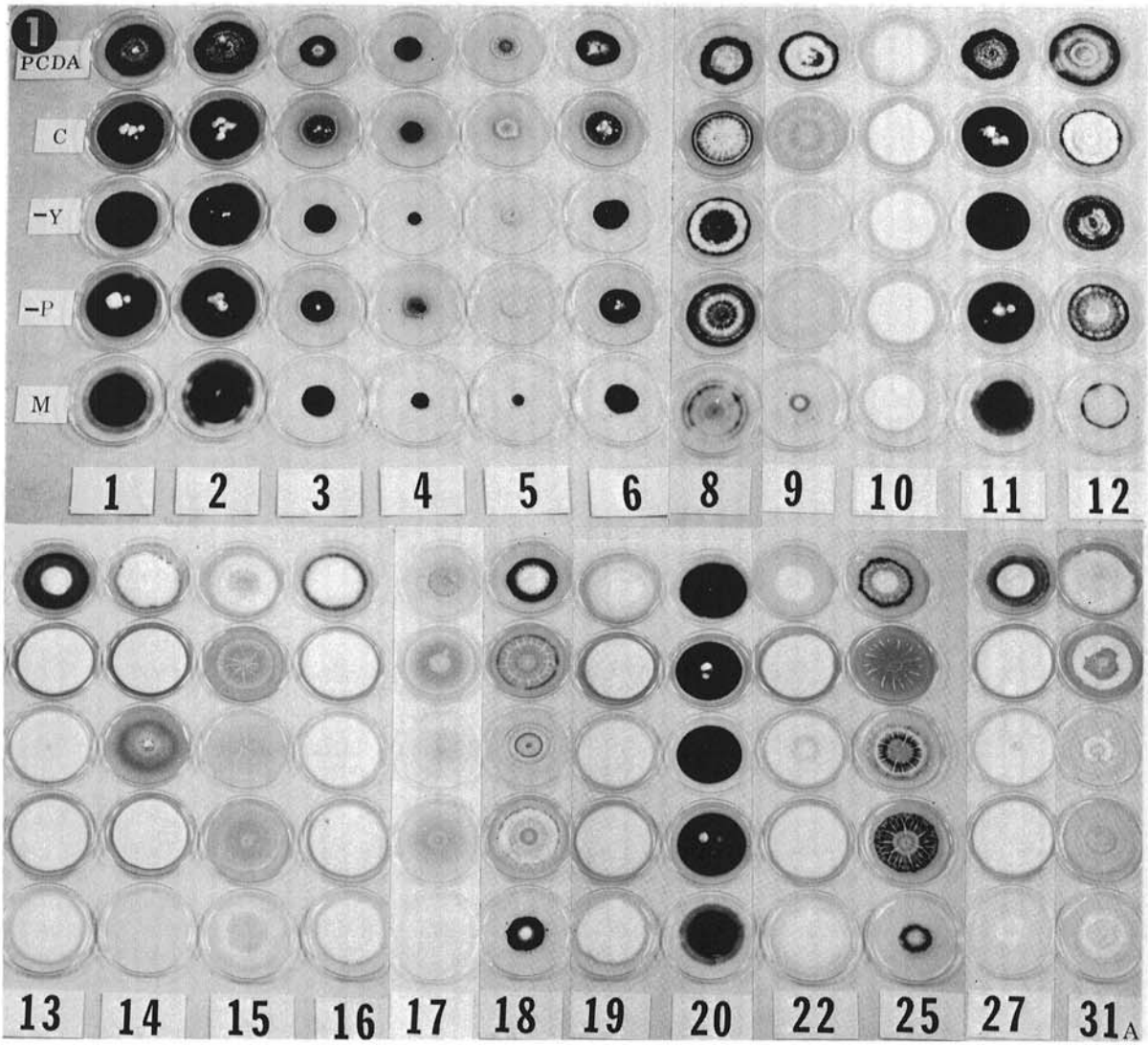

Fig. 1. Isolate T9 (No. 1) of *Verticillium albo-atrum* and first through fifth generation microsclerotial descendants 22 days after hyphal-tip inoculation on potato-carrot-dextrose agar (PCDA), complete medium (C), C minus yeast extract (-Y), C minus peptone (-P), and minimal medium (M).

pipetting different aliquots of the suspension to melted PCDA at 45 C. Five plates of each concentration were made.

Colonies which originated from a sample of conidia in dilution plates, and those which originated from air-dried MS, are hereafter called conidial and MS progeny, respectively. When the conidial and MS progenies were compared for their content of haploid variants within single colonies, conidia were removed and placed in dilution plates of PCDA just before the MS were harvested and dried.

Pathogenicity tests.—*Gossypium hirsutum* L. 'Lankart-57' and 'Acala 4-42' were inoculated by stem-puncture in the field and greenhouse by the method of Bugbee & Presley (3) with 10^6 washed, viable conidia/ml in 10 mM potassium phosphate buffer (pH 7.0). Each of 10 plants was inoculated with ca. 10^4 conidia/isolate. Defoliation and wilting symptoms were noted daily to 20 days after inoculation, and final disease readings were taken at 36 days.

RESULTS AND DISCUSSION.—*Comparison of MS and conidia as a source of haploid variants.*—Isolate T9 gave 248 haploid variants (9.11%) out of 2,722 first-generation MS progeny. Conidia from the same cultures gave 24 variants (0.48%) out of 5,005 single-spore colonies. Isolate V44 gave 63 variants (4.73%) out of 1,333 MS progeny as compared with 0.13% variants in 5,292 colonies that originated from conidia. Similarly, when 1,623 MS progeny were obtained from 17 different first generation haploid variants of T9, 112 of the colonies (6.9%) represented new types of variants, whereas only three new variants (0.26%) occurred in 1,152 conidial progeny from these same 17 variant isolates. Thus, the rate of heritable variability

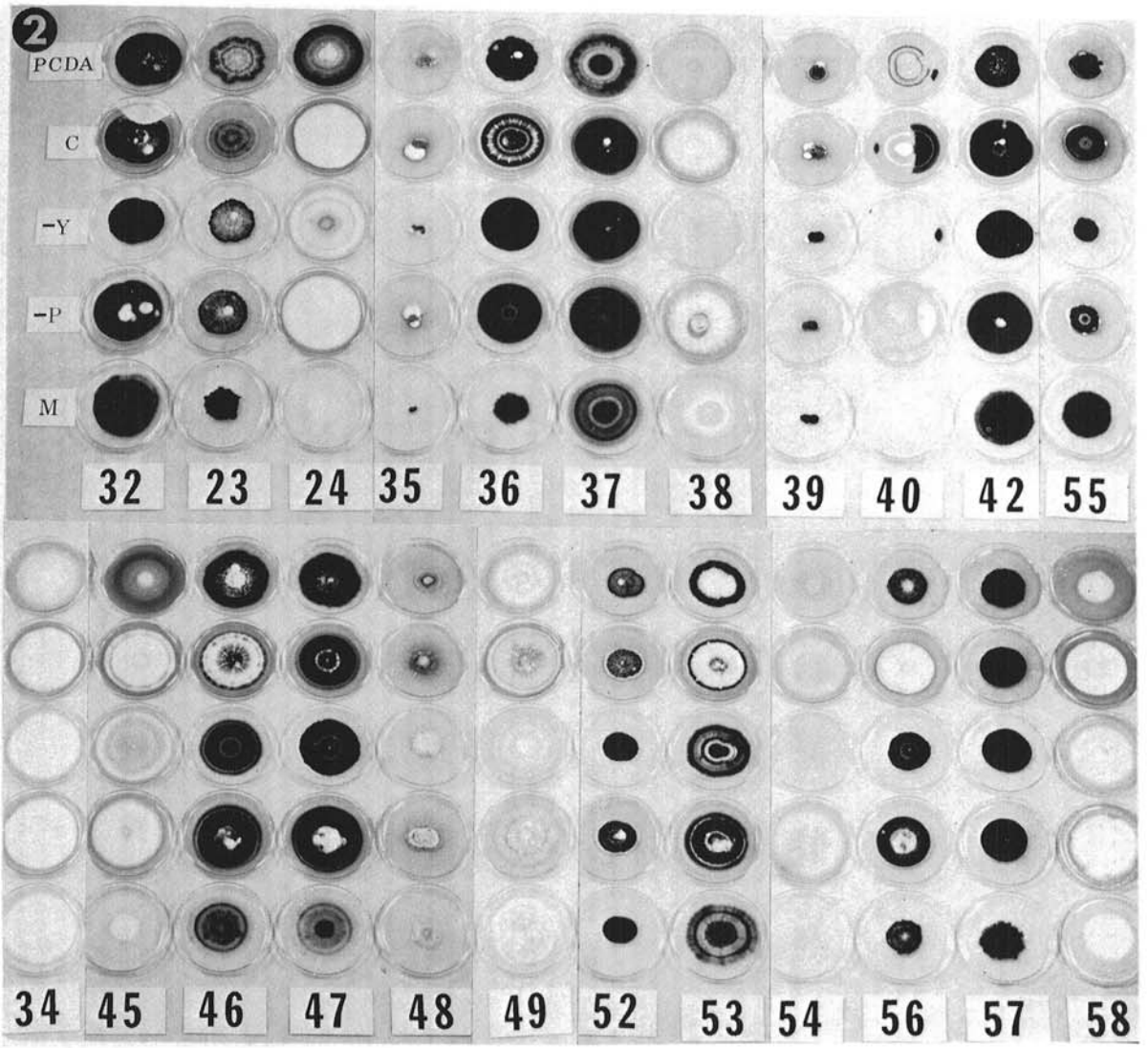

Fig. 2. Isolate V44 (No. 32) of *Verticillium albo-atrum* and first through fifth generation microsclerotial descendants on five media as described in Fig. 1.

remained high within MS populations and low in conidial populations of haploid variants in my studies.

A small sample of the types of haploid variants obtained from first through fifth generation MS progeny of isolates T9 and V44 is shown in Fig. 1-5 on five different media. Use of the five media permitted a finer distinction between isolates which appeared similar on PCDA medium. Isolates 1 and 32 represent T9 and V44, respectively. Figures 1 and 5 show T9 and 27 of its haploid derivatives, whereas Fig. 2 shows V44 and 22 of its haploid derivatives.

Germinated MS from various isolates provided a greater heterogeneity of haploid variants than conidia from the same cultures. For example, the MS cells of dwarf isolate 35 (Fig. 2), which is a first generation MS progeny of V44 (isolate 32), contained numerous new types of haploids, some of which had normal growth rates. Isolates 36 through 55 in the upper column of Fig. 2 represent seven MS progeny of the dwarf. Conidial dilution plates from isolate 35 showed only a small portion of the spectrum of haploid variants present in the colonies, and all had dwarf growth rates.

These observations indicate that MS cells are the major source of haploid variants in cultures of *V. albo-atrum*. The majority of new variants remain quiescent within MS cells in the parent colony where they are formed. Certain types of variants are capable of secondary growth and sporulation within the parent colony. The latter are apparently the types most frequently detected in conidial dilution plates.

Reversion to wild type.—When the MS from 17 different haploid variants of T9 and V44 were germinated, 14 of the isolates regenerated wild type colonies at frequencies of 0.1 to 6.6%. The number of MS progeny examined in each batch of MS from each isolate ranged from 91 to 2,230. The percentage of

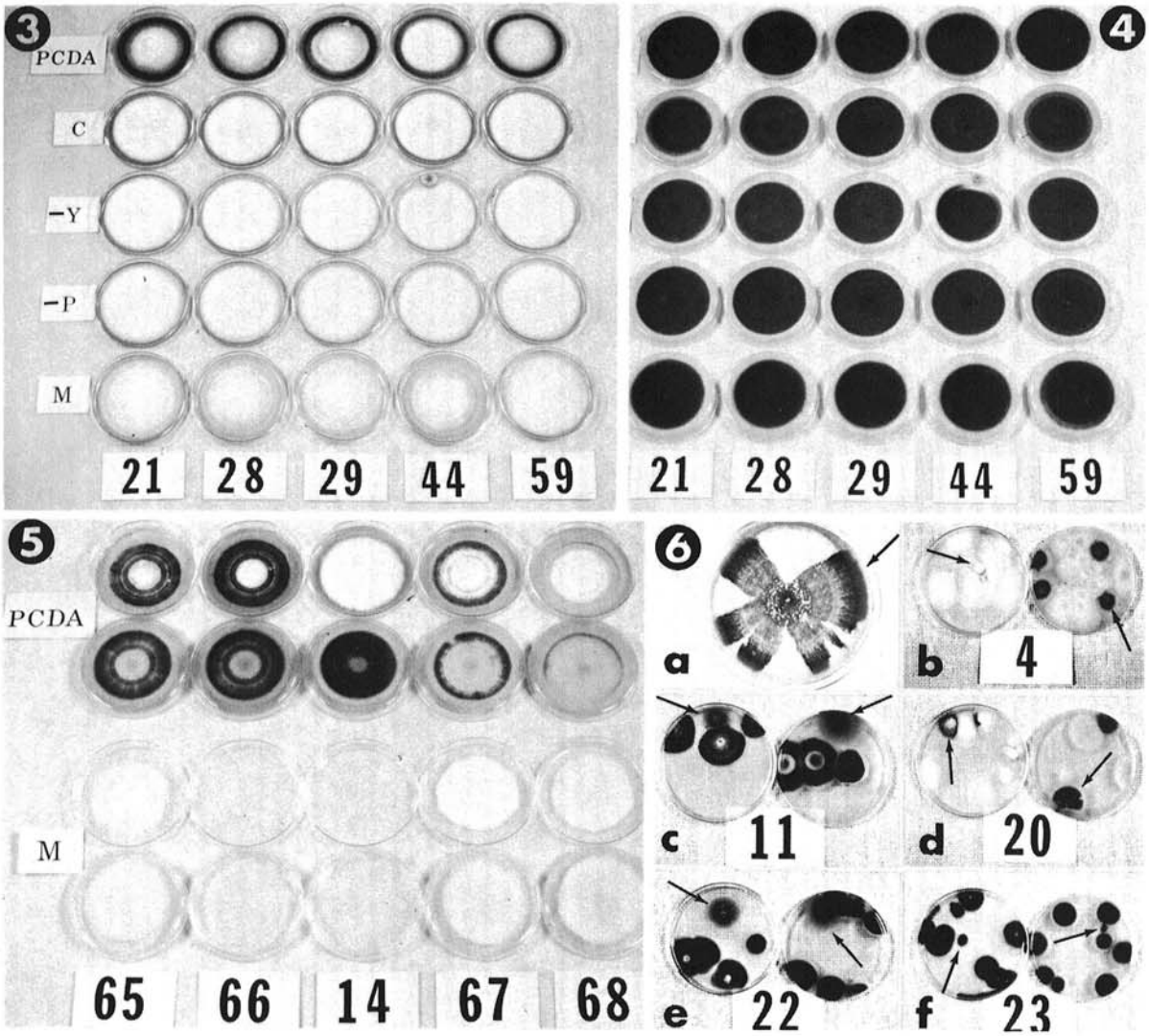

Fig. 3-6. 3, 4) Upper and bottom colony view of a repetitious dark-mycelial haploid variant derived from various second generation microsclerotial progeny of *V. albo-atrum*—isolates 21, 28, 29 from T9, 44 from V44, and 59 from a cotton isolate from El Paso, Tex. Each isolate was grown on the five media as described in Fig. 1. **5)** Auxotrophic haploid variants (No. 66, 14) and two prototrophic sister isolates (No. 67, 68) of 65, which is a second generation microsclerotial descendant of isolate T9; all grown on PCDA and M media. The top row in each set of plates shows upper colony surface; the bottom row shows the underside. **6)** Homozygous diploids of *Verticillium* species, obtained 37-42 days after hyphal-tip inoculation of D medium with haploids, growing on PCDA medium. Arrows point to diploid colonies which are mixed with haploids. The colony in Fig. 6-a was initiated from a single diploid hyphal tip; all others are from conidial dilutions. Plates on the left show the upper colony surface, those on the right show the bottom; a = *V. tricorpus* from *Datura stromonium*, New South Wales; b = *V. dahliae* from cotton, Iran; c = *V. albo-atrum* (MS) from cotton, Missouri; d = *V. albo-atrum* (DM) from potato, South Australia; e = Isolate T9 of *V. albo-atrum* (MS) from cotton, California; f = a haploid variant of isolate T9 (identical to No. 3, Fig. 1).

wild type revertants varied extensively between different MS batches of the same isolates. For example, one white appressed variant which produced MS sparingly gave 16 revertants (6.6%) among 252 progeny in one batch, and four revertants (0.19%) among 2,055 progeny in another batch.

Two examples of reversion to wild type among MS progeny of haploid variants are shown in Fig. 1. Isolate 11 was derived from a white appressed parent, now shown here, which was derived from isolate 9, which in turn was derived from T9 (isolate 1). Isolate 11 appears identical to its great grandparent, T9. The grandparent of isolate 11 (isolate 9) also regenerated the T9 phenotype and gave eight revertants (0.4%) out of 2,230 MS progeny.

Isolate 20 (Fig. 1) is a wild type revertant that was obtained from MS of isolate 19, which in turn is a direct MS progeny of T9. Other variants which regenerated the T9 phenotype through MS germination include isolates 25 and 31A.

Repeating variant types.—In addition to wild type revertants, different types of haploid variants yield common new types of variants as a small percentage of the MS progeny. A single example of this is illustrated in Fig. 3 and 4, which show the upper and lower colony surface, respectively, of five isolates on five media. Each of the five isolates (Fig. 3, 4) descended from a different type of MS parent, but produced black hyphae (DM) instead of MS on the five media. Each of the isolates turned M medium pink in advance of hyphal growth. These isolates are identical in their phenotypic characteristics in spite of the widely separated geographical origins of their three wild type grandparents.

Auxotrophy.—Two complete and twelve incomplete auxotrophs occurred on M medium among 70 haploid variants of T9 and V44 that were derived from germinated MS. The auxotrophs were stimulated in growth and development by yeast extract or peptone. The two complete auxotrophic haploids are shown in Fig. 5 (isolates 66 and 14) along with their parent (isolate 65) and two sister isolates of their parent (isolates 67 and 68). The parent was a second generation MS derivative of T9.

Pathogenicity.—The haploid isolates tested included T9, V44, and 50 of their haploid variants derived from first through fourth generation MS progeny. Among T9, V44, and their variants which are illustrated in Fig. 1-5, pathogenicity ratings were as follows: severe wilt and total defoliation in less than 20 days with isolates 1, 11, 12, 17, 19, 20, 31A, 32, 37, 47, 56, and 57; moderate wilt and incomplete defoliation with isolates 5, 16, 25, 39, and 53; mild wilt with isolates 6, 9, 36, 40, 52, and 65; and slight or no wilt with isolates 3, 4, 35, 38, 48, and 66.

Pathogenicity was absent or reduced in several haploid variants, but was partially or fully recovered in a following MS generation through new variants or wild type revertants. For example, isolate 3 (Fig. 1) was nonpathogenic, whereas one of its MS progeny (isolate 5) was moderately pathogenic. Isolate 37 (Fig. 2) was as pathogenic as its wild type grandparent (V44), whereas its parent (isolate 35) was nonpathogenic.

Homozygous diploids.—The MS progeny of T9, V44, and three other cotton isolates of *V. albo-atrum* from scattered areas in Texas consistently included approximately 1% homozygous diploids. Germinated MS from haploid variants also gave homozygous diploids.

A more efficient method of obtaining homozygous diploids, hereafter simply called diploids, employs D medium (22). Six examples of diploid recovery in dilution plates from D medium are illustrated in Fig. 6-a through f. The arrows point to diploid colonies, showing the upper and lower surfaces. Each plate contains haploid colonies for comparison.

Out of 29 additional MS and DM haploid isolates of *V. albo-atrum* from various hosts and different areas of the world, 23 gave diploids (0.9 to 97%) in PCDA dilution plates after growth and aging in D medium. Single haploid isolates of *V. nigrescens* and *V. nubilum* were also converted to diploids in D medium.

The single morphological feature which consistently distinguished diploids from haploids in the various species and isolates of *Verticillium* was the production of long conidia, usually on simple conidiophores. Diploids were also easily distinguished from their corresponding haploids on PCDA by colony morphology (Fig. 6-a through f).

Auxotrophy was found in 16 out of 18 diploids of *Verticillium* spp. Specific nutritional requirements of certain diploids were adenine, L-leucine, and D-methionine. Creatine and choline permitted the growth of certain diploids on M medium, and betaine was less effective. Requirements for growth of most diploids were not identified.

Prototrophy was restored in the initial flush of haploids regenerated as conidia in aging cultures of auxotrophic diploids.

Eleven diploid isolates derived from T9, V44, and their haploid variants were tested for pathogenicity to cotton. Ten of the diploids were nonpathogenic, and one was moderately pathogenic. The pathogenic diploid was prototrophic and equal in pathogenicity to its haploid component, which was a variant of V44. Most of the nonpathogenic diploids were obtained from highly pathogenic haploids. These diploids were distributed throughout the leaves of inoculated cotton plants without causing visible symptoms, and were reisolated at the end of pathogenicity tests.

Haploids which were regenerated in the initial flush of haploid conidia from nonpathogenic diploids in culture regained their original levels of pathogenicity.

Role of diploids in the life cycle.—Homozygous diploids are involved in the production of MS, DM, and chlamydospores of *Verticillium* spp., regardless of whether a colony is initiated from the haploid or diploid state. The involvement of the diploid in forming the black resting structures is most apparent when colonies of its haploid either fail to form the structures, or form them slowly and sparingly upon aging (Fig. 6-a, b, d). The diploids of white haploid isolates produce the resting structures heavily, but only in the zones of colony development where haploids are being regenerated. A mixture of ploidy within individual cells appears to be required for the formation of the black cells (*unpublished data*).

The participation of homozygous diploids in forming MS suggests that they may play a role in the production of haploid variants. Although the initial flush of haploid conidia produced within diploid colonies consistently reproduces the original haploid phenotype, variants are obtained from the conidial population when MS produced within diploid colonies have aged for several days.

Haploid dominance.—A total of 50 diploid isolates recovered from germinated MS or from haploids grown in D medium were studied. In every case, haploids prevented the independent growth of diploids in mixtures of haploid and diploid conidia. In detailed studies of T9 and its diploid, the haploid

dominated over the diploid during colony development when 5 μliters of a mixture containing 10^6 conidia/ml was spotted on plates of PCDA even though 98% of the conidia were diploid. Various ratios of haploid and diploid conidia at different concentrations showed that haploids are consistently dominant over diploids. In some diploid isolates, the regenerated haploid has a large sphere of dominance and produces V-shaped sectors which compress the diploid colony front (Fig. 6-a).

Alternate homozygous diploids.—An unusual feature of diploid formation in DM and MS types of *V. albo-atrum* is that some haploid isolates are capable of forming two distinct colony types of homozygous diploid. A DM isolate from potato in New Hampshire formed only one type of diploid in D medium, but a slower-growing type which formed black cells later was consistently recovered in conidial dilutions made from PCDA colonies of the first type. Conidia from both types of diploid colonies consisted of a mixture of three types: their own type; the alternate diploid; and the common haploid. Thus, no matter which diploid one starts with on PCDA, the other is a constant member of the conidial population. A similar case was found in a diploid formed from a haploid variant of V44.

In three other cases, including V44, one specific type of homozygous diploid was formed under acid conditions in D medium, and a second type was consistently recovered from germinated MS that were produced under alkaline conditions. In these cases, the alternate diploids did not produce each other through conidia.

Cytology.—Several reports (16) on nuclear studies in *V. albo-atrum* mention the presence of "nuclear granules". Heale et al. (14) concluded that, "The conidial nucleus assumes a horse-shoe shape before forming a ringed constellation in which a number of chromosomes is joined by a fine thread". Different investigators refer to the same small DNA-carrying units as chromosomes or nuclei.

A cytological study of living and stained (DNA) cells with the light microscope (still photographs and time-lapse movies) and with the electron microscope was conducted. Haploid cells of *V. albo-atrum* possessed eight DNA-containing subunits connected in tandem to form a chain. When this chain is at its shortest length (8 μ), each subunit is tadpole-shaped and 1 μ long. The subunit head is rounded and ca. 0.25 μ in diam with a narrow attached tail that is 0.75 μ long. The subunits attach head-to-tail, leaving one end of the chain with a free tail and the other with a free head. The chain of connected DNA-carrying subunits found in conidia, hyphae, and MS of *V. albo-atrum* closely resembles a segment of an intranuclear, interphase chromosome from higher organisms. Because of these cytological observations and the genetic behavior of *Verticillium*, I choose to refer to the chain as a chromosome and the subunits as chromomeres.

Diploids are formed by an end-to-end connection between two haploid chromosomes. It has not been determined whether connections are head-to-tail or tail-to-tail between the two chromosomes in specific diploids; however, both methods of connection have been observed. The two modes of connection might be related to the occurrence of alternate homozygous diploids.

Microsclerotial cells become polychromosomal as they enlarge. A significant cytological event which occurs upon aging in MS cells is a temporary disconnection between chromomeres. The independent tadpole-shaped chromomeres scramble extensively for a short period of time, then rapidly reconnect to form chromosomes. This process may permit repositioning of chromomeres within chromosomes to give new linear sequences of connections. Without gene change, new patterns of gene translation might be established due to the influence of one chromomere upon another (the position effect). Theoretically, total scrambling of eight chromomeres could give 40,320 linear sequences of connection in haploids. However, half of the connections would result in an identical sequence of chromomeres in which the head-to-tail connection pattern is reversed. Depending upon whether this pattern of connection is genetically important, there may be 20,160 possible combinations of eight chromomeres.

Disconnection between chromomeres, scrambling, and a random reconnection to form chromosomes carrying the complete genome could be the basis for the parasexual cycle in *Verticillium* spp. The repositioning of chromomeres would permit both allelic gene exchange and recombination in the haploids formed by heterozygous diploids (9, 10, 11, 12, 13, 15) and new patterns of gene translation due to the position effect. In homozygous diploids, only the position effect would function to produce haploid variants.

DISCUSSION.—The MS cells of *V. albo-atrum* are an important source of haploid variants which appear to carry the same genome but have different patterns of expression. The numerous patterns are heritable, and can be perpetuated by hyphal-tip transfers, but are subject to changes in each generation of MS cells. Within a single batch of MS, the changes occur in a variety of different directions. Some of the changes lead to the regeneration of wild type even though wild type may be two to four MS generations removed by way of different variants; other changes lead to the production of new variants; others result in phenotypically repetitious variants which may arise from different types of parents.

None of the proposed mechanisms for heritable variability in *V. albo-atrum* (7, 8, 9, 10, 11, 12, 13, 15, 18) adequately accounts for the frequency with which new haploid variants are recovered from germinated MS, or the frequency of reversion to wild type. The occurrence of different types of repetitious variants (or wild type revertants) suggests that variants carry the same genome with different patterns of gene repression and derepression. The MS cell appears to be a site where the heritable sequence of gene translation is scrambled each generation. Valadon & Heale (23) described the heritable

derepression and re-repression of carotenoid synthesis in an orange-colored mutant of *V. albo-atrum* (DM), but did not recognize the mechanism as playing a role in normal variability of the fungus.

Gene repression and derepression could play a significant role in the properties of homozygous diploids which are formed by the end-to-end connection of two identical haploid chromosomes. Most of the diploids are auxotrophic, nonpathogenic, and assume various new morphological features which are not evident in the haploids. However, when the diploid chromosome fractures at its original point of fusion during the initial regeneration of haploids, the original characteristics of the haploids are restored, including prototrophy, pathogenicity, and morphology. Thus, the position effect appears to play an important role in gene repression and derepression within diploids.

It is likely that in aging MS cells, which are formed by an interaction of haploid and diploid chromosomes, there is a more complete disengagement of the chromomeres within individual chromosomes and extensive repositioning when they reconnect to form haploid variants. Hastie (9, 10, 11, 12) commented on the unusually high frequency of gene recombination in haploid segregants formed by heterozygous diploids of *V. albo-atrum* (DM) through the parasexual cycle. It appears that the same mechanism which functions to produce extensive gene recombination from heterozygous diploids may also operate to produce haploid variants from homozygous diploids. It is suggested that these two phenomena are related through a temporary disconnection between chromomeres and random patterns of reconnection to form a complete haploid genome.

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