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Mating Type, Vitamin E And Morphogenesis In Ustilago Violacea And Other Smut Fungi

Alan James Castle

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MATING TYPE, VITAMIN E AND MORPHOGENESIS IN *USTILAGO VIOLACEA* AND OTHER SMUT FUNGI

by

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Department of Plant Sciences

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

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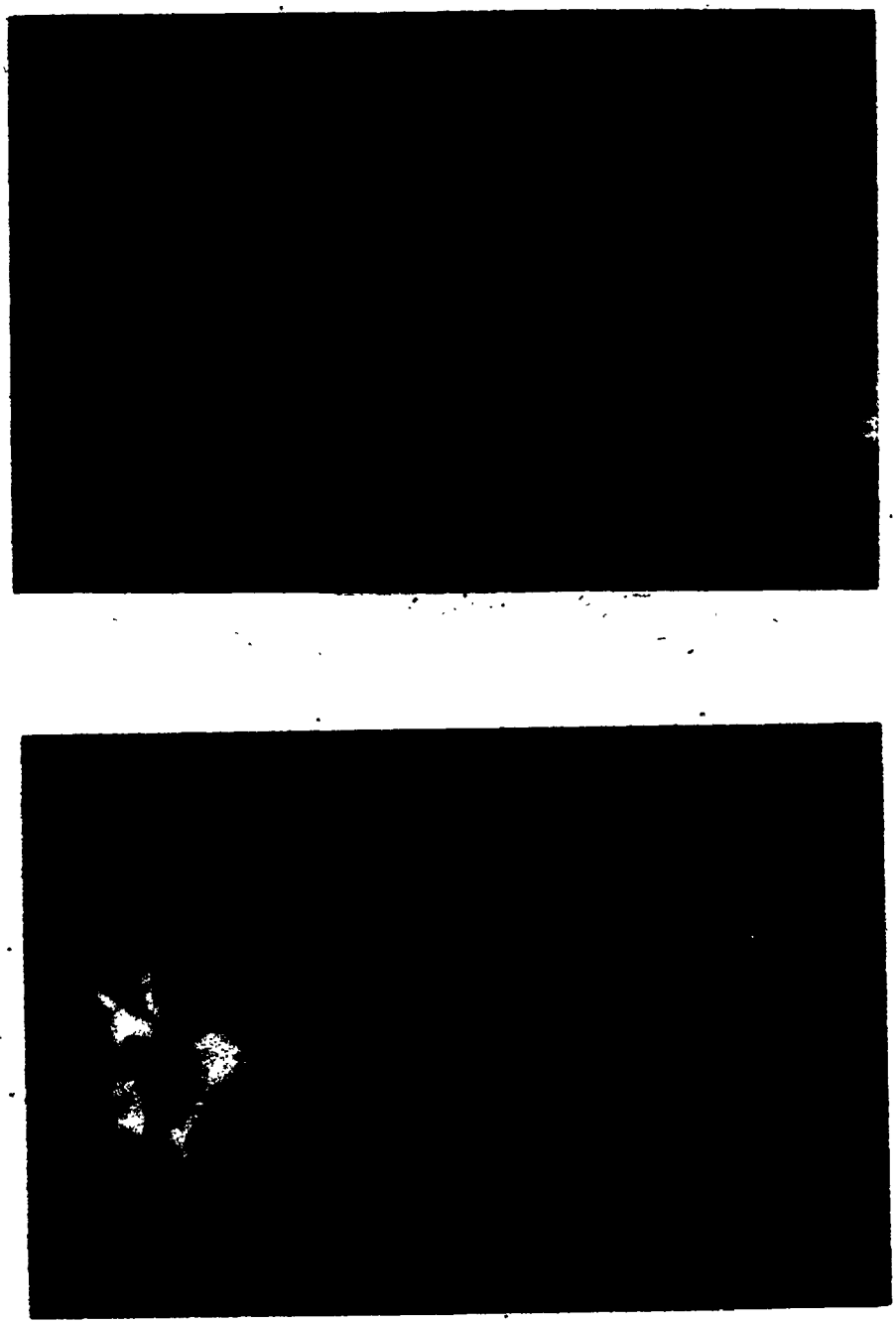

Frontispiece

Male flowers of *Silene alba* not infected (top) and infected (bottom) with *Ustilago violacea*.

ABSTRACT

The anther smut fungus, *Ustilago violacea*, grows either saprophytically (yeastlike cells) or as a parasite of the Caryophyllaceae (mycelial cells). Factors affecting two types of development controlled by the mating type gene, sporulation and myceliation, were examined.

Sporulation: Freshly isolated diploid cells, heterozygous for the mating type alleles, a_1 and a_2, develop into sexual phase precursor cells (SPP) on complete medium at temperatures below 20°C. The a_1/a_2 diploid produces at least four different cell types, termed opaques, spontaneously at high frequencies (>3 x 10^{-3}). These types are: 1) neutral strains (op-N) which do not mate and still initiate sporulation but under altered conditions. 2) Strains which mate as a_1 types (op-a_1) and 3) cells which mate as a_2 types (op-a_2). 4) Constitutively self mating (op-C) strains. Types 2 to 4 have lost the ability to develop into SPP cells. These four strains were shown to remain diploid and to be altered only at the mating type locus or chromosome. Genetic analyses of tetraploid (op-a_1 x op-a_2) and triploid (op-a_1 x haploid a_2) crosses indicated that the mating opaques probably arise after mitotic crossing - over yielding a_1/a_1 (op-a_1) and a_2/a_2 (op-a_2) types. Experiments showing similar increases in opaque frequency and mitotic recombination near marker alleles following UV irradiation support this conclusion.

Myceliation: Aqueous extracts from plants that host *Ustilago* species were found to induce the mycelial stage of *U. violacea* and some other smut species in cells that were potentially pathogenic i.e. expressed both mating type alleles. Aqueous extracts from most non-host species were inactive. However, efficient extraction of all tested plant species with organic solvents indicated that the stimulatory compound was universal in distribution. α-Tocopherol was identified as the major active compound in plant extracts. All tested host species contained amounts of tocopherol above the threshold level (5 x 10^{-8} M) while non-hosts had either 1) above

threshold levels of tocopherol; 2) above threshold levels of tocopherol plus toxins or inhibitors; or 3) sub-threshold levels of tocopherol. These results suggest that the availability of tocopherol may form one basis for the restriction of the host range of *Ustilago* species.

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CHAPTER 1

INTRODUCTION

In recent years, members of the order Ustilaginales have been developed as useful species for genetic research. The presence of an easily cultured haplophase in some species as well as the economic importance of cereal smut fungi has led to this exploitation (Day, 1974). Research on the causative organism of corn smut, *Ustilago maydis*, has provided valuable information on the mechanisms of DNA recombination and gene conversion (Holliday, 1964; 1974), pathogenicity of diploids versus haploids (Holliday, 1961), and the inheritance of cytoplasmic virus - like particles (Pulhalla, 1968; Day and Anagnostakis, 1973).

Ustilago violacea, the anther smut fungus which attacks species in the Caryophyllaceae, was developed as a tool for genetic research by A. W. Day (Day, 1968; Day and Jones, 1968; 1969). Since then, extensive investigations into the biology, structure, genetics, and physiology of this fungus have been carried out in both Dr. Day's laboratory, and more recently in Chicago by Dr. E. D. Garber and his associates. A short review of some of the aspects of our understanding of this species relevant to this thesis is given below.

1.1 Life Cycle of *Ustilago violacea*

Over 70 species of the Carnation family, Caryophyllaceae, are susceptible to infection by the fungus *Ustilago violacea* (Zillig, 1921; Liro, 1924). Within this fungal species, many physiological races defined by the specificity for particular host species have been described (Liro, 1924; Goldschmidt, 1928). The race most frequently used during this work was an isolate from *Silene alba*, known commonly as White Campion.

The life cycle of *U. violacea* (fig. 1), as described in Fischer and Holton (1957), consists of a saprophytic and a parasitic state. Diploid teliospores form in the anthers of infected plants and are the most conspicuous symptom of disease. They appear as a purplish - brown powder replacing the normal creamy - yellow pollen (frontispiece). The teliospores germinate readily on a moist surface to produce a three celled promycelium. Meiosis occurs during germination and the four haploid products bud off as yeastlike cells (sporidia) from the teliospore and the three cells of the promycelium. Continued mitotic divisions occur on nutritive media in the absence of host plant exudates, and many haploid sporidia are therefore produced. The organism can be grown indefinitely on such media by continued budding of these saprophytic sporidia.

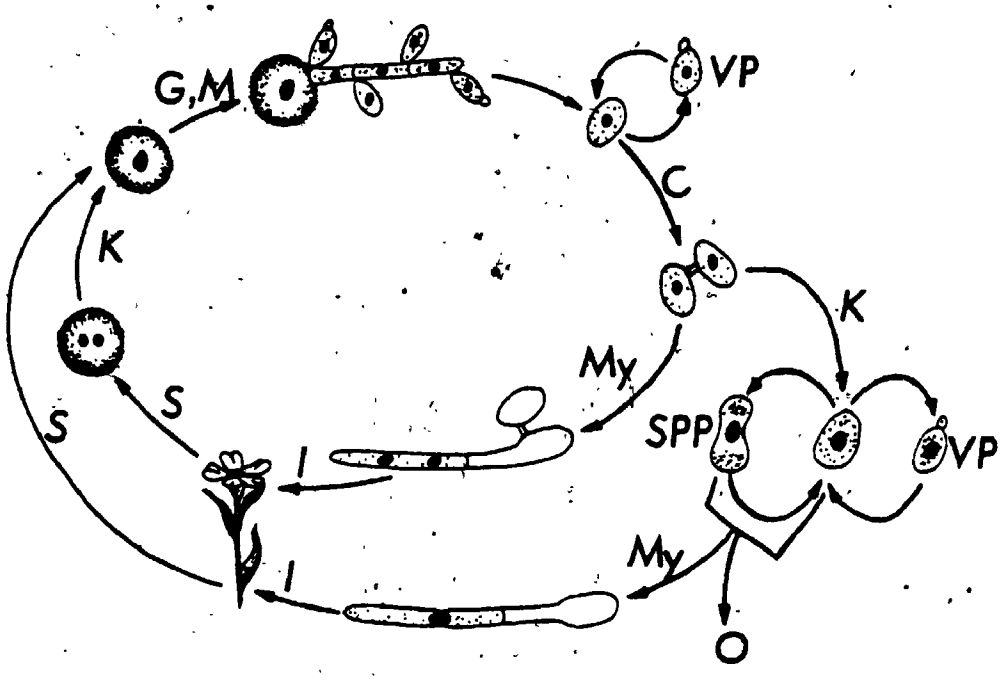
The sporidia are of two mating types, determined by alleles a_1 and a_2 of the mating type locus. On the surface of a suitable host, conjugation of compatible sporidia (a_1 with a_2) initiates the dikaryophase. Infection hyphae, "suchfaden", develop from the conjugated cells on the surface of the host, and form infection structures, an appressorium and a penetration peg. After penetration, continued growth of dikaryotic hyphae occurs primarily intercellularly and the whole plant becomes infected (systemic infection). Persistence of the dikaryon is completely dependent upon the host plant, i.e. this phase is obligately parasitic. Damage to the host during this process is minimal as no foliar symptoms are observed, apart from a mild stunting of growth (White, 1936; Baker, 1947; Spencer and White, 1951).

Teliospore formation is initiated when hyphal growth reaches the sporogenous cells of either male or female (*S. alba* is dioecious) plants (Batcho, Audran, and Zambettakis, 1979). In plants of either sex, the sporogenous cells are completely destroyed by the fungus (Batcho, Jean, and Bouriquet, 1980). Thus, in male plants (XY chromosome type; Warmke, 1946), fungal teliospores completely replace pollen in the anthers. In female plants (XX chromosome type), ovary tissue aborts and some teliospores are formed in this area (Batcho *et al.*, 1980). However, more strikingly, the fungus induces development of the normally vestigial stamens and anthers which then become filled with teliospores. In effect, female plants produce male

Figure 1. Life Cycle of *Ustilago violacea*

Legend:
- G - Teliospore germination.
- M - Meiosis.
- VP - Vegetative growth phase (see Chapter 3).
- C - Conjugation.
- K - Karyogamy.
- SPP - Sexual spore precursor phase (see Chapter 3).
- My - Myceliation (see Chapter 4).
- I - Infection.
- S - Teliopsore formation.
- O - Formation of opaque strains (see Chapter 3).

(Based upon Fischer and Holton, 1957; amended to include results presented in this thesis.)


G,M
VP
C
K
K
My
S
S
SPP
VP
I
My
I
O

flowers in response to parasitic growth, and the fungus effectively takes over the role of the plant Y chromosome. The mechanism of this fungus induced sex change has not been determined.

Sporulation is completed while the hyphae are still dikaryotic. Nuclear fusion, or karyogamy, within the teliospore produces the diploid phase. As reductional division occurs upon germination, the diplophase, therefore, usually occupies a very short segment of the life cycle.

1.2 Use of *U. violacea* for Genetic Research

Ustilago violacea was studied extensively during the late nineteenth and early twentieth century (Fischer and Holton, 1957; Day and Cummins, 1981). During this period, the life cycle, including details of saprophytic growth and parasitic effects on the host plant, was described. More recently, this fungus was developed as a tool for genetic research (Day, 1968). This later work is reviewed here.

The isolation of several auxotrophic and colour mutant strains of race SA-1 (Day and Jones, 1968, 1969; Garber, Baird, and Chapman, 1975) provided the markers for use in mapping by both sexual and parasexual techniques. Random spore analysis (Day and Jones, 1969) and several variants of tetrad analysis (Cattrall, Baird, and Garber, 1978; Garber, Will, and Kokontis, 1981; Garber *et al.*, 1982) have been developed to analyse the meiotic products from germinated teliospores. Vegetative diploid strains, i.e. strains that bud as diploid sporidia, selected by complementation between auxotrophic markers in conjugated haploid cells, have been used for parasexual mapping analysis. This type of analysis involves either haploidization with p-fluorophenylalanine (Day and Jones, 1971) or mitotic crossing - over following irradiation with ultraviolet light (Day, 1968; Day and Jones, 1969). More recently, Day (1978) devised a chromosome transfer technique for assigning newly selected markers to particular linkage groups. Details of each of these procedures can be found in the Materials and Methods section of this thesis (Chapter 2). Further types of analysis are currently being developed by Garber's group at the University of Chicago and are being

used to continue mapping of this organism as well as to study non-disjunction, crossing - over, and polymorphisms in natural populations (Garber *et al.*, 1975, 1978, 1981, 1982; Baird and Garber, 1979a,b).

This mapping work, together with cytological studies, has shown that the haploid chromosome number of this species is more than 12, perhaps in the order of 20 (Day and Jones, 1968, 1969, 1971, 1972; Poon, 1974; Poon and Day, 1976b). These observations refuted earlier studies which assigned a chromosome number of n = 2 for *U. violacea* (Kharbush, 1927, 1928; Wang, 1932a,b; Wang, 1943; Person and Wighton, 1964). Apparently, these earlier estimates were due to confusion with the "two - track" appearance of chromosomes at mitotic metaphase (Day and Jones, 1972; Poon and Day, 1976b).

Cytological studies were also used to provide a detailed description of mitosis in this organism (Day and Jones, 1972; Poon and Day, 1974a, 1976a,b). Particular importance was placed on the structure and function of the spindle pole body as a controlling feature for microtubule formation (Poon and Day, 1976b). Separation of the two new nuclei is initiated in the daughter cell and one nucleus migrates back to the mother cell. Nuclear membrane breakdown is minimal and microtubules can be seen extending through the elongated dividing nucleus to a spindle pole body at each apex (Poon and Day, 1976b). Complete reformation of the nuclear envelope occurs slightly before septum formation completes cytokinesis (Poon and Day, 1976a).

The events controlling formation of the conjugation tube have been described (Poon, Martin, and Day, 1974; Cummins and Day, 1974, 1976; Day and Cummins, 1974). Conjugation occurs only between cells of opposite mating type, a_1 and a_2. The morphogenetic sequence resulting in formation of the conjugated pair is initiated by the exchange of information between mating cells (Cummins and Day, 1974, 1976; Day and Cummins, 1974). Cells can conjugate when touching or when separated by distances of three to five cell widths (up to 20 μm). Long protein fibrils, termed "fimbriae", appear to provide the first connection between the conjugants (Poon and Day, 1974b, 1975). Once a fimbrial connection has been established, the a_2 cells produce conjugation pegs which grow out as conjugation tubes towards

a_1 cells. The a_1 cells develop small protuberances at the eventual site of union, and the membranes and walls of both cells disintegrate at the point of contact and a cytoplasmic bridge is formed (Poon *et al.*, 1974).

Karyogamy (nuclear fusion) does not normally follow immediately after plasmogamy in this organism. The conjugants revert to haploid budding when placed on a nutritive medium, but form hyphae when placed on a suitable plant surface. On media which do not permit growth, eg. not containing a nutritional requirement, diploid sporidia form at a rate of about 1 per 10^4 conjugations. Ultraviolet irradiation (254 nm) increases the frequency of diploid formation to much higher levels, 1 in 10^3 amongst the survivors (Clements, Day, and Jones, 1969; Day and Day, 1974). Apparently, somatic karyogamy is normally repressed in these cells (Day and Day, 1974). This repression appears to be overcome by UV treatment, and is somehow negated in teliospores prior to germination (Day and Day, 1970).

As mentioned previously, diploids can be isolated using a selection system provided by the complementation of various recessive markers. Freshly isolated diploids have sporidial and colonial phenotypes different from either parental haploid. These differences arise from the mating type locus as the diploid phenotype is generated by the summation of both a_1 and a_2 allele activity (Day and Cummins, 1976). A regulatory locus (cc) has been postulated to control expression of the mating type alleles during the cell cycle (Day and Cummins, 1972; Cummins and Day, 1973). This gene appears to be closely linked to the mating type locus and to be cis - dominant. The cc allele normally associated with the a_1 mating type allele permits expression of the a_1 phenotype only during G1, a feature which has been termed "stringent" control. The a_2 allele is expressed throughout the cell cycle and its corresponding cc allele, therefore, exhibits "relaxed" control. One isolate of an a_1/a_2 diploid strain was tested to see how these control systems work when paired together in a diploid. It was found that this diploid mated as an a_2 cell but only in the S and G2 phases. This was explained as an interaction between the relaxed and stringent controls operating on the a_2 and a_1 alleles of this strain (Day and Cummins, 1972; 1975). However, other diploid strains tested since did not show any mating

type activity with either haploid at any time in the cell cycle. This confusion over the mating ability of diploids is explained at least in part by the observations reported in this thesis that a_1/a_2 diploids are unstable and give rise frequently to a variety of derivative types which are specifically altered in mating type activity (Chapter 3).

The role of the host in eliciting or repressing expression of fungal genes and thereby altering fungal morphogenesis has begun to be investigated. Day observed that certain plant extracts induce conjugated cells to develop extensive mycelial growth on artificial media. Previously, all growth on these media had been strictly sporidial and the mycelial stage was only seen in the host plant. This topic is covered in detail in Chapter 4 of this thesis.

1.3 Aims of this Thesis

The aims of this thesis were to investigate factors controlling development and differentiation in *Ustilago violacea*. The two aspects of differentiation that were studied, while involving quite different developmental pathways, share a common feature. Both illustrate the central role of the mating type locus as a developmental master switch.

In the first project, the frequency of production, characteristics and the mechanism of origin of diploid cells altered in sporulative development were investigated. This study concentrated on the nature of the genetic changes in the region of the mating type gene which affect the transition from vegetative growth to teliospore.

In the second project, external hormone - like chemicals found in host plant extracts were shown to act in concert with the two alleles of the mating type locus to initiate development and continued growth of vegetative hyphae while suppressing both sporulation and saprophytic vegetative growth.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture Conditions

The media outlined here were developed by A. W. Day (Day, 1968) and are described in detail in Cummins and Day, 1977. These media are useful for the culture of the sporidial phase of the life cycle and are similar to those used in yeast work. All chemicals were obtained from the Fisher Chemical Co., Fair Lawn, New Jersey, unless otherwise specified.

Media

(1) Water Agar (WA)

Agar	20 - 25 g
Distilled Water	To 1 litre

(2) Complete Medium (CM)

Glucose	10 g
Peptone	10 g
Yeast Extract	3 g
Malt Extract	3 g
Beef Extract*	1 g
Distilled Water	To 1 litre

For agar plates add 25 - 30 g agar.

(* not usually required)

(3) Minimal Medium (MM)

Glucose	10 g
Concentrated Salts	50 mL
Distilled Water	To 1 litre

For agar plates add 25 - 30 g agar.

Concentrated Salts

Sodium nitrate	20 g
Potassium phosphate	10 g
Potassium chloride	5 g
Magnesium sulphate	5 g
Calcium chloride	1 g
Ferrous sulphate	1 - 2 crystals
Thiamine	1 mg
Distilled Water	To 1 litre

The salt solution was autoclaved and then stored at 4°C.

(4) Supplemented Complete Medium

Complete medium supplemented with the following growth inhibitors was used.

i. cycloheximide (Sigma Chemical Co., St. Louis, MO) 50 mg/L

ii. carboxin (Sigma Chemical Co., St. Louis, MO) 10 mg/L

iii. p-fluorophenylalanine (PFP) (Sigma Chemical Co., St. Louis, MO) 300 mg/L

Both cycloheximide and carboxin were added to the CM prior to autoclaving. The PFP and one pellet of NaOH were dissolved in 10 mL H_2O. The solution was sterilized by irradiation with 254 nm UV light for 20 min, a total dose of 1-2 J/m^2. This sterile solution was mixed with the autoclaved CM just before pouring. i.e. after the medium had cooled to about 50°C. The

peptone concentration in the RFP medium was halved, using 5 instead of 10 g/L.

(5) Supplemented Minimal Medium

Supplements (0.5 mL) were added to each Petri plate before the MM was poured. The supplement and molten medium were mixed thoroughly by gently swirling the plate. The concentrations of the supplement stock solutions are as follows:

i. L - amino acids (Sigma Chemical Co., St. Louis, MO)
   2 g/L distilled water
ii. adenine and inositol (Sigma Chemical Co., St. Louis, MO)
   0.2 g/L distilled water

2.2 Stock Cultures

The strains used were derived from an isolate of *Ustilago violacea*, designated UWO-1 (Day, Castle, and Cummins, 1981), from the host plant, *Silene alba*, white campion. The fungus was collected by D. Snow of the University of Reading, Reading, U.K., and the various mutant strains were developed by A. W. Day (Day, 1968; Day and Jones, 1968, 1969). The accepted practice in the literature on fungal genetics is to use the term "haploid" in place of the proper term "monoploid". This convention will be adopted in this thesis.

The storage of cultures was accomplished in two ways. Cultures used on a daily basis were stored on CM plates in a 4°C refrigerator. Every month, the strains were subcultured, grown at 22°C and replaced in the refrigerator. Actively growing cultures were prepared by inoculating the desired medium with a small sample from the cold stored plates.

Stock Cultures

Haploids

Designation	Genotype
WT1Y	a_1 y
WT2Y	a_2 y
1.C2	a_1 y his_1
1.C2u4	a_1 y his_1 uvs_1
1.C2u6cx	a_1 y his_1 uvs_2 cxr_2
2.716	a_2 lys_2
2.716cx	a_2 lys_2 cxr_1
1.716u6	a_1 lys_2 uvs_2
2.716u4cx	a_2 lys_2 uvs_1 cxr_1
1.D2716u4	a_1 his_1 lys_2 uvs_1
2.112	a_2 his_3
1.113	a_1 his_4
2.113	a_2 his_4
2.143	a_2 cit_1
2.4	a_2 arg_1
2.C4	a_2 y arg_1
1.729Su4	a_1 o $inos_1$ uvs_1

Designation	Genotype
2.729Su1	a_2 o $inos_1$ uvs_1
1.D2729C	a_1 y his_1 $inos_1$
1.D3143C	a_1 y lys_3 cit_1
2.D3143C	a_2 y lys_3 cit_1
2.D4110	a_2 arg_1 met_2
1.TM6	a_1 y orn_1 $inos_1$ met_3
1.T10cb_3	a_1 his_1 arg_3 lys_2 cb_3
1.W1	a_1 o his_1 $inos_1$ lys_2 uvs_1

Abbreviations: a_1, a_2 - mating type alleles; y - yellow colony colour; o - orange colony colour; his - histidine requirement; lys - lysine requirement; inos - inositol requirement; met - methionine requirement; arg - arginine requirement; orn - ornithine requirement; cit - citrulline requirement; uvs - ultraviolet light sensitive; cxr - cycloheximide sensitive; cb - carboxin sensitive.

Diploids

Designation	Cross
D10	1.729Su4 x 2.D3143C
C4/118	1.D2716u4 x 2.C4
B32	1.TM6 x 2.143
B33	1.TM6 x 2.112
B45	1.D2729C x 2.D4110

Designation	Cross
X2	1.D3143C x 2.716u4cx
X6	1.W1 x 2.113
X8	1.113 x 2.4
P3	1.D3143C x 2.716u4cx
P4	1.D3143C x 2.729Su1

For very long periods, the various strains (as well as other races of *Ustilago violacea* and several other fungal species) were stored desiccated on silica gel at -10°C. The storage bottles were prepared by adding about 5 g of the silica (Davison Chemicals; Grade 12, 28-200 mesh) and heating to 200°C for 30 minutes to remove as much water as possible. The bottles were tightly capped after a short cooling period and stored for future use. For culture storage, a small volume of packed cells (0.1-0.5 mL) was placed on top of the silica, the bottle was recapped and left at room temperature overnight. The culture bottle was placed in the -10°C freezer. Cultures have been kept for at least 10 years using this procedure. Actively growing cultures were obtained from silica stocks by placing a small piece of the dried fungus on a CM plate and incubating at 22°C for 24 h. The rehydrated cells were spread over the plate and left for 4-5 days at which time a thick layer of sporidial growth was present.

1.3 Mating Tests

Conjugation between haploids of opposite mating types is induced by both low temperature and low nutrient conditions. The recovery of mated dikaryons is greatest after a 24-48 h incubation period at 15°C on water agar (Day, 1968; Day and Jones, 1968). This medium and temperature were used to determine the mating type of any strain or colony. A small sample of the culture was transferred to two spots on a WA plate by means of a sterile wire loop. An approximately equal amount of a known a_1 haploid was mixed into one of the spots and a known a_2 into the other. The

following day the mixed cultures were dispersed into drops of water on a microscope slide and screened for the presence of conjugation tubes. Since mating is only possible between opposite mating types, the presence of conjugated cells means that the unknown must express the mating type allele opposite to the known strain.

2.4 Host Plant Infection

The method used for infecting the host species, *Silene alba*, was based on that described in Day (1968). The cultures to be used were suspended in sterile water at a concentration of about 10^8 cells/mL. Several drops were placed in the leaf axils of young seedlings (6-10 leaves) and the plants were wounded at this site by gently scraping with a flame sterilized dissecting needle. While there are minor symptoms of infection prior to flowering (Baker, 1948), the most obvious symptoms occur in the stamens of the flowers. In normal infection, the anthers were distended and dark purple in colour due to the mass of teliospores (brandspores) produced. The petals were liberally dusted with spores when the flower opened. In certain cases (e.g. inoculation with a single diploid strain), the infection was much less vigorous, at least during sporulation. In these cases, there was only a slight discoloration of the anthers to a dark yellow or light brown. Far fewer teliospores were recovered from these infections. After harvesting, some of the infected flowers were stored in silica gel vials as outlined in section 2.2. Teliospores were used for random spore or tetrad meiotic analyses.

2.5 Sexual Analysis

Two methods of meiotic analyses were used in this work: random spore and tetrad. For random spore analysis, an infected anther was crushed with a sterile glass rod into approximately 1 mL of sterile water. This suspension was plated onto CM and incubated at 22°C for 3-4 days, until small colonies (0.5 mm diameter) appeared. These colonies contain the four genotypes produced during meiosis. The plate was then scraped with a wire loop and the cells suspended in sterile water. The concentration was estimated

roughly from the turbidity of this suspension and an appropriate dilution series was made. Sufficient cells were plated on CM at 22°C to produce about 300 colonies per plate. A representative sample of these monosporidial colonies was removed with a wire loop and placed in rows on a series of master plates (about 30-40 colonies/ plate). After 2 or 3 days further incubation, the master plates were replica plated (Lederberg and Lederberg, 1952) to an appropriate series of supplemented MM and CM plates. The genotypes of the meiotic progeny were determined by inspection of these plates after a 4-5 day incubation period at 22°C.

For unordered tetrad analyses, infected anthers were crushed in sterile water and the suspension was filtered through a small plug of glass wool in a Pasteur pipette to remove clumps of teliospores. The filtrate was examined microscopically to verify that only single teliospores remained. The process was repeated if too many clumps were present. The teliospore suspension was subsequently diluted and plated on CM. After 3 days, the very small colonies containing the four haploid products of meiosis were streaked onto separate CM plates in order to separate out the four progeny. Samples of colonies from the streak plates were transferred to CM master plates which, following incubation, were replica plated to a series of supplemented MM and CM to determine genotypes. Occasionally, only three genotypes were recovered from a single teliospore. In this case, the fourth type was deduced by reasoning that both alleles of every gene must appear twice. When less than three or greater than four genotypes were recovered, the result was ignored.

2.6 Parasexual Analysis: Mitotic Haploidization

The production of haploids from a diploid during the mitotic rather than the meiotic cycle was accomplished using the chemical, para-fluorophenylalanine (PFP). This technique was first described for the ascomycete, *Aspergillus niger* (Lhoas, 1961) and later developed for *U. violacea* (Day, 1968; Day and Jones, 1968). Between 10^8 and 10^9 diploid sporidia were spread evenly over the surface of a PFP plate. The concentration of the chemical (0.3% w/v) was inhibitory to the fungus. The

plate was incubated at 22°C and after a period of 3-4 weeks small, round colonies, termed "papillae" appeared amid a background of slow growing or dead cells. Most of these colonies are monoploid or disomic for one or two linkage groups and can grow on the PFP plate as a result of an acquired resistance to the chemical (Day, 1968). The diploids used in these analyses were heterozygous for a gene determining colony colour. Consequently, papillae expressing the recessive yellow allele (wild type pink is dominant), indicative of some chromosomal loss, were selected. These papillae were transferred to master plates and the genotypes determined by means of replica plating.

2.7 Parasexual Analysis: Mitotic Recombination

Exposure of a diploid to ultraviolet light is known to induce mitotic recombination in *U. violacea* (Day and Jones, 1969). In this work, the diploid D10 (see section 2.2 for genotype) was treated with a 180 J/m^2 dose of 254 nm ultraviolet light. Several dilutions were plated on CM and incubated at 22°C for 1-2 weeks, until large colonies were present. An unirradiated control culture was treated similarly. The total number of colonies on each plate was determined using a model 600 Fisher colony counter or by direct counting. Each plate was replica plated to three plates of CM and one plate of MM. One of the CM plates was incubated at 22°C with no further treatment. This plate was used to detect colour recombinants and any errors in replica plating. A second CM plate was irradiated with 6.0 J/m^2 254 nm UV light in order to detect any ultraviolet sensitive recombinants. The original heterozygous uvs+/uvs diploid was resistant to this dose. The final plate of CM and the MM plate were incubated at 15°C with no further treatment. These plates were used to determine the number of opaques and auxotrophs recovered.

2.8 Mapping with the Chromosome Transfer Method

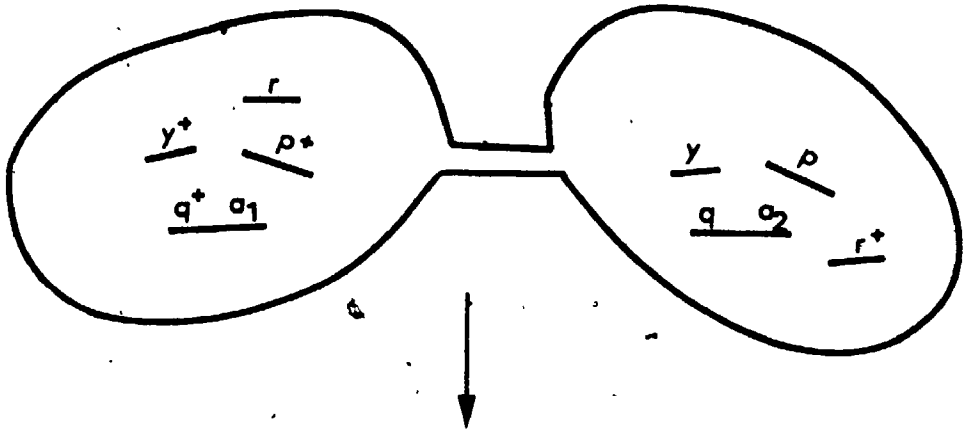
The search for markers linked to the mating type gene involved the production of new mutant strains and the screening of these strains using the chromosome transfer technique (Day, 1978). When conjugated haploid

cells carrying complementary auxotrophic markers are plated on nutritionally deficient media such as MM, one of three events occurs: a) cell death; b) fusion of nuclei to give a diploid strain (frequency about 3×10^{-4}, Day and Jones, 1968); or c) transfer of one to a few chromosomes from one genome to the other (frequency about 1×10^{-3}, Day, 1978). Following event "c", the acquired chromosomes often persist in the recipient cell and this new "aneuploid" strain can be selected and analyzed. Frequently the acquired chromosomes are lost under non-selective conditions, and in this case they may be either lost as a group (Day, 1978) or singly (Day, pers. comm.). Two genes on the same chromosome are always transferred together even when one is unselected (Day, 1978). On the other hand, selection of one marker results in recovery of an unselected unlinked marker only 10-20% of the time. Thus, the phenomenon involves the acquisition of a limited number of whole chromosomes rather than specific loci. This phenomenon was exploited to provide a technique for the screening of markers linked to mating type, as selection for a mating type allele would allow simultaneous selection for any markers linked to this allele (fig. 2).

A yellow a_2 haploid with the unmapped mutation was mated with a pink (wild type, dominant) lysine requiring strain, 1.716u6. Prototrophic colonies were selected by plating the crosses on MM. Yellow prototrophs were presumed to be aneuploid rather than diploid as they had acquired the chromosome carrying the wild type allele for the auxotrophic mutation but not the different chromosome carrying the pink allele. If the prototrophic allele was linked to mt, then all of the yellow prototrophs would be expected to exhibit the colonial and sporidial characteristics of a_1/a_2 heterozygotes (section 3.1). If not, only at most 10-20% would show this phenotype. Thus screening for linkage to the mating type locus was performed by examining the prototrophs arising from a large number of auxotrophic strains carrying the yellow marker. Any strain in which almost all the yellow prototrophs had the translucent colonial morphology of a_1/a_2 cells would be highly likely to contain an auxotrophic marker linked to mt.

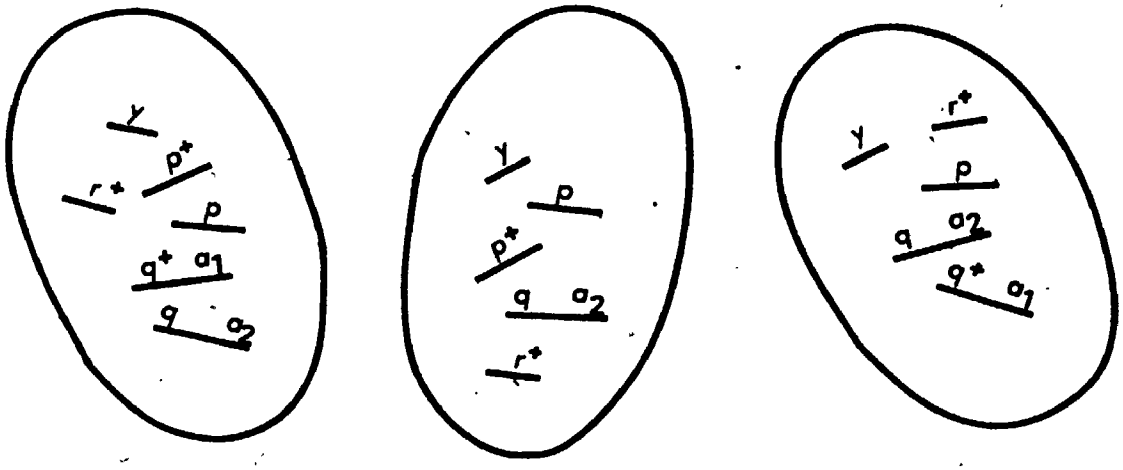
Figure 2. Chromosome Transfer Selection Technique

The expected results for a marker linked (q) and not linked (p) to the a_2 allele of the mating type gene are outlined. Selection for the wild type (q^+) allele results in the appearance of the linked a_1 allele in 100% of the progeny. As the p^+ allele is not linked to the a_1 mating type allele, transfer of the a_1 allele to the progeny occurs randomly when the p^+ allele is selected. In practice when an unlinked marker was selected, the observed percentage of colonies which showed both the wild type phenotype of the unlinked gene and the a_1/a_2 heterozygous phenotype was less than 10%. The r marker is used to ensure that the parental y^+ q^+ p^+ a_1 cell does not grow on the selection plates.



Selection for:

y r+p+ y r+q+



<10% of colonies
y p+ a_1/a_2
phenotype

>90% of colonies
y p+ a_2
phenotype

100% of colonies
y q a_1/a_2
phenotype

2.9 Bioassay: Induction of Hyphal Growth

Two assays were used in the course of this work. Test A was used very infrequently and test B frequently. For test A, extracts were applied before mating had occurred. Cells of opposite mating type were mixed on a WA surface and flooded with 0.2 mL of plant extract (see section 2.10). Following overnight incubation at 15°C, the entire plate was placed on the stage of a Zeiss RA microscope and the cells on the surface examined using a x16 or x40 objective. Two characteristics were scored for: presence of long conjugation pegs ("wild pegs"), usually initiated by a_2 cells; and presence of long infection hyphae arising from cells that had conjugated overnight.

In test B extracts were tested on already mated haploid sporidia or primary diploids. A stock culture, containing 40-80% mated cells, was kept on WA at 4°C for periods of up to 4 weeks. Cells were scraped off these stock plates and spread on a small section of WA or MM before flooding with 0.2 mL of extract or test solution. When several tests were being performed simultaneously, each plate was divided into 4 or 6 sections by removal of narrow strips of agar. One test was then done on each agar section. The plates were incubated at 22°C and examined 24-48 h later for the presence of hyphae. Control cultures were treated with 0.2 mL of sterile water (negative controls). Where different species or conditions were being tested, a control treated with 0.2 mL of an active *S. alba* extract was also used (positive control). For most tests, a simple scoring system was used: +++, 60-100% of the conjugated pairs produced infection hyphae; ++, 10-60%; +, 1-10%; and 0, <1%, or no greater than control cultures treated with water.

For quantitative experiments, 0.1 mL of a suspension of cells from the stock mated culture, containing about 10^7 cells/mL, and at least 60% mated cells, was plated on WA or MM and allowed to dry. A specific volume of the appropriate test solution was then applied to the cells. Twenty-four hours later, each test spot was photographed. The photographs were used to provide accurate measurements of a) the lengths of hyphae and b) the percentage of conjugated pairs of cells with hyphae (percent germination).

A variety of plant extracts were tested in the bioassay. Aqueous extracts were applied directly to the cells without further adjustment. Acidic or basic extracts were first neutralized with sodium hydroxide or hydrochloric acid, respectively, followed by a small volume of 0.2 M phosphate buffer, pH 6.8. Extracts in organic solvents were usually dried by blowing air into a sample followed by resuspension into water. Frequently, organic solvents were diluted 10 or 100-fold into water. This emulsion or solution was vigorously shaken and then applied to the cells. It was found that most organic solvents used (n-hexane, methanol, ethanol, and acetone) were not toxic at these concentrations. Toxicity was assessed as cell lysis or as a shrinking of the cytoplasm, both resulting in a loss of refractivity when viewed microscopically.

Over 200 chemicals were tested in the bioassay during the course of this work. A few crystals or drops of the compound were placed in the centre of the smear of cells. After overnight incubation, the cells were examined and if the compound was toxic, the test was repeated using a diluted, neutralized (if acidic or basic) sample.

2.10 Extraction of Plant Tissue

Several extraction procedures were used to remove the active components from plant tissue. The ones most commonly used were aqueous, cold methanol or acetone extractions.

Plant material used for extraction was obtained from three sources: a) greenhouse and herbarium specimens supplied by numerous members of the department; b) samples collected in the field; or c) bought at local food stores, e.g. parsnip and broccoli.

2.10.1 Aqueous Extracts

Fresh leaf material was macerated in distilled H_2O using either a Waring blender, a Sorvall omni-mixer, or a mortar and pestle. Twenty mL of water per g leaf tissue (fresh weight) was used. The extract was filtered through Whatman No. 1 filter paper or centrifuged at 1250xg for 5 min

to remove debris and tested in the bioassay. This procedure, termed "unconcentrated aqueous" extraction, was used to test a wide variety of plant species (Appendices 1-4). Frequently these extracts were autoclaved at 15 p.s.i. for 15 min. This sterilization did not noticeably destroy activity. For "concentrated aqueous" extracts, 3 mL of H_2O per g tissue was used and the extract was centrifuged at 27000xg for 20 min in a Sorvall RC-5 centrifuge. The pellet was discarded and the supernatant kept for further testing. Aqueous extracts were also concentrated by means of lyophilization. Both the sample and the lyophilizer flask were placed in either a -20°C or -70°C freezer. Once frozen, the sample was attached to the lyophilizer and dried overnight. The resultant powder was extracted/ dissolved with the desired solvent.

2.10.2 Acidic Extracts

Acidic extracts were used for the extraction and assay of ascorbic acid contained in the leaf tissue of a variety of species. Cold (4°C) 0.35 M m-phosphoric acid (as 2:1 $NaPO_3$:HPO_3, Fisher Chemicals) and 1.4 M acetic acid were substituted for the H_2O in the concentrated aqueous extraction procedure. Subsequent dilutions were made in cold 50mM perchloric acid prior to chromatography and were neutralized prior to testing in the bioassay. This protocol is an adaptation of the technique outlined in Pachla and Kissinger (1976).

2.10.3 Methanolic Extracts

Both hot and cold methanolic extractions were used frequently to determine the presence of hyphal inducing compounds in plant tissue (see Chapter 4). Hot extractions involved immersion of plant tissue for 20 min in methanol (1 g tissue/ 20 mL solvent) heated to boiling (63-64°C) over a hot water bath. The methanol was decanted and the procedure repeated until the tissue was white. All solvent washes were pooled and reduced to an aqueous residue in a rotary evaporator with a water bath set at 35-40°C. When stored at 4°C, the unevaporated extracts did not retain activity for more than 3 days even though the activity of these extracts,

when freshly prepared, was equal to the acetone extract (see below).

Cold methanol extractions were carried out with the solvent at room temperature. This procedure was used primarily for the extraction and concentration of the major active component from the leaves and roots of *Silene alba* and, later, *Pastinaca sativa*. The tissue was macerated in a Sorvall omni-mixer with 3 mL of methanol per g of tissue. Batches of up to 30 g were treated this way. The solvent was decanted and centrifuged at 27000xg for 20 min. The pellet and the debris from the maceration were combined and re-extracted. The supernatant was evaporated to an aqueous residue as in the hot methanol procedure. Further centrifugation (27000xg/ 20 min) resulted in the precipitation of the active component from the water. This pellet was washed with 1-2 mL of HPLC grade methanol and recentrifuged as above to remove any large particulate matter prior to chromatography. Two extractions were sufficient to remove virtually all of the active component from the tissue.

2.10.4 Extraction with Other Organic Solvents

Freshly collected leaves were ground with a mortar and pestle and 20 mL of acetone were added per gm of leaf tissue. The extract was decanted to remove larger debris and stored in a tightly capped bottle at 4°C. These extracts were stable for as long as 3 or 4 months and old extracts were commonly used as positive controls in the bioassay. For more extensive procedures, however, only fresh preparations were used. Dried leaf material from herbarium specimens were also extracted using this procedure.

At various times, other organic solvents (n-hexane, n-heptane, n-butanol, chloroform, diethyl ether, or ethyl acetate) were substituted for acetone in the extraction.

2.11 High Performance Liquid Chromatography (HPLC) - General Procedures

Most chromatographic procedures employed an Altex model 330 liquid chromatograph equipped with a fixed wavelength ultraviolet absorbance detector or a Kratos FS970 fluorescence detector. Results were recorded

on a Hewlett-Packard 3390A integrator. Occasionally, a Hewlett-Packard 1084A liquid chromatograph was used. Reverse phase separations were carried out on either an Ultrasphere-ODS column (Beckman Associates), silica gel covered with octadecylsilyl moieties, or a Hamilton PRP column, containing a styrene-divinylbenzene copolymer. One ion exchange procedure required a Zorbax-NH_2 column (Dupont) containing silica gel-NH_2 packing. Normal phase chromatography was run on two silicic acid columns: Hewlett-Packard Si-100 or Brownlee Spheri-5. Specific details on each technique are provided below.

Certain procedures necessary for the prevention of serious damage to a liquid chromatograph and columns were adopted in the course of this work. These procedures centred on both preventative measures such as solvent and sample preparation and reparative measures to correct the inevitable minor deterioration associated with day to day operation.

Proper preparation of solvent systems was found to be crucial for column maintenance and reproducibility of results. All organic solvents were "HPLC grade" purchased from Fisher Chemicals or JT Baker Chemicals. Those used for normal phase chromatography on silica gel columns were stored over molecular sieves (4A, 8-12 mesh; Fisher) to remove as much water as possible. The exception to this precaution was methanol which was found to react with this particular type of sieve. Instead, methanol and pre-mixed solvent systems containing methanol were stored in a desiccator containing anhydrous copper sulphate, $CuSO_4$. Water suitable for HPLC use was prepared by passage of double distilled water through a reverse phase column (Bondapak-C18, Waters Associates) dedicated to this task. Finally, all solvents were filtered through 0.45 µm Millipore filters (HAWP for aqueous solvents, GVWP for organic mixtures) and degassed prior to use.

Samples were dissolved only in solvents miscible with the mobile phase, e.g. no phosphate buffer in sample if methanol was the running solvent. Before injection, all samples were centrifuged for 20-30 min at 27000xg (15000 rpm in an SS-34 rotor, Sorvall RC-5 centrifuge) to remove any large particulate matter. Centrifugation was found to be as good as, if not better

than, Millipore filtration for removing particles and less sample was lost.

Guard columns were employed for most procedures in order to prolong the life of an analytical column. The guard column was prepared by packing empty columns (Column survival kit, Whatman) with either silica gel from a normal phase Sep-pak cartridge (Waters Associates) or particulate silica gel covered with octyldecylsilyl (ODS) residues (Whatman). These packings were suspended in an appropriate solvent and loaded into the column via a Pasteur pipette. Periodically, the packings were flushed out and replaced. No difference in column efficiency was noticed when a guard column was used compared to the same system without it.

Despite these precautions, the equipment and columns occasionally did get contaminated as evidenced by irregular, inconsistent results and/or changes in operating parameters such as flow rate or back pressure. If the column was the source of the irregularity, regeneration was necessary. For reverse phase columns, the system was flooded with a series of solvents decreasing in polarity, e.g. H_2O to 50/50 H_2O/CH_3OH to CH_3OH. Normal phase silica columns were washed with a solvent series increasing in polarity, e.g. n-hexane to 50/50 n-hexane/ethyl acetate to ethyl acetate. This particular sequence was useful for the removal of water which was observed to alter drastically the chromatographic conditions. For both types of chromatography, the columns were cleaned by washing with solvents in an increasing order of elution strength. To return to original conditions the order was reversed. Occasionally, the column inlet frit became plugged and the cleaning series had no effect. In this case, the frit was replaced by either a new frit or one that had been cleaned in a sonicator. The sonication process only partially regenerated the frit but this was usually sufficient to warrant re-use.

In other instances, the Altex chromatograph had to be repaired or cleaned. Usually, this simply involved replacing the seal around the piston responsible for the influx and efflux of solvent within the pump. This problem was manifested by the presence of solvent behind the seal. A more serious problem occurred when the inlet and outlet check valves, also

regulating solvent uptake and output, became soiled. The result of this contamination was an actual flow rate less than the set flow rate, particularly when back pressure, i.e. from the column was applied. Soaking the valves in isopropanol for several hours and sonication in acetone was found to be an effective cleaning protocol.

Final verification of the efficacy of cleaning and repair procedures was by measurement of column efficiency both before and after these measures. Three parameters were calculated: a) efficiency or the number of theoretical plates per metre; b) k' values also known as capacity factors; and c) selectivity or α values. The number of theoretical plates per metre (N) is given by the formula: $N = 5.54\ (t_R/w_{1/2})^2$. Capacity factors are calculated with the formula: $k' = (t_R - t_0)/\ t_0$, and selectivity defined as: $\alpha = k'_2 / k'_1$. In these formulae, t_R = retention time, t_0 = retention time of an unretained compound or the void volume, and $w_{1/2}$ = the peak width at half height.

2.11.1 Chromatography of Ascorbic Acid

Two chromatographic techniques were developed for the isolation of ascorbic acid during the course of this work. The development of both techniques is described in section 4.5.2.

The first procedure utilized tributylamine (TBA) as a hydrophobic counter ion which complexed with ascorbate to increase the affinity of the column for the acid. The solvent system used to initially chromatograph ascorbic acid was 1 mM TBA in 10% methanol in water. For the analysis of plant extracts, the TBA concentration was increased to 10 mM to ensure complete association of ascorbate with this compound. The solvent was prepared by dilution of a stock solution of TBA (Fisher Chemicals) into 100% methanol followed by addition of this solution to water. Any change in this sequence resulted in the TBA remaining insoluble in the water/methanol solution. A flow rate of 2.0 mL/min through the Ultrasphere-ODS column was used to analyse the plant extracts. This procedure is a modification of the technique outlined in Pachla and Kissinger (1979).

The second technique was anion exchange chromatography with a Zorbax-NH_2 column (Dupont). The mobile phase was 0.02 M monobasic potassium phosphate (KH_2PO_4) in water and the flow rate was 2.0 mL/min. Detection was by UV absorbance at 264 nm. During both reverse phase and anion exchange chromatographic procedures, it was necessary to keep all samples on ice prior to analysis.

2.11.2 Chromatography of α-Tocopherol

Two efficient techniques were used for the chromatography of the various tocopherols, particularly α-tocopherol. A reverse phase system was developed by myself and subsequently proved to be almost identical to a protocol previously reported (Hatam and Kayden, 1979). A normal phase separation described by Vatassery, Maynard and Hagen (1978) was adapted to the particular columns used here.

Reverse phase chromatography was initially used to purify a desired component from cold methanol extracts of roots and leaves of various plant species. Both the Ultrasphere-ODS and PRP columns were employed. The solvent was 100% methanol at a flow rate of 2.0 mL/min. Suspecting that α-tocopherol might be the unknown compound in the extracts, it was chromatographed in this system. The k' values for α-tocopherol with the Ultrasphere-ODS and PRP columns were on average 5.1 and 8.7, respectively.

For normal phase chromatography, both the Hewlett-Packard Si-100 and the Brownlee Spheri-5 columns were used. The solvent was 0.5% methanol in n-hexane with the Brownlee column and 0.2% methanol in n-hexane with the Si-100. A flow rate of 2.0 mL/min was used with both columns. The k' values for α-tocopherol were 2.4 with the Brownlee column and 3.3 with the Si-100 column. It was possible to detect and isolate the other tocopherols, β,γ and δ, with these two columns.

2.12 Thin Layer Chromatography of Phytol

A commercial preparation of phytol (Sigma Chemical Co., St. Louis, MO) was chromatographed on thin layer silica gel plates (K5F, 250μm; Whatman).

A mobile phase of n-hexane:ethyl acetate (17:3) was used: this has been reported to produce an RF of 0.35 for phytol and 0.50 for isophytol (Demole and Lederer, 1958). After development, some minor components were visualized by examination of the plate held under an ultraviolet lamp emitting light of 254 or 350 nm wavelength. Some bands were fluorescent, others absorbing. Subsequently, half of the plate was covered with a paper towel and the other half was sprayed with concentrated sulphuric acid. The acid treatment immediately turned the major components of the sample yellow. The corresponding band on the covered side of the plate was scraped into a test tube and eluted with fresh n-hexane:ethyl acetate (17:3). Alternatively, two sets of plates were run in the chromatograph system simultaneously and one set was sprayed with sulphuric acid and heated at 60°C for 20 min. All components appeared as brown bands as a result of this charring procedure. Material at the corresponding positions on the unsprayed plates was scraped off and eluted with fresh running solvent. The insoluble silica was pelleted by centrifugation and the supernatant was tested in the bioassay. Compounds isolated this way were re-chromatographed to verify purity.

2.13 Ultraviolet Spectroscopy

The ultraviolet spectrum from 210 to 400 nm of the peak suspected to be ascorbic acid was obtained using the scanning function of the Hewlett-Packard chromatograph. An aqueous extract of *Zea mays* leaves was separated on a Zorbax-NH_2 column (Dupont) with a mobile phase of 0.02 M KH_2PO_4, pH 5.5. When the appropriate peak appeared, the solvent pumps were stopped and the scan initiated. Spectra of both the leading and trailing slopes of the peak were recorded to test peak purity. Several attempts were made to isolate the peak in order to obtain a better quality spectrum using a conventional spectrophotometer. These were not successful due to the low amount of material that could be obtained this way (more concentrated samples did not chromatograph well) and the instability of the compound.

The UV spectrum of a commercial ascorbic acid preparation (Fisher Chemicals) was measured on a Shimadzu UV250 spectrophotometer. The sample was dissolved in 50 mM $HClO_4$ and was scanned from 210 to 400 nm.

The spectra of compounds isolated from *Silene alba* and *Pastinaca sativa* roots or leaves were determined with the use of a Pye-Unicam 1800 spectrophotometer. The spectra of two compounds thought to be chlorophylls a and b were measured on a Beckman 25 spectrophotometer. Readings were taken at 10 nm intervals in the 400 to 700 nm region.

2.14 Mass Spectrometry

A sample of the active compound, estimated to be 2 mg based upon integrator area units, was isolated from cold methanolic extracts of parsnip root tissue by reverse phase HPLC. Mass spectral analysis was done with a Varian MAT 311A mass spectrometer in the Department of Chemistry, U.W.O. The instrument was set at a potential of 70 eV and a probe temperature of 60°C. A sample of synthetic α-tocopherol (Sigma Chemical Co., St. Louis, MO) was analyzed for comparison. A second sample of the active compound, approximately 0.5 mg, was run at 25 eV and 70°C.

CHAPTER 3

DIPLOID DERIVATIVES WITH ALTERED MATING TYPE PROPERTIES

3.1 Introduction - Diploid Morphology and Alternate Growth Forms As Understood at the Start of this Thesis

Newly produced diploid colonies of *U. violacea* present a translucent, mucoid appearance on MM at 22°C which is very different from the opaque appearance of haploid, aneuploid or diploid colonies in which the mt alleles are not heterozygous (Day, 1979). This characteristic, therefore, is a convenient diagnostic for cells heterozygous for mating type (plate 1). When viewed with the low power objective of a microscope, the sporidia of these translucent colonies are observed to be widely separated unlike the opaque, haploid colonies which contain densely packed cells (plate 1). This separation is due to the secretion of a "gelatinous" matrix by the diploid cell under certain conditions. While the composition of the matrix has not been determined, it is responsible for the translucent appearance of the colony.

Investigations have determined that the sporidial and colonial growth forms of cells heterozygous for mt are dependent on a number of factors, especially temperature and composition of the growth medium (Day, 1979). The heterozygous a_1/a_2 diploid is capable of both vegetative growth and the initiation of sporulation. Vegetative growth (VP) of the diploid sporidium occurs by means of yeast-like budding, in a manner identical to that of haploid cells. The generation time for both cell types is approximately 5-6 h under optimal conditions (Day and Jones, 1972). The diploid cell exhibits this vegetative mode of growth on nutritive media at relatively high temperatures: on CM above 20°C and on MM above 25°C. Below

these critical temperatures the sporidia cease dividing and after 40 h begin to elongate and swell greatly, becoming full of large lipid granules and taking on a dumbbell shape (plate 2). It is during this phase that the gelatinous matrix is produced. The change from vegetative to sporulative cell very closely parallels the initial stages of teliospore formation in the anthers of the host plant (Day, 1979). The term "sexual spore precursor phase" or "SPP" has been applied to these large cells (Castle and Day, 1980). These SPP cells do not complete development into teliospores under all conditions so far tested on artificial media (Day, 1979). Presumably, some factor(s) necessary for the final steps which is present in the anthers of the host is lacking on these agar media. The SPP cell is not committed to remain in this pre-sporulative state. A reversal of growth conditions, such as plating on CM above 20°C results in the reformation of the vegetative cells. The SPP cell produces a short hypha which buds off typical VP cells. This process is apparently the result of mitotic division rather than meiosis, normal or aberrant, as no segregation of markers is observed.

The switch from the vegetative to the sexual stage and vice versa is controlled by the mating type gene. Only a_1/a_2 diploid sporidia and a_1+a_2 dikaryons, i.e. conjugated pairs of cells, are capable of forming the SPP; haploids and homozygous diploids are not (Castle and Day, 1980). As the conditions of temperature and media which permit SPP formation are those that permit conjugation, it has been postulated that SPP cells result when both mt alleles are active in the same cell while VP cells result from inactive mating type alleles (Day and Cummins, 1974; Day, 1979).

In summary, the a_1/a_2 diploid cell can form either VP or SPP sporidia and switch back and forth between these two phases. The phase chosen at any particular time is dependent upon environment, nutrition and temperature, and the switch mediating the response to these stimuli is the mating type gene (Day, 1979).

The cessation of vegetative budding as a population of a_1/a_2 sporidia enter the SPP stage confers a selective advantage to any cell which continues to divide under these conditions. These vegetative cells would

eventually give rise to sectors or whole colonies and come to predominate in the culture. The a_1/a_2 diploid, grown under conditions which induce the non-dividing SPP phase, frequently gives rise to such vegetative sectors and colonies (plate 2). The vegetative areas are recognized by their "opaque" appearance since little or no mucoid matrix is produced by these rapidly dividing cells.. Sometimes the opaque colonies appear to arise directly from a single diploid cell immediately after plating, but more frequently opaque colonies arise as sectors in the minicolonies of swollen sexual cells (plate 2). Cells at the periphery of the minicolony divide occasionally; therefore, opaque sectors can arise at any time during the slow growth of the colony. Opaque sectors were also clearly visible in the spreading "wet" and translucent colonies of large masses of a_1/a_2 diploid cells on MM (plate 2).

Many such opaque colonies were isolated and purified using standard microbiological techniques: dilution or streak plating. Successive rounds of sub-culturing have shown that the ability of these cell types to remain vegetative under conditions favouring sporulation is a stable trait and not a transient phenomenon.

Subsequent analysis by A. W. Day showed that these sectors could be divided into three categories based upon mating and sporulation characteristics. The frequency of each of the categories was dependent upon the culture conditions used to produce the opaques. On CM at 15°C, about 60% of the isolates were termed neutral opaques (op-N) as they did not conjugate with either a_1 or a_2 haploid. Thirty-eight percent were strains which expressed the a_2 allele (op-a_2) and 2% were of the a_1 mating type (op-a_1). On MM at 15°C, 80% were op-a_2 types and the remainder were op-a_1. No neutral opaques were recovered (table 1).

This was the extent of our understanding at the time I began my work. The purposes of this section of this thesis were:

(1) to determine the frequency with which each type of opaque

was formed;

(2)to describe the characteristics of each type of opaque;

(3)to determine the mechanism of opaque origin.

3.2 Frequency of Formation of Opaques

All of the opaque strains obtained up to this point were isolated from plates incubated in conditions favouring SPP growth, as these are the conditions that favour their selection. However, it was not clear whether these conditions were necessary to induce opaques or whether opaques are formed under either VP or SPP conditions. If the former hypothesis were true, opaques would not be produced at 22°C on CM, as all of the diploid cells would be vegetative, but they would be induced if exposed to 15°C. On the other hand, if opaque formation is independent of temperature or state of the cell (VP or SPP) the frequency of opaque formation should be the same at all temperatures. The frequency of opaque formation was therefore measured using freshly isolated VP cells of strain D10 given a 15°C exposure of varying durations before transfer back to 22°C.

After determining the cell concentration and diluting the culture appropriately, equal sized aliquots containing about $5x10^2$ sporidia were spread on each of 60 plates of CM. Twenty plates were immediately placed in a 22°C incubator. The remaining plates were divided into four groups of ten and incubated at 15°C for 5.5, 24, 48, or 72 h before transferring to 22°C. When colonial growth was complete, all plates were replica plated to MM and CM and incubated at 15°C. Total colony numbers were estimated using a Fisher colony counter (model 600) and the numbers of opaques determined by inspection.

The duration of exposure to 15°C did not affect the frequency of opaque appearance on MM and had relatively little effect on CM (table 2). Thus, the switch from VP to SPP did not result in the production of the opaque strains: it only provided the conditions necessary for their selection. The difference between the frequencies on MM and CM was probably due to the

Plate 1. Diploid and Haploid Colonial Appearances

A three day old a_1/a_2 diploid colony (top) grown on CM at 15°C showed characteristic large unbudded cells separated by a gelatinous matrix (x500). A haploid colony after two days growth on CM at 22°C, was characterized by a densely packed appearance (x500).

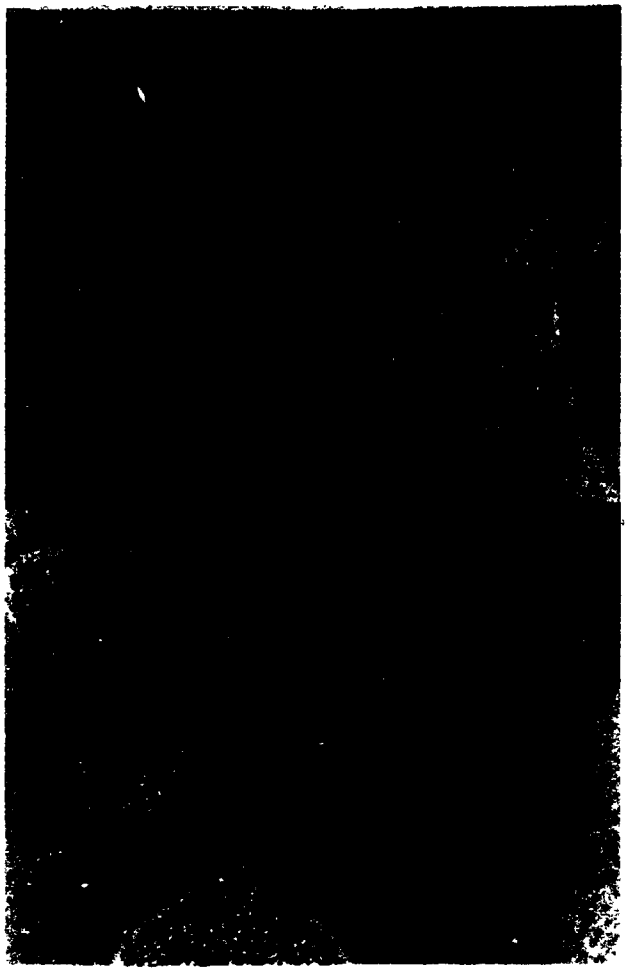


Plate 2. Morphology of the Sexual Cell and Appearance of Opaque Sectors in a Sexual Colony

Opaque sectors can be seen to have arisen from a small colony of freshly isolated diploid cells on CM at 15°C (top left). The loosely packed a_1/a_2 cells gave the colony a translucent appearance as opposed to the dense appearance of the opaque sector (x170). This point is further illustrated in the top right photograph, taken with oblique lighting and the low power of a Wild dissecting microscope. The opaque sectors appeared white while the sexual diploid sectors were translucent (x4.5). The photograph of a replica plate (MM at 15°C) in the lower left shows a single pure opaque colony (arrow) presumably derived from an opaque cell present in the initial inoculum (x0.9). The figure in the lower right is a phase contrast photograph showing the vegetative growth appearance of unmated opaque cells. In addition, conjugated op-a_1 x op-a_2 sporidia cultured on MM at 15°C have ceased vegetative budding and have entered the sexual spore precursor phase (x1500).

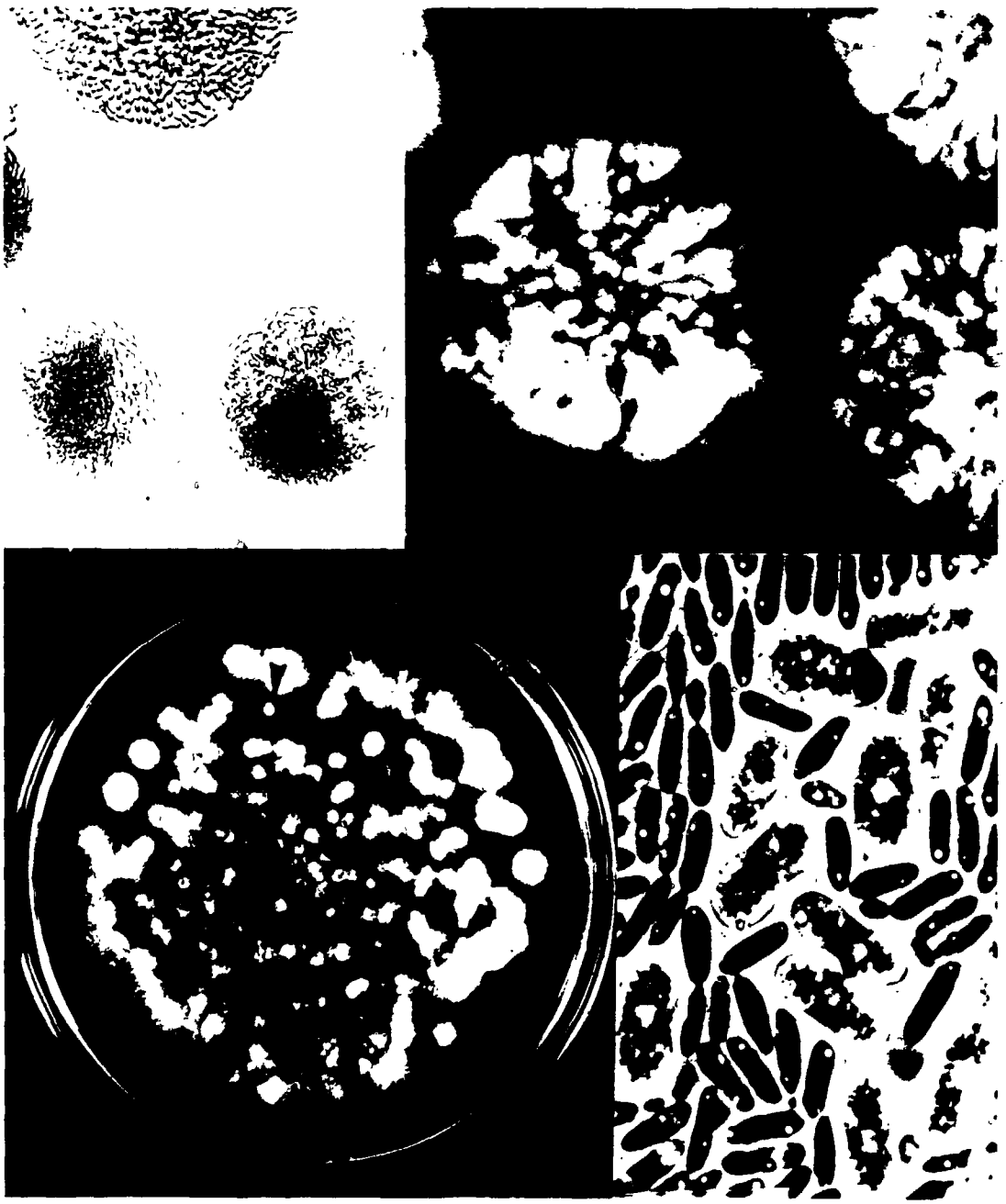


Table 1. Types Of Opaque Vegetative Colonies Isolated From CM And MM At 15°C

Medium	Total No. Opaques	Op-N	Op-a_2	Op-a_1
CM	136	74	59	3
MM	266	0	214	52

Table 2. The Effect of Exposure to 15°C on the Frequency of Formation of Opaques in a_1/a_2 Diploid, D10

Treatment Time at 15°C Before Transferring to 22°C (h)	Total No. of Colonies	No. of Opaques		Frequency of Opaques ($\times 10^{-3}$)	
		MM	CM	MM	CM
0 (control)	9,585	14	32	1.46	3.34
5.5	4,166	9	32	2.16	7.68
24	5,728	10	30	1.75	5.24
48	4,478	6	29	1.34	6.48
72	6,164	13	50	2.11	8.11

ability to detect the op-N class on CM but not on MM under these conditions (see section 3.3.2 and table 1). This was because op-N develop as SPP cells on MM and VP cells on CM at 15°C (see section 3.3.2 and table 3).

Using the ratios of the three opaque types on CM as presented in table 1, the frequencies of each type of opaque recovered on CM were calculated. Of the overall frequency of opaques on CM at 22°C with no 15°C pre-treatment (3.34×10^{-3}, table 2) the frequencies of op-N, op-a_2, and op-a_1 were determined to be 1.9×10^{-3}, 1.2×10^{-3}, and 0.3×10^{-3} respectively.

3.3 Characteristics of Opaques

The principal characteristics of freshly isolated a_1/a_2 diploids (termed primary diploids), their opaque derivatives, and haploids are summarized in table 3. The following sections provide detail concerning these characteristics.

3.3.1 Cell Size and Growth Rate

Transition of the primary diploid from VP to SPP involves an appreciable increase in cell volume and an alteration in shape. Average volume of the unbudded G1 diploid, D10, in VP is 93.1 ± 10.3 μm^3, while in the SPP form it is 813 ± 123 μm^3. In contrast, the 2.716 haploid cell volume is 30.4 ± 3.1 μm^3 during optimal growth conditions. All volumes were calculated using the formula for a prolate ellipsoid. All three opaque types appeared identical in size to the primary diploid in its VP state, with the exception of the op-N once it has entered the SPP phase. For example, an op-a_2 isolate of the diploid, C4/118, had an average cell volume of 90.8 ± 8.0 μm^3, which is not significantly different from the size exhibited by primary VP diploid cells.

For the growth rate measurements, flasks containing 10 mL of liquid CM were inoculated with either the haploid 1.C2, the diploid D10, or one of the D10 opaques, op-N19, op-a_1-1, and op-a_2-11. The initial concentrations were all 1×10^6 sporidia/mL. Samples were taken at approximately 24 h intervals and the cell concentrations were measured

Table 3. Characteristics Of Opaques

	a_1 Haploid	a_2 Haploid	a_1/a_2 Diploid	op-N	Op-a_1	Op-a_2	Op-C
Conjugation	$+a_2$	$+a_1$	-	-	$+a_2$	$+a_1$	?
Self conjugation	-	-	-	-	-	-	+
Growth Form <20°C CM <25°C MM	VP	VP	SPP	SPP'	VP	VP	VP
Growth Form >20°C CM >25°C MM	VP	VP	VP	VP	VP	VP	VP
Solopathogenicity	-	-	+	+	-	-	NT
Pathogenicity when mixed with a_1 or a_2 haploids	$+a_2$	$+a_1$	+	+	$+a_2$	$+a_1$	NT

Note: $+a_1$: possible when mixed with an a_2 strain; $+a_2$: possible when mixed with an a_1 strain;
VP: vegetative growth observed; SPP: sexual spore precursor phase observed; SPP': SPP is altered;
NT: not tested; ?: not known.

using a Neubauer hemacytometer. The results are shown in fig. 3. No differences were observed in the rates of growth of any of the cultures.

3.3.2 Mating and Sporulation

The op-N, like the primary diploid, is neutral in mating type, conjugating with neither a_1 nor a_2 haploid strains. It also resembles the a_1/a_2 diploid in that it can form SPP cells. The two types differ in their response to the environmental stimuli which induce SPP cells. While the primary diploid initiates sexual morphogenesis after about two days on CM at 15°C, the op-N remains vegetative until the nutrients have been depleted (about ten days). On MM at 15°C, SPP cells are formed by both primary diploid and op-N after an incubation period of two to three days. Thus, the change from the primary diploid to the op-N results in a altered response to the environmental signals triggering SPP development. Since selection of opaque derivatives on CM requires incubation of the cultures at 15°C for longer than the time required for the op-N to initiate SPP development, this strain therefore appears amongst the opaque population on CM. However, on MM the op-N enters SPP development more quickly and therefore is not included in the opaque types recovered. The overall rate of opaque production on MM is thus lower than on CM because of this inability to recognize the op-N variants (table 2).

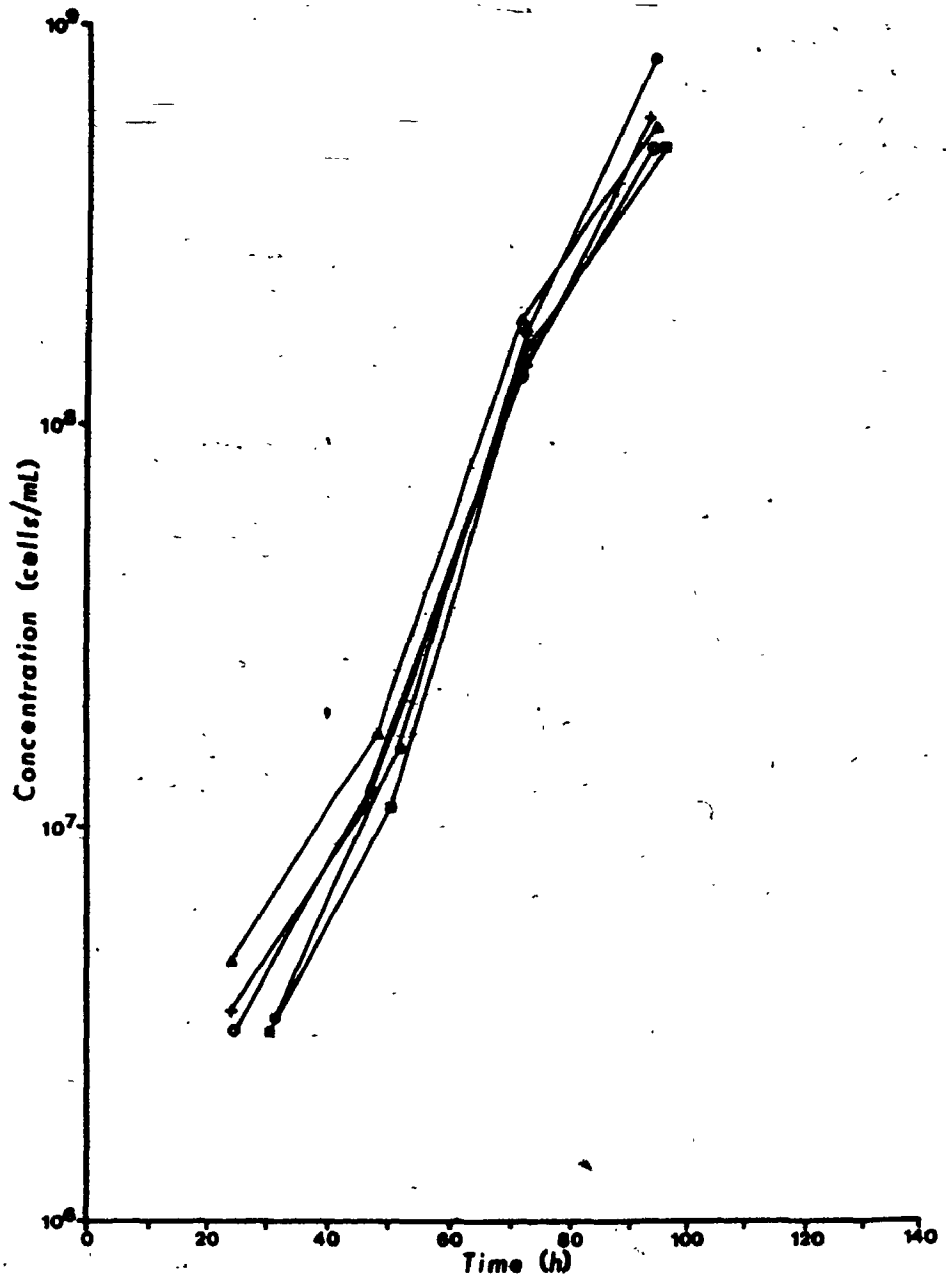
The two mating opaques, op-a_1 and op-a_2, remain vegetative under all tested growth conditions and do not form SPP cells. Dikaryons formed by conjugation between opaques or opaques and haploids were able to initiate sporulation (plate 2) due to complementation between the two mating partners. These mating opaques are identical to a_1 and a_2 haploids, respectively, in ability to conjugate, and in response to the environmental signals which affect conjugation.

3.3.3 Pathogenicity

Mixed haploids of opposite mating type and a_1/a_2 diploids are capable of infecting and sporulating within the host plant, *Silene alba*. When only one mating type is present in the inoculum, as in pure haploid cultures,

Figure 3. Growth Rates of Haploid, Diploid, and Opaque Strains

Complete medium flasks were inoculated with a_1 haploid (•); a_1/a_2 diploid (■); op-N (○); op-a_1 (▲); or op-a_2 (+) strains. The cultures were incubated at 22°C and every 24 h, 0.5 mL aliquots were removed to determine cell concentration.


Concentration (cells/mL)
10^9
10^8
10^7
10^6
0
20
40
60
80
100
120
140
Time (h)

no infection occurs (Day and Jones, 1968; 1969). A study of the pathogenicity of opaque strains was undertaken to determine if these alterations affected the ability to infect and sporulate in the host plant. Groups of four plants were inoculated with either the primary diploid D10, an op-a_1, an op-a_2 or one of two op-N derivatives of the diploid, C4/118.

The primary diploid produced weak infections in the two plants which flowered. This result was consistent with earlier observations of infections produced by single diploids compared to mixed haploid strains (Day and Jones, 1969). The teliospores recovered from these plants germinated normally and study of the haploid products with tetrad and random spore analyses indicated that meiosis was normal.

Only one of the four inoculated plants flowered in each of two op-N tests. The plant inoculated with C4/118 op-N17 initially appeared to be uninfected. The anthers were normal in both size and colour and microscopic examination of the excised tissue failed to reveal any teliospores. However, when two anthers were crushed in sterile water and the entire suspension was plated on CM, a total of nine colonies appeared after a week of incubation at 22°C. Each of these colonies was streaked on CM to isolate any genetically different clones present in the colony produced by the germination of a single teliospore. Twenty-five colonies were isolated from each streak and genotypes were determined by replica plating and by tests with known mating types on WA (table 4). In all the nine teliospore colonies, only two out of four genotypes were present, and all clones were clearly haploid as judged by size and genotype. With the exception of mating type, segregation of the various markers appeared normal and the two alleles for any one gene were present in approximately equal frequencies within the total sample. The only allele not observed in any of the progeny was the a_1 allele of the mt gene. These results indicate that these colonies probably derived from meiosis in rare teliospores in the anthers rather than from the vegetative growth of mycelial fragments.

Thus this op-N retained the ability to infect a host plant (although sporulation was reduced to a barely detectable minimum), a feature which in all previous experience indicates the presence of both a_1 and a_2 alleles; yet in the subsequent meiosis, half of the meiotic products (those containing the a_1 allele) did not survive. The simplest explanation appears to be that formation of this op-N involved alterations to the a_1 allele (e.g. through mutation, recombination, transposon activity, etc.) resulting in the mildly altered phenotype for the diploid and a lethal loss of necessary functions in haploids containing the altered allele.

The resulting half-tetrad analysis of these nine teliospore colonies is shown in table 4. A limited amount of mapping information can be gleaned from these colonies. Inviability of the meiotic products carrying the a_1 allele automatically eliminates one allele of the remaining genes whenever parental ditypes (PD) or non-parental ditypes (NPD) were formed. The appearance of both alleles of any of the other loci in any one of these nine situations therefore implies formation of a tetratype (T) tetrad. As mt was found to be a centromere linked marker (see section 3.4.3), %T can be used to map the gene to centromere distances for the other genes in this cross. Values significantly less than 67% provide useful information and the gene - centromere distance = 1/2 %T (Fincham and Day, 1971). On this basis y and arg_1 particularly are close to their centromeres, while his_1 appears to be relatively distant. The centromere linkage of the y locus has been documented more thoroughly by Garber's group (Cattrall *et al.*, 1978).

A second op-N, C4/118 op-N19, also produced an infection substantially weaker than the primary diploid. Superficially, the plant appeared uninfected but in this case teliospores were observed during microscopic examination of the anthers. In addition, these teliospores yielded approximately equal proportions of a_1 and a_2 cells in the meiotic progeny. Thus, the strain op-N19 appeared to have a less severe alteration than op-N17.

Table 4. Op-N-17 Tetrad Analysis

Genotype of Primary Diploid C4/118:

VII	II	III	?	XII	VI
$\frac{y}{+}$	$\frac{his_1}{+}$	$\frac{lys_2}{+}$	$\frac{uvs_1}{+}$	$\frac{+}{arg_1}$	$\frac{a_1}{a_2}$

NL: Not Linked

Table 4. Op-N-17 Tetrad Analysis

Teliospore	Genotypes Recovered					
1.	y	+	+	his	uvs	a_2
	y	+	+	+	uvs	a_2
2.	y	arg	lys	his	uvs	a_2
	y	arg	lys	+	+	a_2
3.	+	arg	+	+	+	a_2
	+	arg	+	his	+	a_2
4.	+	arg	+	+	uvs	a_2
	+	arg	+	his	+	a_2
5.	+	arg	lys	+	+	a_2
	+	arg	+	his	+	a_2
6.	+	arg	lys	+	+	a_2
	+	arg	lys	his	+	a_2
7.	y	arg	+	+	+	a_2
	y	+	lys	his	uvs	a_2
8.	y	+	+	his	uvs	a_2
	y	+	lys	+	+	a_2
9.	y	+	+	+	uvs	a_2
	y	+	lys	his	uvs	a_2

Segregation as Compared to mt Gene

Gene	PD	NPD	T	Percent Recombination with mt	Gene to Centromere Distance	Recessive Allele Frequency
y	5	4	0	44.4	0.0	55.6
lys_2	3	2	4	44.4	22.2	44.4
his_1	0	0	9	50.0	NL* (>33%)	50.0
arg_1	5	3	1	38.9	5.6	57.9
uvs_1	3	2	4	44.4	22.2	44.4

The results with these two opaques highlight a previously unrecognized problem with sexual crosses involving *U. violacea*. Some crosses, particularly those involving diploid strains, may produce plants which appear uninfected to the naked eye, having apparently normal anthers: clearly in these cases it is worth making microscopic examinations and/ or plating out crushed anther suspensions to detect rare teliospores before discarding these plants as useless.

Neither of the mating opaques, C4/118 op-a_1-1 or op-a_2-11, produced any signs of infection in the six of eight plants which flowered. No teliospores were observed in the anthers examined microscopically; instead they were filled with pollen. The inability of these opaques to produce smutted anthers was probably due to a failure to infect. The alternate possibility that the infection occurred but sporulation was blocked is unlikely as later experiments (see Chapter 4) suggested that the mating opaques were incapable of systemic growth, as they did not respond to host plant extracts which induce parasitic hyphal growth. Regardless of when the block in pathogenic development occurred, it could be overcome through complementation between different opaque strains or between opaque and haploid strains. Mixtures of op-a_1 and op-a_2 cells produced heavily smutted anthers, as did op-a_1 and haploid a_2 mixtures (see section 3.4.4).

3.3.4 Genotypes of Opaques

The principal purpose of my investigation was to determine what genetic alterations occurred in primary diploids to produce these various opaque derivatives. Analyses of: a) the ploidy of opaques, b) the possible changes in chromosomes bearing a variety of marker loci, and c) the possible changes in the mating type chromosome were therefore carried out.

Firstly, a survey of opaque phenotypes derived from multiply marked primary diploids was done by A. W. Day and myself to determine the extent of the alteration of the diploid genomes. Eleven of the twelve linkage groups were assessed for alterations in any of the marked loci during opaque formation. All of the 224 opaques isolated from five diploid strains,

Table 5. Phenotypes of Opaques Obtained from a_1/a_2 Diploids Carrying a Variety of Chromosomal Markers

Diploid	Genotype of Diploid	Phenotype of Opaques (All Classes)
B32	VII I IV V IX VI	
	$\frac{y}{+}$ $\frac{orn_1}{+}$ $\frac{inos_1}{+}$ $\frac{met_3}{+}$ $\frac{+}{cit_1}$ $\frac{a_1}{a_2}$	28 opaques - all prototrophic and y^+
B33	VII I IV V X VI	
	$\frac{y}{+}$ $\frac{orn_1}{+}$ $\frac{inos_1}{+}$ $\frac{met_3}{+}$ $\frac{+}{his_3}$ $\frac{a_1}{a_2}$	22 opaques - all prototrophic and y^+
X2	VII IX III ? ? VI	
	$\frac{y}{+}$ $\frac{lys_2\ cit_1}{+\ +}$ $\frac{+}{lys_2}$ $\frac{+}{uvs_1}$ $\frac{+}{cxr_2}$ $\frac{a_1}{a_2}$	107 opaques - all prototrophic, UV resistant, and cx sensitive[1]
X6	VII II IV III ? XI VI	
	$\frac{or}{+}$ $\frac{his_1}{+}$ $\frac{inos_1}{+}$ $\frac{lys_2}{+}$ $\frac{uvs_1}{+}$ $\frac{+}{his_4}$ $\frac{a_1}{a_2}$	20 opaques - all prototrophic, or^+, and UV resistant
X8	XI XII VI	
	$\frac{his_4}{+}$ $\frac{+}{arg_1}$ $\frac{a_1}{a_2}$	47 opaques - all prototrophic

1 - Eventually cx resistant sectors grew up from these colonies, proving that they were heterozygous for cxr_2.

heterozygous for a variety of auxotrophic, drug resistance, and UV sensitive markers, remained prototrophic and expressed the wild type dominant alleles for colour (two genes), cycloheximide sensitivity, and ultraviolet light resistance (table 5). In addition, many more opaques from different primary diploids have been observed during the course of other experiments. The vast majority of these opaques gave similar results to those presented in table 5. Occasionally, recessive markers were expressed in the somatic descendants of various primary diploid and opaque strains. Most of these recombinants, expressing a recessive allele, retained the a_1/a_2 phenotype while virtually all opaques remained wild type and phenotypically identical to the primary diploid (apart from the sporulative and conjugative differences already mentioned).

Secondly, in order to confirm that the opaques did indeed remain heterozygous at the marker loci, several opaque isolates were induced to haploidize on PFP medium (table 6a,b). In all except one case all recessive alleles were recovered in a sample of haploid segregants from each opaque strain. The one exception, an op-a_2 derived from diploid B32, did not produce any haploids expressing the $\underline{cit}_1$ marker. However, this is most likely due to the small sample size and the epistatic masking of this marker by the $\underline{orn}_1$ marker also present in this cross. Thus, the double mutant $\underline{orn}_1\underline{cit}_1$ would be scored as $\underline{orn}_1$.

Finally, the haploids recovered from these and other treatments were tested for mating type (table 7). Three primary diploids produced haploids of either mating type, as expected. The two mating opaques showed no evidence of the presence of the opposite unexpressed $\underline{mt}$ allele. Thus, op-a_1 produced only a_1 haploids and op-a_2, a_2 haploids. The results obtained from the op-N were varied. Two opaques had retained both a_1 and a_2 alleles. One produced only a_1 haploids and the remaining seven yielded only a_2 haploids. In some instances, a few haploids recovered from the PFP treatment of the various opaque strains were subsequently mated to known a_1 and a_2 haploid test strains and diploids were selected. These diploids showed a normal a_1/a_2 rather than an opaque phenotype. Thus, a PFP-induced haploid from an op-N, for example, when mated with a haploid test

strain of the opposite mating type, produced an a_1/a_2 diploid rather than a new op-N.

These genetic analyses establish several important facts concerning opaque formation in primary diploids. Firstly, the opaques clearly remain essentially diploid, retaining the heterozygosity of the primary diploid in at least 11 of the 12 marked chromosomes, and are altered only in the chromosome carrying the mating type locus, chromosome VI; Day and Jones, 1969). Secondly, op-a_1 and op-a_2 each appear to have lost the mt allele that is not expressed; the remaining allele being apparently unaltered in function. Thirdly, op-N are variable in constitution, sometimes carrying two different functional mating type alleles, other times apparently only having one functional mating type allele. This variety of op-N types revealed by mitotic haploidization parallels the variety revealed earlier by analysis of chromosomal reduction at meiosis (see 3.3.3).

3.4 Mechanism of Origin of Mating Opaques

The phenotypes of the opaques and the results from the mitotic haploidization tests left little doubt that genetic alterations during opaque formation were restricted to chromosome VI carrying the mt locus. Also the large cell volume and the vigorous growth rate supported a diploid state rather than an aneuploid condition. It was decided to restrict detailed investigations of the mechanisms of opaque formation to the op-a_1 and op-a_2 derivatives as these seemed to provide the better chance of success.

Several events could potentially have occurred in primary diploids to produce op-a_1 or op-a_2 derivatives which are diploid but altered at chromosome VI, so that they now express a single mating type allele. These events are:

(1) chromosome VI may remain disomic and heterozygous in these strains but one mating type allele may be deleted;

(2) as in (1) except that one mating type allele may be inactivated;

(3) as in (1) except that one of the two homologues of chromosome VI may be inactivated in its entirety;

Table 6a. PFP-Induced Haploidization of Diploids and Opaque Derivatives

A. Primary Diploid B32 Genotype: VII $\frac{y}{+}$ I $\frac{orn_1}{+}$ IV $\frac{inos_1}{+}$ V $\frac{met_3}{+}$ IX $\frac{+}{cit_1}$ VI $\frac{a_1}{a_2}$

Possible Haploid Genotypes	Primary Diploid	Haploids Derived From: Op-N Derivative	Op-a_1 Derivative	Op-a_2 Derivative
y o i m c	0	0	0	0
y o i m +	3	1	1	1
y o i + c	0	0	0	0
y o i + +	0	1	0	0
y o + m c	0	0	0	0
y o + m +	2	2	2	5
y o + + c	0	0	0	0
y o + + +	0	2	0	0
y + i m c	0	0	0	0
y + i m +	7	3	4	0
y + i + c	0	0	1	0
y + i + +	2	1	1	0
y + + m c	2	0	0	0
y + + m +	8	2	2	1
y + + + c	0	3	0	0
y + + + +	0	0	0	0
Totals	24	15	11	7
	Allele Frequencies			
orn_1	.21	.40	.27	.86
$inos_1$	.50	.40	.64	.14
met_3	.83	.53	.82	1.0
cit_1*	.08	.20	.09	0.0

*orn_1/cit_1 double mutant scored only as ornithine requiring due to epistasis

Table 6b. PFP-Induced Haploidization of Diploids and Opaque Derivatives

B. Primary Diploid 118/C4 Genotype: VII $\frac{y}{+}$ II $\frac{his_1}{+}$ III $\frac{lys_2}{+}$? $\frac{uvs_1}{+}$ XII $\frac{+}{arg_1}$ VI $\frac{a_1}{a_2}$

Possible Haploid Genotypes	Haploids Derived From: Op-N Derivative	Op-a_1 Derivative	Op-a_2 Derivative
y h l a u	0	0	0
y h l a +	0	0	0
y h l + u	1	2	7
y h l + +	0	4	2
y h + a u	1	0	0
y h + a +	0	0	0
y h + + u	0	3	4
y h + + +	1	2	4
y + l a u	2	1	2
y + l a +	1	1	2
y + l + u	4	2	4
y + l + +	3	0	0
y + + a u	1	1	3
y + + a +	1	1	5
y + + + u	2	2	5
y + + + +	3	4	6
Totals	20	23	44
Allele Frequencies			
his_1	.15	.48	.39
lys_2	.55	.43	.39
arg_1	.30	.17	.27
uvs_1	.55	.48	.57

Table 7. Distribution of Mating Type Alleles Following PFP-Induced Haploidization of Diploids and Opaques

Primary Diploid		Primary Diploid		Op-N		Op-a_1		Op-a_2	
		a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2
B32	i.	1	2	i. 2	5	i. 14	0	i. 0	8
118/C4	i.	7	16	i. 18	0	i. 17	0	i. 0	17
	ii.	23	19	ii. 0	11	ii. 21	0	ii. 0	18
				iii. 0	29			iii. 0	15
				iv. 0	35			iv. 0	18
				v. 0	12			v. 0	26
				vi. 0	20				
P3	i.	7	17	i. 2	12	i. 69	0	i. 0	43
	ii.	7	5	ii. 0	19	ii. 3	0	ii. 0	6
	iii.	2	7	iii. 0	16			iii. 0	5
	iv.	5	3						
	v.	2	1						
	vi.	5	10						

Roman numerals indicate results from different isolates of each type.

(4) chromosome VI may be monosomic in these strains, one homologue being lost entirely;

(5) chromosome VI, or at least that section of it containing the mating type locus, may now be homozygous due to either nondisjunction, mitotic crossing-over, or mating type interconversion.

Several experiments designed to distinguish between these various possibilities are now described.

3.4.1 Isolation of Markers Linked to Mating Type

Markers linked to the mt locus would be of great value in elucidating the mechanism of opaque formation, but none were available in *U. violacea* at the time of this investigation. Preferably, these markers would lie on both sides of the centromere, allowing unequivocal differentiation between mechanisms involving non-disjunction, mitotic recombination, or allele inactivation. Allele inactivation would have shown only the mt gene altered. Mitotic recombination would also have resulted in changes in genes distal to the mating type locus, while with non-disjunction, all of the markers along the chromosome would have been affected. Previous work with 33 mutant strains had not resulted in the discovery of any markers other than mt on chromosome VI (Day, 1968). Consequently, a number of new mutants were isolated and screened for linkage to the mating type gene. A total of 60 auxotrophic mutants and one carboxin resistant mutant were tested using the chromosome transfer method (Day, 1978; see section 2.8 of this thesis). No markers linked to mating type were found in spite of this intensive screening. As a result, other less direct methods for determining the mechanism(s) of opaque formation had to be employed. It may be noted here that the failure to find any markers linked to mt in such a large sample may indicate that there is a special sex chromosome carrying only the mating type gene and other genes related to sexual morphogenesis. If so, this situation would be unique in fungi.

3.4.2 Haploids and Mating Type Interconversions

So far, only diploid cultures had been observed to undergo changes involving mt expression. It was possible that formation of the mating type opaques, op-a_1 and op-a_2, could have resulted from the switching of one mating type allele for the other. Changes of mating type in haploid cells of the yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been well documented (reviewed in: Hicks, Strathern, and Herskowitz, 1977; Egel, 1977). One of the mechanisms hypothesized for these fungi might have been applicable to *U. violacea*. This seemed unlikely in view of the extensive previous work on mating in this species in which no mating type switches had ever been observed. However, experiments to determine if switching in mating type occurred in haploid cultures were performed.

In the first experiment, a haploid culture was plated on WA and incubated at 15°C overnight. Microscopic observation of over 1.3×10^4 cells showed no conjugation tubes, and therefore no indication that any sporidia switched mating type. If spontaneous changes did occur in haploids, the altered cells would have had to be present at a frequency of less than 7.7×10^{-5}. By comparison, mating opaques appeared in a diploid culture at a frequency of 1.46×10^{-3} (table 2), or twenty times greater.

Mutagenic treatments were employed to see if switching in mating type expression could be induced. At the same time, a powerful selection system was devised to select for any mating type interconversions. The mutagen used was N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Sigma Chemical Co., St.Louis, MO) with the protocol outlined in Cummins and Day, 1977. A dense mixture of two haploids, 1.C2u6cx and 1.D2716y4, both the same mating type (a_1) and both in the logarithmic growth phase, was made in sterile phosphate buffer (0.1 M Na_3PO_4, 0.15 M NaCl, pH 5.3). Two final concentrations of MNNG were used: 0.55 and 0.0055 mg/mL. The sample was treated with the mutagen at room temperature for 5 min and then centrifuged at 1000xg for 5 min. The pellet was washed with fresh buffer and recentrifuged. After resuspension in 0.5 mL of phosphate buffer, the cells were plated on WA for 24 h. Any cell in which the a_1 mating type allele had switched to

a functional a_2 allele could have conjugated with one of the unaltered cells under these conditions. The selection system employed complementing uvs markers: one of the strains carried the uvs_1 allele and the other, uvs_3. These two genes complement in the mated dikaryon to confer UV resistance (Day and Day, 1970). If the mutated cell conjugated with the strain carrying the complementary uvs gene (50% chance, as equal concentrations of haploids were used), then the dikaryon could be selected using a low dose of ultraviolet light. This dose was sufficient to kill any sensitive strain but weak enough to permit growth of the complementing partners of the dikaryon. The cultures were then plated on CM and incubated at 22°C until the surviving colonies appeared. Nineteen of the colonies were tested for mating type and all were typical haploids of a_1 mating type. Presumably these colonies originated as either a result of non-lethal damage to a uvs cell or reverse mutation to uvs+. No evidence of any change from one mating type to the other was obtained.

Based upon these results, it was clear that the mt alleles of *U. violacea* are indeed stable and do not change to the other allele, at least in haploid strains. Thus, the alterations in mating type expression which occur during opaque formation appear to be restricted to diploids.

A problem with the above tests, however, is that they assay the activity of the opposite mating type allele. Inactivation of one mt allele in an a_1/a_2 diploid would result in the formation of a mating opaque but the corresponding event in a haploid cell would have gone unnoticed. While such inactivation was not specifically tested for, no haploid culture has been observed to spontaneously lose its mating ability during several years of research using this organism.

3.4.3 The Effect of Ultraviolet Light on Opaque Frequency

One of the most plausible mechanisms for the formation of opaques, in particular, op-a_1 and op-a_2, was mitotic recombination resulting in homozygosis at the mt locus. Ultraviolet light has been shown to be an efficient inducer of mitotic crossing-over in this organism (Day and Jones,

1969). If irradiation with UV had no effect upon the frequency of opaque formation, then mitotic crossing-over would not likely be responsible for the production of these strains. The procedure used is outlined in section 2.7, and the results are presented in table 8.

In the control unirradiated population, the average frequency of strains homozygous for any one of the recessive markers was about 0.03%. The opaque frequency was much higher, 0.41% on CM and 0.30% on MM. Following irradiation, the frequency of homozygosis of the genetic markers increased on average about 20 fold to approximately 0.6%. Similarly, the frequency of opaque formation also increased about 20 fold, 9.2% on CM and 7.3% on MM. During this experiment, a fourth type of opaque, designated as opaque-constitutive (op-C), was first noticed. The properties of this op-C type are described below (see 3.4.4).

This experiment showed that UV induced similar increases in both mitotic crossing-over near marker genes and in opaque formation. These results support mechanism 5: that mitotic crossing-over is the mechanism responsible for opaque formation. A later experiment also showed that the frequency of opaques after UV treatment could be reduced by photoreactivation, as also occurs with mitotic recombination (Holliday, 1962). The much higher overall frequencies of opaque formation as compared to the other genetic markers suggest that there is a hot spot of spontaneous mitotic recombination in the interval between the centromere and the mt locus on chromosome VI. Tetrad analysis indicated that this interval is a short one as mt is very close to its centromere (see section 3.4.5).

3.4.4 A New Class of Opaque: Op-C

A fourth type of opaque, op-C, was observed following the induction of mitotic recombination by UV light. This section summarizes observations concerning these strains.

Initially, the op-C was detected by its very slow growth at low temperatures and was thought to be a low temperature sensitive mutant. No growth was observed on either CM or MM replica plates incubated at

Table 8. Effect of UV on the Frequency of Mitotic Crossing-over and on the Frequency of Appearance of Opaque Strains in Diploid D10

Treatment	Total No. of Colonies Examined	Mitotic Crossing-over: No. (%) Homozygous for: y	or	auxo	uvs	Opaque Formation: No. (%) of Opaques on: CM	MM	Op-C strains
No irradiation	3,672	1 (.03)	1 (.03)	0 (0)	2 (.05)	15 (.41)	11 (.30)	0 (0)
Irradiation (180 J/m^2)	9,291	58 (.62)	55 (.59)	61 (.66)	50 (.58)	799 (9.2)	630 (7.3)	33 (.38)
Increase (Irr./No Irr.)			22.6			22.4	24.3	-

Note: Genotype of diploid D10:

VII	IX	IV	?	VI
$\frac{or\ +}{+\ y}$	$\frac{+\ +}{lys_3\ cit_1}$	$\frac{inos_1}{+}$	$\frac{uvs_1}{+}$	$\frac{a_1}{a_2}$

15°C. At 22°C on CM, growth was permitted though the colony had a much rougher appearance than the normal diploid. Microscopic examination of the sporidia incubated at 15°C showed an absence of vegetative budding. Instead, long conjugation tubes were produced by the cells (plate 3). At 22°C, most of the sporidia exhibited vegetative growth identical to the primary diploid or the other opaques, but a few of the cells produced conjugation tubes, resulting in the rough colonial appearance. Thus, conditions which elicit expression of the mating type alleles, i.e. less than 25°C on MM or 20°C on CM, resulted in the constitutive production of conjugation tubes (self-mating phenotype) by the op-C. Above the critical temperatures on these media, normal vegetative growth occurred and the cells were of typical VP diploid size. SPP development was not initiated under all tested conditions (table 3). These and later observations led to the conclusion that this new strain was an opaque and not a low temperature sensitive mutant as the initial isolation conditions suggested.

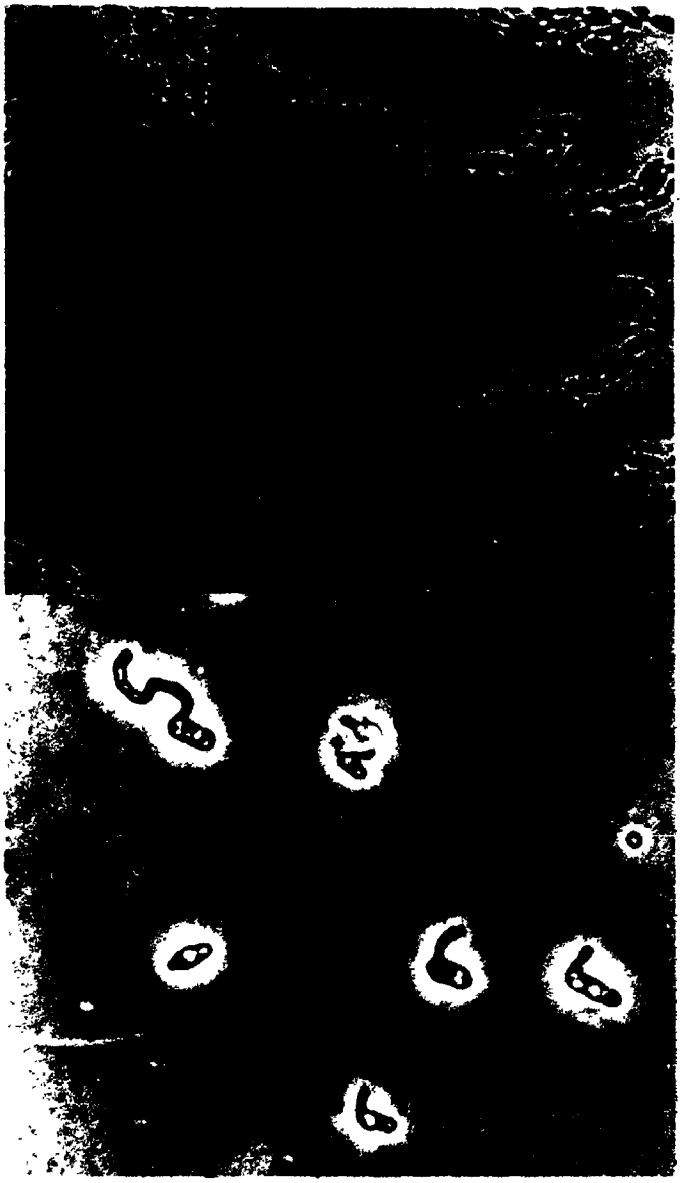
The op-C was initially isolated from a culture which had been exposed to UV light. The induced frequency of production was 3.6×10^{-3}. A subsequent test determined that the spontaneous frequency, i.e. not induced by UV, was 0.2×10^{-3}. The ratio of induced to non-induced, therefore, was 18, very similar to the ratios obtained for the other opaques (table 8). Thus, mitotic recombination, possibly responsible for the production of the mating opaques, might also result in op-C production, as well. Adequate tests which would support or disprove this hypothesis, however, have not yet been initiated. Some preliminary observations on the properties of op-C types follow.

Op-C isolates were not as stable as the other three opaque types, particularly the mating opaques. Vegetative colonies appeared amid the background of non-dividing, self mating cells on plates incubated at 15°C. Several of these sectors were isolated and all (73/73) proved identical to op-a_1 produced directly from primary diploids.

When haploidized on PFP medium, all recessive markers were recovered from several op-C, again confirming that like other opaques they are diploid

Plate 3. Morphology of Constitutive Opaques

Constitutive opaque cells grown on CM at 22°C budded vegetatively in a manner identical to a_1/a_2 primary diploid cells (x500). When grown at 15°C on CM, vegetative growth ceased and appendages closely resembling conjugation tubes were formed in the absence of any conjugation partner (x750).



(table 9a). Of the 119 haploids chosen from these tests, 109 expressed the a_1 mating type allele and none were a_2 (table 9b). The remaining 10 isolates retained the self mating phenotype of the parental op-C, even though expression of auxotrophic and colour markers indicated that a reduction in ploidy had occurred. A likely possibility is that the self mating haploids were, in fact, aneuploid having remained disomic for chromosome VI carrying the mt gene. When these self mating "haploids" were plated on CM at 15°C, they produced a_1 but not a_2 sectors, paralleling the behaviour during haploidization of op-C strains and consistent with the 73 op-a_1 types produced by the op-C under the same conditions. These a_1 haploid sectors might have been produced by the loss of the homologue carrying the a_2 allele or by mitotic recombination to yield a homozygous a_1/a_1 disomic strain.

An auxotrophic self mating haploid was mixed with complementary a_1 or a_2 haploids and plated on MM at 22°C to try to reform the diploid condition. After two weeks, many prototrophic diploid colonies were visible on those plates where the self-mating strain had mated with the a_2 haploid and karyogamy had occurred. As expected, the self mating haploid mated readily as an a_1 strain, perhaps unaltered, but possibly by producing a_1 sectors prior to conjugation. However, a few diploid colonies were also seen on plates inoculated with the self mating and a_1 haploid mixture. The self-mating strain was therefore also capable of acting as an a_2 type, permitting conjugation with a_1 haploids. This was particularly interesting in view of the failure to recover stable a_2 types from op-C and self-mating haploids. The diploids produced by the fusion of the self mating strain with either of the haploid test strains appeared normal, not op-C in phenotype.

In summary, a fourth opaque type was observed in the mitotic recombination experiment with the diploid D10. Subsequent studies showed that this opaque was also produced spontaneously but was not isolated with the three other opaques because of its lower frequency of production and the selection protocol used. This selection procedure was carried out at 15°C, a temperature at which the self mating phenotype rather than than vegetative growth was exhibited by the op-C. Mitotic haploidization tests

Table 9a. Mitotic Haploidization of Constitutive Opaques

Possible Haploid Genotypes						Isolate 4	Isolate 7	Possible Haploid Genotypes						Isolate 4	Isolate 7
+	y	i	l	c	u	2	3	o	+	i	l	c	u	1	0
+	y	i	l	c	+	1	2	o	+	i	l	c	+	1	0
+	y	i	l	+	u	0	0	o	+	i	l	+	u	0	0
+	y	i	l	+	+	0	0	o	+	i	l	+	+	0	0
+	y	i	+	c	u	0	0	o	+	i	+	c	u	0	0
+	y	i	+	c	+	0	0	o	+	i	+	c	+	0	0
+	y	i	+	+	u	6	6	o	+	i	+	+	u	3	4
+	y	i	+	+	+	4	6	o	+	i	+	+	+	2	1
+	y	+	l	c	u	1	2	o	+	+	l	c	u	2	1
+	y	+	l	c	+	3	1	o	+	+	l	c	u	0	0
+	y	+	l	+	u	0	0	o	+	+	l	+	u	0	0
+	y	+	l	+	+	0	0	o	+	+	l	+	+	0	0
+	y	+	+	c	u	0	0	o	+	+	+	c	u	0	0
+	y	+	+	c	+	0	0	o	+	+	+	c	+	0	0
+	y	+	+	+	u	3	3	o	+	+	+	+	u	1	1
+	y	+	+	+	+	0	3	o	+	+	+	+	+	1	2

Total haploids scored: isolate 4, 31; isolate 7, 35.

Allele Frequencies

Gene	Isolate 4	Isolate 7
y	.65	.74
orn_1	.35	.26
$inos_1$	.65	.63
uvs_1	.61	.57
lys_3	.35	.26
cit_1	.35	.26

All isolates recovered from the primary diploid, D10 (see table 8 for genotype).

Table 9b. Mitotic Haploidization of Constitutive Opaques: Distribution of Mating Type

Op-C Isolate	Phenotype a_1	Phenotype a_2	self
i.	29	0	2
ii.	33	0	2
iii.	17	0	3
iv.	30	0	3
Totals	109	0	10

All isolates recovered from the primary diploid, D10.

indicated that only chromosome VI carrying the mating type locus was altered in the production of the op-C as all other recessive markers remained unchanged. The mating type alteration was specifically on the a_2 rather than the a_1 allele. Op-a_1 and haploid a_1 recovered from these strains were normal as far as could be determined. Expression of the a_2 mating type could only be indirectly observed by the fusion of a self mating haploid with an a_1 haploid under highly selective conditions. Finally, the frequent production of op-a_1 sectors from the unstable op-C leads to the possible, and unproven, hypothesis that these op-C types may be intermediates in the production of op-a_1.

3.4.5 Meiotic Analysis of a Haploid x Haploid Cross

The position of the mating type locus relative to its centromere is an important factor in determining segregation patterns. This distance was therefore mapped to provide background information necessary to interpret the results of crosses involving opaque strains, as reported below.

Teliospores from the haploid cross, 1.C2u4 x 2.716, were germinated and the meiotic products subjected to both random spore and tetrad analyses (see section 2.5). All genes observed in this cross assorted independently, as indicated by the recombination values close to 50% (table 10) and as expected based upon earlier work with these markers (Day, 1968). When two genes are examined by means of tetrad analysis and one or both of the genes assorts independently of its centromere, the frequency of tetratype segregation patterns approaches a value of 67%. If both genes are tightly linked to their respective centromeres, this percentage approaches zero as tetratypes are formed solely by recombinational events within the gene to centromere regions. The closer <u>both</u> genes are to their centromeres, the lower the frequency of tetratypes (Fincham and Day, 1971).

In this experiment, three gene combinations showed tetratype frequencies of less than 67%. In particular, the mating type and yellow loci showed a very low percentage (2.1), meaning that both genes are very close to their centromeres on linkage groups VI and VII, respectively. This

close linkage of y to its centromere was also demonstrated earlier in table 4 and has been reported by Cattrall *et al.* (1978). In this type of analysis, the percent recombination and therefore the map distance between a gene and its centromere is one half the percent tetratype. Consequently, the mating type locus like the yellow locus appears to be within a maximum of one map unit of its centromere. A third gene, lys_2, is also linked to its centromere on chromosome III, based upon these observations, although the linkage did not appear to be as close as the two other genes.

3.4.6 Infection of Plants with Crosses Involving Opaque Strains

The best method of determining which of the 5 postulated mechanisms is responsible for opaque formation appeared to be meiotic analysis of crosses involving opaque strains. Mechanisms 1 and 4 imply that the mating opaques would be hemizygous (op-a_1 = a_1/- and op-a_2 = a_2/-). Mechanisms 2 and 3 imply that both alleles were retained but only one was active (op-a_1 = a_1/[a_2] and op-a_2 = [a_1]/a_2), while mechanism 5 means that the mt locus would be homozygous (op-a_1 = a_1/a_1 and op-a_2 = a_2/a_2).

As mentioned earlier, mating opaques in pure culture were not capable of producing an infection resulting in teliospore formation. However, mixtures of op-a_1 and op-a_2 or mating opaque and haploid showed vigorous infections as characterized by an abundance of teliospores, quite different from the weak infections produced by primary diploids or op-N strains. The characteristics of the teliospores produced by op-a_1 x op-a_2, op-a_1 x haploid a_2, solopathogenic diploid a_1/a_2, and dikaryotic haploid a_1 x haploid a_2 infections are listed in table 11.

The volumes of teliospores increased in an approximate 1:2:3:4 ratio in the (1n+1n):(2n):(putative 2n+1n):(putative 2n+2n) classes. Thus, the crosses involving opaques yield much larger teliospores, a result which is in keeping with the suspected triploid (op-a_1 x haploid a_2) and tetraploid (op-a_1 x op-a_2) nature of these spores. However, the reasons for the particular ratio of spore sizes in this series are not obvious. If spore size is determined by total DNA content, then the 2n and 1n + 1n spores should have been

Table 10. Meiotic Analysis of a Haploid a_1 x Haploid a_2 Cross

Gene Pair A/B	% Recombination: Random Spore Analysis	Tetrad Analysis	Percent Tetratype	Gene B to Centromere Distance
y/lys_2	52.2	54.3	14.9	7.5
y/his_1	46.6	45.8	82.9	NL
y/uvs_1	50.0	55.3	72.3	NL
lys_2/his_1	56.2	48.9	80.9	N/A
lys_2/uvs_1	52.8	51.1	72.3	N/A
his_1/uvs_1	49.4	53.2	59.6	N/A
mt/y	46.1	52.1	2.1	1.1
mt/lys_2	48.9	57.5	12.8	6.4
mt/his_1	45.5	57.5	80.9	NL
mt/uvs_1	53.4	52.1	70.2	NL

Cross: 1.C2u4 x 2.716, a_1 y his_1 uvs_1 x a_2 lys_2.
A total of 178 meiotic haploids were scored in the random spore analysis and 47 teliospores were scored in the tetrad analysis.
NL - Not linked: recombination value is greater than 33%.
N/A - Analysis is not applicable as both genes are not linked to their centromeres.

of equal volume. Alternatively, because the spores are normally formed before nuclear fusion occurs (Day, pers. comm.), spore volume may be determined by the ploidy of the dikaryotic nuclei. Once again, the observed ratio does not fit this hypothesis as there seems to be no reason why 2n, 2n + 1n, and 2n + 2n teliospores should differ in size. Until more work on the molecular biology of spore formation is done, the reasons for the size ratio shown by this polyploid series are likely to remain obscure.

Germination was normal in all four categories i.e. 3 celled promycelia producing four sporidial products were observed. However, the sizes and growth rates of sporidia from the putative triploid op-a_1 x haploid a_2 cross were very variable, indicating the presence of many aneuploids, whereas sporidia from the putative tetraploid spores (op-a_1 x op-a_2) were uniform and large enough to be diploid. Thus, these results strongly suggest that the op-a_1 x op-a_2 cross did indeed result in tetraploid teliospores and that the op-a_1 x haploid a_2 gave triploid teliospores. This deduction was confirmed by random spore analysis and by examination of a few tetrads (see section 2.5).

3.4.7 Meiotic Analysis of an Op-a_1 x Op-a_2 (Tetraploid) Cross

The results of a random spore analysis of the op-a_1 x op-a_2 cross are presented in table 12. The expected segregation frequencies of markers in this putative tetraploid were based upon two assumptions: 1) that, because of genetic homology, tetravalents would be formed in this cross, and 2) the chromosome segregation pattern was 2:2 yielding diploid products rather than 3:1 or 4:0 yielding aneuploids. For a gene in the triplex condition (one copy of the recessive allele and three of the dominant) the expected frequency of the expression of the recessive allele in the diploid products of meiosis ranges from 0% (no crossing-over between the gene and its centromere) to about 4% (100% of teliospores with crossing-over in this region). In the duplex state (2 recessive, 2 dominant) the values range from 16.7% to 22%. Much of the relevant theory has been worked out during the analysis of tetraploid meiosis in *Saccharomyces cerevisiae* (Roman, Phillips, and Sands, 1955). The results presented here (table 12) coincide

Table 11. Teliospore Size and Germination in a Polyploid Series

Cross	Teliospore Diameter (μm)[1]	Volume (μm³)	Ratio of Volumes	Percent Germination	Sporulation Ability[2]	Germination Morphology
1n + 1n: Hap. a_1 x hap. a_2 (diploid teliospores)	sample 1: 5.10 sample 2: 5.34 (spherical)	69 79	1.00 –	80 – 90 88	– good	Normal teliospore + 3-celled promycelium buds off 4 haploid products of meiosis
2n: a_1/a_2 Dip. B45 (diploid teliospores)	6.37 (spherical)	135	1.95	80–90	poor	Normal, as above
2n + 1n: Op-a_1 x hap. a_2 (triploid teliospores)	7.08 (spherical)	186	2.70	90	good	Normal initially product variable in size and growth rate; probably mostly aneuploids
2n +2n: Op-a_1 x op-a_2 (tetraploid teliospores)	8.47 (slightly ellipsoid)	275	4.00	79	good	Normal initially; most of meiotic products shown to be diploid

1 – Mean of 50 teliospores.
2 – Good sporulation: anthers purple, distended; petals dusted with teliospores.
Poor sporulation: anthers yellow to brownish yellow.

Table 12. Tetraploid Meiosis and Random Spore Segregation in the Diploid Op-a_1 x Diploid Op-a_2 Cross

Marker	Colonies Expressing Marker	Total Colonies	Allele Frequency (%)	Expected Allele Frequency from Tetraploid Segregation (%)
$inos_1$	1	111	.9	0-4
cit_1	4	111	3.6	0-4
lys_3 and lys_2	4	111	3.6	0-8
his_1	2	111	1.8	0-4
arg_1	3	111	2.7	0-4
y	33	200	16.5	17-33
o	4	150	2.7	0-4
uvs_1	16	111	14.4	17-33
			Phenotypic Frequency (%)	
a_1/a_2	88	128	69	
a_1/a_1 or a_2/a_2	40	128	31	
	In sample of 19:			
	9-a_1/a_1	19	47	
	10-a_2/a_2	19	53	

Cross: D10 op-a_1-1 x 118/C4 op-a_2-11. (?: unknown mating type condition).

op-a_1-1: $\frac{or\ +}{+\ y}$ $\frac{inos_1}{+}$ $\frac{+}{+}$ $\frac{uvs_1}{+}$ $\frac{+}{+}$ $\frac{+}{+}$ $\frac{+\ +}{lys_3\ cit_1}$ $\frac{a_1}{?}$

op-a_2-11: $\frac{+\ y}{+\ +}$ $\frac{+}{+}$ $\frac{lys_2}{+}$ $\frac{uvs_1}{+}$ $\frac{his_1}{+}$ $\frac{+}{arg_1}$ $\frac{+\ +}{+\ +}$ $\frac{?}{a_2}$

The lower allele frequency is expected if no crossing - over occurs, and the higher frequency is expected if very frequent crossing - over occurs in the region between the gene and its centromere.

closely to the expected values as predicted by these analyses. All triplex markers were expressed in the expected 0 - 4% range while the two duplex markers both had values close to the expected 16.7%. These results suggested that the two assumptions mentioned above were valid and that a normal tetraploid meiosis producing diploid products had occurred. To confirm this, several pink meiotic products were treated with PFP to induce mitotic haploidization (see section 2.6). Yellow and orange papillae were produced from each pink isolate, proving that these colonies were not haploid but were disomic for chromosome VII (y +/+ o) at least and, therefore, were probably fully diploid.

The segregation of the marker genes in this op-a_1 x op-a_2 cross establishes that a tetraploid meiosis producing diploid products had occurred. The segregation of the mating type locus is the principal point of interest as this depends on the state of this locus in these opaques. Sixty-nine percent of the meiotic isolates showed the characteristic colonial and sporidial morphology of SPP cells and, therefore, were a_1/a_2 genotypically. The remaining 31% were diploid in cell size but did not give the a_1/a_2 phenotype. Nineteen of these types were tested for mating type and approximately 50% were a_1 and 50% a_2 (table 12). These types were indistinguishable from the original parental op-a_1 and op-a_2 strains. In accord with the 5 putative mechanisms for mating opaque production listed earlier (section 3.4), three segregation patterns for mating type were possible (table 13). Deletion of one mating type allele, mechanism 1, would have resulted in the "hemizygous" pattern. Loss of one complete homologue of chromosome VI in each of the opaques, mechanism 4, would have produced a "diploid" pattern. Mechanism 5 implied that normal tetraploid segregation would occur ("tetraploid" pattern). The patterns predicted by mechanisms 2 and 3 were uncertain as they depended on whether the inactivation was permanent or temporary. If the inactivation could have been reversed by passage through the host plant, then the tetraploid pattern would have been observed while no reversal of the inactivation would have produced the hemizygous pattern of segregation. Since the mating type locus was shown earlier to be tightly linked (less than 1 map unit) to its centromere,

Table 13. Possible Opaque Genotypes and Their Expected Segregation Patterns Following Meiosis in Op-a_1 x Op-a_2 Crosses

	Hemizygous Pattern			Diploid Pattern			Tetraploid Pattern		
	Op-a_1	Op-a_2		Op-a_1	Op-a_2		Op-a_1	Op-a_2	
Opaque Constitutions	$\frac{a_1}{a_1}$ ——	$\frac{a_2}{a_2}$ ——		$\frac{a_1}{a_1}$ ——	$\frac{a_2}{a_2}$ ——		$\frac{a_1}{a_1}$ —— $\frac{a_1}{a_1}$ ——	$\frac{a_2}{a_2}$ —— $\frac{a_2}{a_2}$ ——	
Pachytene Configuration	$\frac{a_1}{a_1}$ —— —— $\frac{a_2}{a_2}$ ——			$\frac{a_1}{a_1}$ —— $\frac{a_2}{a_2}$ ——			$\frac{a_1}{a_1}$ —— $\frac{a_1}{a_1}$ —— $\frac{a_2}{a_2}$ —— $\frac{a_2}{a_2}$ ——		
Segregation Pattern in Meiotic Progeny	Gen.	Phen.	Exp. %	Gen.	Phen.	Exp. %	Gen.	Phen.	Exp. %
	$a_1/-$	op-a_1	33.3(40)	a_1	op-a_1	50	a_1/a_1	op-a_1	16.7
	$-/a_2$	op-a_2	33.3(40)	a_2	op-a_2	50	a_2/a_2	op-a_2	16.7
	a_1/a_2	primary diploid	16.7(20)				a_1/a_2	primary diploid	66.7
	$-/-$	unknown	16.7 (0)						

Estimated frequencies uncorrected for effects of crossing-over.
Frequencies in brackets are estimated assuming that the -/- type is lethal.
Gen. = Genotype; Phen. = Phenotype; Exp. % = Percent Expected

crossing-over between mt and its centromere will not greatly affect any of these predicted patterns. Furthermore, even if bivalents were formed instead of tetravalents, no change in any of the patterns would result e.g. the predicted ratio for the tetraploid pattern would remain at 16.7:16.7:66.7, $a_1{:}a_2{:}a_1/a_2$.

The results obtained for mt segregation (15.5:15.5:69, table 12) clearly fit the tetraploid pattern and thus show that both of the mt alleles were present in two copies in the mating opaques. This observation eliminates mechanisms 1 and 4 and strongly supports mechanism 5. Mechanisms 2 and 3 are only tenable in the event that the postulated inactivation process is reversed during plant infection.

3.4.8 Meiotic Analysis of an Op-a_1 x Haploid a_2 (Triploid) Cross

As described mechanism 5 is favoured by the above results but, mechanisms 2 and 3 remain possibilities in the event that the inactivated mating type allele is reactivated during growth in the plant or upon germination of the teliospore. If this is so, the op-a_1 parent in the op-a_1 x haploid a_2 cross would change from being $a_1/[a_2]$ to a_1/a_2 and result in triploid spores of the genotype $a_1/a_2/a_2$. On the other hand, mechanism 5 holds that the op-a_1 is homozygous a_1/a_1 and, therefore, the triploid spores would be $a_1/a_1/a_2$. Analysis of triploid spores should allow a clear distinction between these hypotheses based on the very different expected ratios of a_1 and a_2 products of meiosis.

The meiotic products of the teliospores of this op-a_1 x haploid a_2 cross were very variable in size, shape, and growth rate, indicating the presence of many aneuploids. The results from the random spore analysis of triploid teliospores are given in table 14. The segregation of seven unlinked loci, including mt, should yield 128 different genotypes. Seventy-five were recovered in the 190 progeny studied and the segregation patterns were consistent with no linkage between most of the genes (see below).

The allele frequencies of the five recessive markers, arg$_1$, [illegible], y, uvs$_1$, and cxr$_2$, were between 22.1% and 32.1%, compared to the expected allele

frequency for triploid segregation of 16.7% to 33.3% (depending upon the survival of the disomic segregants, see table 15). $\underline{Lys_2}$, present on two of the three chromosomes, was recovered in 53.2% of the progeny, compared to an expected recovery rate of between 50.0% and 66.7% (table 15). All of the marker genes carried in this cross, therefore, segregated as expected on the basis of a normal triploid segregation pattern. It was noted that crossing-over would have had very little effect upon these expectations, particularly as many of the genes are closely linked to the centromere. The evidence of frequent aneuploidy together with the recovery, in the expected ratios, of all recessive markers carried by both the opaque and the haploid, confirmed that these teliospores were triploid (tables 14 and 16).

As in the tetraploid cross, several different segregation patterns can be predicted consistent with the postulated mechanisms. Loss of one copy of chromosome VI (mechanism 4)would result in a 50:50:0, a_1:a_2:a_1/a_2 ratio in the meiotic progeny. Deletion of one mating type allele (mechanism 1) would yield a 40:40:20 as would inactivation of either chromosome VI or just one $\underline{mt}$ allele if reversal of inactivation was not possible. Reactivation of a silenced a_2 allele in the op-a_1 would produce a triploid nucleus with a genotype of $a_1/a_2/a_2$. Segregation of these alleles in turn would result in a 17:50:33, a_1:a_2:a_1/a_2 pattern if 100% of the disomics were viable and 33:67:0 at 0% survival of disomic progeny. Mechanism 5, which implies that the op-a_1 genotype is a_1/a_1 and,therefore, the triploid nucleus is $a_1/a_1/a_2$, would yield a 50:17:33 or a 67:33:0 segregation pattern at 100% and 0% disomic survival, respectively (table 15). The observed segregation was 54:36:10 which fits approximately with the 40:40:20 pattern (best fit is around 40% disomic survival with a predicted ratio of 45:45:10) or with the 50:17:33 pattern (best fit at 20% disomic survival and a ratio of 61:29:10, table 15). Thus, either mechanisms 1 and 5 are possible on the basis of these results. The inactivation hypotheses are tenable only if reactivation was not possible. The other hypotheses, including any inactivation/ reactivation mechanism or loss of one homologue of chromosome VI (mechanism 4) can be excluded.

Table 14. Triploid Meiosis and Random Spore Segregation in the Diploid Op-a_1 x Haploid a_2 Cross

Marker	Colonies Expressing Marker	Total Colonies	Allele Frequency (%)	Expected Allele Frequency from Triploid Segregation (%)
arg_1	61	190	32.1	17-33
lys_2	101	190	53.2	50-67
his_1	44	190	23.2	17-33
uvs_1	42	190	22.1	17-33
y	48	190	25.3	17-33
cxr_2	59	190	31.1	17-33
			Phenotypic Frequency (%)	
a_1 or a_1/a_1	100	186	54	
a_2 or a_2/a_2	67	186	36	
a_1/a_2	19	186	10	

Cross: 118/C4 op-a_1-5 x 2.716cx. (?: unknown mating type condition).

op-a_1-5: $\frac{y}{+}$ $\frac{lys_2}{+}$ $\frac{his_1}{+}$ $\frac{uvs_1}{+}$ $\frac{+}{arg_1}$ $\frac{+}{+}$ $\frac{a_1}{?}$

2.716cx: + lys_2 + + + cxr_2 a_2

The lower allele frequency is expected if 100% of the disomics survive, and the higher frequency is expected if 0% of the disomics survive.

Table 15. Expected Random Spore Segregation as a Result of Triploid Meiosis

Chromosomes	Possible segregation patterns at meiosis I		Genotype of tetrad	
I $\frac{a}{a}$	1. I	II,III	a,a	AA,AA
II $\frac{A}{A}$	2. II	I,III	A,A	Aa,Aa
III $\frac{A}{A}$	3. III	I,II	A,A	Aa,Aa
			Monosomic Segregants	Disomic Segregants

Random Spore Analysis

Frequency of Each Genotype in Random Meiotic Products (%)

Event	A	a	AA	Aa	aa
1. All disomics viable	33.3	16.7	16.7	33.3	0
2. All disomics inviable	66.7	33.3	0	0	0

Application to Data

1. Markers $\underline{arg}_1$, $\underline{his}_1$, $\underline{uvs}_1$, $\underline{y,cxr}_2$: Let A = dominant, a = recessive. Expected % recessive phenotype = 16.7 to 33.3.

2. Marker $\underline{lys}_2$: Let A = recessive, a = dominant. Expected % recessive phenotype = 50.0 to 66.7.

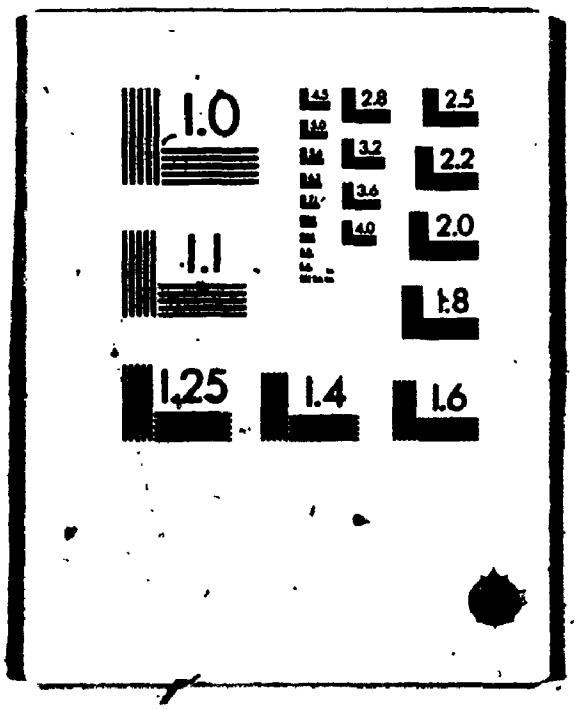
3. Mating type: Let A = a_1, a = a_2. Expected phenotypic frequencies as follows:

Phenotype	Observed Frequency (%)	Expected Frequency at: 0% Disomic Survival	20% Disomic Survival	100% Disomic Survival
a_1 (a_1 or a_1/a_1)	54	66.7	61	50.0
a_2 (a_2 or a_2/a_2)	36	33.3	29	16.7
a_1/a_2	10	0	10	33.3

Only one mechanism of opaque origin, number 5, was consistent with the observations obtained in both tetraploid and triploid analyses. The inactivation/ reactivation hypotheses, which were consistent with the tetraploid data, were not possible on the basis of the triploid results. Similarly, the deletion and inactivation/ no reactivation hypotheses, which were in agreement with the triploid observations, were not consistent with the tetraploid results. Thus, the op-a_1 and op-a_2 types, therefore, can be assumed to be diploid and homozygous for a section of chromosome VI bearing the mating type locus. The size of this section, altered during opaque formation, remains to be determined. This determination depends upon the isolation of mating type linked genes.

The most likely mechanisms of origin of these opaques are mitotic crossing-over or non-disjunctional events involving chromosome VI. The evidence that UV light induces opaque formation to the same extent as it induces mitotic crossing-over near other marker genes supports the former possibility. If so, the spontaneous frequency of mitotic exchange near the mating type locus on chromosome VI in a_1/a_2 diploids is at least 10 times higher than that occurring near other loci so that as many as 0.5% of the mitotic products have a re-arrangement of the genes in this region.

Seventy-five different genotypes were detected in the 190 meiotic segregants from the triploid cross compared to the 128 possible combinations of seven genes. Many more segregants would have had to have been scored to have detected all 128 types. The 75 genotypes recovered were examined for any evidence of linkage between these markers. Such an analysis could only be approximate because it was not certain what proportion of the disomics would survive and whether or not this proportion might vary for different chromosomes. All genes, however, appeared to be segregating independently with the exception of the $\underline{cxr_2}/\underline{his_1}$ combination. The possibity of linkage between these two genes should therefore be checked by meiotic or mitotic analyses of appropriate haploid x haploid crosses or diploids.



3.5 Discussion

Previous work on the mating type properties of diploid strains of *Ustilago violacea* was based on the assumption that these types were stable. The results presented here clearly show that this assumption is not valid. Samples of cultures from stock plates kept at 4°C and then grown at 22°C superficially will appear to be the same as the original diploid but in fact have undergone changes in the region of the mating type locus at high frequency. These altered types - "opaques" will be detected only after the cultures are incubated below 20°C on CM. Then the cultures will show a preponderance of cells that remain vegetative rather than initiate SPP development. The finding that diploids undergo changes which are selected by refrigeration explains partially some of the confusion about the mating properties of diploids. Initially, diploids were described as neutral (non - mating [Day and Jones, 1968]). Subsequent studies suggested that mating with a_1 haploids could occur in these strains and was a function of the age of the culture as well as position in the cell cycle (Day and Cummins, 1973, 1975). However, the diploid strain, HD2, used in these studies was lost and other a_1/a_2 diploids have not been induced to mate with either haploid under any conditions. At present, the most likely explanation is that the HD2 diploid used in the work reported in Day and Cummins (1973, 1975) was in fact an opaque derivative rather than a primary diploid. This uncertainty emphasizes the hitherto unexpected problems of genetic instability in diploid strains and points out the need for stringent precautions during the maintenance and re-isolation of such strains. Future experiments concerning mating and morphogenesis in diploids should include repeated assessment of the colonial growth characteristics. Continuous subculturing at 22°C on CM would minimize the proportion of opaques in the culture substantially since the selective pressure for altered cell types would not be as great as at lower temperatures. However, it must be realised that pure a_1/a_2 diploid cultures do not exist as opaques are produced at a rate of about 10^{-3} in both VP and SPP. At present, the best procedure is to avoid low temperature selection of opaques and to reisolate the desired cell type frequently. If such precautions are taken, the analysis of mating

type changes during the cell cycle in a variety of opaques as begun in Day and Cummins' (1973, 1975) work may yield very interesting results.

Observations made on the four opaque strains indicated that only the mating type locus or chromosome VI carrying this gene was altered in the transition from primary diploid to opaque. Thus, the four opaques showed various changes in sporulation, pathogenicity, and mating. At the same time, these strains still proved to be diploid and unaltered for genes on various chromosomes controlling ultraviolet sensitivity, drug resistance and nutritional requirements. Mitotic haploidization, mitotic crossing - over, and meiotic analyses showed that all recessive traits were retained in the opaque strains. The alteration which results in the change from primary diploid to opaque type must therefore be restricted to chromosome VI.

The most efficient means of determining the mechanism of opaque formation would be to determine the effect of the change on other genes carried on chromosome VI. However, the lack of any marker linked to the mt gene, despite an extensive attempt to identify one, meant that other methods had to be used. The procedures consisted of determining a) if mating type changes occurred in haploid cells; b) if ultraviolet irradiation had any effect on the frequency of changes; and c) the number of copies of each mating type allele present in mating opaque strains by means of polyploid segregation patterns.

No evidence of any change in the mating type of a haploid strain of *U. violacea* was obtained in either this work or in many years of observation of this organism. Alterations of the mt gene or chromosome VI were observed only in diploid cells. This situation, therefore, is clearly different from the mechanisms of mating type switching seen in haploid clones of the yeasts, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis* (Winge, 1935; Leupold, 1959; Herman and Roman, 1966). In each of these organisms, it has been hypothesized that strains capable of switching mating types carry at least one silent copy of one mating type allele and an active copy of the other allele.

The switch from one mating type to the other, a to α or α to a, in *S. cerevisiae* occurs when a copy of the inactive allele is inserted into the MAT locus, displacing the previously active allele. This mechanism has been termed the "cassette model" (Oshima and Takano, 1971; Harashima, Nogi, and Oshima, 1974; Hicks, Strathern, and Herskowitz, 1977). Copies of inactive alleles are maintained at two equivalent loci termed HMR and HML. Silent α or a alleles may be carried at either of these two genes (Naumov and Tolstorukov, 1973; Harashima et al., 1974; Arima and Takano, 1979; Strathern *et al.*, 1980). Cassette exchange is mediated by the HO allele of the homothallism gene, HO (Winge and Roberts, 1949; Hawthorne, 1963).

In *S. pombe*, crosses involving homothallic (h90) and heterothallic (h^+ and h^-) strains showed that mating type is determined by two very closely linked loci, termed mat_1 and mat_2 (Leupold, 1959; Gutz and Doe, 1973). Mat_1 contains information for the minus (M) mating type and mat_2 for the plus (P) mating type (Leupold, 1959). In homothallic strains, switches between the two mating types may occur by the inversion of the intervening sequence between mat_1 and mat_2 (the flip - flop model, Egel, 1977). Alternatively, or perhaps in conjunction, insertion of P information from mat_2 into the mat_1 locus results in P expression and M silence. Reversion to M mating type would then occur by the exact excision of the P sequence (Leupold, 1980).

Kluyveromyces lactis has two mating types, a and α. Conversion from α to a is dependent upon the gene, Hα, while a to α changes require a separate gene, Ha (Herman and Roman, 1966). Superficially, this system closely resembles the *S. cerevisiae* situation but more information is necessary before more definite comparisons can be made. However, as stated earlier, the situation in the basidiomycete, *U. violacea*, is quite different from that in these yeasts as no mating type interconversions occur in haploid strains. Instead, mating type changes only occur when the mating type alleles are brought together in the same nucleus, i.e. as a diploid.

Five mechanisms, based upon the requirement for the diploid state, could have been responsible for the production of the mating opaques (section

3.4). Two of the hypotheses (1 and 4) would have resulted in the mating type gene being hemizygous in the opaques. Two others (2 and 3) implied that the mating type gene was functionally hemizygous with only one of the two alleles in an active configuration. Mechanism 5 meant that the mating type gene had become homozygous for either a_1 or a_2 mating type.

Evidence in favour of the fifth hypothesis was obtained using ultraviolet light to increase mitotic crossing - over in the primary diploid, D10. The induction of opaques was of a similar magnitude (20 fold) to the stimulation of mitotic crossing - over near marker genes. The equal stimulation by UV of production of all four opaques suggests that each type was formed by similar mechanisms, namely mitotic crossing - over. As the overall frequencies of opaque production were much higher than mitotic crossing - over near marker genes, it appears that there is a hot spot of mitotic crossing - over in chromosome VI near the mating type locus.

Analysis of the meiotic products of triploid (op-a_1 x haploid a_2) and tetraploid (op-a_1 x op-a_2) crosses established that the mating opaques were homozygous for mating type. Thus, mechanism 5 again appears to be responsible for the production of mating opaques. It should be noted that only one op-a_1 and one op-a_2 were analysed in these polyploid crosses. Any of the five proposed mechanisms could give rise to a strain with a mating opaque phenotype. Several other mating opaques would have to be examined in meiotic analyses similar to the ones already carried out before it can be unequivocally stated that only one mechanism is responsible for opaque origin. These strains may very well prove to be a phenotypic class produced by a variety of mechanisms.

Little can be said about the origin of op-N or op-C types as no relevant experiments were performed using these strains. However, the UV induction experiment makes it likely that these types arise by the same recombinational mechanism but that the precise position of the crossing - over event may determine whether or not mt regulatory loci also become homozygous. As altered mating type alleles in both op-N and op-C strains were detected earlier, it is also possible that the recombinational events

may be occurring within the mating type gene in these cases. The pronounced difference in frequency of appearance of op-a_1 and op-a_2 types (table 1) cannot be explained satisfactorily yet. The large excess of op-a_2 types may, perhaps, indicate that the a_1 allele is coupled to a marker which would confer lethality when homozygous. The surviving op-a_1 and possibly the op-C which spontaneously produce op-a_1 types would presumably have avoided this as a result of the position of the crossing-over or as a result of a second cross-over event. Clearly, there are several unsolved problems remaining here.

The mating phenotype of the opaque strains on artificial media provide further evidence that the stage termed "sexual spore precursor phase" or "SPP" is completely analogous to the initial stages of teliospore formation in the host plant (Day, 1979). In particular, the op-N types showed both a delayed response to the environmental conditions which induce SPP development and a drastic decrease in the number of teliospores produced in the anthers, as compared to the primary diploid. In Chapter 4 of this thesis it is shown that this decrease is not due to a block in the initial stages of infection as infection hyphae were readily produced by the op-N strains. Thus, the control of teliospore formation may be studied on artificial media with a high degree of confidence that one is actually examining this process and not some response peculiar to the laboratory situation.

The crosses involving opaque strains also shed new light on the role of the mating type locus in pathogenicity. Thus, a_1/a_2 strains are solopathogenic although they undergo no nuclear fusion, sporulate weakly, and go through meiosis as a diploid, yielding haploid sporidia. This poor pathogenicity of solopathogenic diploids has also been reported for other smut and rust fungi (Caten and Day, 1977). The a_1/a_1 or a_2/a_2 strains, on the other hand, are not solopathogenic, and when combined with a strain of opposite mating type establish a dikaryotic infection which sporulates strongly. They produce triploid or tetraploid teliospores which segregate aneuploids or diploids at meiosis. Clearly, the interaction of two mating type alleles within the same nuclear membrane is very different from their

interaction in the two separate nuclei of the dikaryon. These different interactive situations enable the mating type locus to do far more than direct conjugation. In effect, the mating type locus acts as a master developmental switch controlling plasmogamy, karyogamy, sporulation, pathogenicity, vegetative budding, and response to environmental signals such as cations, temperature and a host plant product (Day, 1979; see Chapter 4 of this thesis). Changes in the mating type locus of the type described here thus have important effects on development of this fungus.

CHAPTER 4

INDUCTION OF THE PARASITIC STATE BY HOST PRODUCED COMPOUNDS

4.1 Introduction

One of the central problems faced by plant pathologists is the high degree of specificity in host - parasite relationships. How is it determined? Virtually all plant pathogenic fungi attack only a very few of the total number of plant species. Thus, *Ustilago maydis* attacks only the grasses, *Euchlaena mexicana* and *Zea mays* (corn) (Fischer, 1953), of the more than 285,000 species of angiosperms (Arms and Camp, 1982). Why? In general, two possibilities exist. Either immune plant species have physical or chemical defences which prevent infection or they lack specific compounds which would be required by the pathogen for successful infection. Most research to date has been focussed upon the role of host defense mechanisms to pathogens. While some defense mechanisms are a permanent or normal aspect of the structure of a particular species, others are formed only in response to wounding or infection.

There are many examples of permanent or constitutive resistance factors, both chemical and physical. Thus, in some varieties of plums, resistance to the brown rot fungus, *Monilinia fructicola*, is due to a thickened cuticle which resists mechanical penetration by the fungus (Curtis, 1928). Similarly, resistance to the onion smudge pathogen, *Colletotrichum circinans*, occurs in onion varieties with pigmented yellow or red bulb scales while varieties with white scales are susceptible. This resistance of coloured varieties is determined by water soluble phenolic compounds (catechol and protocatechuic acid) which occur only with the yellow flavone and red anthocyanin pigments. These phenolic substances

diffuse into the infection drop preventing germination and penetration by the fungus (Walker, 1923; Angell, Walker and Link, 1930; Link and Walker, 1933).

Many examples of toxic substances which are induced by wounding or infection have been identified since the term, "phytoalexin", was coined to describe these compounds (Muller and Borger, 1940; cf. Cruickshank, 1980). Rapid accumulation of a phytoalexin in response to an attack by a parasite commonly results in localized necrosis of the resistant plant tissue. This feature, termed "hypersensitivity" (Stakman, 1915), is a common and an effective form of resistance to fungal and bacterial parasites. The pathogen is either killed by the hypersensitive response or remains isolated in a necrotic patch of host tissue and cannot spread further. The first phytoalexin chemically identified was "pisatin", a pterocarpan isolated from *Pisum sativum* (Perrin and Bottomley, 1962). Since then, many chemically diverse compounds (Stoessl, 1982) from a wide range of plant families have been described. Phytoalexins in the Leguminosae and the Solanaceae have been particularly thoroughly documented (Ingham, 1982; Kuc, 1982). Certain generalizations about the characteristics of these compounds can be made. Firstly, they are secondary metabolites - compounds which are not essential to normal growth and metabolism of the plant but are produced in stress situations (Martin and Demain, 1980). Secondly, they may be induced by a number of biotic stimuli, including bacteria and fungi which are non-pathogenic to the plant species observed, as well as by abiotic stimuli, such as wounding, freezing and exposure to heavy metals (Bailey, 1982). Quite often this induction does not result in sufficient concentrations of the phytoalexins to produce the hypersensitive reaction. Instead, the host tissue becomes sensitized to further induction which results in an even greater response. Thus, prior exposure to a non-pathogenic fungus, for example, can provide resistance to subsequent pathogenic attack on an otherwise susceptible host plant (Deverall, 1982). Coupled with this observation is the third generalization: most phytoalexins exhibit little specificity and inhibit a wide range of fungal and bacterial species (Smith, 1982).

In spite of these generalizations, the role of phytoalexins in resistance is far from clear. In many cases the difference between a susceptible and resistant plant of a particular species cannot always be attributed to the absence of phytoalexin production in the susceptible cultivar. Instead, it appears that susceptible varieties may not respond rapidly enough to ward off attack and the pathogen then penetrates to surrounding tissue (Mansfield, 1982). In some cases, although phytoalexins are produced, they may be metabolized to a less active form by the invading organism (VanEtten, Matthews and Smith, 1982), e.g. *Botrytis fabae* can catalyze the reduction of wyerone acid to a less active compound in culture (Mansfield and Widdowson, 1973) and possibly during infection (Mansfield and Deverall, 1974).

These examples imply that susceptibility is a negative attribute of a plant and resistance a positive one since the inhibitory factor is generally considered to be either absent or present in concentrations too low to be effective in susceptible plants (Stranga, Majer and Smith, 1974). While this concept is clearly important, carried to an extreme it leads to the view that all pathogens can attack any host species, and only the chemical and physical resistance mechanisms of a particular host limit its potential pathogens to those known today. Intuitively, this seems unlikely. Positive or promotory interactions between host and parasite may also play a role. Thus, susceptibility in some cases may depend on the presence of one or more plant products essential for fungal growth and development and, therefore, be a positive attribute of the plant. Resistant plants would lack these products or have them in lower concentrations. Evidence for this in some plant - pathogen relationships is accumulating.

Brown (1922) found that old spores of *Botrytis cinerea* are capable of germinating and attacking *Cereus* spp. which exude large quantities of nutrients, presumably sugars, but are not parasitic on *Gloxinia* spp. which do not exude similar quantities. Similarly, exudates of turnip roots were found to stimulate the germination of oospores of *Pythium mamillatum* (Burton, 1957). A compound, possibly allyl isothiocyanate (Hooker, Walker, and Link, 1945), produced by crucifer roots but not by roots of non-cruciferous species stimulated the germination of resting spores of

Plasmodiophora brassicae (Macfarlane, 1952). More recently, choline and its oxidation product, betaine, were identified as two components in wheat anthers that stimulate the growth of the headblight organism, *Fusarium graminearum*, (Strange and Smith, 1971; Strange, Majer, and Smith, 1974). Similar situations in which host compounds increase growth and virulence were described for the pathogens *Helminthosporium sokorianum* (Endo and Oertti, 1964), *Cladosporium herbarum* (Fokkema, 1968), *Alternaria brassicola* (Channon, 1970), and *Phoma betae* (Warren, 1972).

In each of these examples, the plant product appears to act as a nutrient and a growth stimulant. The fungus may be considered to be auxotrophic for the particular compound and the host may be considered to be a growth medium (Garber, 1960). Very few cases in which specific developmental changes are induced in the fungus by host products have been described. Anthranilic and chlorogenic acid, as well as an unidentified volatile compound in plant tissue, soil and some fungal spores, cause *Ustilago nuda* to switch from sporidial to mycelial growth (Nielsen, 1966; 1968). Similarly, a volatile compound produced by host tissue induces the wheat stem rust fungus to form infection structures (Grambow, 1977). Host plant products, such as these compounds, which act as fungal hormones have been termed "mycoboethins", literally "fungal aiders" (Day, Castle, and Cummins, 1981).

As outlined in the general introduction to this thesis, *Ustilago violacea*, is a dimorphic fungus. It can grow either in a mycelial form, parasitizing systemically a suitable host plant, or in a yeastlike manner, proliferating by budding. During saprophytic growth, in the absence of any contact with the host, growth is predominantly in the form of yeastlike sporidia. The few hyphae produced in some cultures grow to only 10 - 20 µm in length before budding recommences at the hyphal tip. Growth in the host, in contrast, is exclusively mycelial in form with intercellular penetration of host tissue by hyphal strands. Sporidia are not observed in any part of the parasitized host. When sporidia potentially pathogenic, i.e. heterozygous for mating type, approach closely to, or contact a host plant, infection hyphae termed "suchfaden" (Bauch, 1922) are formed (Day

et al., 1981). Thus, the stimulus required for the switch from yeastlike to mycelial growth appears to come from the host plant. As contact was not an absolute requirement, the stimulus must have been diffusible. The compound exuded by the leaf appeared to act as a fungal hormone which resulted in a highly specific redirection of morphogenesis in the fungus, leading to the induction of the parasitic stage in previously saprophytic cells.

The purposes of this part of my thesis were:

(1) to describe the effect of host products on development in various races of *U. violacea* and other species of smut fungi;

(2) to determine how widely distributed the active compounds are in angiosperm plant species;

(3) to isolate and identify the compound(s) involved.

4.2 Response of *Ustilago violacea* Race SA-1 to *Silene alba* Extracts

In the initial stages of this investigation, leaves of white campion, *Silene alba*, were crudely macerated in water and the debris was removed by filtration or centrifugation (1250xg). The effects of such extracts and also acetone and methanol extracts on cells of different nuclear condition of race SA-1 are described in the next sections.

4.2.1 Haploid Clones

An acetone extract of *S. alba* leaves was dried and resuspended in the same volume of water. One millilitre was added to 10 mL of liquid CM containing approximately 5 x 10^6 sporidia/ mL at 0, 24, and 48 h during the incubation period. Sterile water was added to the control cultures at the same times. The extract did not alter the growth rate of either a_1 or a_2 cells (fig. 4) and did not induce hyphae or other morphological changes in either haploid in this test or in similar tests using solid media

Figure 4. Growth Rates of Haploids in Response to *Silene alba* Extracts

Effect of an acetone extract of *Silene alba* leaves on the growth rate of (a) a_1; (b) a_2; and (c) a_1 mixed with a_2 cells of race SA-1. One millilitre of the extract was added to 10 mL of CM containing 5×10^5 sporidia/ mL at 0, 24, and 48 h during the incubation period (▲). Water was added to the control cultures (◇) in place of the extract.

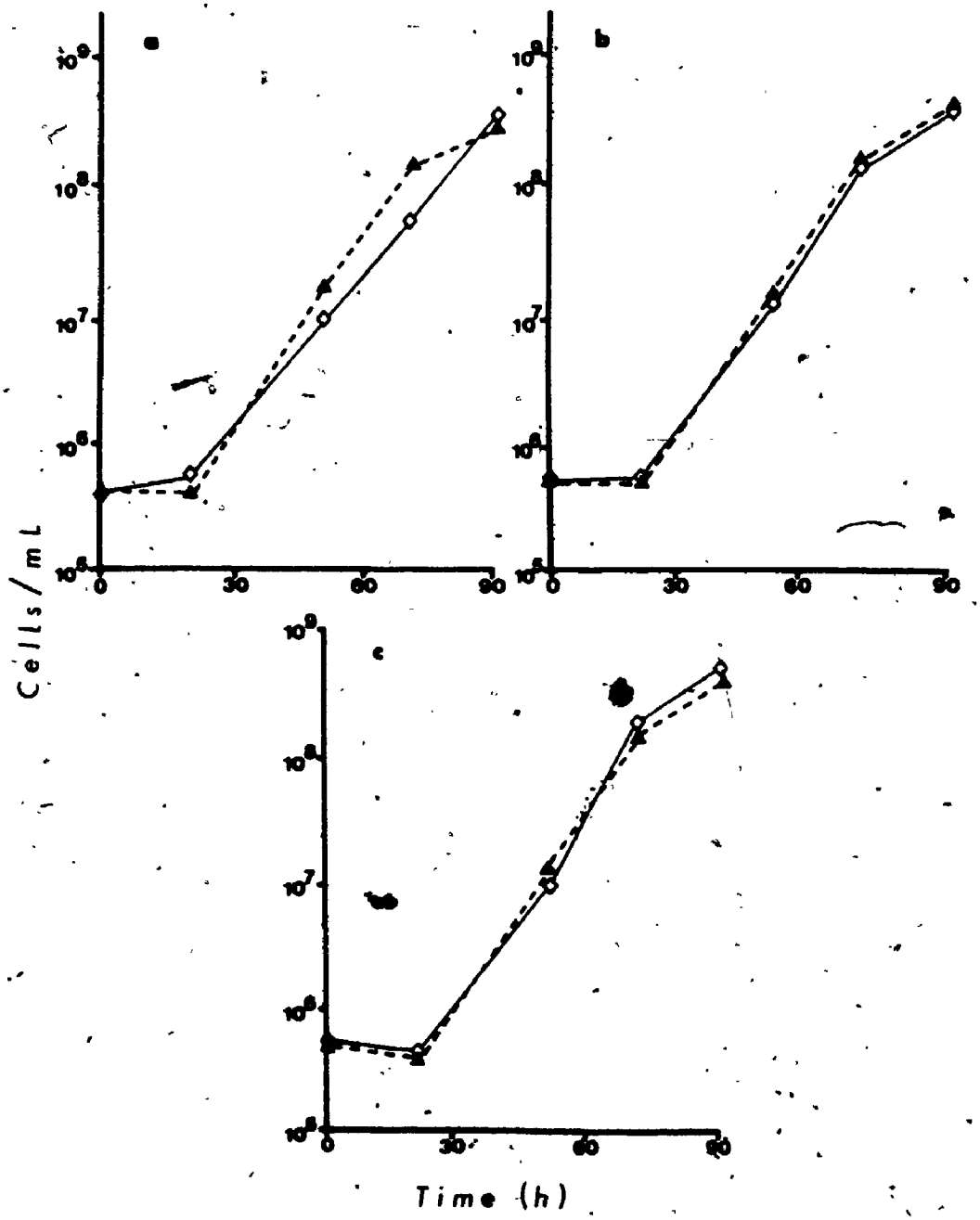
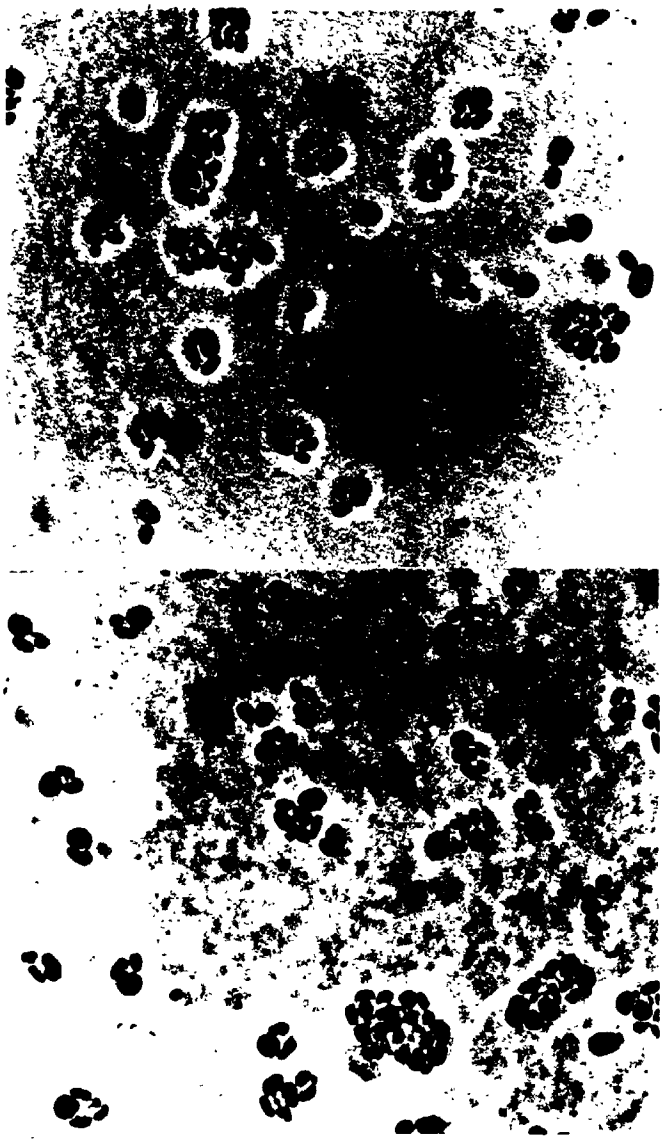

a
b
c
Cells / mL
Time (h)
0
30
60
90

Plate 4. Response of Haploid Clones to Extract of *Silene alba* Leaves

Pure cultures of a_1 haploids did not show any difference in growth pattern when treated with *S. alba* extract (bottom, x500) as compared to the same culture treated with water (top, x500).



(plate 4). To be certain that the extract contained the hyphal growth stimulus, it was tested at the same final concentration on mated haploid cells (see section 4.2.3). The production of hyphae by these cells indicated that the extract was indeed active.

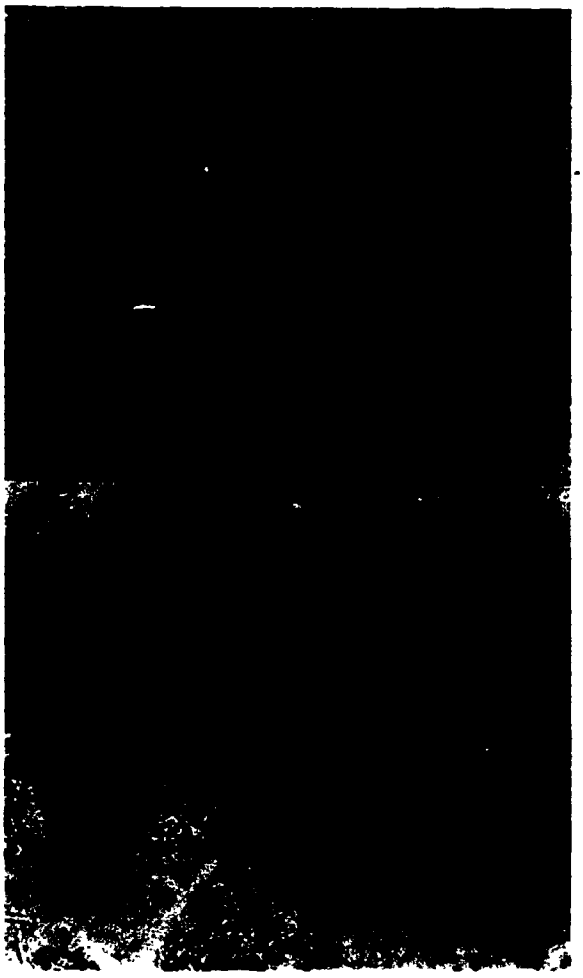
4.2.2 Mixed Cultures of Both Mating Types

Mixed haploid cultures of both mating types were not affected by host leaf extracts under conditions which inactivated the mating type alleles, i.e. high cation levels and high temperature (Day, 1979; also see section 3.1). Thus, plant extract did not alter the growth rate or morphology of mixed $a_1 + a_2$ cultures in liquid CM at 22°C (fig. 4) and neither mating nor subsequent formation of infection hyphae was detected.

Under conditions which are permissive to mating type activity, i.e. MM at 15°C, up to 80% of the a_1 and a_2 mate by forming conjugation tubes. The a_2 cells produce short (<20 µm) conjugation pegs which grow in a directed manner toward a_1 cells. In response, the a_1 cell produced a short protuberance opposite this peg (Day, 1976). Plant extracts greatly stimulated this process (plate 5) particularly in race DC-1 (table 16a; section 4.3), causing (a) multiple peg formation in both mating types, many cells having three to five pegs; (b) random, non-directed peg initiation and growth; and (c) very long pegs (up to 50 µm in a_2 and 10 µm in a_1 cells). The distinctive long pegs from the a_2 cells were termed "wild pegs" and grew in a typical helical manner when a_1 cells were proximal but not close enough for conjugation (plate 5). When a block of WA inoculated with a_2 cells was placed against a block with a_1 cells, host extracts stimulated the rapid growth of wild pegs from a_2 cells and short pegs from a_1 cells. Frequently, the a_2 pegs branched and changed direction before beginning directed growth toward an a_1 peg (plate 5). Once the cells had conjugated in the presence of plant extract, they initiated infection hyphae which elongated at a rate of about 2 µm/h and frequently became aerial (plate 5).

Plate 5. Response of Mixtures of a_1 and a_2 Haploids to Extracts of *Silene alba* Leaves

An acetone extract of *S. alba* leaves induced long conjugation tube formation in conjugating a_1 and a_2 cells on water agar (supplemented with 0.1% glucose) at 15°C. The a_2 colonies produced long narrow conjugation tubes (wild pegs) which at first grew randomly rather than directly to an a_1 cell. The a_1 cells produced shorter pegs. Occasionally, the wild pegs split (top, arrow) before reaching the a_1 colony. After conjugation, hyphae were formed and frequently became aerial (bottom, arrow). Both, x500.



4.2.3 Previously Conjugated Haploid Sporidia

Crude aqueous extracts of *S. alba* stimulated conjugated cells on WA or MM to initiate hyphal growth either from the conjugation tube or from one of the conjugants. The hyphae were initiated about 6 - 8 h after treatment with extract and grew at about 2 µm/h, reaching 30 µm long after 24 h (plate 6). Usually at least 80% of the cells developed these long hyphae, compared with control cultures treated with sterile water in which 2 - 3% of the conjugated pairs produced a short (ca. 10 µm) hypha after 24 h. Initiation and growth of hyphae from these conjugated cells occurred in liquid or solid, nutritive or non-nutritive media at temperatures from 10 to 25°C. Thus, induction of hyphae was not limited to those conditions permissive for mt gene activity. On MM, after treatment with plant extract, the hyphae grew to about 75 µm after 48 h but then reverted to sporidial budding (plate 6). However, continued additions of 0.5 mL of crude extract at 24 h intervals prevented this reversion and stimulated continued hyphal growth, producing hyphae over 200 µm long (plate 6).

Acetone extracts stimulated rapid growth of the hyphae (up to 4 - 5 µm/h) so that the hyphae reached 50 - 80 µm in 24 h. As the dilution of the acetone extract was increased to about 3×10^{-3} of the initial concentration, both mean hyphal length and the percentage of cells with hyphae decreased to the control levels (fig. 5).

4.2.4 Diploid Cells

Primary a_1/a_2 diploid sporidia responded to extract exactly as did conjugated haploid cells (plate 7). The response was independent of both nutritive conditions (WA, MM, or CM) and temperature in the 10 - 25°C range. A third effect of an active plant extract was noticed with the diploid cells. Extracts suppressed the development of SPP cells under conditions which normally favour SPP formation (see section 3.1). Mainly VP cells were observed in the presence of the extract and SPP cells in the absence. Thus, active extracts inhibited sporulation and, therefore, favoured vegetative growth in addition to inducing hyphal growth and affecting conjugation.

Plate 6. Response of Mated $a_1 \times a_2$ Haploids to Extracts of *Silene alba* Leaves

Conjugated haploid sporidia were spread on water agar and treated with water (top, x550) or an aqueous extract of *Silene alba* (middle, x625). In response to the extract, very long hyphae were formed and many became aerial. In the bottom photograph, an aerial hypha has reverted to haploid budding when the extract was exhausted (x500).

Plate 7. Response of a_1/a_2 Diploid Cells to Extracts of *Silene alba* Leaves

Primary a_1/a_2 diploids treated with acetone extract of *S. alba* leaves produced hyphae on MM at 15°C (top). Cells treated with water instead of extract initiated sporulation under these conditions (bottom). Both x 625.

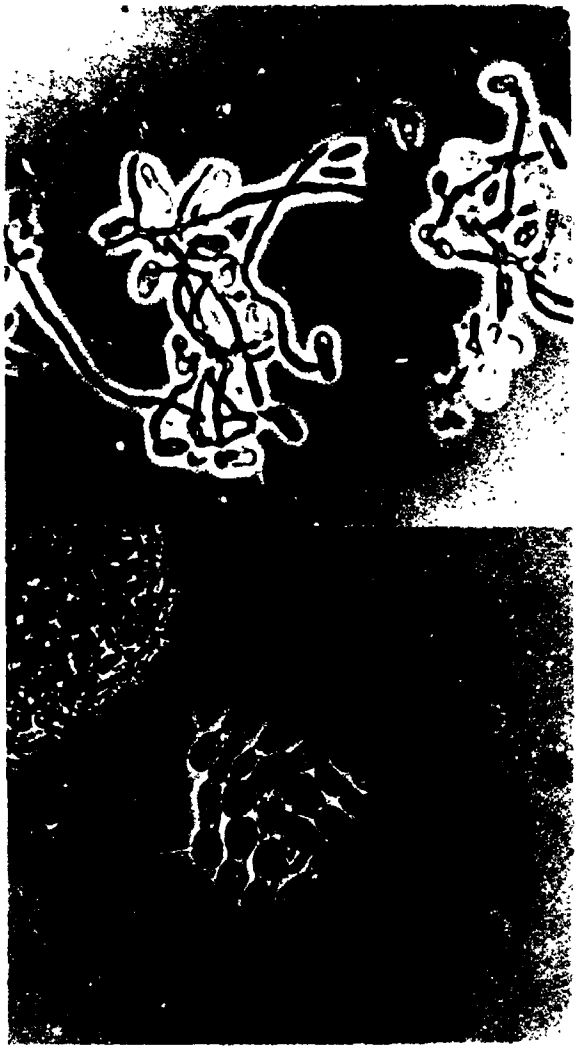
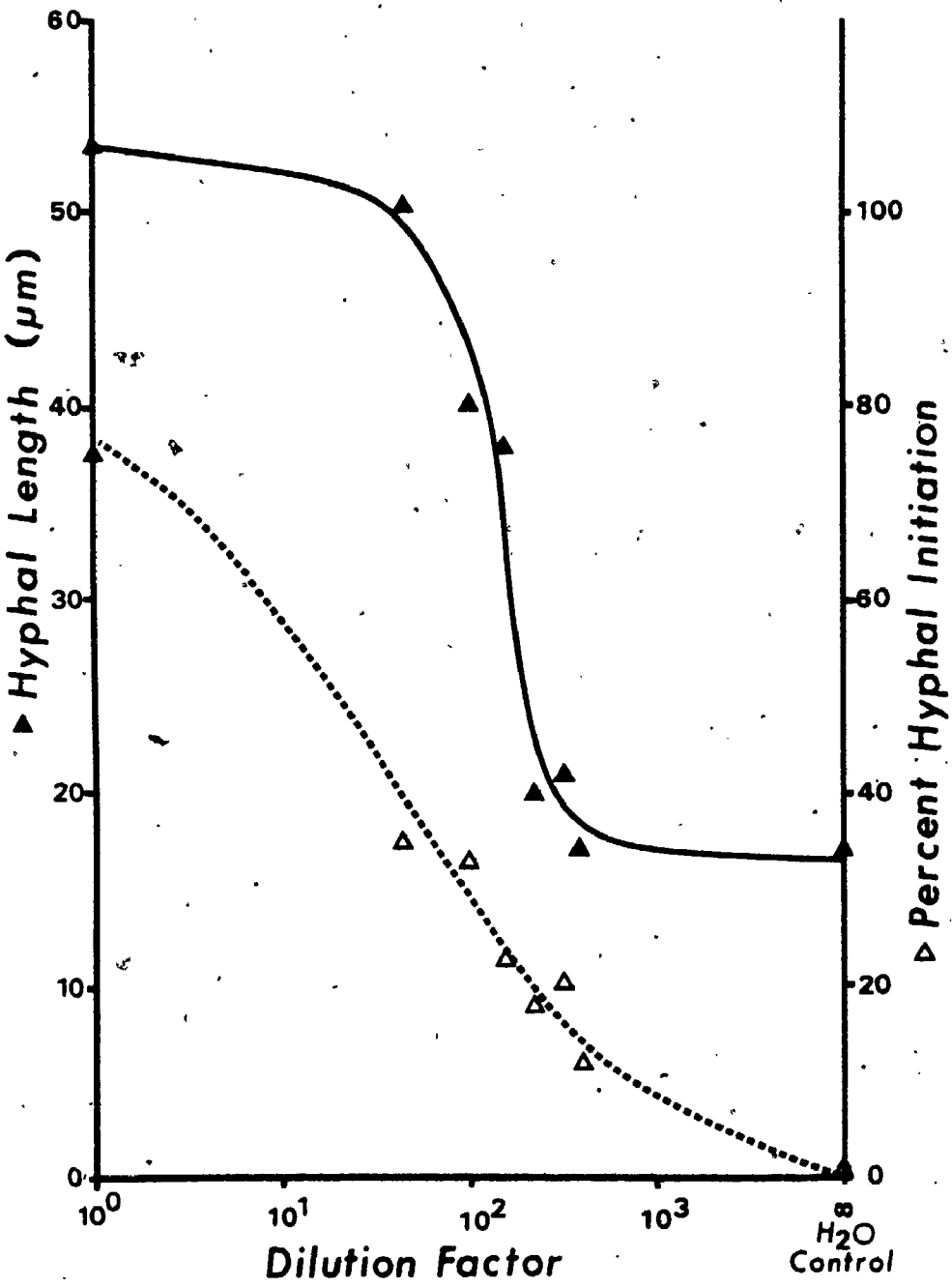


Figure 5. Effect of Dilution of Acetone Extract of *Silene alba* on Hyphal Length and Percentage of Cells with Hyphae in Race SA-1

Acetone extracts (3 mL/gm fresh weight of leaf tissue) were evaporated to an aqueous residue and sufficient distilled water was added to return the samples to the starting volumes. Subsequent dilutions were in water. The extracts were applied to conjugated cells on MM at 22°C and the cultures were examined after 24 h. Each point is the mean of three determinations.


60
50
40
30
20
10
0
▲ Hyphal Length (μm)
100
80
60
40
20
0
△ Percent Hyphal Initiation
10^0
10^1
10^2
10^3
∞
H_2O Control
Dilution Factor

Neutral opaque (op-N) strains also produced hyphae when treated with plant extract. Diploid cells homozygous for mating type, i.e. op-a_1 and op-a_2 isolates, behaved as the equivalent haploid strains: no visible responses in pure cultures; production of conjugation pegs and dikaryotic hyphae when cultures of opposite mating type were mixed under mating - permissive conditions. Thus, response to plant extracts is limited to cell types which contain both mating type alleles in a functional state.

4.2.5 Nuclear Division in Hyphae Formed in Response to Extract

The hyphae from conjugated cells or a_1/a_2 diploids developed in the typical smut manner (Fisher and Holton, 1957; Day, 1974) by laying down septa and retaining cytoplasm only at the growing tip, leaving behind long hyphal filaments that were more or less devoid of cytoplasm. Most of the hyphae produced 24 - 48 h after treatment had begun this process, but only a few had undergone nuclear division. When the plant extract was supplied at 24 h intervals for 4 days, the long hyphae (over 200 µm) formed had cytoplasm in the terminal 30 - 50 µm. Most of the hyphal tips produced from mated haploid cells were binucleate (plate 8), but a few contained four nuclei when stained and observed by acridine orange (Poon and Day, 1974a) or Hoechst 33258 fluorescence microscopy (Lemke *et al.*, 1978). Hyphae produced from diploid a_1/a_2 cells remained uninucleate. Thus, very little nuclear division occurred in these hyphae under these conditions; yet they seemed capable of indefinite growth when periodically supplied with plant extract.

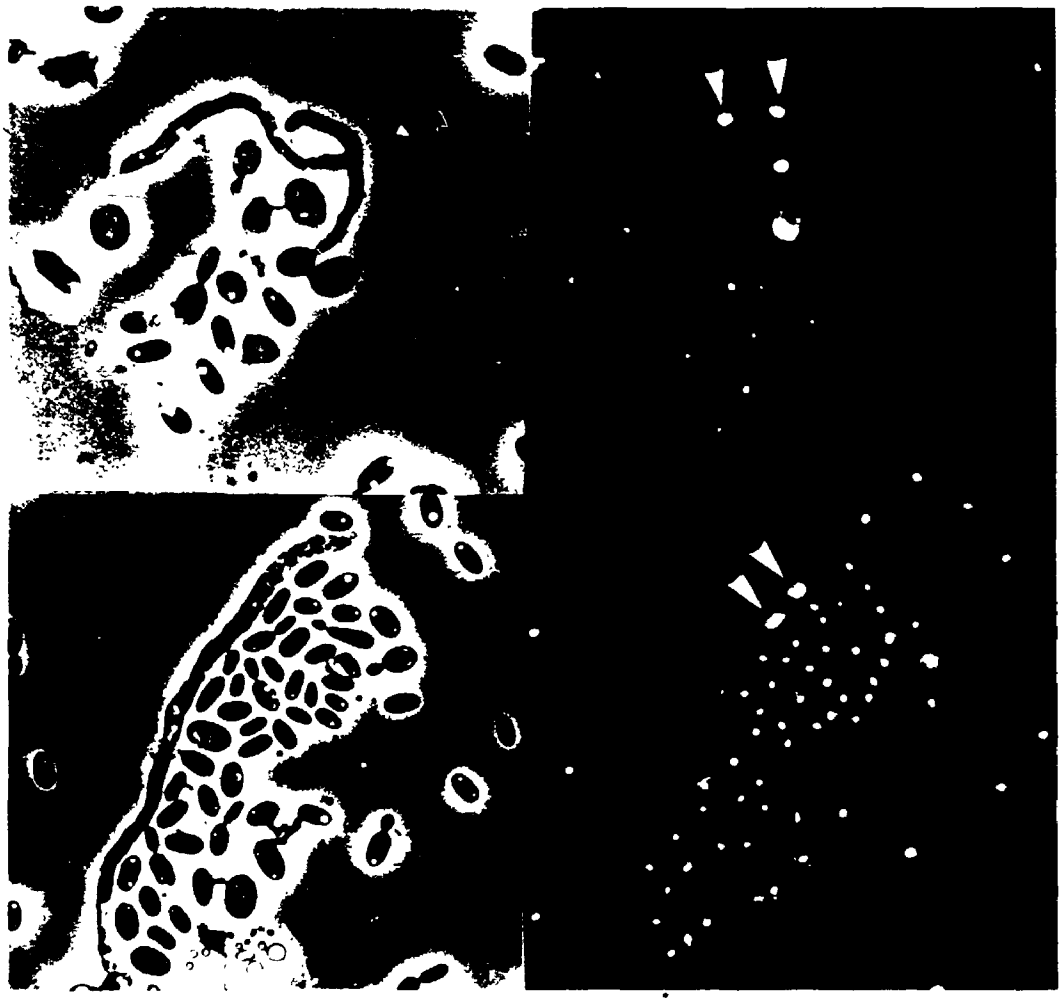
4.2.6 Comparison of Hyphae Produced in Response to Extract with Hyphae Produced During Infection

Hyphae produced on artificial media were compared by A. W. Day to infection hyphae on host leaves. As this work underlines some of the premises of this thesis, it is included with Dr. Day's permission.

Epidermal strips from *S. alba* were supported on WA and inoculated with a mixed suspension of a_1 and a_2 cells. Conjugation occurred on the epidermal surface, and infection hyphae identical to those on WA or MM

Plate 8. Fluorescent Staining of Hyphal Nuclei

Corresponding phase contrast and fluorescent photographs of hyphae show persistence of the dikaryon and no nuclear division, even after branching of the hypha (top). The nuclei (arrows) were visualized by staining with 25 µg/mL Hoescht 33258 fluorescent stain. Top x1000, bottom x800.



were formed. After growing for 5 - 100 µm, the hyphae formed an appressorium from which an infection peg penetrated the cuticle. Extensive hyphal growth occurred within the leaf within 2 - 3 days. Occasionally, hyphae from conjugated cells on the agar surface grew across and penetrated the epidermal surface by an appressorium and penetration peg. The principal point stressed here is that the hyphae induced by plant extracts appear identical to those on the host surface up to the time of initiation of the appressorium.

4.3 Response of Other Races of *U. violacea*

Haploids of both mating types of isolates SD-8, SA/D-1, DC-1, LF-1, and SM-1 (see table 16a for host species) were used to investigate the responses of these races to extracts from their host species and from other host species in the Caryophyllaceae.

As with race SA-1, pure cultures of one mating type of any of these races did not respond to host extracts. Response was limited to mating, mated or primary a_1/a_2 diploid cells. Apart from the similar requirements for the presence of both mating type alleles, distinct differences between races were apparent with mated dikaryons or diploids using test B (see section 2.9). Sporidia from all of the SA, SD, SM, and SA/D races responded strongly to extracts from *Silene alba*, producing hyphae from most conjugated pairs. Races LF-1, LF-2, and DC-1, however, did not respond at all, i.e. they remained strictly sporidial. A plausible explanation for this observation was that these races were adapted to compounds produced only by their own host species in the genera *Lychnis* and *Dianthus*. Subsequent tests showed that this hypothesis was incorrect as these races did not respond even to extracts from their own host species under test B conditions (table 17).

When extracts were applied before conjugation (test A), all races responded well to *S. alba* extract and to extracts from the other hosts, producing vigorous aerial hyphae. All races except races LF-1 and LF-2 produced wild pegs in this test. All races responded under some conditions

Table 16a. Origin of the Physiological Races of *Ustilago violacea*

Isolate (UWO Code)	Host Species	Race Designation	Site of Origin
UWO 1	*Silene alba*	SA-1	Reading, Berkshire, UK
UWO 2	*S. alba x S. dioica* hybrid	SA/D-1	Goodwood, Sussex, UK
UWO 3	*S. alba*	SA-2	Goodwood, Sussex, UK
UWO 4	*S. alba*	SA-3	Huntingdon, Cambridgeshire, UK
UWO 5	*S. dioica*	SD-1	Packington, Warwickshire, UK
UWO 6	*S. dioica*	SD-2	Packington, Warwickshire, UK
UWO 8	*S. dioica*	SD-3	Auchenheath, Lanarkshire, UK
UWO 9	*S. dioica*	SD-4	Auchenheath, Lanarkshire, UK
UWO 10	*S. dioica*	SD-5	Strontian, Argyll, UK
UWO 11	*Lychnis flos-cuculi*	LF-1	Taynuilt, Argyll, UK
UWO 12	*S. dioica*	SD-6	Ayr, Ayrshire, UK
UWO 13	*S. dioica*	SD-7	Ardchattan, Argyll, UK
UWO 14	*S. dioica*	SD-8	Benderloch, Argyll, UK
UWO 15	*L. flos-cuculi*	LF-2	Durness, Sutherland, UK
UWO 16	*S. dioica*	SD-9	Dollar, Fife, UK
UWO 17	*S. dioica*	SD-10	Colinton Dell, Edinburgh, UK
UWO 19	*S. maritima*	SM-1	Sheigra, Sutherland, UK
UWO 21	*S. dioica*	SD-11	Bolberry, Devon, UK
UWO 22	*S. maritima*	SM-2	Aveton Gifford, Devon, UK
UWO 23	*S. dioica*	SD-12	Bolberry Head, Devon, UK
UWO 24	*S. dioica*	SD-13	Bolberry Down, Devon, UK
UWO 26	*Dianthus carthusianorum*	DC-1	Clermont-Ferrand, France

Note: UWO 1 collected by D. Snow; UWO 2 - UWO 24 collected by A. W. Day; UWO 26 collected by A. C. Newton.

Table 16b. Origin of Other Species of Smut Fungi

Species	Host Plant	Source
From Dicotyledonous Hosts:		
Ustilago scabiosae	*Knautia arvensis* (Dipsacaceae)	CMI
U. utriculosa	*Polygonum persicaria* (Polygonaceae)	CBS #178(a_2) CBS #179(a_1)
From Monocotyledonous Hosts - Cyperaceae:		
Farysia olivacea		
From Monocotyledonous Hosts - Liliaceae:		
U. heufleri	*Erythronium americanum*	CMI
U. vaillantii	*Muscari comosum*	CMI
From Monocotyledonous Hosts - Gramineae:		
U. aegilopsidis	*Agropyron fibrosum*	JN
U. avenae	*Avena sativa*	JN
U. bullata	*Bromus catharicus*	REF
U. cynodontis	*Cynodon dactylon*	JN
U. hordei	*Hordeum vulgare*	JN
U. hypodites	*Agropyron trichophorum*	JN
U. kolleri	*Avena sativa*	JN
U. nigra	*Hordeum vulgare*	JN
U. nuda	*Triticum aestivum*	JN
U. maydis	*Zea mays*	AWD
U. tritici	*Triticum aestivum*	JN
U. turcomanica	*Agropyron tsukushiense*	JN

Note: AWD: A. W. Day; CBS: Centraalbureau voor Schimmilcultures, Baarn, Netherlands; CMI: Commonwealth Mycological Institute, Kew, England; JN: J. Nielsen, Canada Department of Agriculture, Winnipeg; REF: R. E. Falloon, DSIR, Palmerston North, New Zealand.

Table 17. Response of Races of *Ustilago violacea* to Three Host Extracts

Fungal Races	Tests of Plant Extracts from: *Silene alba* or *S. dioica* A1	A2	B	*Lychnis flos-cuculi* A1	A2	B	*Dianthus caryophyllus* or *D. carthusianorum* A1	A2	B
SA-1 to SA-3	+	+	+	+	+	+	NT	NT	+
SD-1 to SD-13	+	+	+	+	+	+	NT	NT	+
SA/D-1	+	+	+	+	+	+	NT	NT	+
LF-1, LF-2	-	+	-	-	+	-	NT	NT	-
SM-1, SM-2	NT	NT	+	NT	NT	NT	NT	NT	NT
DC-1	+	+	-	+	+	-	+	+	-

Note: Test A: Extract applied during conjugation; A1: presence/ absence (+/-) of wild pegs; A2: presence/ absence (+/-) of infection hyphae from the cells following conjugation. Test B: Extract applied to conjugated cells or to diploid a_1/a_2 cells; presence/ absence of infection hyphae; NT: not tested.

to extracts from any of the host plants. This observation eliminates the possibilty that individual races are adapted to specific products from their particular host species.

4.3.1 Interracial Conjugants

Cells of race SA-1 and cells of races LF-1 and DC-1 were conjugated in all combinations. The hybrid conjugants and non-hybrid combinations as a control were treated with extracts, using test B (table 18). The SA-1 x SA-1 conjugants produced hyphae, but LF-1 x LF-1 or DC-1 x DC-1 conjugants did not. In hybrid combinations, e.g. SA-1 x LF-1, as long as one conjugant was derived from race SA-1, the conjugated pair produced hyphae like those of the SA-1 x SA-1 matings. Thus, the ability of race SA-1 to form hyphae from premated cells is inherited as a genetically dominant characteristic. The responses of the hybrid SA-1a_1 x DC-1a_2 and non-hybrid SA-1a_1 x SA-1a_2 combinations to a dilution series of an acetone extract of *S. alba* (fig. 6) further suggested that this trait is completely dominant. The hybrid combination responded as well as the non-hybrid in both percent germination and length of the hyphae produced, at all dilutions.

4.3.2 Attempt to Analyse the Genetic Basis of Racial Differences in Response to Extracts

Cells from an a_1 isolate of race SA-1 carrying the recessive yellow colony colour marker were conjugated with wild type a_2 cells from the LF-1 race, and the conjugated cells were used to infect three plants each of *S. alba* and *L. flos-cuculi*. Five plants did not flower in their first season, but the remaining *S. alba* plant did flower and was heavily infected. Teliospores from this plant were plated on CM to obtain the haploid products of meiosis and to determine the genetic basis of the response to extract and possible linkage to the mating type locus. Unfortunately, the teliospores did not develop normally and did not produce any haploid products. Most of the germinating spores produced a two - or three - celled promycelium, which rarely budded sporidia in the usual way. Frequently

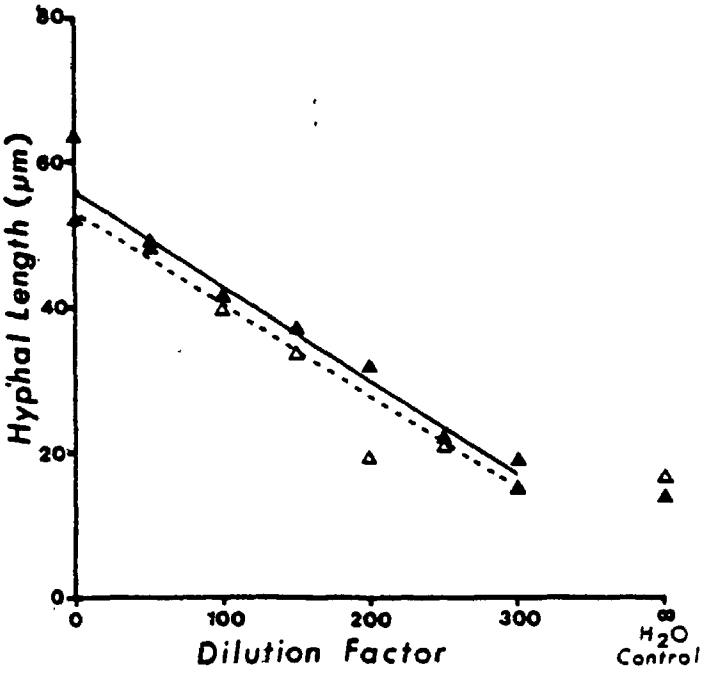
Table 18. Response of Hybrid Conjugants of Races SA-1, LF-1, and DC-1 to Extracts from *Silene alba*, *S. dioica*, and *Lychnis flos - cuculi*

Cross	Extract from: *S. alba*	*S. dioica*	*L. flos - cuculi*
SA-1a$_1$ x SA-1a$_2$	+	+	+
SA-1a$_1$ x LF-1a$_2$	+	+	+
LF-1a$_1$ x SA-1a$_2$	+	+	+
LF-1a$_1$ x LF-1a$_2$	−	−	−
SA-1a$_1$ x DC-1a$_2$	+	+	+
DC-1a$_1$ x SA-1a$_2$	+	+	+
DC-1a$_1$ x DC-1a$_2$	−	−	−

Note: +, myceliation; −, no myceliation. Extracts were tested on mated cells (test B).

Figure 6. Effect of Dilution of Acetone Extract of *Silene alba* on Hyphal Length and Percentage of Cells with Hyphae in a Cross between Races SA-1 and DC-1

An acetone extract of leaf tissue (3 mL/gm fresh weight) was evaporated and resuspended in sufficient distilled water to return the sample to the starting volume. The extract was applied to cells of a DC-1 a_1 x SA-1 a_2 cross (▲) or of an SA-1 a_1 x SA-1 a_2 cross (◇). The MM plates were incubated at 22°C and were scored after 24 h.


Hyphal Length (μm)
80
60
40
20
0
0
100
200
300
∞
H2O
Control
Dilution Factor

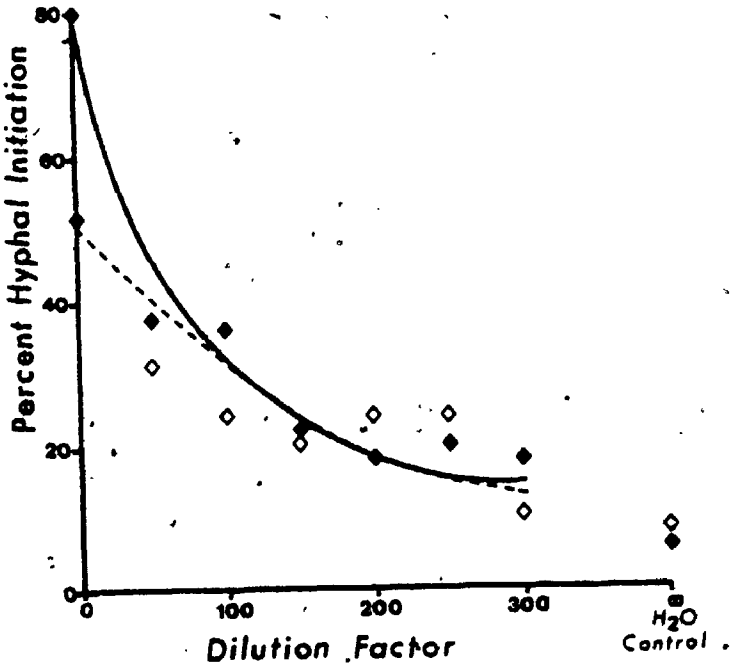
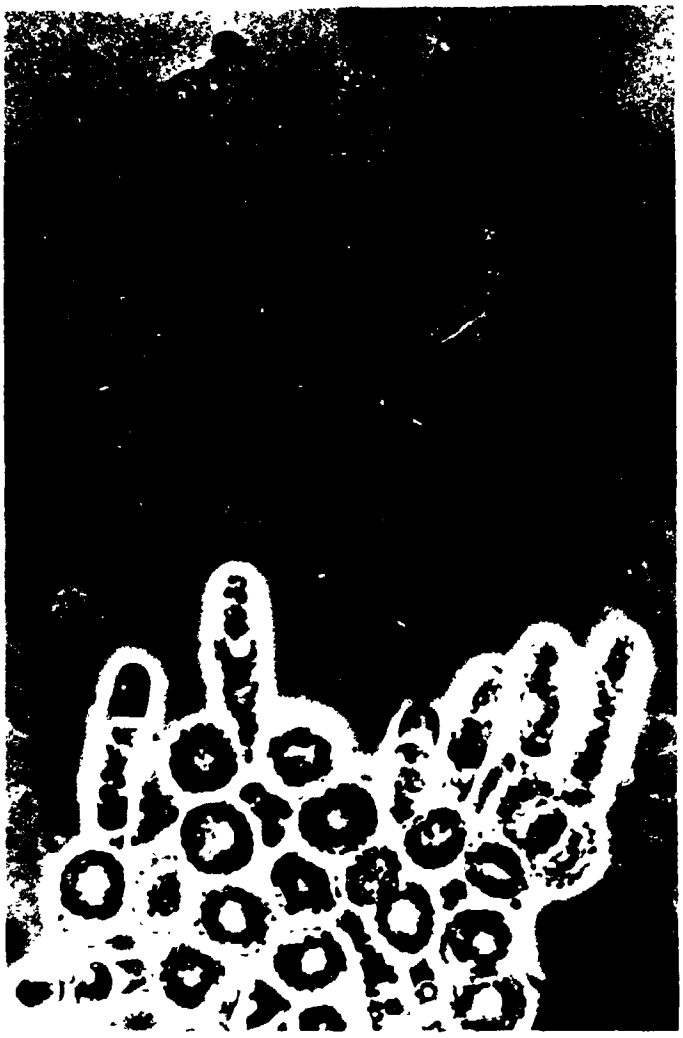

Percent Hyphal Initiation
80
60
40
20
0
0
100
200
300
∞
H2O
Control
Dilution Factor

Plate 9. Promycelium Formation by Hybrid SA-1 x LF-1 Teliospores

The promycelium (P) produced by teliospores (T) recovered from a *Silene alba* plant infected with a_1 cells of race SA-1 and a_2 cells of race LF-1 was initially normal in appearance (bottom, x1800). Later production of swollen diploid primary sporidia (D; top) indicated that meiosis did not occur (both top photographs x800).



one, rarely two, of the cells in the promycelium became swollen, especially at the poles, and filled with refractive granules (plate 9), closely resembling the SPP a_1/a_2 diploid cells (Day, 1979; Castle and Day, 1980). The contents of the promycelium eventually degenerated. The swollen cell behaved like an a_1/a_2 diploid sporidium, budding off other sporidia, under conditions which permit budding in a_1/a_2 cells. Acridine orange fluorescence observations revealed usually only one large nucleus in each promycelium, located in the swollen cell. The large swollen sporidia were neutral in mating type and did not express the recessive yellow marker. Their phenotype matched in every respect the distinctive phenotype of a_1/a_2 cells (Day, 1979) and they were therefore identified as diploid sporidia. It appears that meiosis failed in these hybrid teliospores and 1 - 2 diploid sporidia were produced instead. Attempts to induce these diploid sporidia to haploidize using PFP (see section 2.6) which works well on normal diploids, failed as no viable haploids were recovered. A few poorly growing aneuploids which expressed the yellow marker were recovered, confirming that the large sporidia were at least heterozygous for some markers and therefore probably diploids. Also a few sporidia had poor mating ability with one or the other haploid mating type, but no genetic analysis was possible. Even this poor recovery of markers, however, confirms that the teliospores were hybrid for races SA-1 and LF-1, because the $\underline{y}$ allele from SA-1 and the $\underline{y^+}$ allele from LF-1 were recovered.

The ease of interracial hybridization and the high virulence combined with an inability to perform meiosis in the hybrid spores are all commonly reported in smut fungi (Fisher and Holton, 1957; G. C. Kirby, personal communication to A. W. Day). Interracial diploids of the type described here may give rise to new races that: 1) can attack either of the hosts of both parental species (Goldschmidt, 1928); 2) do not undergo meiosis and therefore do not segregate; 3) yield neutral non-mating sporidial lines; and 4) have larger than normal sporidia. Strains with some or all of these properties have been described (Kniep, 1919; Zillig, 1921; Bauch, 1922; Goldschmidt, 1928). Races from *Dianthus* spp., *Saponaria officinalis*, and *Silene vulgaris* produced large neutral sporidia (Zillig, 1921). Therefore, some naturally

occuring races of *Ustilago violacea* may be sterile diploids arising from interracial hybridization.

4.4 Response of Race SA-1 to Extracts of Various Plant Species

The results presented in section 4.3 indicated that race SA-1 responded not only to extracts from its host, *S. alba*, but to extracts from other species as well. Consequently, a large number of species from a wide range of plant families was collected and acetone or water extracts of these species were tested for ability to induce hyphal growth of fungal race SA-1. The species tested are listed in appendices 1 - 4 and a summary of the results is presented here.

4.4.1 Caryophyllaceae

Acetone or water extracts from all of 27 tested species in the Caryophyllaceae stimulated hyphal growth from mated haploid or a_1/a_2 diploid cells. The stimulatory effect of different species ranged from weak to as strong as *S. alba* extracts. Surprisingly, extracts from dried herbarium specimens, some over 50 years old, were active. These extracts were generally weaker than those from fresh tissue but all 39 tested species still retained activity (appendix 1).

4.4.2 Non - Caryophyllaceae

Extracts from a total of 36 species from families other than the Caryophyllaceae were tested on mated haploids of race SA-1 (appendix 2). Only 4 of the 36 extracts showed weak to moderate activity and induced infection hyphae. The remaining 32 extracts were only very slightly active or were inactive. Thus, it was quite apparent that host species for *Ustilago violacea*, i.e. members of the Caryophyllaceae family produced a compound or a group of compounds to which the fungus reacted by producing infection hyphae, and that most of the non-hosts were apparently deficient in this chemical stimulus. However, as shown later, tests with more concentrated extracts indicated that all tested plant species contain at least small amounts of the active agent (section 4.6).

Closer examination of the non-host group indicated that the four species which yielded active extracts were the only tested species that were hosts for other species of the genus *Ustilago*. These plant species and their respective *Ustilago* pathogens were: *Zea mays*, host for *U. maydis*; *Scabiosa perfecta*, host for *U. scabiosae*; *Oxalis europaea* and *O. cernua* (*U. oxalidis* attacks this genus, though we have not been able to determine whether these particular species are susceptible). This correlation between activity and status as a host of a *Ustilago* species suggested that many, if not all, species of *Ustilago* may respond to the same or similar chemical stimulus and that the many and varied hosts of diverse *Ustilago* species may share a common characteristic viz. production or availability of the active compound. In this case, the host range of this entire fungal genus may be determined in part by the distribution of these particular plant products. A more extensive survey of plant species was conducted to test this hypothesis.

4.4.3 *Ustilago* Host Versus Non-host Species

Extracts from 65 dicotyledonous and 39 monocotyledonous species were tested for their effects on race SA-1 (appendices 3 and 4), and the results are summarized in table 19. The results support the hypothesis that *Ustilago* hosts in general produce a compound which triggers hyphal formation in *U. violacea*, and that non-hosts either lack this compound or have it in lower concentration. Thus, all 38 of the plant species that host *Ustilago* species had active extracts, while only 6 out of 43 plant species without smut pathogens had active extracts. Of particular note were members of the Gramineae family. All species which host a *Ustilago* pathogen yielded active extracts (24 out of 24) while all non-hosts produced inactive extracts (4 out of 4). In a third category of plants, those species which host smut fungi other than *Ustilago*, about one - third of the species (8 out of 25) had active extracts.

Four species in the Polygonaceae (*Polygonum persicaria* and three *Rumex* species) deserve special mention. Although they host *Ustilago* species they had inactive crude extracts; however, these extracts were toxic to

Table 19. Summary of the Effect of Plant Extracts on the Induction of Infection Hyphae in *Ustilago violacea*

Plant Type	Effect of Extract on *U. violacea*: No Induction (inactive)	Induction (active)
Dicotyledonous Species[1]		
Not host to a smut fungus	33	6
Host to smut fungus other than *Ustilago*	13	3
Host to *Ustilago* species	0	37
Monocotyledonous Species[2]		
Not host to a smut fungus	2	0
Host to smut fungus other than *Ustilago*	4	5
Host to *Ustilago* species	0	28
All tested Species[1,2]		
Not host to a smut fungus	35	6
Host to smut fungus other than *Ustilago*	17	8
Host to *Ustilago* species	0	65

Note: 1 - Taken from Appendices 1 and 3.
2 - Taken from Appendix 4.

the cells of *U. violacea* and lysed them. A chloroform/ water partitioning of the extract from *P. persicaria* established that active compounds were indeed present in the chloroform fraction but that they were masked by toxins which separated into the aqueous fraction. Similarly, crude extracts of the non-host species, *Lycopersicon esculentum*, were also inactive and toxic but were found to have the active agents after partitioning to separate the toxic compounds. Extracts from *L. esculentum*, however, did not induce myceliation in *U. violacea* until 24 h after application instead of the usual 6 h. Very few extracts from other species gave any indication of toxins which mask the active compounds. Partitioning experiments similar to those outlined above failed to show any toxic or active compounds in inactive crude extracts of *Pelargonium* sp. or *Saintpaulia* sp. With the exception of the few cases mentioned above, toxins were not an impediment to the screening of extracts of the various plant species for the presence of the hyphal inducing stimulus.

The results obtained with this larger survey were consistent with the hypothesis that the genus *Ustilago* was adapted to parasitize plant species containing sufficient quantities of a particular compound or group of compounds. Although extracts from a few non-host species scored as active in these tests, this was not particularly surprising. Many factors besides the plant products under study here must contribute to the determination of host susceptibility. These species presumably are not hosts of *Ustilago* species because they are deficient in one or more of these other requirements.

Two other observations suggest that the development of mycelium in the host depends on the localization of the active compound. Firstly, twenty male and female *S. alba* plants were divided up into leaves, roots, stems, and floral parts. Extracts of each type of tissue were prepared using approximately equal quantities (1.1 - 1.8 g) of material and hot methanol extraction (see section 2.10.3). The root, stem, and leaf extracts were all highly active and induced good hyphal growth, but the extracts from the total floral parts of either male or female plants had low activity. Secondly, leaf extracts of *Erythronium americanum*, a host of *U. heufleri*, were inactive and not toxic while extracts of bulb tissue did induce hyphal growth

of race SA-1. *U. heufleri* produces a vegetative mycelium in the bulb and sporulates in the leaves each spring. In both of these cases, the sites of vegetative mycelium yielded active extracts whereas the sites of sporulation apparently contained little of the active compound. It is possible, therefore, that the presence of these compounds not only stimulates growth of dikaryotic hyphae, but also inhibits sporulative development. This view is supported by the suppression by leaf extracts of SPP development mentioned earlier (section 4.2.4). On this hypothesis, sporulation would be triggered at least in part by entry into tissues deficient in the hyphal inducing compounds.

4.5 Responses of Other Species of Smut Fungi

A number of species of *Ustilago* and one of *Farysia* (tables 16b and 20) were examined to see if hyphal development required host produced compounds, as suggested by the plant survey. Where possible, monosporidial stocks of separate mating types were established and conjugated cells or diploid strains were tested with extracts from a variety of plant species. The effects on myceliation and sporulation are described below.

4.5.1 Dicot Parasites

Two other species which parasitize dicotyledonous host plants were available for study: *U. scabiosae*, which attacks species in the Dipsacaceae family; and *U. utriculosa*, a pathogen of the Polygonaceae family.

Isolates of *U. scabiosae* grew in a strictly sporidial fashion in the absence of extract and resembled *U. violacea* closely in other respects, such as morphology and polymorphism for colony colour (yellow, white, or pink colonies) and for thiamine prototrophy/ auxotrophy in wild type isolates (Garber, Baird, and Weiss, 1978).

Conjugated cells of *U. scabiosae* behaved very similarly to cells of *U. violacea* and formed hyphae in response to extracts from the tested species (all hosts of smut fungi) (table 20). The response to extract from *Polygonum persicaria* paralleled that of *U. violacea* (see section 4.3.2). Thus,

Table 20. Response of Smut Fungi to Plant Extracts

Fungal species	*Juncus brachy-cephalus*	*Silene alba*	*Scabiosa perfecta*	*Polygonum persicaria*	*Triticum aestivum*	*Zea mays*
Ustilago violacea	+	+	+	−(+)[1]	+	+
U. scabiosae	+	+	+	−(+)[1]	+	+
U. utriculosa	+	+	+	+	+	+
Farysia olivacea	+	−	−	−	−	−

Note: +, myceliation; −, no myceliation.
1 - Crude extract was toxic and therefore scored as inactive. Chloroform/ water partitioning yielded an active chloroform fraction and a toxic aqueous fraction.

the cells of *U. scabiosae* were lysed by the crude extract, but chloroform/ water partitioning of the extract separated the toxic component from a component which stimulated mycelial growth.

In the absence of plant extracts, *U. utriculosa* produced elongated sporidia with occasional very short hyphae. Plant extracts, however, induced conjugated cells to develop long hyphae. Crude extracts from *P. persicaria*, a host of *U. utriculosa*, were not toxic to this species and strongly induced aerial hyphae. Thus, it is clear that the species of *Ustilago* which is adapted to grow on *P. persicaria* has a mechanism to avoid or counteract the toxin which lyses other *Ustilago* species. Further investigation of the nature of this toxin and the avoidance mechanism in *U. utriculosa* would provide new insights into host/ parasite interaction.

In summary, all of the three studied species of *Ustilago* derived from dicot hosts remained strictly sporidial on agar media and showed the same response (induction of hyphae) to the same range of plant extracts. It is clear, therefore, that they share the same requirement for the same host produced compound(s).

4.5.2 Monocot Parasites

Species that grow in a strictly sporidial manner.

Farysia olivacea, a common pathogen of the Cyperaceae family, resembled *U. violacea* and *U. scabiosae* in being strictly sporidial in the absence of plant extract. Two mating types were isolated, and conjugation occurred frequently on WA or MM at 15 or 22°C. Extracts from the plant species that host *Ustilago* did not induce myceliation, but an extract from *Juncus brachycephalus*, a member of the Juncaceae family, often regarded as being close to the Cyperaceae family, did induce mycelial growth (table 20). Thus, it appears that *F. olivacea* responds to a different host product than that which induces the three species of *Ustilago* covered in the previous section. Further investigation of this situation was not carried out, but would be well worthwhile.

Species producing both sporidia and mycelia.

Mixed mating type isolates of *U. aegilopsidis*, *U. avenae*, *U. bullata*, *U. heufleri*, *U. hordei*, *U. kolleri*, *U. maydis*, *U. nigra*, *U. turcomanica*, and *U. vaillantii* produced both mycelia and sporidia on nutritive media in the absence of plant extracts (plate 10). Typically, hyphae developed from conjugated cells and grew at the terminal tip, leaving a filament of empty cells behind. New septa were constantly laid down behind the terminal cell, forming a series of empty compartments. Occasionally, the nuclei in the tip divided and two daughter cells were formed. The terminal tip cell then continued to grow and lay down new septa, so that eventually the hypha had a terminal tip cell and, separated by long stretches of empty cells, occasional intercalary cells with cytoplasm and nuclei. Plant extracts from host or non-host species did not noticeably alter this growth habit or the rate of elongation of hyphae.

On nutrient poor medium (MM at 22 - 30°C), the intercalary cells of all of these species fragmented into few to many dumbbell shaped cells very similar in appearance to the SPP cells of *U. violacea*. Brown pigmentation formed in the walls of these SPP cells in most species. In *U. turcomanica*, the SPP cells developed into normal teliospores, and the whole colony became dark brown to black. These teliospores were very similar in appearance to those formed in the host plant, and some germinated normally.

In our isolates of *U. maydis*, *U. nigra*, *U. aegilopsidis*, *U. heufleri*, and *U. avenae*, enough brown pigmentation was laid down in the SPP cells to colour the whole colony a light brown on MM at 25 - 30°C. However, the teliospores were abnormal in appearance and did not germinate. In the other species, occasional abnormal teliospore - like cells were seen, but again these did not develop further. The development of SPP cells in all species and their later development into complete teliospores in *U. turcomanica* was inhibited by extracts from the tested species (*Zea mays*, *Triticum aestivum*, and *Hordeum vulgare*).

Species with a mycelial growth form.

Teliospores of *U. hypodites*, *U. cynodontis*, *U. nuda*, and *U. tritici* germinated, produced hyphae, and grew in a predominantly mycelial manner on MM or CM at 15 - 30°C (plate 10). The two former species produced aerial chains of sporidia, while *U. nuda* and *U. tritici* developed intercalary dumbbell shaped SPP cells in old cultures. In the latter two species, these SPP cells eventually became deeply pigmented and rounded off into cells similar in size and shape to the normal teliospores of these species. They appeared to be abortive teliospores as their wall development was abnormal and they did not germinate. Plant extracts from *Z. mays* or *T. aestivum* did not affect mycelial growth, but again these extracts inhibited formation and further development of the intercalary SPP cells.

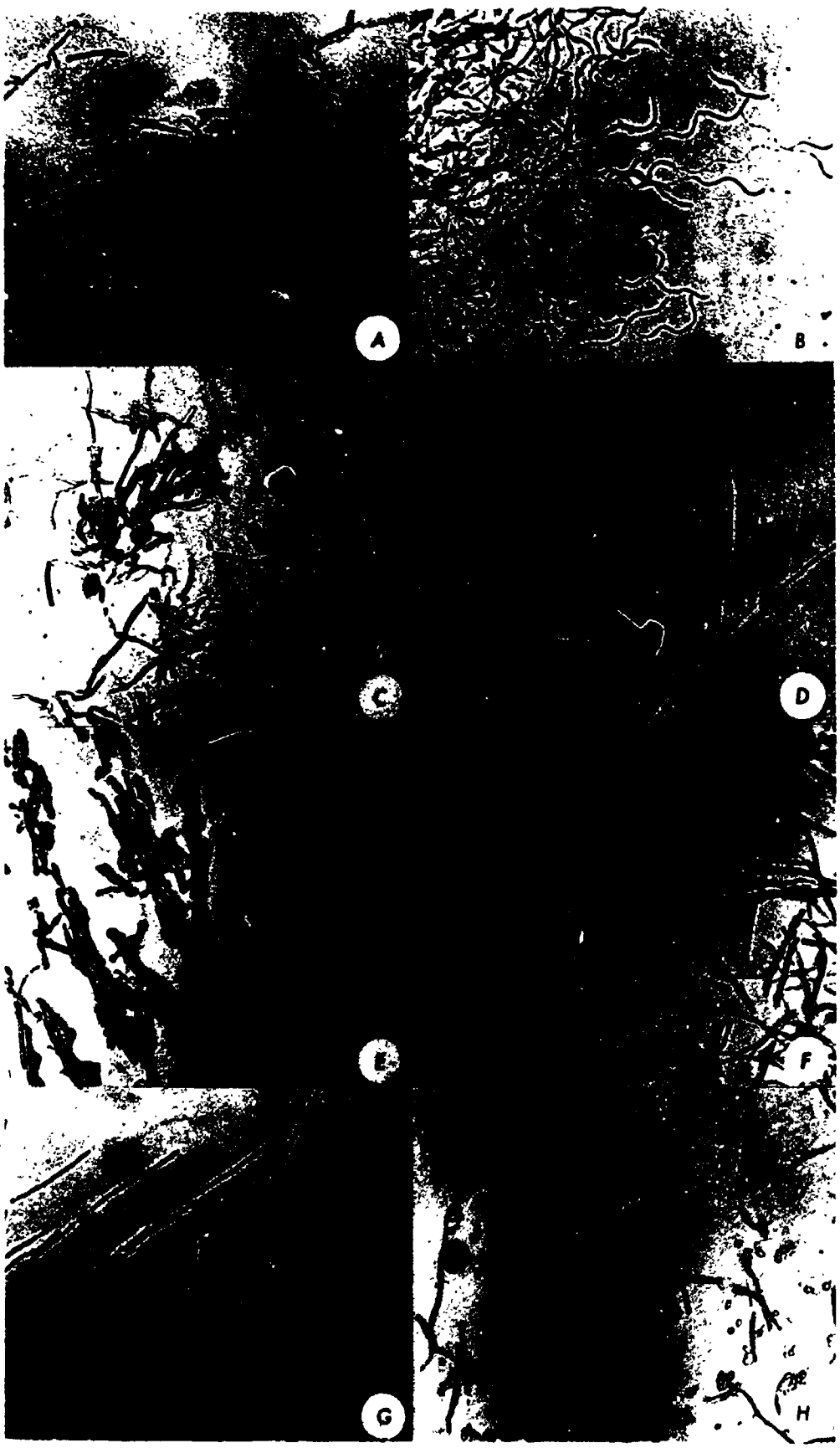
In summary, of the "monocot" smut fungi, only *Farysia olivacea* was strictly sporidial and this species produced hyphae in response to a different plant product from that which induced the "dicot" *Ustilago* species. Fourteen species of *Ustilago* attacking monocots produced hyphae in the absence of plant extract and did not respond noticeably to plant extract, except that development of sporulation cells was inhibited. Thus, there appears to be a major difference between species of *Ustilago* with monocot hosts and those with dicot hosts.

4.6 Isolation and Identification of Active Compounds

Continued work with various plant species and attempts to purify the chemicals responsible for hyphal induction revealed that the difference between host and non-host plants was not due simply to presence or absence of the active agents. Many of the species which scored as negative in water extracts, in fact, induced a little mycelial growth. Consequently, it seemed likely that the difference between active and inactive extracts was due largely to concentration differences. This was confirmed by making highly concentrated extracts from several "negative" species. In general, even water extracts from host species were only weakly active after 10 fold dilution. Extraction of fresh material with methanol or acetone and

Plate 10. Growth Forms of Some Monocot Smut Fungi on Artificial Medium

Several species of *Ustilago* which attack monocotyledonous hosts were plated on minimal medium and observed after 2 to 4 days growth. Every tested species produced extensive hyphal growth in the absence of any plant extract. Pictured are: A. *Ustilago hordei*; B. *U. tritici*; C. *U. turcomanica*; D. *U. maydis*; E. *U. nigra*; F. *U. kolleri*; G. *U. avenae*; H. *U. aegilopsidis*.


A
B
C
D
E
F
G
H

extraction of freeze dried tissue with ether or chloroform yielded samples with greater activity than the water extracts. As shown in sections 4.2.3 and 4.3.2, activity of acetone extracts could be detected even after a two to three hundred fold dilution. Methanol gave the most active extracts and was usually used for further work. Several plant species which were scored as inactive as crude aqueous extracts were found to give active methanolic extracts (table 21). The difference between active host and inactive non-host species was therefore in the concentration or accessibility of the active agent. This compound appeared to be universal or at least very widespread in plant species.

4.6.1 Isolation Procedure - Methodology

Isolation of the active compound from a variety of extracts was attempted using several different procedures. Paper and thin layer (silica, silica C-18, alumina, and cellulose) chromatography were completely ineffective. Once the extract was applied to the chromatogram, it appeared to have been bound irreversibly. After drying (before or after development of the chromatogram), no activity could be eluted or even detected when cells were placed in contact with the chromatogram. Similar results were obtained with various column chromatographic media: Dowex 1 and 50 or DEAE cellulose ion exchange media, Sephadex G50, G25, G10, or LH50 size exclusion media. At various times, irreversible binding of the active agent was observed with several proteins in a native state or denatured by boiling, polyvinylpyrrolidone (PVP), Norit charcoal, fatty acids (palmitic and oleic acid), and both hydrophilic and hydrophobic Millipore filters (perhaps as a result of binding to large particulate matter within the extract). Partitioning experiments gave ambiguous results, possibly due to the affinity of the compound for a diverse collection of molecules: in one extract the activity would partition into the organic phase, in another tested simultaneously, it would stay in the aqueous phase. The binding of the compound to most substrates was found to be not affected by pH. Thus, paper chromatography gave the same negative result when the chromatogram was developed with acidic (4:1:5, n-butanol: acetic acid: water; top layer used), neutral (4:1:2.2, n-butanol: ethanol: water; top layer) or basic (1:1, n-butanol:

Table 21. Comparison of Water and Methanol as Extraction Solvents

Species	Activity of water extract	Activity of methanol extract
Ustilago Host		
Avena sativa	+++	+++
Hordeum vulgare	+++	+++
Scabiosa perfecta	+++	+++
Silene alba	+++	+++
Zea mays	++	+++
Non-Hosts		
Cichorium intybus	-	+++
Glechoma hederacea	-	+++
Hesperis matronalis	-	+++
Lactuca sativa	-	+++
Lobelia siphilitica	-	+++
Lycopersicon esculentum	-	+++
Malva neglecta	-	+++
Plantago major	-	+++
Taraxacum officinale	-	+++
Thalictrum dioicum	-	+++
Trifolium pratense	-	+++

Scale: +++, 60-100% of conjugated cells produce hyphae;
++, 10-60%; +, 1-10%; -, <1% or no greater than water control.

2 M NH_4OH; top layer) solvents.

A second problem, in addition to binding, was the instability of the active component. This attribute was also quite variable. A crude water extract appeared to be as active after autoclaving (15 min at 15 p.s.i.) as before, but the same extract left at room temperature lost most activity within four hours. If the extract was kept on ice, it was still quite active after four hours. Samples were, therefore, stored at -20°C or -70°C to alleviate this instability problem, thought to be due to oxidization of the active compound.

The most effective isolation procedure that was developed was reverse phase high performance liquid chromatography or HPLC. Small Seppak cartridges (Waters Associates) containing a reverse phase chromatographic medium: silicic acid covered with octadecylsilyl (ODS) residues, were initially used to find a solvent which eluted the active component. This procedure was then applied to analytical HPLC. Perhaps, the reason why HPLC isolation proved successful while other procedures failed was the rapidity with which a sample could be analysed, avoiding or reducing much of the instability problem. HPLC analysis of various extracts proved to be a reliable, reproducible technique, when coupled with an extraction procedure that minimized non-specific binding. A total of four compounds, termed peaks A to D, were identified as contributing to the activity of the plant extracts.

During the period while the HPLC procedure was being developed, attempts were made to identify the active compound by testing a wide range of known plant compounds. Over 200 such compounds were screened for activity in the bioassay (appendix 5). A large number of common plant chemicals were ruled out using this survey. Only a few compounds induced any hyphal growth and only one of these (ascorbic acid) was detected in *S. alba* extracts. This compound and the others (cysteine, sorbic acid, and iron ions) were all only weakly active and induced hyphal growth only after a much longer delay (24 h) than found with plant extracts (6 h). Other active chemicals were identified after the isolation and identification of

α-tocopherol as the main inducing component of *S. alba* and other plant species extracts.

4.6.2 Aqueous Extracts of Leaf Tissue

Due to the problems encountered with *Silene alba* extracts and the relative shortage of material of this species, particularly during the winter, an alternate species, *Zea mays*, the host for *U. maydis*, was used to identify a component of the active extracts. The effective technique was then applied to extracts of *S. alba* and other species.

The first peak (A) was found only in aqueous leaf extracts and was highly polar. It was moderately active in the bioassay and characteristically induced mycelia only after an unusually long delay: at least 24 h after treatment compared to 6 h with crude extracts. During the survey of various commercial chemical preparations (appendix 5), it was noted that synthetic L - ascorbic acid (Fisher Chemicals) induced moderate mycelial growth with the same long delay as peak A. Both ascorbic acid and peak A were poorly retained on the Ultrasphere - ODS reverse phase column when eluted with water. Several systems involving three different columns, therefore, were used to chromatograph L - ascorbic acid and related compounds, isoascorbic acid (D - ascorbic acid) and dehydroascorbic acid. Two of these procedures were acceptable and were subsequently used to examine extracts from several plant species.

Three methods employing a reverse phase column, either the Ultrasphere - ODS or Hamilton PRP column (see section 2.11), were tested. Firstly, elution with different concentrations with water and methanol. Secondly, ion suppression: maintenance of ascorbic acid in the un-ionized state by the addition of 50 mM $HClO_4$ to either the sample or the mobile phase (only used with the PRP column since it is tolerant to extreme pH). Thirdly, ion - pairing: addition of a more hydrophobic counter ion, tributylamine or tetrabutylammonium phosphate (Pic A reagent, Waters Associates), which complexed with ascorbic acid resulting in an increase in retention by the column. Of these methods, only ion - pairing using tributylamine was found

suitable (table 22). Every other system resulted in poor retention of ascorbic acid, as indicated by the low k' values (<2). Pic A reagent was deemed unsuitable since it appeared to enhance the degradation of ascorbic acid, a problem not encountered with the tertiary amine.

Ascorbic (AA) and dehydroascorbic acid (DHA) were also efficiently chromatographed using anion exchange chromatography with the Zorbax NH_2 column. Various concentrations of monobasic potassium phosphate (KH_2PO_4) were tested as eluting solvents (table 23) and 0.02 M was found to give the best combination of speed and efficient chromatography. The poor separation of AA and DHA was not considered to be important since it is thought that DHA occurs in most extracts only as an oxidation product of ascorbic acid following inefficient isolation procedures (Barker and Mapson, 1959).

Co-chromatography of ascorbic acid and peak A on the Zorbax NH_2 column (fig. 7) and the Ultrasphere - ODS column using ion pair chromatography supported the conclusion that peak A contained ascorbic acid. Substantiation of this hypothesis was obtained by UV spectroscopy. Both spectra of peak A and ascorbic acid showed a strong absorbance peak at 264 nm (fig. 8).

Several plant extracts were chromatographed in the ion pair system in order to determine the ascorbate content of various plant species. A standard curve correlating integrator area units to µg amounts of ascorbic acid was prepared (fig. 8). The area units of the peak corresponding to ascorbic acid in the plant extracts were compared to the graph and then were converted to mg of ascorbic acid per 100 g of fresh tissue. The values obtained in this survey (table 24) were quite close to values cited in Olliver, 1967. However, no correlation between extract activity in the bioassay and ascorbate concentration in the plant tissue could be made. The strong activity of the *Zea mays* extract was not consistent with an ascorbate content marginally above the threshold of 10^{-3} M for the induction of hyphae by commercially available ascorbic acid (fig. 10). (10^{-3} M ascorbic acid is equivalent to 17.6 mg/ 100 g if one assumes that the 100 gm of

Table 22. Chromatography of Ascorbic Acid on the Hamilton PRP Column

Mobile Phase[1]	Sample Solvent	k' Value	Chromatographic Method[2]
100% CH_3OH	i) 50 mM $HClO_4$ in H_2O	1.29	IS 1
	ii) 45 mM $HClO_4$ in 9:1 H_2O:CH_3CN	1.31	IS 1
99% CH_3OH + 50 mM $HClO_4$	i) 50 mM $HClO_4$ in H_2O	1.32	IS 1 + 2
	ii) 99% CH_3OH + 50 mM $HClO_4$	1.07	IS 1 + 2
50% CH_3OH in H_2O	i) 50% CH_3OH in H_2O	1.32	
	ii) 50 mM $HClO_4$ in H_2O	1.32	IS 1
	iii) 50% CH_3OH in H_2O + Pic A	1.39	IP 1
50% CH_3OH in H_2O + Pic A	50% CH_3OH in H_2O + Pic A	1.18	IP 1 + 2
50% CH_3OH in H_2O + Pic A + 50 mM $HClO_4$	50% CH_3OH in H_2O + Pic A + 50 mM $HClO_4$	1.51	IP 1 + 2 + IS 1 + 2
50% CH_3OH in H_2O + 1 mM TBA	50% CH_3OH in H_2O + 1 mM TBA	1.51	IP 1 + 2
10% CH_3OH in H_2O	10% CH_3OH in H_2O	1.21	
10% CH_3OH in H_2O + 1 mM TBA	i) 10% CH_3OH in H_2O + 1 mM TBA	6.43	IP 1 + 2
	ii) 50 mM $HClO_4$ in H_2O	6.86	IP 2 + IS 1
100% H_2O	50 mM $HClO_4$	2.14	IS 1

Conditions: 1.0 mL/min flow rate; Altex model 330 chromatograph UV absorbance detector at 280 nm; 0.1 mL injection of 0.1 mg/mL solution

1) CH_3OH - methanol; CH_3CN - acetonitrile; $HClO_4$ - perchloric acid; Pic A - tetrabutyl ammonium phosphate (Water's); TBA - tributylamine

2) IS - Ion Suppression; IP - Ion Pairing
IS 1, IP 1 - effective agent in sample; IS 2, IP 2 - effective agent in mobile phase

Table 23. Chromatography of Ascorbic Acid and Dehydroascorbic Acid on the Zorbax-NH_2 Column

Mobile Phase	System*	k' Value AA	k' Value DHA	α Value
0.2 M KH_2PO_4 pH 4.5	1	2.80	1.73	1.62
0.1 M KH_2PO_4 pH 5.1	2	3.17	3.27	1.03
0.05 M KH_2PO_4 pH 5.2	2	4.00	4.17	1.04
0.025 M KH_2PO_4 pH 5.4	2	5.77	5.85	1.01
0.02 M KH_2PO_4 pH 5.5	1	7.62	-	-
0.01 M KH_2PO_4 pH 5.6	2	9.42	9.47	1.01

*Conditions: 2.0 mL/min flow rate; 20 µL injection of 10^{-3} M solutions
1 - Hewlett - Packard 1084A chromatograph; UV absorbance detector at 264 nm; oven temperature 30°C
2 - Altex 330 chromatograph; UV absorbance detector at 280 nm; room temperature
AA - Ascorbic Acid; DHA - Dehydroascorbic Acid

Table 24. Ascorbate Content of Various Plant Species

Species	Ascorbate content (mg/100g)	Cited value in literature[1] (mg/100g)	Extract activity[2] after 24 h
Ustilago Host			
Lychnis coronaria	21.0		+++
Lychnis flos-cuculi	43.5		+++
Silene alba	56.5		+++
Silene maritima	25.5		+++
Tragopogon pratensis	65.0		++
Zea mays	18.5		++
Non-Hosts			
Apium graveolens (celery leaves)	35.5		-
Apium graveolens (celery stalk)	3.0	8	-
Brassica oleracea (broccoli)	107.5	120	NT
Brassica oleracea (brussels sprouts)	115.0	100	NT
Brassica oleracea (kohlrabi)	49.5	70	-
Chenopodium album	212.0		++
Echium sp.	4.0		NT
Erysimum cheiranthoides	125.0		-
Gentiana crinita	157.0		+++
Glechoma hederacea	55.0		+
Lemna sp.	51.0		++
Lobelia siphilitica	152.0		++
Parnassia glauca	86.0		-
Petroselinum pastinaca (parsley)	185.0	170	NT
Rheum rhaponticum	54.0		++
Taraxacum officinale	52.0		-

1 - Olliver, 1967.
2 - Scale: +++, 60-100% of conjugated cells produce hyphae;
++, 10-60%; +, 1-10%; -, <1% or no greater than water control;
NT, not tested in this experiment.

Figure 7. Co-chromatography of Peak A and Ascorbic Acid

Aliqouts of ascorbic acid and an aqueous extract of *Zea mays* leaf tissue were chromatographed using a Hewlett-Packard 1084A HPLC equipped with a Zorbax-NH_2 column. The solvent was 0.02 M KH_2PO_4 and the flow rate was 2.0 mL/min. The tracing on the left is ascorbic acid, centre is ascorbic acid plus plant extract, and on the right is plant extract alone. The peak of interest appears at a retention time of 5 min.

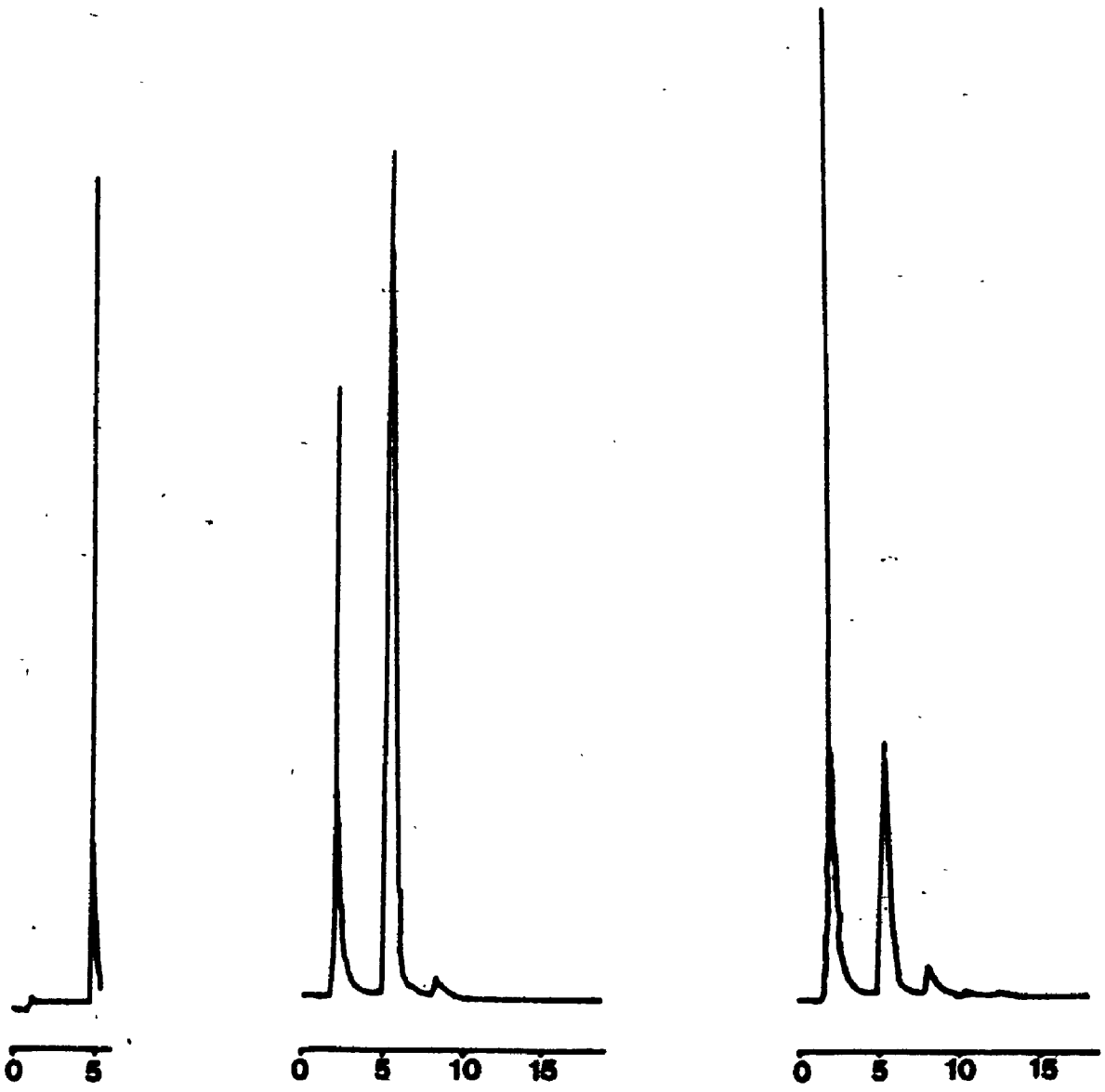

0
5
0
5
10
15
0
5
10
15
Retention Time (min)

Figure 8. UV Spectra of Peak A and Ascorbic Acid

The stepped UV absorption spectrum of material from peak A was obtained using the scanning function of the Hewlett-Packard chromatograph. Chromatographic conditions were as outlined in fig. 7. The lower curve is the spectrum of L-ascorbic acid in 50 mM $HClO_4$ run on a Shimadzu UV250 spectrophotometer. Both spectra show an absorption maximum at 264 nm.

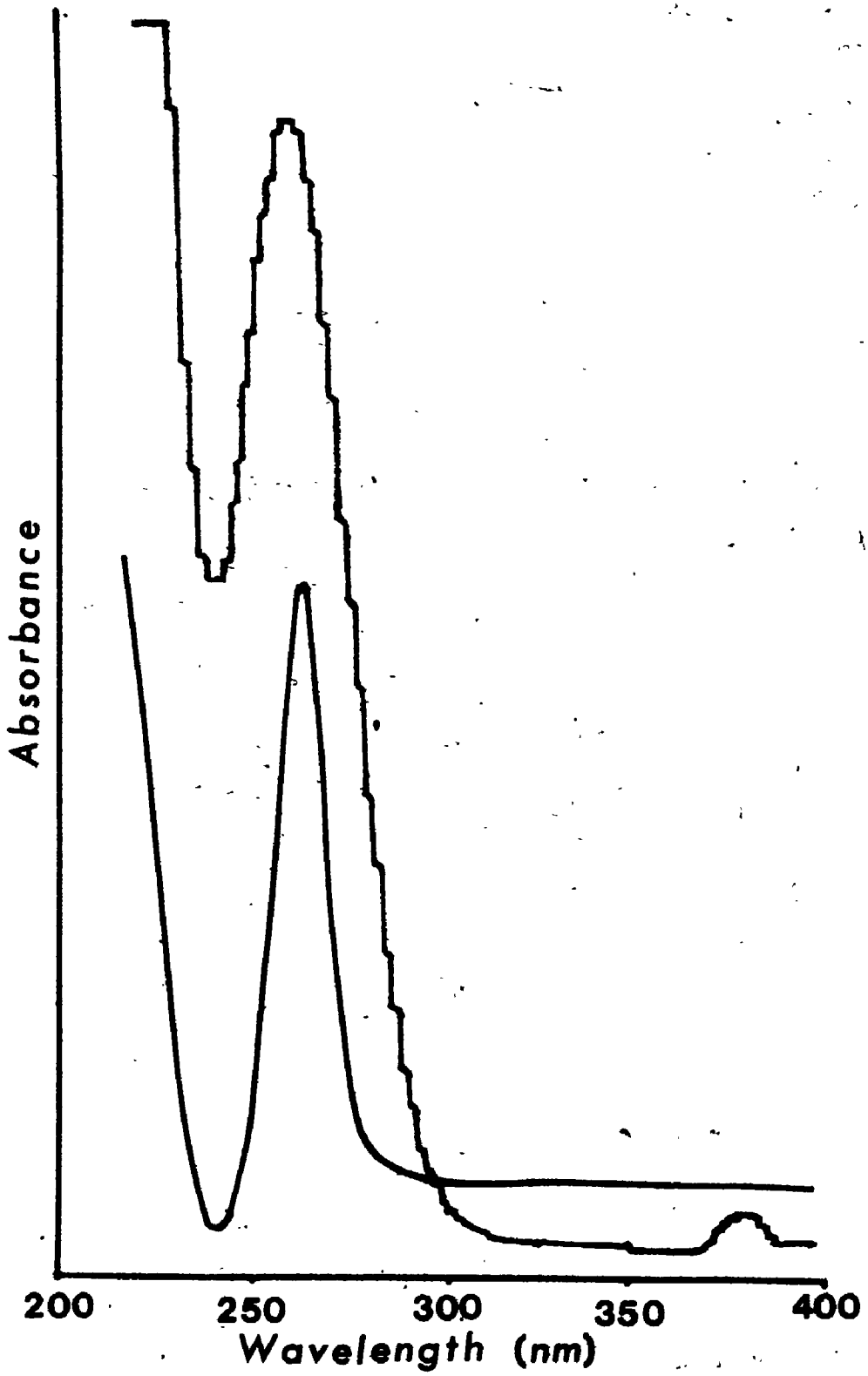

Absorbance
200
250
300
350
400
Wavelength (nm)

Figure 9. Comparison of HPLC Integrator Area Units to Amount of Ascorbic Acid Injected

Aliqoùts of known concentrations of L-ascorbic acid were chromatographed with the Beckman HPLC equipped with an Ultrasphere-ODS column. The mobile phase was 10mM tributylamine in 10% methanol in water. The flow rate was 2.0 mL/min and the eluent was monitored at 254 nm using a fixed wavelength detector. Results were recorded using a Hewlett-Packard 3390A integrator. Area units for the peak corresponding to ascorbic acid were plotted against amount of ascorbic acid injected. The different symbols indicate results from separate determinations.

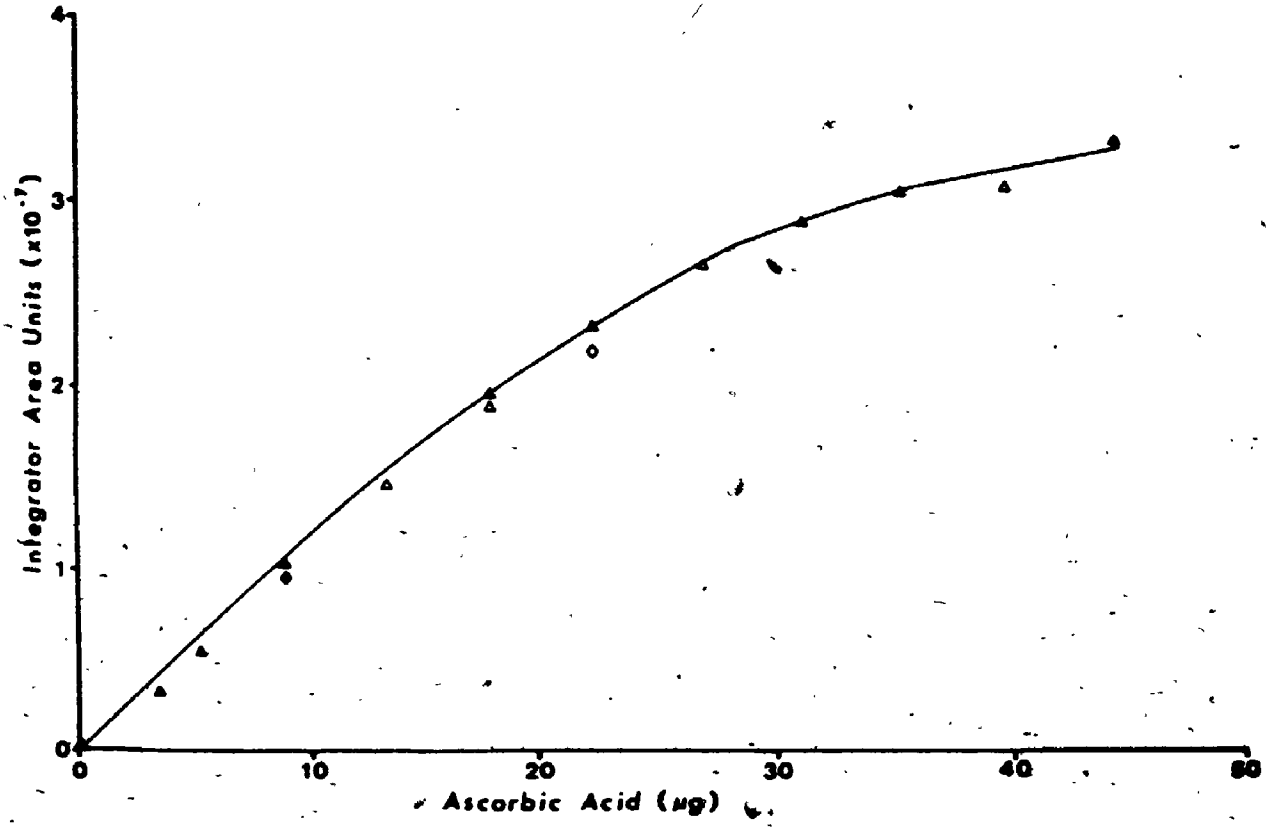
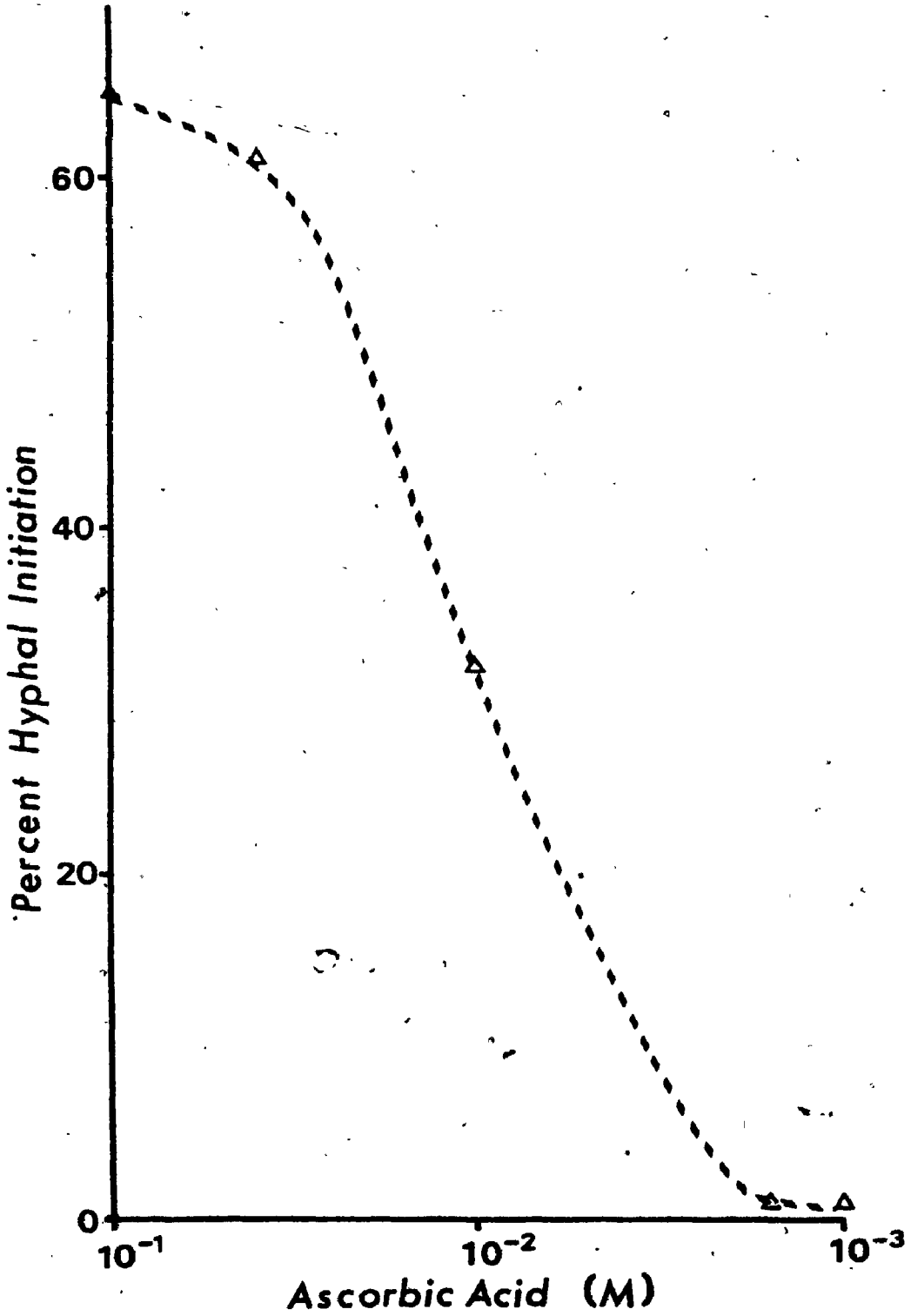

Integrator Area Units (x10⁻⁷)
0
1
2
3
4
0
10
20
30
40
50
Ascorbic Acid (µg)

Figure 10. Frequency of Hyphal Induction in Race SA-1 by Various Concentrations of Ascorbic Acid

L-ascorbic acid dissolved in distilled water was applied as 0.1 mL aliquots to mated $a_1 \times a_2$ cells on MM at 22°C. After overnight incubation, 100 mated pairs were scored for the percentage of dikaryons producing hyphae. Each spot represents the mean of three determinations.


60
40
20
0
Percent Hyphal Initiation
10^{-1}
10^{-2}
10^{-3}
Ascorbic Acid (M)

fresh tissue is completely water). Alternatively, some extracts did not induce hyphae even though the measured concentrations were comparatively high e.g. *Parnassia* and *Taraxacum*. Also, as noted earlier, plant extracts in organic solvents such as chloroform and acetone were highly active, even though ascorbic acid is insoluble in these solvents. These results together with the observation that ascorbic acid is only a weak inducer of myceliation following a long lag period make it clear that peak A is not the main contributor to extract activity.

4.6.3 Methanolic Extracts of Leaf Tissue

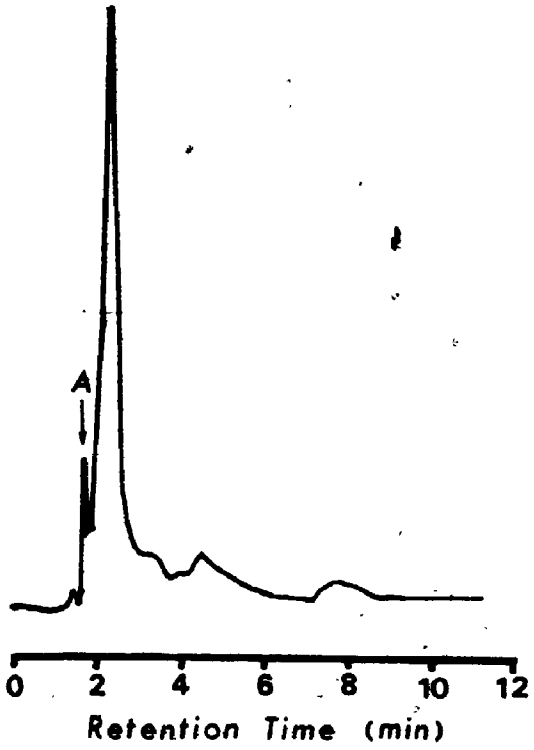
Methanol was the solvent chosen to extract leaf tissue since it provided a highly active extract and was less damaging to the reverse phase columns than the other organic solvents. Three peaks, B, C, and D, of varying activity were found in extracts of *S. alba* chromatographed with 100% methanol on the reverse phase columns. All three were similar to the crude extract, inducing hyphal growth after only a 6 h lag period, but peak D appeared to be the most effective inducer.

Peaks B and C (k' values 1.8 and 3.1, respectively, on the Ultrasphere - ODS column; fig. 11) were green solutions in white light and fluoresced red under 350 nm UV light. The visible spectra (fig. 12) of these two compounds corresponded closely to chlorophylls b and a, respectively. A commercial preparation of chlorophyll a (Sigma Chemicals) was also capable of inducing mycelial growth. This preparation, however, did not induce hyphae at concentrations found in leaf extracts, so it seemed likely that peaks B and C represented mixtures or complexes of an active agent with these chlorophylls. The active component could not be separated from the chlorophylls in several attempts. For example, precipitation of the chlorophylls with 50 mM $HClO_4$ also resulted in precipitation of the activity.

Peak D chromatographed at k' values of 5.2 and 10.5 on the Ultrasphere - ODS and Hamilton PRP columns, respectively. It was clearly more active than either peaks B or C. In these leaf extracts, peak D was quite small and was usually detected by activity in the bioassay rather than as a visible

Figure 11. Chromatography of Aqueous and Methanolic Extracts of *Silene alba* Leaves and Appearance of Peaks A to C

The upper tracing was obtained by chromatographing an aqueous extract using the Beckman chromatograph and a Hamilton-PRP column. The solvent was 50 mM $HClO_4$ in H_2O at a flow rate of 1.0 mL/min. The UV absorbance detector was set at 254 nm and the results were recorded on a Hewlett-Packard 3390A integrator. The lower tracing is of a methanolic extract analysed with the same equipment except that the column was an Ultrasphere-ODS and the mobile phase was 100% methanol. The eluent was monitored at 280 nm. "Peak" D, although not observed as a peak, was detected by the induction of hyphae in the bioassay by a sample of the eluent from the indicated region.


A
0
2
4
6
8
10
12
Retention Time (min)

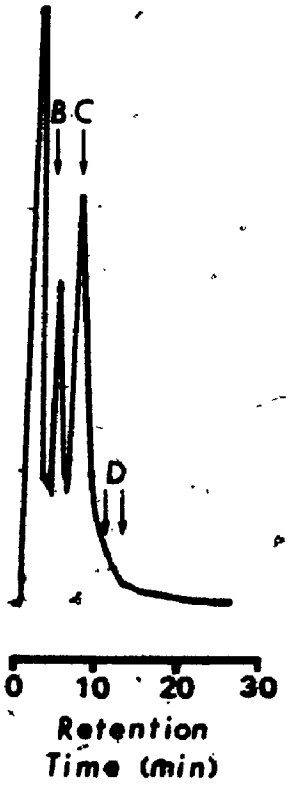
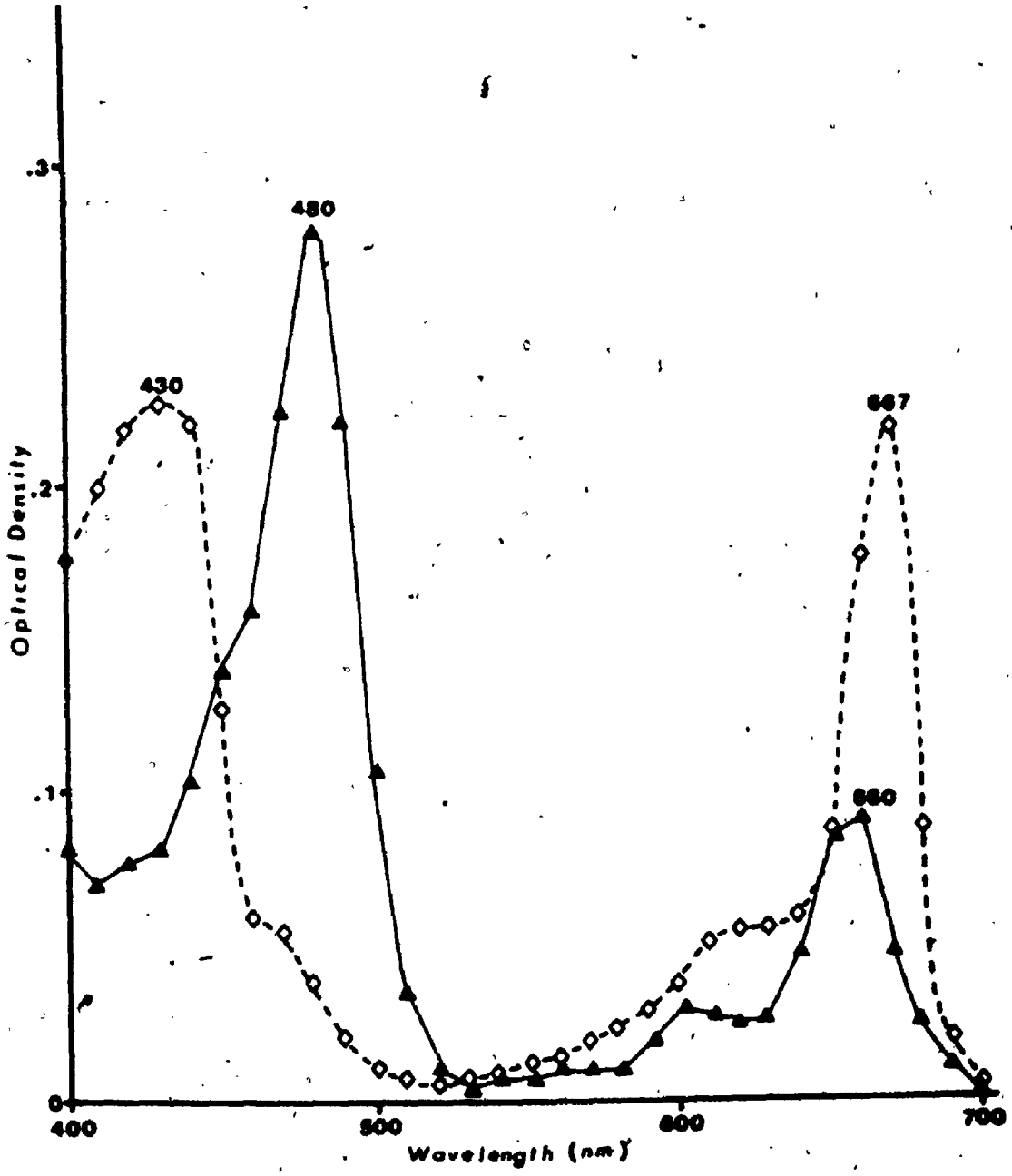

B
C
D
0
10
20
30
Retention
Time (min)

Figure 12. UV Spectra of Peaks B and C

The ultraviolet absorption spectra of material collected from peaks B and C were recorded with a Beckman 25 spectrophotometer. The methanolic extract from which these samples were obtained was chromatographed as described in fig. 11 and the spectra were measured in the running solvent, 100% methanol. Peak B (▲) had a spectrum quite similar to chlorophyll b and peak C (◇) to chlorophyll a.


.3
.2
.1
0
Optical Density
480
430
667
660
400
500
600
700
Wavelength (nm)

peak on the integrator tracing. It was therefore difficult to purify in addition to the possible binding to the chlorophylls and/ or other components of the extract. Root tissue, therefore, was examined in an attempt to circumvent these difficulties.

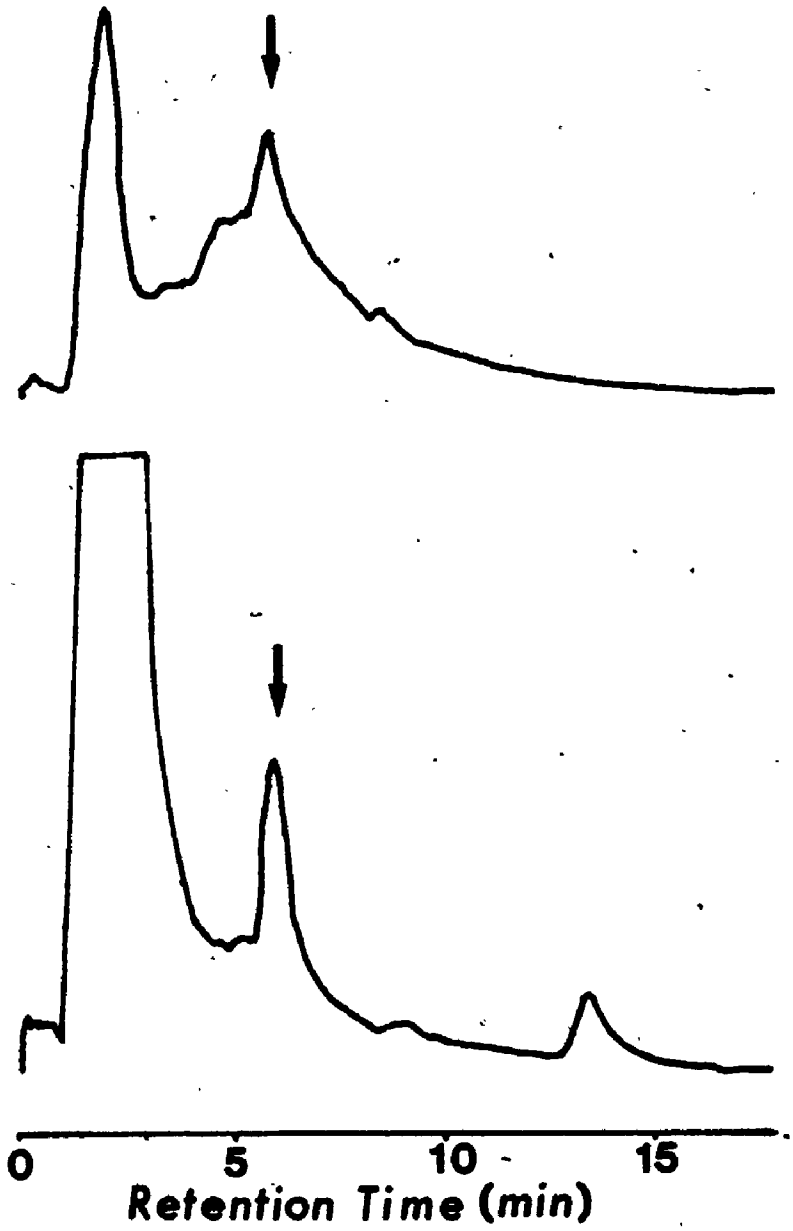
4.6.4 Methanolic Extracts of Root Tissue

Methanol extraction (see section 2.10.4) of roots allowed a concentration step which was not successful with leaf extracts due to the chlorophylls. Consequently, an extract of 30 - 40 gm of root tissue could be reduced to a 2 - 3 mL volume prior to chromatography. When analysed with the same chromatographic conditions used with leaf extracts, extracts of *S. alba* roots contained several peaks, one of which was large and highly active (fig. 13). No other active peaks were found. This peak corresponded closely to peak D of the leaf extracts in terms of retention time (k' value of 5.9 on the Ultrasphere - ODS column, and 10.2 on the PRP column). Extracts of *S. alba* and other species that had lost activity because they had been stored for some time no longer showed this peak when chromatographed. Thus, peak D appeared to be the main, if not sole, determinant of activity in these extracts as none of the other peaks or HPLC fractions showed more than trace levels of activity.

During the winter months, a shortage of sufficient material of *S. alba* forced the use of an alternative species. Commercially available parsnip, *Pastinaca sativa*, roots were found to produce highly active methanolic extracts when concentrated with the same process used for *S. alba* extracts. Reverse phase HPLC analysis showed that parsnip root extracts contained a peak with the same retention time (fig. 13) and UV absorption spectrum (fig. 14) as peak D from *S. alba* roots or leaves. The absorption maxima were noted to be at 212 and 294 nm. The k' values of the active peaks from these two species remained very similar in both the PRP and normal phase chromatographic systems. Thus it appeared very likely that the same compound was responsible for activity in extracts of both species. This was confirmed later when the compound was identified.

Figure 13. Chromatography of Methanolic Extracts of *Silene alba* and *Pastinaca sativa* Roots

Methanolic extracts of *Silene alba* (top) and *Pastinaca sativa* (bottom) were chromatographed on the Ultrasphere-ODS column with a mobile phase of 100% methanol at a flow rate of 2.0 mL/min. The UV absorbance detector was set at 280 nm. The active peak D appears at a retention time of 6 min and is indicated by the arrows.


0
5
10
15
Retention Time (min)

The fractions of the HPLC eluates containing the active peak from *P. sativa* were pooled before running twice more through the same chromatographic system. At the end of these runs, a single large apparently homogeneous peak was obtained containing approximately 2 mg of material.

4.6.5 Identification of Peak D

Purified peak D was analysed by mass spectrometry (fig. 15) and the spectrum obtained was characterized by a strong peak at *m/e* 430 along with a much smaller signal at *m/e* 446. Precise molecular weight determinations gave the values 430.3811 and 430.3807 for the former peak corresponding to the molecular formula of $C_{29}H_{50}O_2$ (calculated precise molecular weight 430.3811). This molecular formula together with the presence of three other ion peaks at *m/e* 446, 205, and 165 and the UV maxima at 212 and 294 nm (fig. 14) identified α-tocopherol as a major constituent of peak D. The *m/e* 446 ion arises from the presence of a small amount of the oxidation product, α-tocopherolquinone ($C_{29}H_{50}O_3$), which is commonly found in samples of α-tocopherol after exposure to air, and the *m/e* 204 and 165 peaks represent the known major ion fragments of α-tocopherol (Waller, 1972). As can be seen in fig. 15, there were a number of discrepancies in the ion peaks of both peak D, and commercially available α-tocopherol, as compared to the published spectrum for this compound (Waller, 1972). A likely explanation for these differences is that both the commercial preparation and peak D contained compounds in addition to α-tocopherol. Subsequent normal phase HPLC analysis indicated that both samples did indeed contain various unidentified contaminants. Despite these impurities, the mass spectra obtained gave sound evidence that α-tocopherol was a major constituent of peak D. Several peaks greater than 20% relative intensity in the commercial α-tocopherol spectrum were present in the spectrum of peak D.

Confirmation of this identity was obtained in two further ways. Firstly, aqueous emulsions of synthetic α-tocopherol (Sigma Chemicals) were found to be highly active and induced mycelia 6 h after treatment (plate 11). Secondly, α-tocopherol and purified peak D from *S. tuber* leaves or roots or

Figure 14. UV Spectra of α-Tocopherol and Peak D from Methanolic Extracts of *Silene alba* and *Pastinaca sativa* Roots

Material from peak D from *S. alba* (bottom) and *P. sativa* (middle) and a sample of α-tocopherol were purified chromatographically using a Beckman HPLC with a Brownlee Spheri-5 column. The isolated peaks, dissolved in the mobile phase (0.5% methanol in n-hexane), were scanned with a Pye-Unicam 1800 spectrophotometer. All spectra showed a characteristic maximum at 294 nm.

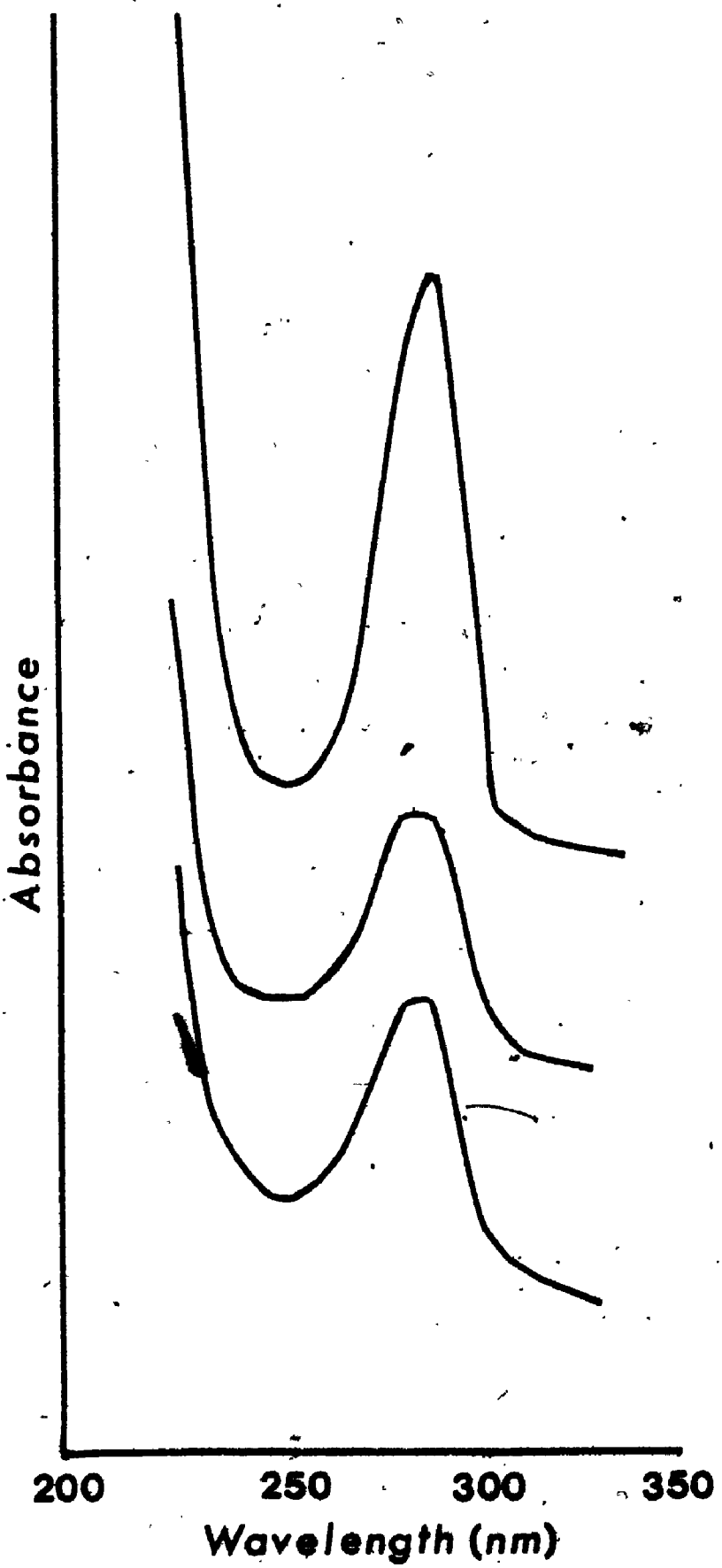
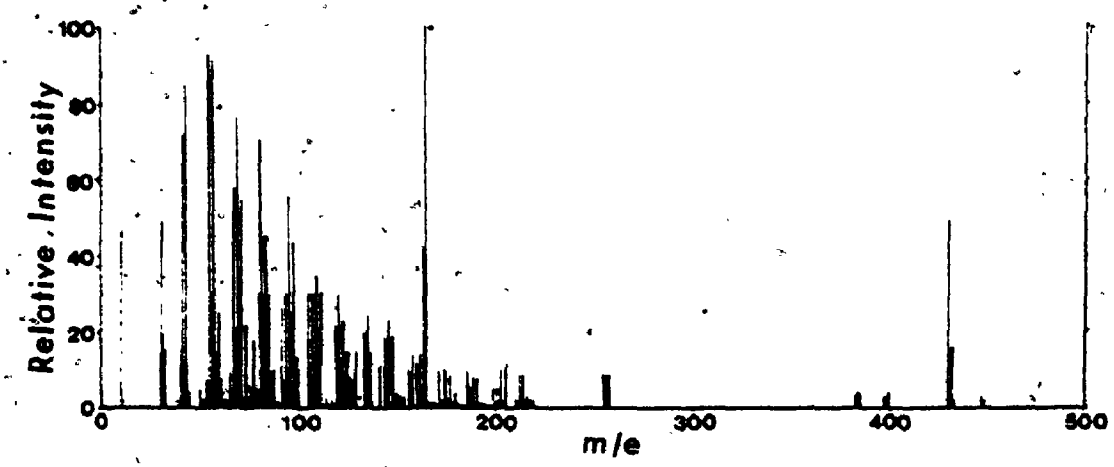
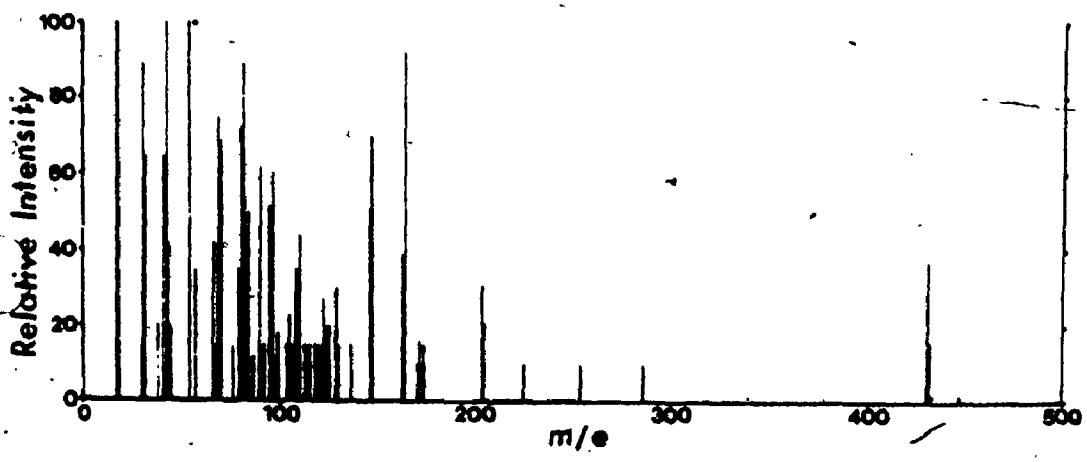
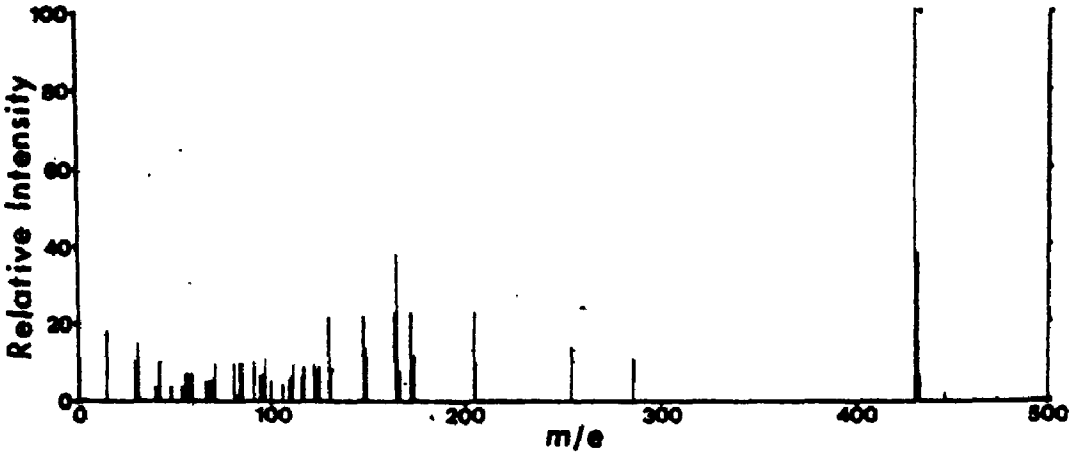

Absorbance
200
250
300
350
Wavelength (nm)

Figure 15. Mass Spectra of Peak D and α-Tocopherol

Mass spectral analysis of a sample of material isolated from peak D (bottom) and of α-tocopherol (top) were done with a Varian MAT 311A mass spectrometer. The instrument was set at probe temperature of 60°C and at a potential of 70 eV. A second sample of material from peak D (p. 160, top) was measured at 25 eV and 70°C. A published spectrum for α-tocopherol (Waller, 1972) is included for comparison. The peaks marked with a dot were set at a value of 100% and all other signals were reported as a percentage relative to that peak.


Relative Intensity
100
80
60
40
20
0
0
100
200
300
400
500
m/e


Relative Intensity
100
80
60
40
20
0
0
100
200
300
400
500
m/e


Relative Intensity
100
80
60
40
20
0
0
100
200
300
400
500
m/e

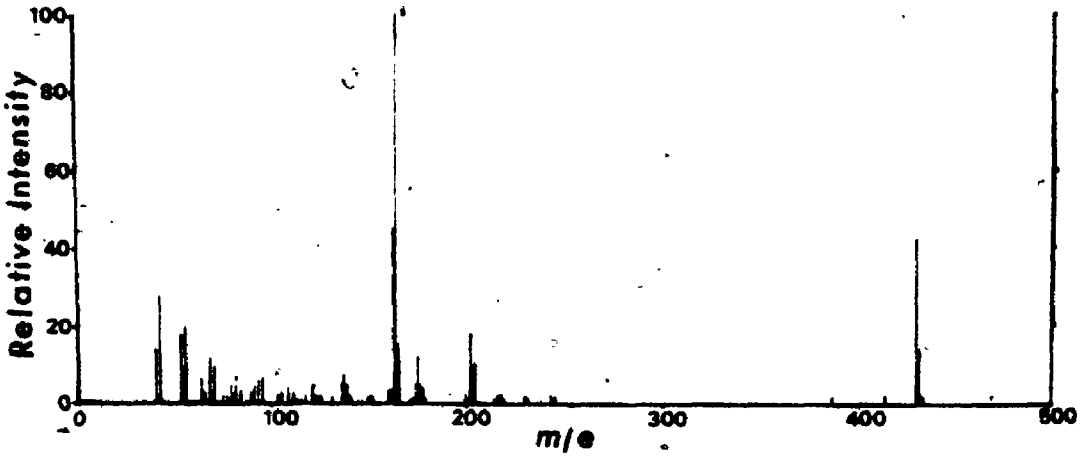

Relative Intensity
100
80
60
40
20
0
0
100
200
300
400
500
m/e

Table 25. Co-chromatography of α-Tocopherol and the Active Compound Isolated from Parsnip Extracts

Reversed Phase[1]	k'	Area Units
10 µL α-tocopherol	5.14	55840
25 µL parsnip extract	5.13	101560
25 µL parsnip extract + 10 µL α-tocopherol	5.14	189710
Normal Phase[2]	**k'**	**Area Units**
10 µL α-tocopherol	3.18	142350
100 µL parsnip extract	3.30	167080
100 µL parsnip extract + 10 µL α-tocopherol	3.30	335200

1. Ultrasphere-ODS column, 2.0 mL/min 100% methanol.
2. Si-100 column, 2.0 mL/min 0.2% methanol in n-hexane.
   Altex model 330 chromatograph with UV absorbance detector set at 280 nm was used for both normal and reverse phase procedures.

P. sativa roots had the same k' values under several reverse phase and normal phase HPLC running conditions. Impurities contained in both peak D isolated by reverse phase chromatography and in the commercial α-tocopherol sample were separated from tocopherol by normal phase chromatography. Only the α-tocopherol peak (identified by measuring the UV spectrum of the isolated compound) was found to be active in the bioassay. Moreover, "spiking" experiments in which α-tocopherol and the active peak were run at the same time gave a single peak approximately equal to the combined integrator areas of the two peaks run separately (table 25). In later experiments, an active peak with the same k' value as α-tocopherol was found in i) methanolic root and leaf extracts of *S. alba* and ii) aqueous leaf extracts of a number of species (section 4.7). No active peaks other than the ones mentioned (peaks A - D) were detected in any plant extract of any plant species during these later experiments.

In summary, the active peak in root extracts was found to contain α-tocopherol. Further purification of peak D and tests of commercial α-tocopherol preparations indicated that the activity of peak D in the bioassay was due to the presence of this compound.

4.6.6 Effective Concentration of Tocopherols and Other Related Compounds

Although synthetic α-tocopherol was highly active, it was necessary to determine if the concentration of this compound found in plant extracts was sufficient to account for the activity of these extracts. This was particularly necessary as α-tocopherol is highly insoluble in water (Karrer *et al.*, 1938). When commercial α-tocopherol was diluted in ethanol before emulsifying in water, it proved to be active from undiluted (hyphae grew right through the oily layer) down to 5×10^{-8} M (fig. 16). Estimations of the concentration of α-tocopherol in active aqueous leaf extracts gave values in the order of 10^{-6} to 10^{-8} M (see below). Thus, it is clear that the levels of α-tocopherol in active extracts are more than sufficient to account for the activity of these extracts.

Other forms of tocopherol (β,γ, and δ) were isolated from a mixed isomer oil (Sigma Chemicals) by normal phase HPLC (fig. 17) and all were highly active at an estimated concentration of approximately 3×10^{-6} M. Other phytyl containing compounds also showed activity in the bioassay. The most potent of these was a commercial preparation of phytol, active down to 10^{-7} M (fig. 16). As this phytol preparation was crude (estimated 60% phytol, Sigma Chemicals), thin layer chromatography was used to purify the components (see section 2.13; plate 12). An isolated sample containing both phytol and isophytol accounted for most of the activity of the extract. Lesser activity was also associated with minor bands of higher Rf values. While the identification of these bands was not pursued further, analysis of the crude phytol preparation by normal phase HPLC showed that none of these components were tocopherols (α, β, γ, or δ).

As mentioned earlier, chlorophyll a was weakly active. Similarly, vitamin K_1 (3 - phytylmenadione) showed marginal activity: no myceliation was induced by either chlorophyll a or vitamin K_1 at concentrations less than 10^{-2} M. In contrast to vitamin K_1, vitamin K_3 (menadione) which lacks the phytyl side chain showed no activity at any tested concentration. Selenium dioxide and sodium selenide, compounds known to partially relieve the symptoms of vitamin E deficiency in animals (reviewed in Draper, 1980), showed weak activity with a long delay before hyphal induction, as did other compounds mentioned earlier (section 4.6.1; appendix 5).

Overall, the active compounds so far identified can be divided into three categories: 1) highly active: tocopherols and phytol; 2) weakly active: vitamin K_1 and chlorophyll a; and 3) weakly active with a delay in the induction of hyphal growth: ascorbic acid, cysteine, sorbic acid, Fe^{2+}, Fe^{3+}, selenium dioxide, and sodium selenide. Certain combinations of these compounds acted synergistically to produce hyphae, e.g. ascorbic acid and iron II sulphate, while other combinations were antagonistic, cancelling the activity of each other, e.g. ascorbic acid and cysteine.

Figure 16. Frequency of Hyphal Induction in Race SA-1 by Various Concentrations of α-Tocopherol and Phytol

For each test, approximately 10^6 conjugated haploid pairs were spread over a small surface of water agar. Stock solutions of α-tocopherol or phytol were serially diluted in 80% ethanol. Each stock dilution was diluted a further 100X in distilled water and 0.1 mL of these emulsions were used to flood the cells. After 24 h, 200 mated pairs were scored for the percentage of conjugated cells producing hyphae. Each spot represents the mean of three determinations. ▲- tocopherol; ◇- phytol.

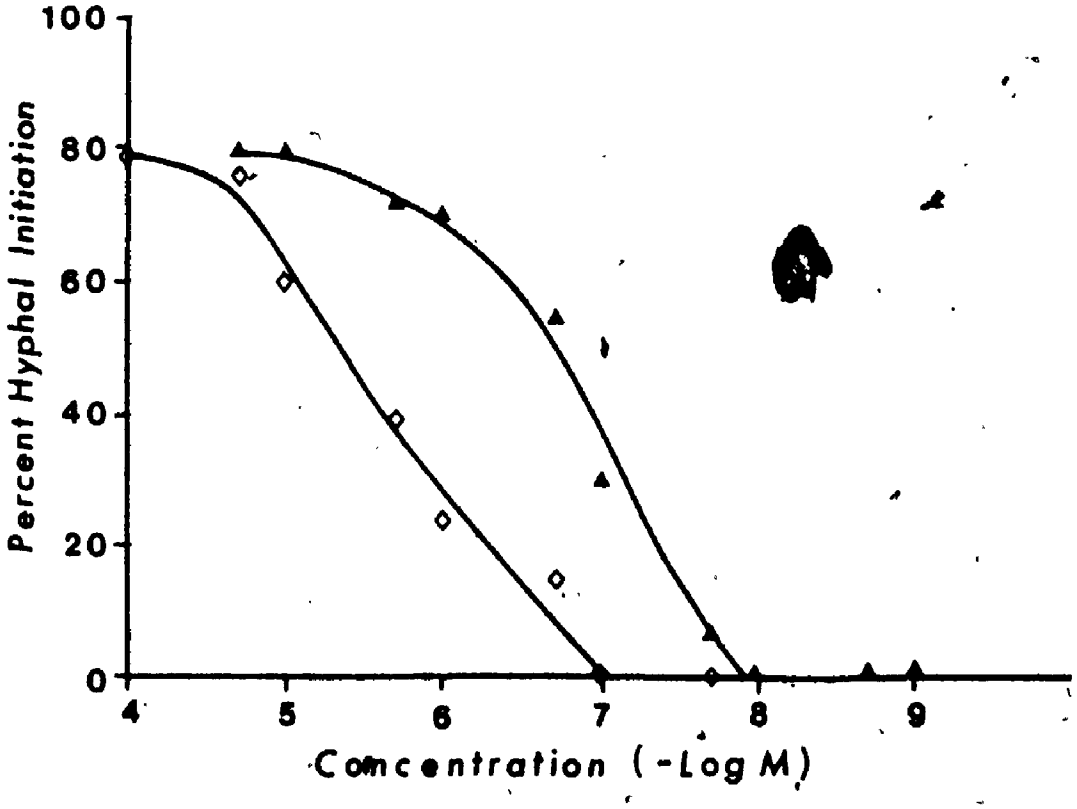

100
80
60
40
20
0
Percent Hyphal Initiation
4
5
6
7
8
9
Concentration (-Log M)

Figure 17. HPLC Normal Phase Separation of α,β,γ, and δ-Tocopherols

A mixed isomer preparation of the four forms of tocopherol (estimated to be 67% α, 1-2% β,γ, and δ by Sigma Chemicals) was dissolved in 0.5% methanol in n-hexane. This solution was injected onto a Brownlee Spheri-5 silica column and was eluted with 0.5% methanol in n-hexane at a flow rate of 2.0 mL/min. Peaks were detected by UV absorbance at a wavelength of 280 nm. The k' value for each compound was: α, 1.9; β, 5.5; γ, 6.6; and δ, 8.6. Peaks were identified according to the elution pattern (Vatassery *et al.*, 1978) and the UV spectra of the individually collected peaks.

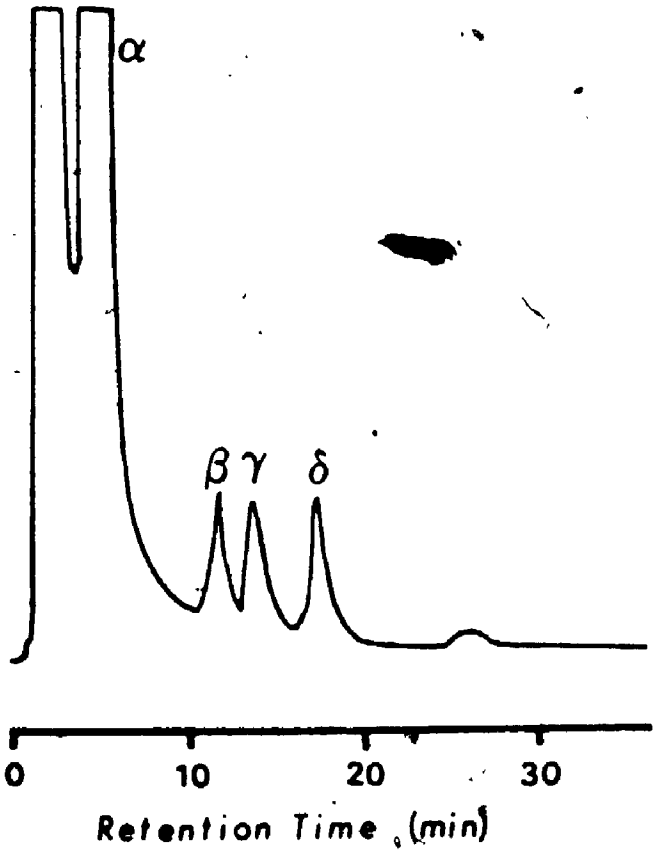

α
β γ δ
0
10
20
30
Retention Time (min)

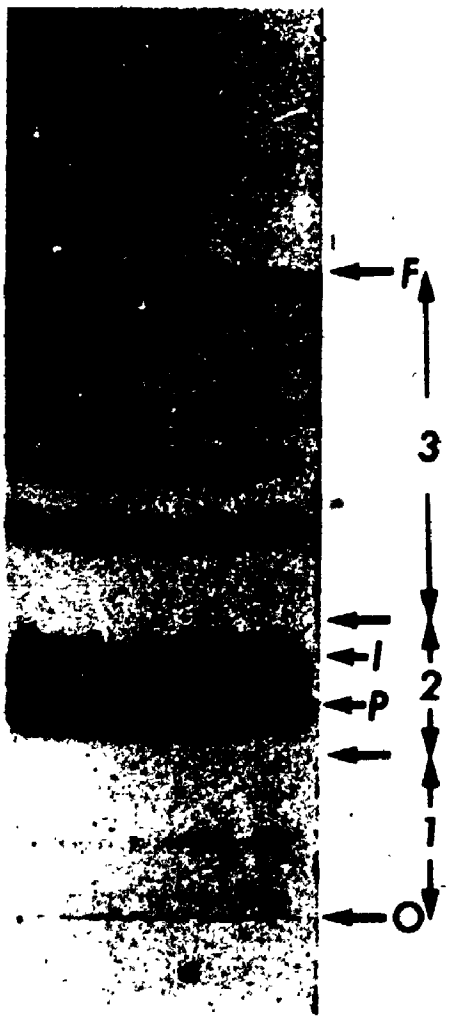
Plate 11. Induction of Hyphal Formation in *Ustilago violacea* by α-Tocopherol

Hyphae were formed by mated haploid cells in response to 10^{-6} M α-tocopherol (top left) and an aqueous extract of *Silene alba* leaves (top right) on WA at 22°C. Cells treated wtih water (bottom) did not show this response.


A
B
C

Plate 12. Thin Layer Chromatographic Separation of Components of a Phytol Preparation

An ethanol solution (10^{-2} M) of a commercial phytol preparation was separated on Whatman silica gel plates (K5F, 250 μm). The mobile phase was n-hexane: ethyl acetate, 17:3 v/v (Demole and Lederer, 1958). Following development and drying, the plate was divided into three sections and the silica was scraped into test tubes. Washes of the silica with the mobile phase were tested in the bioassay. The sample from section 1 induced 5% of conjugated cells to form hyphae, section 2, 60%, and section 3, 19% (water control, 0%). Thus, the majority of the "activity" of the preparation was derived apparently from isophytol (I) and phytol (P). F: front, O: origin.


F
3
I
2
P
1
O

4.7 Differences between Aqueous Extracts of Host and Non-host Species

α-Tocopherol was clearly the main factor determining the activity of methanolic extracts of *S. alba* and *P. sativa*. However, the early results showing differences between a "host" group of plants and a "non-host" group (table 26) were done with aqueous extracts of crudely crushed leaves. Consequently, it was decided to determine whether the activity in the host group and the inactivity of the non-host group was due to concentrations of α-tocopherol ranging above and below, respectively, the threshold level (about 5×10^{-8} M) for hyphal induction in the bioassay. Differences in concentration of this kind using a common extraction technique would indicate differences in overall concentration or in availability or accessibility of α-tocopherol in *Ustilago* host and non-host species.

A standard curve comparing integrator area units to nanogram amounts of α-tocopherol was prepared by injecting known amounts of a tocopherol standard solution (fig. 18). With a Kratos 970 fluorometer, as little as 5 ng was detected, while with a UV absorbance detector set at 280 nm, 50 ng could be measured. In practice, with the plant extracts the lower limit of detection was approximately 40 ng per injection. The area of the tocopherol peak for each injection was correlated to ng of tocopherol via the curve and the amount of tocopherol per gram fresh tissue was calculated.

Aqueous extracts (20 mL H_2O/ g tissue; see section 2.10.1), centrifuged to remove particulate matter, of various host and non-host species were partitioned against an equal volume of acetone: n-hexane, 1:2 v/v. The resulting two layer system was centrifuged at 1250xg for 5 min to break up any emulsion and the upper hexane layer was removed. The process was repeated once more. All hexane samples were combined and evaporated to dryness in a rotary evaporator. The evaporator flask was washed with 2 x 1 mL of 0.4% methanol in n-hexane, the washes were pooled, and stored at -20°C. Just before chromatography, 10 -15 molecular sieves (4A, 8 - 12 mesh; Fisher Chemicals) were added to the sample to remove any trace of water. After 5 min, the sample was decanted from the sieves into a clean

dry test tube. Longer periods of storage in the presence of the sieves resulted in loss of activity in the extract and also in tocopherol standard solutions. For example, a 2 mL sample of 10^{-4} M α-tocopherol in 0.4% methanol in n-hexane, to which 15 molecular sieves had been added, retained only about 0.2% of the tocopherol after overnight storage at -20°C (as estimated by HPLC integrator area units). The solution over the sieves produced only a weak response in the bioassay whereas the sieves, when crushed and placed on the cells, produced a strong response. The following day, neither supernatant nor sieves were active. Similar observations were made with *S. alba* extracts. In contrast, the same 10^{-4} M tocopherol preparation stored under the same conditions in the absence of any sieves retained 90% of the tocopherol for the next seven days as measured by HPLC and was very active in the bioassay throughout this period. Clearly, the molecular sieves both bound the tocopherol and enhanced its degradation. Unfortunately, the sieves were essential since chromatographing extracts not treated with them resulted in the rapid deterioration of the silica column due to the binding of water. Only two or three injections were possible before column regeneration became necessary (see section 2.11). With the molecular sieve treatment, twenty to thirty runs could be carried out prior to cleanup.

The results of analysis of α-tocopherol levels in a small scale sampling of "host" and "non-host" plant species are presented in table 26. Although it will be necessary to test many more species before firm conclusions can be made, several important trends are evident.

Firstly, considering only the host group of species, activity was directly correlated with amount of α-tocopherol. The four extracts from *S. alba* were particularly variable, partly because the extraction technique was still being perfected at this time, and the concentration of α-tocopherol varied from about 10^4 ng/g fresh tissue to about 10^5 ng/g. The threshold for activity was about 1 to 2 x 10^3 ng/g fresh tissue, and the corresponding aqueous extract would have a concentration of 50 to 100 ng/mL as 20 mL of H_2O per g tissue was used for extraction. This observation approximates closely to the estimated threshold for commercial α-tocopherol

Figure 18. Comparison of HPLC Integrator Area Units to Amount of α-Tocopherol Injected

A standard curve was prepared by chromatographing known amounts of commercial α-tocopherol. The Beckman chromatograph with a Brownlee Sheri-5 column was used with a mobile phase of 2.0 mL/min 0.5% methanol in n-hexane. Fluorescence of the eluent was monitored with a Kratos FS970 detector set at an excitation wavelength of 295 nm. Results were recorded using a Hewlett-Packard 3390A integrator and the area units for the tocopherol peak were plotted against the amount of tocopherol injected. Each point is the mean of three determinations.

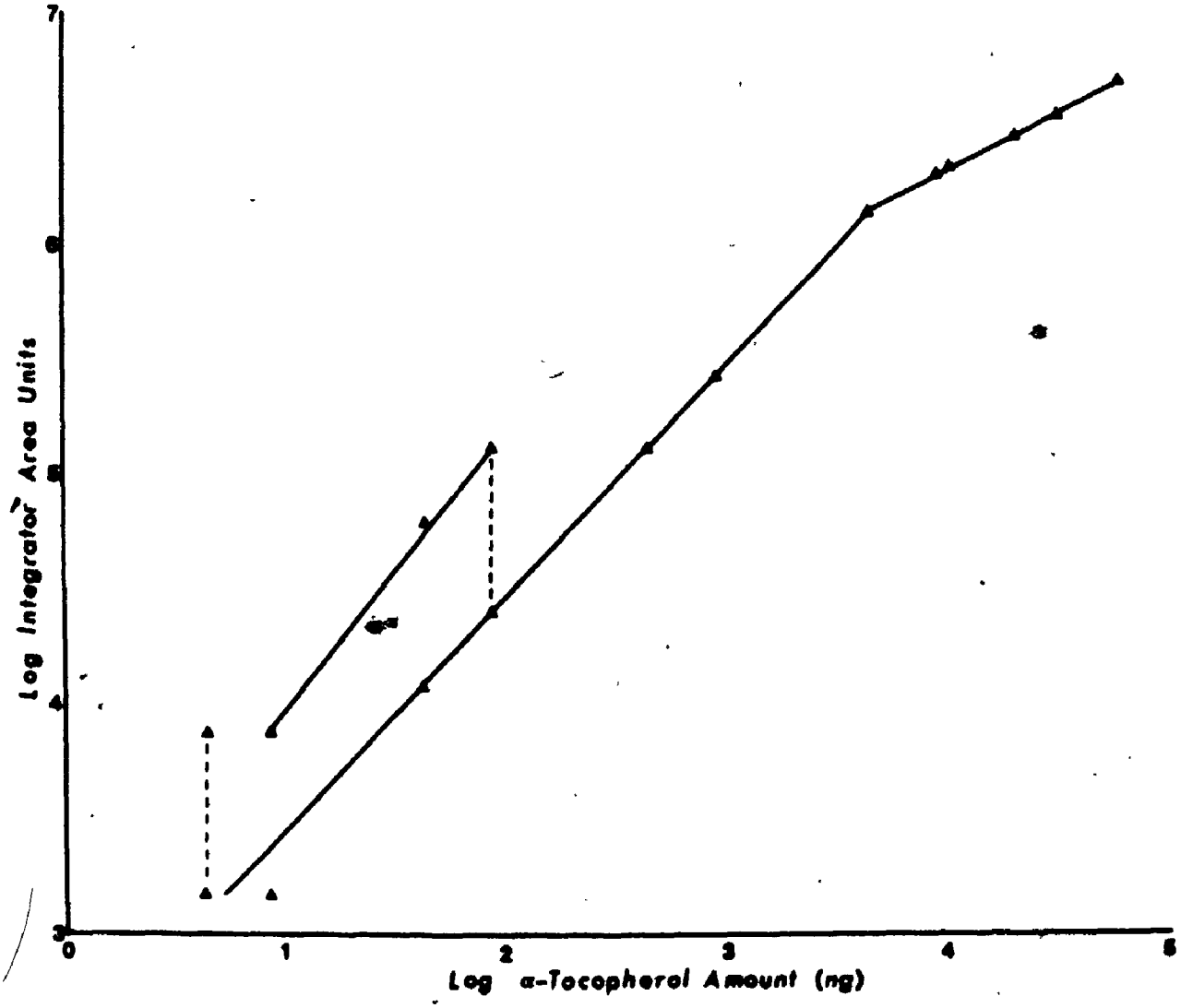

Log Integrator Area Units
7
6
5
4
3
0
1
2
3
4
5
Log α-Tocopherol Amount (ng)

Table 26. α-Tocopherol Content of Aqueous Extracts of Various Plant Species

Species	Sample	Tocopherol Content (ng/g)	Average (ng/g)	H_2O Extract Activity
Ustilago Hosts			2.41×10^4	
Silene alba	i.	4.31×10^4		+++
	ii.	1.07×10^5		+++
	iii.	1.26×10^4		+++
	iv.	6.67×10^4	5.59×10^4	++
Lychnis flos-cuculi	i.	1.81×10^4		+
	ii.	1.04×10^4		++
	iii.	6.19×10^4	3.01×10^4	++
S. armeria	i.	4.46×10^3		++
S. maritima	i.	3.66×10^3		++
Avena sativa	i.	2.61×10^3		+
	ii.	2.25×10^3		+
	iii.	ND(<1×10^3)	1.95×10^3	-
Non-hosts			1.16×10^4	
Gomphrena globosum	i.	3.73×10^3		-
	ii.	9.84×10^3		-
	iii.	1.79×10^4	1.05×10^4	+
Saintpaulia sp.	i.	7.85×10^4		+
	ii.	8.77×10^3		-
	iii.	3.36×10^3	3.02×10^4	-
Taraxacum officinale	i.	ND(<1×10^3)		-
Pelargonium sp.	i.	ND(<1×10^3)		-
	ii.	ND(<1×10^3)		-
	iii.	ND(<1×10^3)	<1×10^3	-
Hedera helix	i.	5.29×10^3		(T) -

Note: T: extract was toxic to the sporidia; concentrations are reported as ng/g fresh weight; ND: no tocopherol detected in extract

Scale: +++, 60-100% of conjugated cells produce hyphae; ++, 10-60%; +, 1-10%; -, <1%, or no greater than water control.

of slightly less than 5 x 10^{-8} M, or 200 ng/mL in an aqueous emulsion. Thus, the activity of aqueous extracts from hosts can be attributed entirely to α-tocopherol. This conclusion was supported by the failure to detect any other active compound in either the aqueous phase of the plant extracts after partitioning the tocopherol into the acetone/ hexane phase or in a 1:1 v/v, ethyl acetate: n-hexane wash of the HPLC column after several injections of active extracts.

Secondly, extracts from all host species had above threshold concentrations of α-tocopherol and all were highly active in the bioassay as shown earlier. The average concentration of α-tocopherol in fresh leaf tissue was 2 x 10^4 ng/g in these "host" species, well above the threshold level. On the other hand, the "non-host" group was more variable and several categories emerged:

Type 1: *T. officinale* and *Pelargonium* sp.

There were no detectable levels of α-tocopherol in aqueous extracts and correspondingly no activity in the bioassay. Extraction with methanol did give active extracts, so it appears that α-tocopherol is present in these species but is either unavailable to water extraction or is present in lower concentrations in these plants.

Type 2: *Saintpaulia* sp., and *G. globosum*.

These extracts contained above threshold levels of α-tocopherol, but little or no activity was observed in the bioassay. In these species it is possible that the α-tocopherol is bound to other components in the extracts and so is inactive in the bioassay. Partitioning with acetone: n-hexane may free the tocopherol and thus produce the large values observed (mean for these two species was 2 x 10^4 ng/g, the same as the host group). While no toxic effects were detected with these extracts, it is also possible that the failure to respond in the bioassay was due to inhibitors lacking in visible effects on the cells. Clearly, these species have relatively high levels of α-tocopherol but unknown factors render this ineffective to the fungus. Interestingly, extracts of *Saintpaulia* sp. spiked with extra α-tocopherol

3 3

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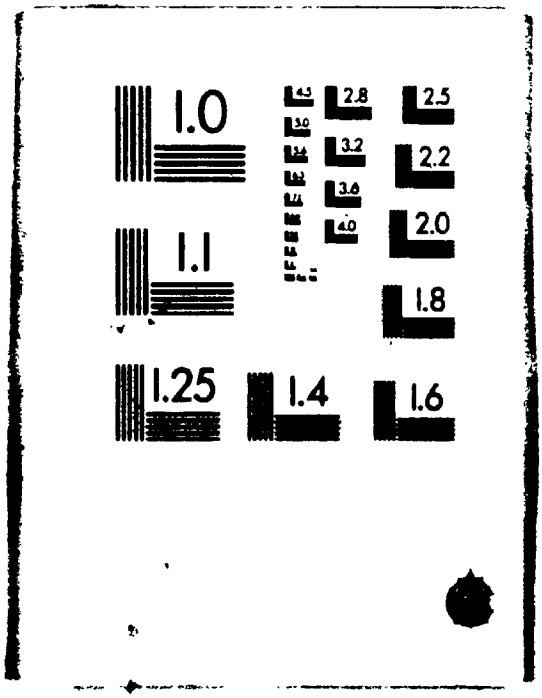

(10^{-4} M) were highly active.

Type 3: *H. helix.*

There was no activity in the bioassay even though detectable levels of α-tocopherol were present (5×10^3 ng/g). The main difference from type 2 species was the presence of a potent toxin which lysed the test cells before hyphae could develop. A similar situation was described earlier for extracts of the host species *Polygonum persicaria* which also lyse *U. violacea.*

Type 4: *Tradescantia obiensis* and *Lepidum sativum.*

This category is not apparent from the data presented in table 26, but can be hypothesized based upon the results listed in appendix 3. These species yield aqueous extracts which presumably contain α-tocopherol at above threshold levels and are active in the bioassay. They are, therefore, suitable as hosts for *Ustilago* species in this respect, but probably are immune to these pathogens because they lack other required compounds or they have effective physical or chemical resistance mechanisms.

A much more extensive survey of non - host species and identification of inhibitors or toxins in the extracts of some of these species would be required as tests of these hypotheses. It is apparent, however, that non - host species cannot be treated as a single group and that there is no one reason for the lack of activity in aqueous extracts of these species.

4.8 Discussion

The work presented in this chapter establishes that cells of *U. violacea* are induced to change from saprophytic yeastlike growth to the parasitic mycelial form by phytyl - containing compounds e.g. α-tocopherol and phytol. α-Tocopherol was found to be present at above threshold concentrations in aqueous plant extracts from species that host a *Ustilago* pathogen, but was either masked by inhibitors/ toxins or present at below threshold concentration in extracts of non-host species.

The MS and UV spectra clearly identified α-tocopherol as a major component present in plant extracts which induced mycelial formation. It was detected as the main active peak in both root and leaf extracts of two tested species that were active in the bioassay. The only other active peaks corresponded to ascorbic acid (leaves, aqueous extracts) and chlorophylls a and b (leaves, methanolic extracts). Ascorbic acid was weakly active and a response was observed only after a long delay, while commercially available chlorophyll a was not active at the concentration present in plant extracts. Therefore, the activity of the chlorophyll a and b peaks isolated from leaf extracts was most probably due to tocopherols or other phytyl containing compounds complexed with the phytyl moiety of the chlorophyll molecule. While phytol and some other tocopherols (β,γ, and δ) were shown to be highly active inducers, they were not specifically detected in plant extracts. α-Tocopherol clearly was the active agent in plant extracts and thus is likely to be the active agent in the plant which induces *Ustilago violacea* to change to its parasitic mycelial form. However, this hypothesis cannot be proven conclusively and it remains possible that some other compound (probably with a phytyl moiety) is the plant compound which comes into contact with the pathogen during host infection. Inducers would have to be located on the leaf surface as myceliation is initiated prior to penetration (A. W. Day, personal communication). Continued growth of mycelia depends on a continued supply of inducer so that later colonization of the plant would require inducer molecules within the plant tissue. This second inducer need not be the same compound as the leaf surface inducer.

If tocopherols are the major inducers *in vivo*, the following questions arise: i) do tocopherols occur on the leaf surface, at least in host plants? ii) how does tocopherol availability/ accessability differ in plants, and thus account for the observed differences between host plants (active water extracts) and non-host plants (inactive water extracts)? While little is known of the distribution and role of α-tocopherol within plant cells and tissues, the following points are relevant. Firstly, α-tocopherol appears to be present universally in angiosperms at concentrations far above the

5×10^{-8} M threshold required for the *U. violacea* bioassay (Dicks, 1965). Secondly, it is located mainly in the chloroplasts and how much is outside these organelles is uncertain (Janiskowska and Pennock, 1976).

What then is the basis of the difference between the aqueous extracts of host and non-host species? The results presented in table 26 and appendix 4 begin to provide an answer to this question. Several categories of plants were proposed on the basis of these observations. Thus, there is clearly one direction which future research must take. The cellular location and form of α-tocopherol within host and non-host species must be thoroughly documented, and the extent to which toxic or inhibitory substances endogenous to some non-host species are interfering with the response of tested smut cells should be determined. At present, the results point to previously unsuspected differences in the cellular location of α-tocopherol between plant species. Differences in tocopherol concentration in different plants have been catalogued previously (Dicks, 1965), but the relevance of these differences to the pathology of the plants has received no attention. A possibility arising from this work is that a diverse group of angiosperms are at least partly suitable as hosts for *Ustilago* species because they have readily available α-tocopherol or other phytyl containing compounds.

These speculations may be developed more formally as a series of postulates which begin to explain the basis for specialization of related pathogens on a very limited number of host species, and the limited sites of sporulation.

1. The genus *Ustilago* is adapted to grow on hosts that are rich in available phytyl containing compounds. The strong correlation between the status of a plant species as a host or non-host of *Ustilago* sp. and the ability of aqueous extracts to induce hyphal formation in *U. violacea* (table 19) firmly supports this postulate. In spite of the apparent absence of *in vitro* effects of plant extracts on the smut fungi which attack monocotyledonous hosts, the correspondence of aqueous extracts and ability to host an *Ustilago* species is still evident. Most temperate Gramineae are

attacked by *Ustilago* species. Four notable exceptions are *Nardus stricta*, *Cynosurus cristatus*, *Deschampsia flexousa*, and *Anthoxanthum odoratum*. These four species were the only ones of the 28 tested species in the Gramineae yielding inactive extracts. It is possible therefore that these four species owe their immunity to *Ustilago* to their low production of available phytyl compounds.

2. Phytyl containing compounds may constitute basic hormonal or nutritional requirements common to a large taxon, i.e. the genus *Ustilago*. Other factors superimposed on these basic needs may act to govern resistance and susceptibility at later taxonomic levels, i.e. species and physiologic races. At any of these taxonomic levels the metabolic requirements of the pathogen are likely to have both positive and negative components: positive in the requirements for certain growth factors and negative in the need to avoid chemical or physical resistance factors. Host specificity based on requirements for plant compounds is entirely compatible with gene for gene and protein for protein (Vanderplank, 1982) mechanisms of host/ parasite specificity which apply more particularly at the cultivar/ physiologic race level. Requirements for plant products would limit a pathogen to certain species; gene for gene and protein for protein considerations would further restrict the number of suitable hosts.

3. Distribution and availability of phytyl compounds within the host plant may be important in determining in which tissues sporulation will occur. In both *Silene alba* and *Erythronium americanum*, active extracts were obtained from plant tissues which support vegetative growth of the pathogen but not from tissues in which sporulation occurs (section 4.4.3). This distribution pattern, along with the inhibitory effect of host extracts on sporulation in *Ustilago* sp. (section 4.5.2), suggest that sporulation may be limited to tissues that are low in phytyl containing compounds.

The dependence of particular pathogenic taxa on specific plant compounds may occur in other groups of fungi, particularly obligately parasitic species. Thus, it may be significant to note that the group of apparently unrelated species which act as hosts to *Ustilago* species form

a subset of the larger group of plants species which host the uredial/ telial stages of the heteroecious *Puccinia* and *Uromyces* species. In contrast, the aecial hosts of these fungi rarely have *Ustilago* pathogens (cf. lists of host species in Arthur [1934] and Fisher [1953]). An attractive and testable hypothesis deriving from the present work is that *Ustilago*, *Puccinia*, and *Uromyces* species (uredial/ telial stages) are adapted to infect the same group of plants because they are rich in similar required compounds. Work on the effects of various chemicals on several species of rust fungi has shown that some potassium salts and sugars induce infection structure formation by these organisms (Kaminskyj and Day, 1984a,b; Staples, Grambow, and Hoch, 1983). However, α-tocopherol and related compounds had no effect (Kaminskyj, personal communication) on this aspect of parasitic development in these rust fungi. It remains possible, however, that phytyl compounds or other host metabolites may act as hormones for other specific morphogenetic steps in these fungi. As research in this area continues, other pathogen systems may show similar dependencies on host products.

The main effect of plant extracts studied here was that induction of mycelia from either conjugated $a_1 + a_2$ cells or diploid a_1/a_2 cells. In all cases, both mating type alleles were required for the hyphal response and, therefore, α-tocopherol and similar compounds exert their effects via an interaction with the mating type locus. This effect fits well with the current concept of the mating type gene as a developmental master switch (Day, 1979; and see Chapter 3). However, in addition to the induction of hyphae plant extracts also produced two other developmental changes. Firstly, cells of both mating types produced much longer conjugation pegs than untreated controls. The pegs from a_2 cells, in particular, appeared to develop into short haploid hyphae which grew at least initially in a random undirected manner instead of the usual direct approach to a cell of opposite mating type. While, in the crowded conditions of the plate tests, these effects did not increase the percentage of conjugating cells, it is possible that in less crowded conditions, e.g. on the leaf itself, the active compound might significantly increase the likelihood of conjugation. There

is also a real possibility that, as in *U. maydis* (Hanna, 1929), infections by haploid hyphae induced by host metabolites may sometimes occur before conjugation, as well as by the usual method of dikaryotic infection.

Secondly, development of SPP cells was inhibited by plant extracts, even under those conditions which normally favour SPP formation by conjugated $a_1 \times a_2$ haploids or a_1/a_2 diploids. Vegetative hyphal growth was observed in the presence of plant extracts and SPP formation in its absence. Development of SPP cells was also inhibited by plant extracts in several other species of *Ustilago*, even those which showed no induction of myceliation.

A marked difference in response to plant extracts between species of *Ustilago* derived from dicotyledonous hosts and those from monocotyledonous hosts was observed. In addition to *U. violacea* the only other two tested species from dicotyledonous hosts, *U. scabiosae* and *U. utriculosa*, also required plant extracts for hyphal formation. On the other hand, all of the tested species which attack monocotyledonous plant species produced some hyphae in the absence of extracts and were apparently unaffected by extract treatment, except for the inhibition of development of SPP cells. Other investigators have also noted differences between smut fungi from dicot and monocot plants in other quite different characteristics, so that there may well be a clear taxonomic division between thse two groups (Baird and Garber, 1979a; Will *et al.*, 1983).

There are indications that at least one species of the genus *Farysia*, *F. olivacea*, which attacks species in the Cyperaceae, responds to a different plant compound. Extracts from hosts of *Ustilago* species did not induce myceliation in this species which, like *U. violacea*, is normally strictly sporidial. However, extracts from a species in the Juncaceae (closely related to the Cyperaceae) which does not host a *Ustilago* species did induce mycelial development in both *F. olivacea* and *U. violacea* (table 20). The simplest explanation for this observation is that the plant contained two distinct inducing compounds, one of which induced *U. violacea* and the other, *F. olivacea*.

The mode of action of vitamin E in plant and animal systems is still controversial despite many years of research (McCay and King, 1980). While an important antioxidant role of α-tocopherol is established (Tappel, 1962; Diplock, 1974; McCay and King, 1980), other more specific mechanisms, including control of gene transcription (Catignani, 1980) have been postulated. If α-tocopherol does indeed act as an antioxidant in regulating development in *U. violacea*, then the activity of ascorbic acid, cysteine, and iron ions, all redox agents, is understandable. However, phytol which is non-oxidizing is a potent inducer, while other common redox agents (reduced glutathione, dithiothreitol, mercaptoethanol) are inactive. It appears more likely that α-tocopherol acts in this system by affecting gene regulation more or less directly, a view that is supported by i) the very low concentrations required (0.1 mL of a 5×10^{-8} M solution per 10^8 cells is equivalent to about 10^{-17} gm per treated cell); ii) preliminary data showing changes in protein synthesis patterns 3 - 6 h after tocopherol treatment (unpublished data from an undergraduate project by Beverly Dickson); iii) the requirement for heterozygosity at the mating type locus, the gene controlling development in this organism (Day, 1979; Castle and Day, 1980). In this respect, the effect of vitamin E on *U. violacea* is very similar to the effect on the rotifers, *Asplancha sieboldi* and *Brachionus calciflorus*, where again low concentrations (5×10^{-7} M) induce striking developmental changes (Gilbert, 1974). These concentrations are much lower than those required for effects on mammalian cells (Gilbert, 1974).

The weak and delayed effects of ascorbic acid and cysteine could be explained by a sparing effect on α-tocopherol, as these compounds can form an oxidation - reduction chain with this vitamin (Tappel, 1962). However, so far no endogenous α-tocopherol or any other inducing agent has been found in *U. violacea* and several other fungi so that explanations involving sparing effects may be of dubious validity. Clearly, much remains to be done to investigate the specific developmental changes induced in *U. violacea* by α-tocopherol. Indeed, the rapid synchronous response to α-tocopherol and the ease of manipulation of the cells of this simple eucaryote make this an ideal system in which to investigate the mode of action of this vitamin.

Furthermore, the *U. violacea* system could be developed into a very sensitive bioassay for detecting tocopherols or phytols at concentrations as low as 10^{-8} M, at present beyond the workable range of HPLC detection.

The requirement for phytyl compounds for parasitic development in several species of *Ustilago* is unique in fungi. Vitamin E responses or requirements of any kind have not been reported previously in fungi. As mentioned in section 4.1, the term "mycoboethin" was coined to describe host products which act specifically to induce morphogenetic changes favouring the parasitic state. Several examples of plant and animal compounds which induce growth of the parasite *in vivo* are listed by Strange, Majer, and Smith (1974). Many of these compounds involve nutritional effects and serve to increase the growth rate. However, α-tocopherol acts as a true mycoboethin on *U. violacea*, and a few other *Ustilago* species, inducing morphogenetic changes at hormonal concentrations. In all of these cases, several obvious questions arise. Why do host plants produce compounds such as fungal growth stimulants and mycoboethins which aid particular pathogens? Why have mutant strains lacking these products not been selected during the evolution of the host species? One obvious answer to these problems is that the particular compounds are indispensible or serve other valuable functions, e.g. resistance to insects or to grazing animals. Although the role of α-tocopherol in plants is not understood, it apparently serves an indispensible function as it occurs ubiquitously in chloroplasts. Clearly, detailed investigations of the chemistry of plant produced phytyl products, their role in hosts, the differences in location in non-hosts, and their mode of action in *Ustilago violacea* and other dicot smut fungi are needed. Strange *et al.* (1974) suggested that, in addition to the present work on resistance factors, "it may prove profitable . . . to expend some effort on the complementary endeavour of identifying susceptibility factors and eliminating them from important crops". As pointed out above, this view may be oversimplistic if these factors are important or indispensible to the host. However, a better understanding of all of the factors affecting plant susceptibility including the role and importance of mycoboethins, may well lead to improved disease control methods.

APPENDICES

Appendices 1 - 4. Effect of Crude Aqueous Extracts from a Variety of Plant Species on the Induction of Hyphae from Cells of *Ustilago violacea* Race SA-1

Appendix 1. Plants in the Caryophyllaceae: Hosts of *U. violacea*

Species	Relative Activity
Agrostemma githago	+
Arenaria serpyllifolia	++
Cerastium arvense	++
C. viscosum	+++
C. vulgatum	+++
Dianthus armeria	+++
D. caryophyllus	+++
D. deltoides	+++
Lychnis coronaria	+
L. flos-cuculi	++
Saponaria officinalis	+++
Silene alba	+++
S. antirrhina	+++
S. armeria	++
S. compacta	+++
S. cucubalus	+
S. dichotoma	+++
S. dioica	+++
S. fabaria	+
S. maritima	+
S. noctiflora	+++
S. vespertina	++
Stellaria graminea	+++
S. holostea	++
S. media	+++
S. pubera	+
Vaccaria segetalis	+
Herbarium specimens	*

Note: Scoring system: +++, 60-100% of the conjugated pairs produce hyphae in response to "unconcentrated" aqueous extracts (section 2.10.1); ++, 10-60%; +, 1-10%; -, <1%, or no greater than water controls. *: Material

from the herbarium of the University of Western Ontario, collected from 1900 to 1970. Extracts from the following species were tested, and all had some activity: *Agrostemma githago, Arenaria serpyllifolia, Cerastium arvense, C. vulgatum, Dianthus armeria, D. barbatus, D. caesius, D. caryophyllus, D. chinensis, D. deltoides, D. plumarius, D. superbus, Gypsophila paniculata, Lychnis affinis, L. alpina, L. chalcedonica, L. coelirosa, L. coronaria, L. flos-cuculi, Saponaria officinalis, Silene acaulis, S. alba, S. antirrhina, S. armeria, S. caroliniana, S. cucubalus, S. dioica, S. latifolia, S. maritima, S. menziesii, S. nivea, S. pendula, S. virginica, Stellaria graminea, S. holostea, S. longifolia, S. media, S. pubera,* and *Viscaria vulgaris*. In these plant extracts, activity ranged from weak to moderate, but in view of uncertainty concerning the loss of activity during prolonged storage, quantitative differences between species were not assessed.

Appendix 2. Plants in Other Families: Not Hosts of *U. violacea*

Species	Family	Activity
Hedera helix	Araliaceae	-
Impatiens wallerana	Balsaminaceae	-
Cynoglossum viginianum	Boraginaceae	-
Lonicera oblongifolia	Caprifoliaceae	-
Tradescantia obiensis	Commelinaceae	-
Erigeron pulchellus	Compositae	-
Gynura aurantica	Compositae	-
Solidago canadensis	Compositae	-
Tanacetum vulgare	Compositae	-
Taraxacum officinale	Compositae	-
Convolvulus arvensis	Convolvulaceae	-
Barbarea vulgaris	Cruciferae	-
Hesperis matronalis	Cruciferae	-
Scabiosa perfecta	Dipsacaceae	++
Geranium maculatum	Geraniaceae	-
Zea mays	Gramineae	+
Coleus blumei hybrid	Labiatae	-
Glechoma hederacea	Labiatae	-
Coronilla varia	Leguminosae	-
Medicago lupulina	Leguminosae	-
Smilacina racemosa	Liliaceae	-
Malva neglecta	Malvaceae	-
Oenothera brennsis	Onagraceae	-
Oxalis cernua	Oxalidaceae	+
O. europaea	Oxalidaceae	+
Corydalis flavula	Papaveraceae	-
Rheum rhaphonticum	Polygonaceae	-
Anemone canadensis	Ranunculaceae	-
Ranunculus acris	Ranunculaceae	-
Gallium mollugo	Rubiaceae	-
Antirrhinum majus	Scrophulariaceae	-
Lycopersicon esculentum	Solanaceae	-
Solanum dulcamara	Solanaceae	-
Aegopodium podagraria	Umbelliferae	-
Zizea aervea	Umbelliferae	-
Viola striata	Violaceae	-

Note: Scoring system: +++, 60-100% of the conjugated pairs produce hyphae in response to "unconcentrated" aqueous extracts (section 2.10.1); ++, 10-60%; +, 1-10%; -, <1%, or no greater than water controls. Species are arranged after the classification of Engler, given in Willis (1957).

Appendix 3. Larger Survey of Plant Extracts: Dicotyledons

Species	Family	Pathogen	Toxicity	Activity
Hedera helix	Araliaceae		+	-
Impatiens wallerana	Balsaminaceae		-	-
Cynoglossum virginianum	Boraginaceae		-	-
Symphytum officinale	Boraginaceae		+	-
Lobelia sp.	Campanulaceae	E	-	-
Lonicera oblongifolia	Caprifoliaceae		-	-
Chenopodium album	Chenopodiaceae		-	+
Tradescantia obiensis	Commelinaceae		-	++
Aster sp.	Compositae	E	-	-
Centaurea scabiosa	Compositae		-	-
Cichorium intybus	Compositae		-	-
Chrysanthemum indicum	Compositae		-	-
Echinops sp.	Compositae		+	-
Erigeron pulchellus	Compositae	E	+	-
Gynura aurantica	Compositae		+	-
Helianthus tuberosus	Compositae	E	-	+++
Lactuca sativa	Compositae	E	-	-
Solidago canadensis	Compositae	T	-	-
Tanacetum vulgare	Compositae		-	-
Taraxacum officinale	Compositae		-	-
Tragopogon pratensis	Compositae	Us	-	+++
Convolvulus arvensis	Convolvulaceae	T	-	-
Barbarea vulgaris	Cruciferae		-	-
Cheiranthus cheiri	Cruciferae		-	-
Hesperis matronalis	Cruciferae		-	-
Lepidum sativum	Cruciferae		-	+++
Scabiosa perfecta	Dipsacaceae	Us	-	+++
Euphorbia esula	Euphorbiaceae	T	+	-
Geranium maculatum	Gerianaceae		-	-
Coleus blumei	Labiatae		+	-
Glechoma hederacea	Labiatae		+	-
Coronilla varia	Leguminosae		-	-
Medicago lupulina	Leguminosae		-	-
Trifolium pratense	Leguminosae	T	-	++
Althea rosea	Malvaceae		+	+++
Malva neglecta	Malvaceae		-	-
Oenothera brennsis	Onagraceae		-	-
Oxalis cernua	Oxalidaceae	Us	-	++
O. europaea	Oxalidaceae	Us	-	+++
Corydalis flavula	Papaveraceae		-	-
Dicentra cucullaria	Papaveraceae		+	-
Plantago major	Plantaginaceae		-	-
Polygonum persicaria	Polygonaceae	Us,S,M	+	-(+++)*
Rheum rhaponticum	Polygonaceae		+	-

Appendix 3. Continued

Species	Family	Pathogen	Toxicity	Activity
Rumex crispus	Polygonaceae	Us,S,M	+	-
R. obtusifolia	Polygonaceae	Us,S,M	+	-
R. crispus x obtusifolia	Polygonaceae	Us,S,M	+	-
R. acetosella	Polygonaceae	Us,S,M	-	+++
Clematis patens	Ranunculaceae		-	++
Delphinium elatum	Ranunculaceae	U,E	-	++
Hepatica americana	Ranunculaceae	U	-	-
Thalictrum dioicum	Ranunculaceae	E	++	+++
Ranunculus acris	Ranunculaceae	U,E,D	-	-
Anemone canadensis	Ranunculaceae	E,D	-	-
Spiraea x vanhouttei	Rosaceae		-	-
Galium mullugo	Rubiaceae		-	-
Antirrhinum majus	Scrophulariaceae		-	-
Lycopersicon esculentum	Solanaceae		+	-(+)*
Solanum dulcamara	Solanaceae	E	+	-
Aegopodium podagraria	Umbelliferae		+	-
Petroselinum sativa	Umbelliferae		-	+++
Zizea aervea	Umbelliferae		-	-
Viola striata	Violaceae	U	-	-

Note: Activity scoring system: +++, 60-100% of the conjugated pairs produce hyphae in response to "unconcentrated" aqueous extracts (section 2.10.1); ++, 10-60%; +, 1-10%; -, <1%, or no greater than water controls. Toxicity scoring system: +, toxic; -, nontoxic. Genera of smut fungi: D, *Doassania*; E, *Entyloma*; M, *Melanopsichum*; S, *Sphacelotheca*; T, *Tilletia*; U, *Uromyces*; and Us, *Ustilago*. Details of smut fungi and their hosts as in Ainsworth and Sampson (1950), and Fischer (1953), applying to Britain and North America. The pathogens are capable of infecting the given plant genus, though not necessarily the particular species. *: The crude extract was toxic and therefore scored as inactive. Chloroform/ water partitioning yielded a chloroform fraction which was active in promoting hyphal growth and a toxic aqueous fraction.

Appendix 4. Larger Survey of Plant Extracts: Monocotyledons

Species	Family	Pathogen	Toxicity	Activity
Carex nigra	Cyperaceae	Sc,U,Th,F,C,P	-	+++
Carex pennsylvanica	Cyperaceae	Sc,U,Th,F,C,P	-	+++
Luzula acuminata	Cyperaceae	Us,C	-	+++
Agropyron repens	Gramineae	Us,T,U	-	+++
Ammophila arenaria	Gramineae	Us	-	+++
Anthoxanthum odoratum	Gramineae	T	-	-
Arrenatherum elatius	Gramineae	Us	-	++
Arundo donax	Gramineae	Us	-	++
Avena sativa	Gramineae	Us	-	+++
Bromus inermis	Gramineae	Us,T,U	-	+++
Cynosurus cristatus	Gramineae		-	-
Deschampsia flexuosa	Gramineae	U,T,E	-	-
Digitaria sanguinalis	Gramineae	Us,T,S	-	+++
Dactylis glomerata	Gramineae	Us,U	-	++
Echinochloa crus-galli	Gramineae	Us,To,S	-	+++
Festuca elatior	Gramineae	Us,T,U	-	+++
Festuca ovina	Gramineae	Us,T,U	-	+++
Festuca obtusa	Gramineae	Us,T,U	-	+++
Holcus lanatus	Gramineae	Us,T,E	-	+
Hordeum vulgare	Gramineae	Us,T,U	-	+++
Lolium perenne	Gramineae	Us	-	++
Nardus strictus	Gramineae		-	-
Panicum virgatum	Gramineae	Us,T,To,S,So,E	+	++
Phqlaris arundinacea	Gramineae	Us	-	+++
Phleum pratense	Gramineae	Us	-	++
Poa annua	Gramineae	Us,T,E,U	-	+++
Poa trivialis	Gramineae	Us,T,E,U	-	+++
Secale cereale	Gramineae	Us,T,U	-	+++
Setaria viridis	Gramineae	Us,S	-	++
Triticum aestivum	Gramineae	Us,T,U	-	++
Zea mays	Gramineae	Us,S	-	++
Allium cepa	Liliaceae	U	-	+
Colchicum autumnale	Liliaceae	U	-	++
Erythronium americanum	Liliaceae	Us,U		
leaves			-	-
bulbs			-	+++
Muscari botryoides	Liliaceae	Us	-	+++
Smilacina racemosa	Liliaceae	U,Tu	-	-
Trillium grandiflora	Liliaceae	U,Tu	-	-
Hyacinthus orientalis	Liliaceae	Us	-	+++
Juncus brachycephalus	Juncaceae	C,U,To	-	++

Note: Activity scoring system: +++, 60-100% of the conjugated pairs produce hyphae in response to "unconcentrated" aqueous extracts (section 2.10.1); ++, 10-60%; +, 1-10%; -, <1%, or no greater than water controls. Toxicity

scoring system: +, toxic; -, nontoxic. Genera of smut fungi: C, *Cintractia*; E, *Entyloma*; F, *Farysia*; P, *Planetella*; S, *Sphacelotheca*; Sc, *Schizonella*; So, *Sorosporium*; T, *Tilletia*; Th, *Thecaphora*; To, *Tolyposporium*; Tu, *Tuburcinia*; U, *Uromyces*; and Us, *Ustilago*. Details of smut fungi and their hosts as in Ainsworth and Sampson (1950), and Fischer (1953), applying to Britain and North America. The pathogens are capable of infecting the given plant genus, though not necessarily the particular species.

Appendix 5. Commercially Available Chemicals Tested in the Bioassay

Compounds which induce hyphal formation.

Strongly active (effective at $>10^{-5}$ M); hyphae observed after 6 h.

α,β,γ, and δ-tocopherol; α-tocopherol acetate; phytol.

Weakly active (not effective at $>10^{-5}$ M); hyphae observed after 6 h.

chlorophylls a and b; vitamin K_1 (3-phytyl menadione).

Weakly active; hyphae observed only after 24 h.

D and L-ascorbic acid; dehydroascorbic acid; cysteine; sorbic acid (only after neutralization); iron III sulphate; iron II chloride; iron III chloride; iron III acetate; iron III acetate basic; sodium selenite; selenium dioxide.

Compounds which do not induce hyphal formation.

Amino acids (all L- unless otherwise specified).

alanine; aspartic acid; asparagine; glutamic acid; glutamine; arginine; cystine; glycine; threonine; lysine; methionine; serine; ornithine; citrulline; homocystine; homoserine; DL-ethionine.

Antioxidants.

dithiothreitol; mercaptoethanol; NADH; reduced glutathione; butylated hydroxytoluene.

Carboxylic acids and related salts and esters.

acetic acid; formic acid; oxalic acid; oxaloacetic acid; oxamic acid; oxalomalic acid; chlorogenic acid; phospoglycolic acid; glycolic acid; thioglycolic acid; glyoxylic acid; chelidonic acid; lactic acid; tartaric acid; pyruvic acid; succinic acid; fumaric acid; malic acid; citric acid; butyric

acid; shikimic acid; isovaleric acid; quinic acid; cis-aconitic acid; trans-aconitic acid; ethylene diamine tetraacetic acid; adipic acid; mucic acid; itaconic acid; α-ketobutyric acid; α-ketoglutaric acid; α-ketocaproic acid; α-ketovaleric acid; α-ketoisovaleric acid; α-ketoisocaproic acid; α-ketoadipic acid; ketomalonic acid; tartronic acid; dihydroxyfumaric acid; squaric acid; glutaric acid; tricarballylic acid; citramalic acid; saccharic acid; hydroxyglutaric acid; para-aminobenzoic acid; diaminobenzoic acid; anthranilic acid; folic acid; para-aminosalicylic acid; cholic acid; folinic acid; tetrahydrofolic acid; dihydrofolic acid; thiotic acid; palmitic acid; oleic acid; linolenic acid; methyl linoleate; cholesteryl linoleate; methyl tetrahydrofolate; oxamic acid ethyl ester; oxaloacetic diethyl ester; glucuronic acid lactone; isocitric acid lactone; calcium oxalate; magnesium oxalate; ammonium oxalate; potassium oxalate; tin II oxalate; ammonium acetate; magnesium acetate; calcium formate; sodium malonate; monobasic potassium isocitrate; monobasic isocitrate lactone.

Inorganic salts.

aluminium chloride; sodium chloride; potassium chloride; lithium chloride; palladium chloride; nickel chloride; magnesium chloride; calcium chloride; calcium carbonate; calcium nitrate; manganese II sulphate; monobasic sodium phosphate; dibasic sodium phosphate; copper II sulphate.

Nitogenous bases, nucleosides, and nucleotides.

adenine; guanine; cytosine; thymine; uracil; xanthine; hypoxanthine; AMP; cyclic AMP.

Phenolics.

phenol; resorcinol; orcinol; inositol; phlorglucinol; m-cresol; vanillic acid; vanillin; syringic acid; coumaric acid; coumarin; quercetin kaempherol; rutin; quercitrin; cyanidin chloride; gallocyanin; arbutin; salicin; vitexin.

Plant growth regulators.

3-indole acetic acid; GA-3; GA-7; abscisic acid; kinetin.

Steroids.

cholesterol; stigmasterol; progesterone; cortisone; pregen-3β-ol-2-one; 21-acetoxypregenolone; 17β-estradiol; deoxycorticosterone; Δ^4-androsten-3,17-dione; testosterone; camesterol; 11α-acetoxyprogesterone; ergosterol; prednisone; 1-hydroxypyridine-2-thione; 5α-androstan-17β-ol-3-one; epiandrosterone.

Sugars.

glucose; sucrose; fructose; lyxose; xylose; arabinose; galactose; maltose; mannose; rhamnose; ribose; melizitose.

Triterpenoids.

β-amyrinacetate; icterogenin; oleanolic acid; lantadene A; moronic acid; erythrodiol.

Miscellaneous.

acetaldehyde; acrolein; menadione; menadione $NaHSO_3$; paraquat; diquat; trifluralin; sorbitol; glycoaldehyde; hemin; urea; ubiquinone 50; ubiquinone 30; galactosyl diglyceride.

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