ABSTRACT

Ph.D. C.M.R. Hennessy Plant Pathology

STUDIES ON VARIATION IN *PUCCINIA HELIANTHI*

Rust was collected on putative ancestors of the cultivated sunflower in Texas. Races least pathogenic on standard differentials were most common. Races 1 and 2 predominated on *Helianthus annuus* and *H. debilis*; race 2 on *H. praecox* and race 4 on *H. petiolaris*. Most host species were heterozygous for resistance. *Puccinia helianthi* showed considerable variability; local specialization seemed unimportant.

Races were not distinguishable serologically or chromatographically. No antigenic affinity was found between *P. helianthi* and sunflower differentials. There were antigenic differences and similarities between *P. helianthi* and *P. graminis* f.sp. *tritici*. Chromatograms of rust-infected sunflower leaves revealed one phenolic compound that was characteristic of necrosis and another of virulent rust infections on *H. annuus*. Infections by other fungal parasites also showed specific spots.

Wall pigment in uredospores and teleutospores was evidently determined by one or a small number of genes; cytoplasmic pigment was unstable. Aecial cups contained products of multiple fertilizations. Somatic segregation occurred in colour hybrids.

STUDIES ON VARIATION IN *PUCCINIA HELIANTHI*

by

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SHORT TITLE

VARIATION IN *PUCCINIA HELIANTHI*

HENNESSY

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I. INTRODUCTION

Studies on the inheritance of pathogenicity in sunflower rust, Puccinia helianthi Schw. (Miah, 1967; Miah and Sackston, 1970b; Hennessy, 1967), made little progress for a number of reasons. Teleutospore germination was capricious, differential host varieties were few and those available proved to be heterozygous for resistance, and the manipulation of sufficient numbers of progeny cultures for valid statistical tests was difficult.

Miah found the "Canadian" races of P. helianthi to be heterozygous for pathogenicity. In some races, on some varieties, virulence was determined by a recessive gene; in others virulence appeared to be inherited as a dominant factor(s) with virulence hypostatic to avirulence. In the case of one differential variety (Morden Cr29) there was evidence of a cytoplasmic-nuclear interaction. Hennessy (1967) corroborated the findings of heterozygosity and epistasis, and subsequently (unpublished) of cytoplasmic inheritance of virulence on Morden Cr29. Neither Miah nor Hennessy, however, were able to substantiate their observations from F_2 segregation in controlled crosses.

In-breeding has lately achieved homozygosity for

pathogenicity in "Canadian" races 1 and 2 of P. helianthi on varieties CM90RR and Morden Cr29 and for resistance to the four "Canadian" races in Morden Cr29 (Hennessy unpublished). Miah's "multiple inoculation" technique (Miah and Sackston, 1967) permits several pathogenic races to be tested simultaneously on the same plant, allowing host variability to be discounted. Teleutospore dormancy was overcome by culturing the rust on detached leaves at low temperature (Hennessy and Sackston, 1970). These developments should facilitate genetic studies but some fundamental difficulties remain. Controlled environmental conditions are essential and space is limited; the "multiple inoculation" technique is tedious and time-consuming, and it is not possible to inoculate large numbers of plants at one time. Furthermore, there are still only two reliable differentials for distinguishing race types of P. helianthi and the inheritance of resistance in these is not clearly understood (Miah, 1967; Miah and Sackston, 1970a).

Genetic research on sunflower rust was instigated to determine whether the gene-for-gene hypothesis (Flor, 1956) was applicable to the sunflower-rust host-parasite system. This theory has been criticised by Laubscher (1963) and Laubscher, Combrink and Lombard (1966) and additional proof is needed. The difficulty lies not in the principle of host-parasite inter-dependence, of which there can be little

doubt, but in the mechanism by which it operates. Laubscher and his co-workers favoured a functional system incorporating regulator, operator and inductor elements in preference to the simple "complementarity of fit" (Flangas and Dickson, 1961b) implicit in the gene-for-gene concept. Convincing evidence for an active product-for-active product hypothesis was presented in a study of wheat stem rust on isogenic lines of wheat possessing single gene differences for rust resistance by Kao and Knott (1969). At present, available sunflower varieties do not permit a comparable study on sunflower rust.

This thesis reports the results of a series of experiments in which attempts were made to study variation in P. helianthi apart from the influence of its host. Additional markers, it was hoped, would aid understanding of the mechanisms of variation and gene function which are fundamental to parasite adaptation and pathogenicity.

Puccinia helianthi has not yet been cultured in vitro. Serological differences were sought between physiological races in the belief that antigens represent the phenotypic expression of gene activity (Schultz, 1959) and a chromatographic study was undertaken because phenolic compounds, particularly flavonoids (Moore, Harborne and Williams, 1970), have identified allelic differences in higher plants. A

study in host specialization on wild sunflower species in Texas was included for the insight it could provide into the evolution of pathogenicity and the potential for diversification in P. helianthi. A colour mutant afforded a readily detectable marker for an apparently simple Mendelian trait and an attempt was made to resolve the problem of the unit of fertilization in the rusts.

The four topics: Host Specialization, Serology, Chromatography and Inheritance of Spore Colour are presented and discussed as separate sections.

II. SPECIALIZATION IN *PUCCINIA HELIANTHI* ON WILD SUNFLOWERS IN TEXAS

Literature Review

Puccinia helianthi is an autoecious, macrocyclic rust. Described in North Carolina and Pennsylvania by Schweinitz in 1822 (Arthur and Bisby, 1918) it is now considered worldwide in distribution (Anon., 1969; Putt and Sackston, 1957; Sackston, 1962). It has been reported on 35 species in the genus *Helianthus*, on *Heliopsis helianthoides* (L.) Sweet and *Viguiera* spp. in North America (Arthur and Cummins, 1962), on *Xanthium strumarium* L. in Russia (Eremeyeva, 1923; Eremeyeva and Karakulin, 1929) and may occur on *Fluorensia* spp. and *Wedelia* spp. in South America (Sackston, 1956; 1959). Baxter (1959) suggested that *P. xanthifoliae* E. and E. on *Iva xanthifolia* Nutt. should be treated as a variety of *P. helianthi*.

Studies on host specialization in *P. helianthi* date from the beginning of the century (Arthur, 1903; 1904; 1905; 1906; Kellerman, 1903; 1905). Woronin (Kellerman, 1905) identified rust from *Helianthus tuberosus* L. as *P. helianthorum* Schw., believing it distinct from *P. helianthi* on *H. annuus* L. but this distinction was not accepted and the two names are now considered synonymous (Arthur and Cummins, 1962).

Bailey (1923) distinguished three "biologic forms" on the basis of their virulence on eight species of sunflower. Brown (1936) considered that uredial cultures from H. annuus, H. tuberosus, H. subtuberosus Britton and H. petiolaris Nutt. constituted four separate strains on the basis of reactions on H. annuus, H. tuberosus, H. subtuberosus, H. petiolaris, H. subrhomboideus Rydb. and H. maximiliani Schrader. Brown further determined that cultures from the annual species of sunflower were intersterile with those from the perennials, while within the annual and perennial groups cultures crossed readily. This suggested a possible varietal sub-division of P. helianthi analagous to that in P. graminis Pers. in which similar intersterile groups are recognized (Brown, 1936).

Later workers have also identified physiological races in P. helianthi. Sackston (1962) distinguished four races from reactions on two differential varieties of cultivated sunflower and reported their occurrence in several countries. Hoes and Putt (1962) described nine races on 14 inbred lines of sunflower. The propensity of the rust fungi to differentiate physiologically distinct forms has been recognized since Stakman and Piemeisel (1917) described biological forms of P. graminis. The number of distinguishable races is a function of the number of varieties on which they are tested (Person, 1959; Sackston, 1962). Using

Sackston's (1962) differentials plus an additional two lines, the number of recognized races was increased to ten (Miah, Hennessy and Sackston, 1967). Hoes and Putt (1962) used different lines and their races are not comparable to those of Sackston and Miah et al.

The concept of coevolution in the rusts and their hosts originated in the teachings of de Bary, of Fischer and of Gäumann (Leppik, 1965). It was recently reviewed by Leppik (1970) and Savile (1971). Pathogen specificity as an indicator of host evolution was discussed by Vavilov (1951) and Savile (1954).

The cultivated sunflower is believed to have originated from Indian domestication of H. annuus in eastern North America (Heiser, 1951c; 1955). The ancestry of H. annuus is uncertain. It is one of the most variable species in the genus although probably monophyletic (Heiser et al., 1969). Its closest relative appears to be H. argophyllus Torrey and Gray (Heiser, 1951a; 1951b) which is indigenous to the gulf coast of Texas. Helianthus annuus is, however, considered to have been introduced to eastern Texas by man and its form there differs markedly from elsewhere in its range (Heiser, 1951b). Variation in eastern Texas is in the direction of H. debilis Nutt. The phylogenetic connection between H. annuus and H. debilis, if indeed there is one,

has not been stated but the two are known to hybridize freely (Heiser, 1951b; 1956). The evolution of H. debilis and its relationship with the other annual species mentioned in this investigation were discussed by Heiser (1956).

The ecological distribution of the species of Helianthus used in this study is given by Heiser et al. (1969). Helianthus annuus is distributed throughout the U.S. with the greatest concentration east of the Mississippi, extending into Canada and Mexico. Helianthus debilis Nutt. ssp. cucumerifolius (T. and G.) Heiser occurs in southeastern Texas; H. debilis Nutt. ssp. silvestris Heiser in northeastern Texas, grading into cucumerifolius in the southern part of its range. Helianthus petiolaris ssp. petiolaris grows mostly in the Great Plains east of the Rocky Mountains, southern Canada to northern Texas. Helianthus praecox Engelm. and Gray ssp. hirtus Heiser is known only from the area of Carrizo Springs, Dimmit Co., Texas and H. praecox Engelm. and Gray ssp. praecox only from Galveston Island, Texas and the adjacent mainland. Helianthus praecox Engelm. and Gray ssp. runyonii Heiser inhabits the coastal "prairies" of southern Texas, intergrading with H. debilis ssp. cucumerifolius. Helianthus argophyllus occurs in eastern Texas and is adventive in Florida and perhaps elsewhere.

The above are annual species. Of the perennials, H.

tuberosus occurs from Nova Scotia to Manitoba, south to northern Florida and Texas. Helianthus grosseserratus Martens extends from New England to South Dakota and south to Texas. Helianthus giganteus L. is common from Nova Scotia to Minnesota, south to northern Georgia and west to Illinois.

Texas appears to be at least a secondary centre of origin for the cultivated sunflower and would be expected, therefore, to be a centre of diversity for P. helianthi also. Variation in this pathogen on the putative ancestral species in this region is thus of interest to a study of parasite evolution, to an understanding of the potential for pathogenicity in P. helianthi, and to extending resistance in the cultivated sunflower. With this in mind, rust samples were collected from wild species of Helianthus in Texas in July and August, 1969 and tested for pathogenicity under greenhouse and controlled environmental conditions at Macdonald College. The summer of 1969 was unfortunately dry in the area surveyed, and rust on sunflowers proved to be relatively scarce.

Materials and Methods

Uredospores were collected from H. annuus, H. debilis ssp. cucumerifolius, H. debilis ssp. silvestris, H. petiolaris ssp. petiolaris, H. praecox ssp. praecox, H. praecox

ssp. hirtus, H. praecox ssp. runyonii in Texas (Figure 1); from H. annuus in Kansas, Oklahoma, Iowa and Nebraska, and from H. petiolaris ssp. petiolaris in Kansas and Oklahoma. Uredospores were also collected from one plant each of the perennial species H. tuberosus in Nebraska, H. grosseserratus in Iowa and H. giganteus in Indiana.

Collections were made from roadside populations which rarely extended more than a hundred yards from the highway. Where rusted sunflowers were abundant samples were taken at 10 to 20 mile intervals. More often infections were sporadic and collections were made wherever rust was found. Seldom were more than one or two infected plants obtained from a particular population. The search for rust in states other than Texas was made during the return to Canada and was less thorough.

Rust-infected leaves were dried in a plant press for 12 to 24 h, placed in paper or cellophane envelopes and kept in a refrigerator until transferred to Montreal.

Seed was collected from infected plants when available to provide hosts for cross-infection studies. The perennial species had not set seed when these collections were made but some seed of H. grosseserratus was obtained from Dr. C.B. Heiser Jr., University of Indiana. No rust was found on H. argophyllus but seed obtained from Dr. M.L. Kinman, College

Figure 1

Figure 1. Map showing collecting sites and ecological Zones of Texas (adapted from Thomas, 1969). A. Rolling Plains and High Plains. B. Pineywoods, Blackland Prairies and Cross Timbers and Prairies. C. Post Oak Savannah, Blackland Prairies and Gulf Prairies and Marshes. D. South Texas Plains and Gulf Prairies and Marshes. E. Edwards Plateau and Trans-Pecos, Mountains and Basins. 1. College Station. 2. Galveston. 3. Carrizo Springs.

Symbols for species:

- ● *H. annuus*
- ■ *H. debilis cucumerifolius*
- □ *H. debilis silvestris*
- ○ *H. petiolaris*
- △ *H. praecox praecox*
- ⊕ *H. praecox hirtus*
- ▲ *H. praecox runyonii*

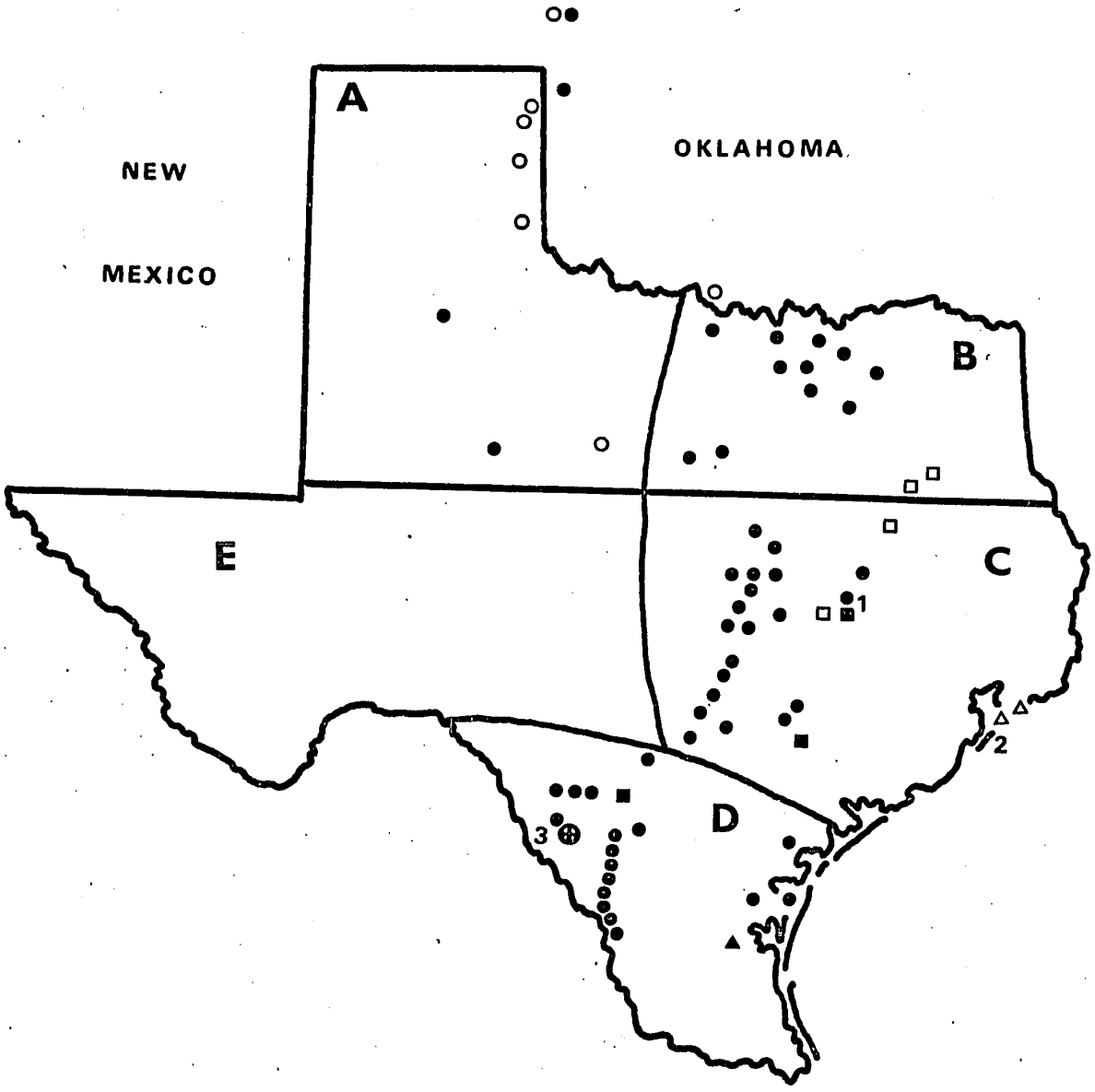


Figure 1

Station, Texas, was used in determining the pathogenicity of some of the cultures on this species.

Rust collections were increased on the "universal suscept" in the greenhouse at Macdonald College and compared with the four "Canadian" races (Sackston, 1962) for pathogenicity on the differential varieties CM9ORR and Morden Cr29 which will be referred to as the "Canadian" differentials. CM9ORR carries resistance factor R_1 and is resistant to races 1 and 2 and susceptible to races 3 and 4; Morden Cr29 carries resistance factor R_2, is resistant to races 1 and 3 and susceptible to 2 and 4 (Sackston, 1962; Putt and Sackston, 1963).

Some cultures were also tested on Miah's supplemental lines M62-2672-2-r1 and M62-2685-14-I (Miah, 1967; Miah and Sackston, 1970a). Cross-infection studies were made on plants of the wild species grown in the greenhouse and controlled environment growth chamber. Cultures were compared by multiple inoculation of several cultures, together with the "Canadian" races, simultaneously on the same plant (Miah and Sackston, 1967), with controls inoculated on the "Canadian" differentials. Reactions were assessed 11 to 12 days after inoculation on a numerical scale 0 to 4 (Sackston, 1962; Hennessy, 1967). "Canadian" differentials used for infectivity tests were grown at 22 ± 2°C day and 20 ± 2°C

night temperature, the wild species at 25 ± 2°C day and 22 ± 2°C night temperature with a daylength of 16 h and light intensity in both cases of 13200 ± 2200 lux (1200 ± 200 ft-c) from VHO cool white fluorescent tubes supplemented with incandescent lamps.

Collection sites are identified in Appendix Tables I and II by the name of the nearest town or conspicuous geographic feature and a number when more than one site is listed for the same area. Individual plants at the same site are indicated by a letter, e.g. (a), following the site number.

Experimental Results

The distribution of rust races on annual species of Helianthus in Texas and Oklahoma is summarized in Table 1 condensed from Appendix Table II and Figures 2, 3 and 4 drawn from Appendix Table II. Races 1 and 2 predominate when all sites and all species are considered. On individual species, races 1 and 2 predominate on H. annuus and H. debilis, race 2 on H. praecox and race 4 on H. petiolaris. There is no significant difference between the distribution pattern on H. annuus in Texas and that on H. annuus over the range of this survey.

Miah's supplemental lines indicated differences in

Table 1. Distribution of race types of *Puccinia helianthi* on annual species of *Helianthus* in Texas and Oklahoma.

Ecological Zone[1]	Host Species	Race Type 1	2	3	4
A	*H. annuus*	4[2]			
	H. petiolaris			1	5
B	*H. annuus*	1	2	4	4
	H. debilis silvestris		2		
	H. petiolaris				1
C	*H. annuus*	8	9		2
	H. debilis cucumerifolius	2			1
	H. debilis silvestris		2		
	H. praecox praecox		2		
D	*H. annuus*	10	5	1	1
	H. debilis cucumerifolius	1			
	H. praecox hirtus		1		
	H. praecox runyonii			1	
Total number of sites		26	23	7	14

[1]See Figure 1 for description of zones.

[2]Number of collection sites sampled.

Figure 2. Distribution of the "Canadian" races of *Puccinia helianthi* on four annual species of *Helianthus* in Texas.

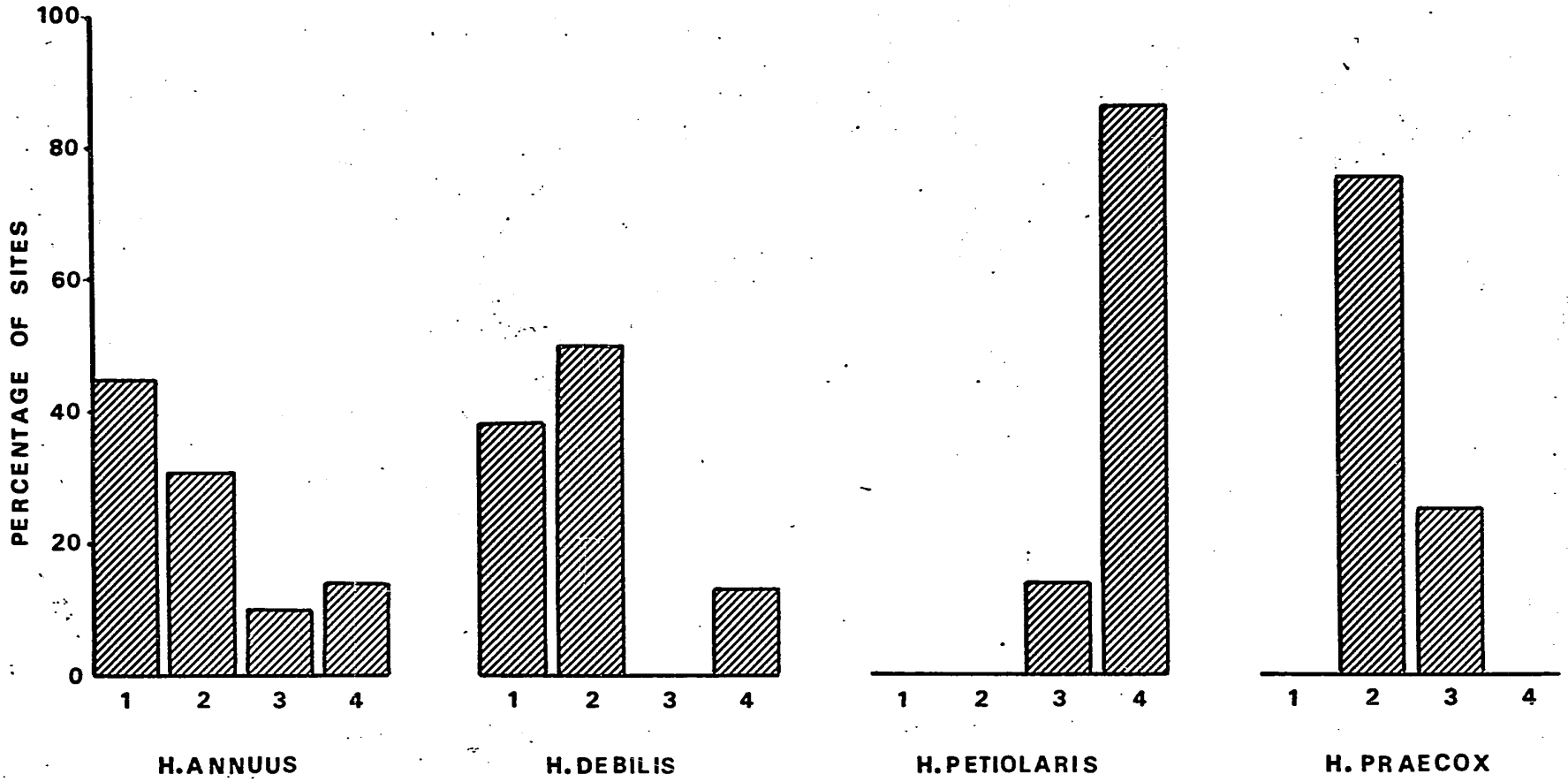
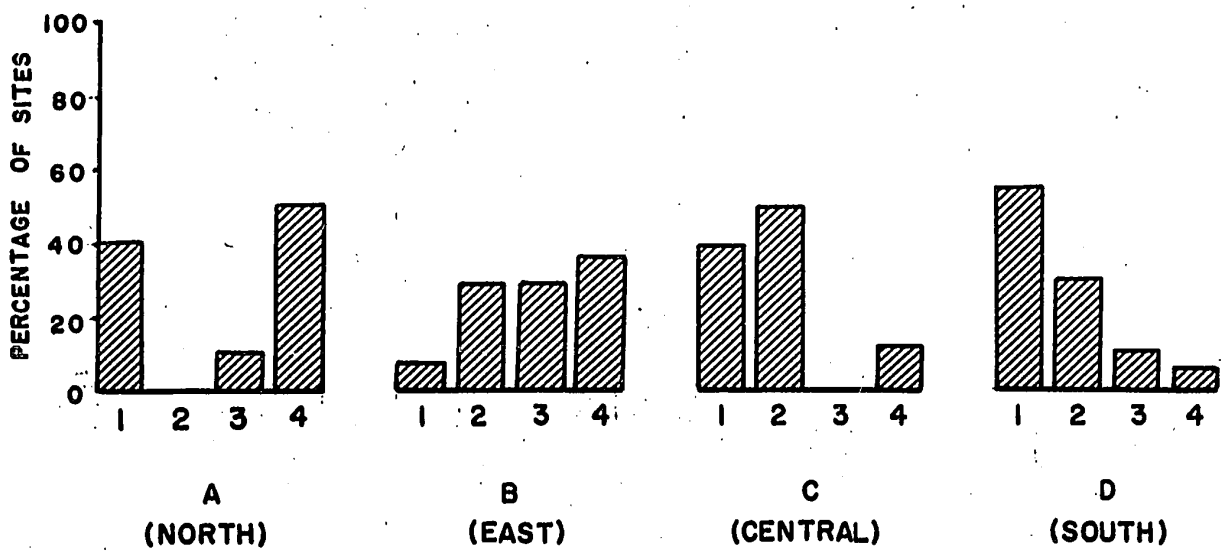

PERCENTAGE OF SITES
100
80
60
40
20
0
1 2 3 4
H.ANNUUS
1 2 3 4
H.DEBILIS
1 2 3 4
H.PETIOLARIS
1 2 3 4
H.PRAECOX
RUST RACES AND HOST SPECIES

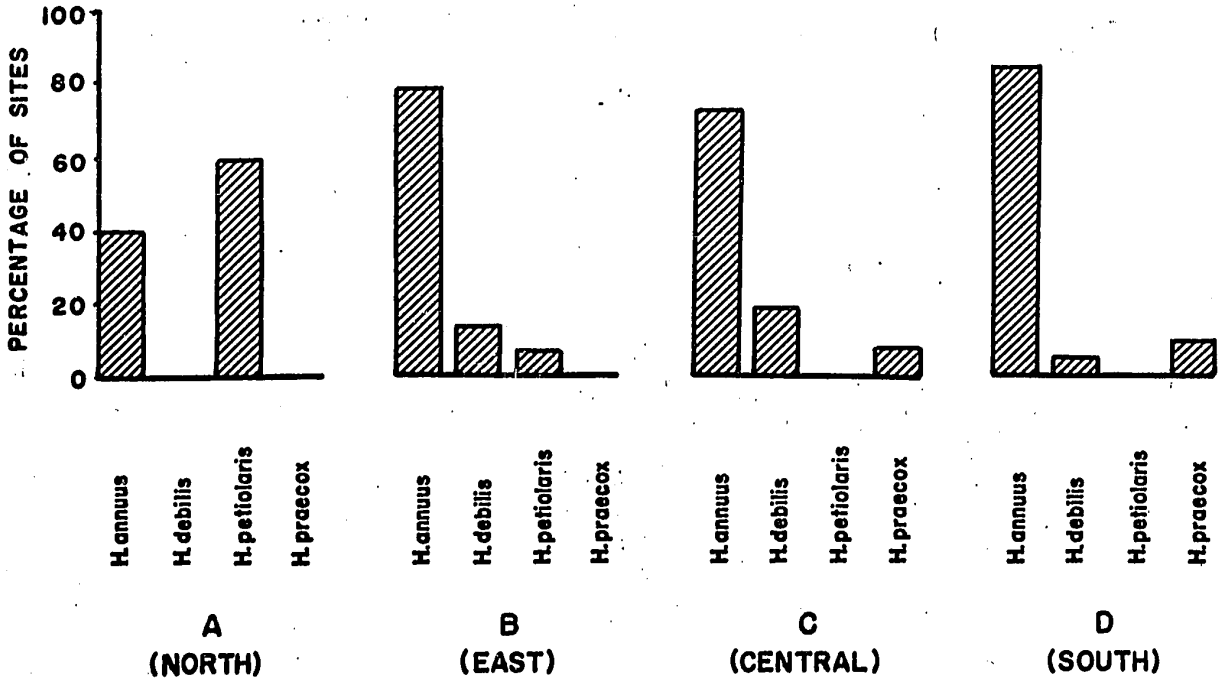
Figure 2

Figure 3. (i) Distribution of the "Canadian" races of *Puccinia helianthi* in the different ecological zones of Texas.

(ii) Distribution of four annual species of *Helianthus* in the different ecological zones of Texas.



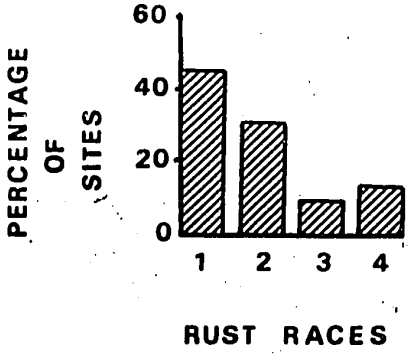
(i) RUST RACES AND ECOLOGICAL ZONES

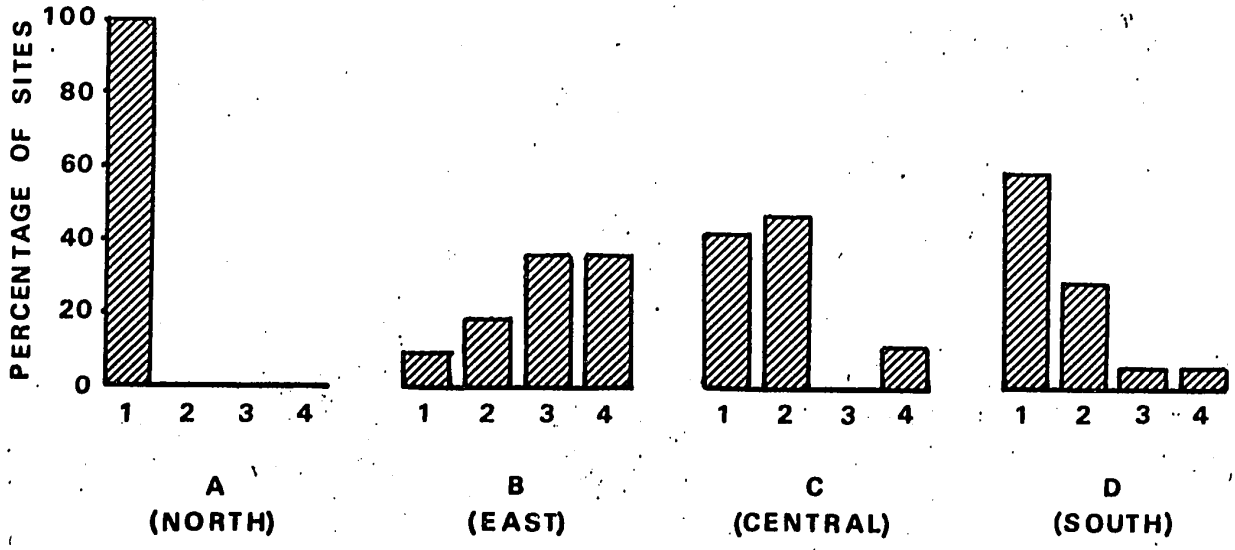


(ii) HOST SPECIES AND ECOLOGICAL ZONES

Figure 3

Figure 4. Distribution of the "Canadian" races of *Puccinia helianthi* on *Helianthus annuus* in Texas.





RUST RACES AND ECOLOGICAL ZONES

Figure 4

pathogenicity not detectable on the "Canadian" differential lines (Appendix Table I) but the data are incomplete. Seed stocks of these lines proved to be variable and tests on them were not continued. Discussion of distribution patterns is, therefore, confined to the "Canadian" race types identified on the standard "Canadian" differentials.

The map of Texas (Figure 1) is divided into sectors coinciding with Thomas' (1969) ecological zones. The northern sector (A) north of Waco, west of Wichita represents Rolling Plains, with the single site Cone situated in High Plains; sector (B) north of Waco, east of Wichita comprises Pineywoods, Blackland Prairies and Cross Timbers and Prairies; sector (C) south of Waco, north of San Antonio is largely Post Oak Savannah and Blackland Prairies, with the two Gilchrist sites (near Galveston) in Gulf Prairies and Marshes; sector (D) south of San Antonio comprises the South Texas Plains except for sites Driscoll, Padre Island and Refugio in the Gulf Prairies and Marshes zone. Sector (E) essentially comprises the low rainfall areas of Edwards Plateau and the Trans-Pecos, Mountains and Basins which were not considered profitable to visit.

Race distribution in the northern, panhandle, area of Texas differs from that over the rest of the state (Figures 3 and 4) due, perhaps, to the preponderance of H. petiolaris.

Differences between other sectors where collections were from H. annuus, H. debilis and H. praecox are not appreciable but samples were too few for statistical analysis.

Reactions of the wild species to Canadian isolates of rust are summarized in Table 2 (condensed from Appendix Table III). Most wild seed collections proved to be heterozygous for rust resistance. The annuals: H. annuus from Carrizo Springs, H. petiolaris, H. praecox ssp. hirtus and H. praecox ssp. runyonii, and the perennial H. grosseserratus, appeared to be resistant to the four "Canadian" races; H. debilis ssp. silvestris from Jewett and Oakwood (Appendix Table III) appeared to be susceptible. The number of plants tested in all these cases was too small to assume homozygosity.

Reactions of the wild species to collections of rust from each of the other wild species are summarized in Table 3 and Appendix Table III. Cultures from H. debilis were avirulent on H. annuus except for one population of this species from San Antonio. Cultures from H. petiolaris induced resistant and one or two intermediate reactions on H. praecox ssp. praecox, and were considered avirulent on this species. Cultures from H. praecox ssp. praecox induced resistant or intermediate reactions on H. annuus. Helianthus argophyllus, while evidently heterozygous, appeared equally

Table 2. Reactions of the "Canadian" differential varieties and wild species of *Helianthus* to Canadian isolates of *Puccinia helianthi*.[1]

Host Lines	Race Types 1	2	3	4
Differentials				
S37-388	+	+	+	+
CM90RR	-	-	+	+
Morden Cr29	-	+	-	+
Wild species				
H. annuus	±	±	±	±
H. argophyllus	±	±	±	±
H. debilis cucumerifolius	±	±	±	±
H. debilis silvestris	±	±	±	±
H. petiolaris	-	-	-	-
H. praecox praecox	±	-	±	±
H. praecox hirtus	-	-	-	-
H. praecox runyonii	-	-	-	-
H. grosseserratus	-	-	-	-

[1] + indicates susceptibility

- indicates resistance

± indicates stocks were heterozygous, some plants giving susceptible, others resistant reactions.

Table 3. Cross-infectivity of *Puccinia helianthi* on annual species of *Helianthus*.[1]

Rust Cultures Isolated from	Host Species Inoculated							
	H. annuus	*H. argophyllus*	*H. debilis cucumerifolius*	*H. debilis silvestris*	*H. praecox praecox*	*H. praecox hirtus*	*H. praecox runyonii*	*H. petiolaris*
H. annuus	+	+	+	+	+	-	-	-
H. debilis cucumerifolius	-	+	+	+	+	-	0	-
H. debilis silvestris	-	+	+	+	+	-	0	0
H. praecox praecox	-	0	0	+	+	-	0	-
H. praecox hirtus	+	0	0	+	+	+	0	-
H. praecox runyonii	-	+	-	+	0	0	0	0
H. petiolaris	+	+	-	+	-	-	+	+

[1] + indicates susceptible reactions

\- indicates resistant reactions

0 indicates no test data available

susceptible to all rust cultures tested on it. The perennial H. grosseserratus was immune to all cultures including that collected from H. grosseserratus in Iowa. Tests with rust from the perennial species were few but suggested a narrower host range than that for the annual species.

Rust was found on cultivated sunflowers in Texas only at McGregor, about 10 miles from Moody, on two plants of Peredovic, and on a hybrid line at College Station Nursery. The isolate from McGregor proved to be race type 2, similar to that on H. annuus at Moody. The isolate from College Station Nursery was race type 3, whereas the two collections from H. debilis at College Station, nearby, were race types 1 and 4. The isolate from H. annuus at College Station Nursery was pathogenic on wild H. annuus, H. praecox ssp. praecox, and H. argophyllus. It was avirulent on H. grosseserratus. The McGregor isolate was not tested on the wild species. As with other cross inoculations which should have been made, lack of viable seed of the wild species made this impossible.

Discussion

Infection in the field at the time of this survey was low so that collection sites were fewer than had been hoped for and variation in P. helianthi within populations of

sunflowers could not be assessed. Most of the host species proved to be heterozygous, limiting host-specificity estimates to rather broad generalizations and preventing any assessment of regional specialization within H. annuus. The number of differentials on which race classification is based was inadequate to distinguish differences in pathogenicity in many cases. Table 2 shows H. praecox ssp. praecox to be resistant to "Canadian" race 2, yet the culture collected from it was identified on the "Canadian" and supplemental differentials as race 2 (Appendix Table 1). Infection studies of this type are subject to variation in the fungus, due to contamination or mutation in culture, so that when several uredial generations intervene before testing the isolate may no longer be the same as that occurring in the field. Such changes may explain discrepancies between Appendix Tables I and III, e.g. Claflin (b) (Kansas), Gilchrist 1 (Texas) and Red Oak (~~Nebraska~~ Iowa). Other aberrant reactions, when the same culture showed different characteristics in different tests, e.g. Gilchrist 2 and Refugio (Texas) are less easily explained. Results were also confused by "intermediate" reactions which could not readily be ascribed to either the "resistant" or "susceptible" class. Ambiguous reactions of this sort were attributed to environmental factors rather than inherent properties of either host or

pathogen.

This study is based on a single collecting trip. Fluctuations in race distributions over a period of years are well documented in P. graminis Pers.f.sp. tritici (Erikss. and Henn.) Guyot (Green, 1971) and Melampsora lini (Pers.) LéV. (Kerr, 1959) and the significance of a particular distribution pattern in one year must be limited.

The number of plants tested in these trials was unsatisfactorily small due to poor seed germination. Indeed, only two plants of H. petiolaris were obtained for inoculation. It was not possible to determine differences between populations of H. annuus at the various collection sites, or the extent to which rust from H. praecox ssp. runyonii may differ from that collected from H. praecox ssp. praecox. Data for rust from the perennial species were also meagre but suggested a narrower host range than was found for cultures from the annual species. The seed of H. grosseserratus used in these tests was obtained from Dr. C.B. Heiser, Jr. and the resistance shown in Table 2 does not represent the population from which rust was collected on this species.

It is tempting to draw phylogenetic implications from these data, suggesting that H. debilis and H. praecox ssp. praecox are more closely related than the others; that H. annuus has some affinity with them and is perhaps closer to

H. debilis, while H. petiolaris and H. praecox ssp. hirtus are at some distance removed (cf. Heiser, 1956). This would be presumptious. It seems more likely that adaptation in the pathogen reflects the distribution of these species. Helianthus annuus is more or less sympatric with all of them. Helianthus debilis ssp. silvestris may be sympatric with H. praecox ssp. praecox, and H. debilis ssp. cucumerifolius is sympatric with H. praecox ssp. runyonii at the extremity of its range. Helianthus praecox ssp. hirtus occupies a more or less isolated position in the south and H. petiolaris is separated in the north from all except the ubiquitous H. annuus. Helianthus argophyllus has a rather restricted distribution but is sympatric with H. annuus and possibly also with H. debilis ssp. cucumerifolius in some areas. The degree of isolation in this context may be debatable, however, for there are no geographic barriers to the passage of rust spores over this entire region.

Heiser (1956) states that a number of organisms in addition to H. praecox ssp. hirtus, including a sub-species of the pocket gopher, are unique to the area of Carrizo Springs. It would be interesting to know the extent to which an airborne pathogen like P. helianthi may differ in this area from elsewhere, and also whether resistance in H. annuus is different here from elsewhere in its range. Unfortunately,

the data do not answer these questions.

The influence of host genotype on rust race occurrence is copiously documented. In northern Texas and Oklahoma race types 3 and 4 were found on H. petiolaris and race 1 on H. annuus, sometimes within a few feet of each other. Whether this represents evolutionary specialization in the fungus or a transitory adaptation is not known.

Wind-borne spores from Argentina initiated an outbreak of sunflower rust in Uruguay (Sackston, 1957). Epiphytotics of P. helianthi in Manitoba were of local origin, however, starting from overwintering teleutospores on sunflower stalks (Sackston, 1952). If sunflower rust uredospores move northwards over Texas and adjacent areas in the manner of wheat stem rust, virulence genes may be selected afresh each year and the pathogen adapt to the host species it encounters. It would remain relatively unspecialized, with a high degree of adaptive variability. In areas where local overwintering occurs, specialization may be expected to develop.

Factors such as temperature influence the survival of rust biotypes (Katsuya and Green, 1967; Kerr, 1959). Races of sunflower rust differed in temperature ranges for germination, appressorium formation, and infection, and also in longevity at various temperatures (Sood, 1968; Sood and

Sackston, 1971). Additional surveys and much more information on host specificity are required, however, to separate the effects of climatic factors and host relationships in sunflower rust.

The most abundant races found in this study were 1 and 2 as identified on the "Canadian" differentials. According to Putt and Sackston (1963) race 1 carries no genes for virulence; race 2 carries a single factor matching resistance gene R_2 in the host; race 3 carries a matching factor for gene R_1; and race 4 carries both factors. Although this is an oversimplification (Miah and Sackston, 1970a; 1970b), it appears to offer some support for van der Plank's (1968; 1969) contention that the least pathogenically specialized races are the most successful. It does not explain the relative scarcity of race 3 in the survey. Van der Plank's theory was not supported by recent observations on wheat stem rust (Green, 1971).

Resistance in the "Canadian" differentials is derived from H. annuus from Renner, Texas (Putt and Sackston, 1963) and that in the supplementary lines from H. annuus in Chicago (Miah and Sackston, 1970a). These represent rather limited gene pools and clearly the resistance genes in the differentials are not the same as those in the wild hosts of this study. Resistance in wild H. annuus and the cultivated

differentials is dominant over susceptibility; in H. argophyllus, H. debilis and H. petiolaris resistance is recessive (Putt and Sackston, 1957; Kinman, personal communication). While race 1 was the most prevalent on H. annuus, H. annuus was far from being devoid of resistance. It may be supposed that "vertical" or oligogenic resistance (van der Plank, 1968) is less important in natural populations than "horizontal" resistance, but even this may be an unjustified assumption at this stage.

From a practical point of view this study has shown that rust resistance is widespread in populations of wild sunflowers. Resistance from H. annuus, H. argophyllus, H. debilis ssp. cucumerifolius, and H. petiolaris has been incorporated in breeding programs in North America, Argentina, and the U.S.S.R. (Anon., 1966; Putt and Sackston, 1957; Sackston and Miah, 1963). There appears, however, to be an abundant potential for plant breeders yet to be exploited; H. praecox ssp. hirtus, in particular, may have useful genes to contribute. It is also evident that P. helianthi has a considerable adaptive capacity and resistance from a single source is unlikely to give lasting protection.

III. SEROLOGY

Literature Review

Serology has received rather less attention from mycologists than from virologists and bacteriologists and its application to fungi has been largely confined to those of medical importance. Nevertheless, investigations in fungal serology were being conducted early in this century and there have been over a thousand publications dealing with the serology of fungi and mycotic infections (Seeliger, 1960; 1968). Serology in phytopathology was reviewed by Badami (1960).

Serology was applied to the problem of phylogeny in the rust fungi at an early date and antigenic properties were recognized in the cereal rusts from their allergenic potential in man, although no differences between species were detected (Arthur, 1929). Protein differences between physiological races of wheat leaf rust and between varieties of wheat were demonstrated by Fedotova in 1938 (Chester, 1946), linking susceptibility of certain wheat varieties and physiological specialization in the rust with protein metabolism in the host.

Similarities between antigens of host and parasite have been reported in flax and M. lini (Doubly, Flor and

Clagett, 1960), cotton and Xanthomonas malvacearum (E.F.Sm.) Dows. (Schnathorst and DeVay, 1963) and in watermelons and Fusarium semitectum Berk. and Rav. (Abd-el-Rehim et al., 1971). In flax and flax rust, this was considered support for the gene-for-gene theory (Doubly et al., 1960). No common antigens were, however, found in wheat and P. graminis f.sp. tritici (Knott, 1963) or in cabbage and Fusarium oxysporum Schlect. f. conglutinans (Wr.) Snyd. and Hans. (Heitefuss et al., 1960).

Races of P. graminis f.sp. tritici, P. graminis Pers. f.sp. avenae Erikss. and Henn., P. recondita Rob. ex Desm. (formerly P. rubigo-vera Wint.), P. coronata Corda. and P. sorghi Schw. were characterized serologically by Bahn (1957). He observed that races of P. graminis f.sp. tritici comprised serological groups rather than serological units and that increasing genetic purity improved serological distinctions.

Flangas and Dickson (1961a) in a genetic study of P. sorghi concluded that control of antigen formation and pathogenicity was of an indeterminate nature, selfing for three generations failing to induce homozygosity. Beno (1964) substantiated earlier findings in P. sorghi but failed to show a correlation between antigenicity and pathogenicity.

Bahn (1957) and Beno (1964) employed the precipitin, complement-fixation and tannic acid haemaglutination tests,

as did Flangas and Dickson (Allen et al., 1961). Since then, immunodiffusion has largely supplanted other serological techniques. It is simpler and reputedly offers a higher degree of specificity and resolution (Crowle, 1961). Immunodiffusion has been applied to a separation of closely related species of Cronartium (Gooding and Powers, 1965) and to a study of the serological relationship between wheat and wheat stem rust (Knott, 1963).

Gel-immunodiffusion, developed by Ouchterlony (Ouchterlony, 1949; 1958), incorporates the principle of the precipitin reaction with diffusion of antibodies and antigens through a semisolid medium such as agar. Precipitin bands are formed where antigen and antibody make contact. Diffusion allows the reactants to attain concentrations compatible with specific precipitation. In most instances both antibody and antigen are impure and both reactants may be mixtures of substances of differing specificity. Any precipitin band which forms is thus likely to be the result of a range of antigen-antibody reactions and, in a given test, more than one band may be constituted by a single reactant combining either with another single reactant or varieties of it (Crowle, 1961). Interpretation of agar-gel diffusion patterns was discussed by Wilson and Pringle (1955; 1956), Korngold (1956) and Feinberg (1957; 1960). The resolving

power of immunodiffusion is exemplified by the work of van Regenmortel (1967) and others (Mathews, 1970) who detected single amino acid differences between virus derivatives.

The purpose of the present study was to determine whether pathological races of P. helianthi are distinguishable by immunodiffusion. Studies on pathogenesis in obligate parasites are hampered by the fact that they cannot be separated from their hosts. The serotype is a phenotypic expression of gene activity (Schultz, 1959) and serology in these organisms may provide data comparable to in vitro studies in saprophytes. In sunflower rust this is of particular interest since races are but poorly defined on a small number of differentials.

Materials and Methods

Cultures of the four "Canadian" races of P. helianthi and hybrids between races 1 x 2 and 1 x 3, which phenotypically resembled race 1, were grown on the appropriate differential or the "universal suscept" in the greenhouse under plastic covers to prevent cross-contamination between races. Races 15B and 56 of P. graminis f.sp. tritici were grown on wheat cultivar Kharkov, in separate greenhouses.

Antisera were prepared by injecting uredospore extracts of P. helianthi race 2 (culture S16R4b1) and P.

graminis f.sp. tritici race 56 into 2-month-old female New Zealand white rabbits. Inject antigen was prepared from ungerminated uredospores. Approximately 500 mg of uredospores were ground in a pestle and mortar for 15 min with about 2 ml of phosphate buffered saline (PBS) (0.85% NaCl-0.01 M K_2HPO_4-KH_2PO_4) (pH 7.0). The volume was made up to 10 ml with PBS and passed through a French press at 16,000 psi (1088 atm). This procedure effected disruption of 95 to 99% of spores. The resulting homogenate was centrifuged in a Sorvall RC2-b centrifuge with a No. SS-34 rotor at 3000 g for 20 min to remove cellular debris. The supernatant was mixed 50:50 with Freund's incomplete adjuvant (Difco) and injected at the rate of 0.5 ml intramuscularly into each thigh.

Two rabbits (Nos. 230 and 5) were injected with P. graminis f.sp. tritici and two (Nos. 210 and 211) with P. helianthi spore extract as above. Two additional rabbits (Nos. 212 and 213) were injected with a purified antigen of race 2, prepared by a modification of Gooding and Powers (1965) procedure. To 1.0 ml of the supernatant from the initial centrifugation, 1.2 ml chloroform were added and the mixture blended in a tissue homogeniser in an ice bath for 5 to 10 min. An additional 1.0 ml PBS was added, blending continued for a further minute, and the mixture was centrifuged

at 5000 g for 20 min. The resulting aqueous phase was mixed 50:50 with Freund's incomplete adjuvant and injected at the rate of 0.5 ml intramuscularly into each thigh.

Rabbits injected with P. helianthi were given three injections at 10- to 14-day intervals (No. 210 received an additional injection of 1/8 ml antigen intravenously in the ear 8 days before the course of intramuscular injections began) and were then bled for antiserum from the marginal vein in the ear. They were bled again a week later, given another injection one month after the second bleeding, and bled again ten days later. Those injected with P. graminis f.sp. tritici, were given three injections at 14-day intervals and bled once for antiserum two weeks after the last injection.

Test antigen was initially prepared by homogenising ungerminated uredospores as above, or uredospores germinated in calcium phosphate-potassium phosphate buffer (pH 7.0) containing 1×10^{-4} M nonyl alcohol and 0.01% Tween 20 (polyoxyethylene sorbitan mono-laurate) (Maheshwari and Sussman, 1970). Germination over water of stored P. helianthi uredospores had previously been negligible. This procedure achieved a germination of 15 to 40%. It was also found that almost 99% disruption of spores could be obtained by grinding them with carborundum in a pestle and mortar. Passage

through the French press was dispensed with in later tests with P. graminis f.sp. tritici and P. helianthi hybrid antigens and the volume of homogenate before centrifugation was reduced to about 5 ml.

Sunflower antigens were prepared by homogenising leaves from greenhouse grown 31-day-old plants of the"universal suscept" S37-388 (8.6 g) and the differentials, CM90RR (11.5 g) and Morden Cr29 (11.1 g) in a Waring blendor with 50 ml PBS and centrifuging at 3000 g for 20 min. Proteins were precipitated with acetone (Doubly et al., 1960), separated by centrifugation, and resuspended in PBS.

Puccinia helianthi antigens had UV absorption spectra with an optical density (OD) 260:280 ratio of about 1.6 (max at 257 nm, min at 237 nm). The OD at 260 nm is due to the purine and pyrimidine components of nucleic acid, and that at 280 nm to the aromatic amino acids of the protein (DeMoss and Bard, 1957). A ratio of 1.6 indicates a concentration of about 20% nucleic acid. The OD 260:280 ratio for P. graminis f.sp. tritici antigens varied from 2.5 (max at 258 nm, min at 236 nm) to 1.8 (max at 255 nm, min at 243 nm) (Figure 5). Inject antigen of P. helianthi had a concentration of 40 OD units per ml (280 nm) and that of P. graminis f.sp. tritici, 6 to 9 OD units per ml (280 nm).

Normal and immune sera to P. helianthi were tested

Figure 5. UV absorbance spectra of inject antigen.

(a) Puccinia helianthi race 2 diluted x 1/40.

(b) and (c) P. graminis f.sp. tritici race 56 diluted x 1/20.

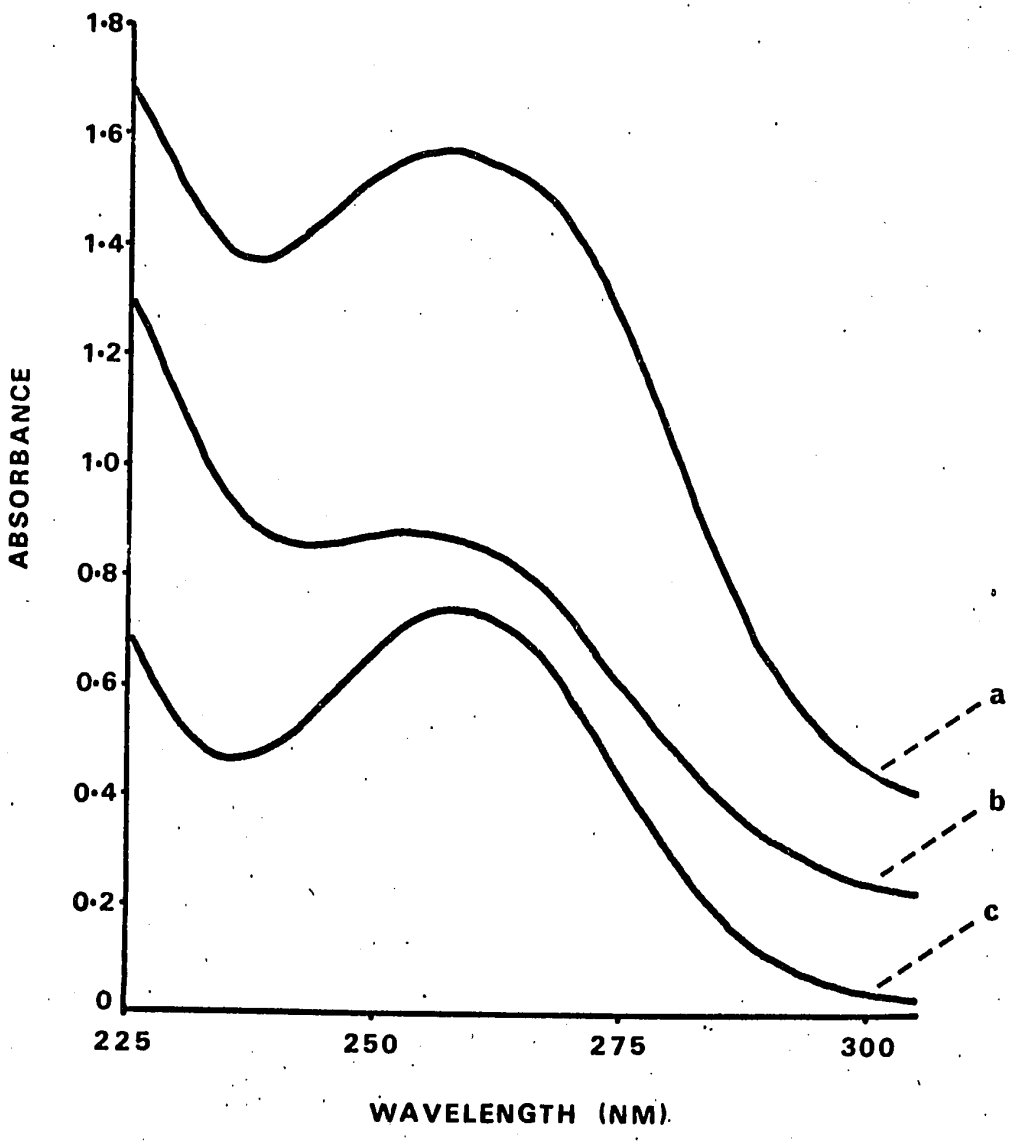

1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
ABSORBANCE
225
250
275
300
WAVELENGTH (NM)
a
b
c

Figure 5

against homologous and heterologous antigens in microprecipitin tests (van Slogteren, 1954; Ball, 1961) to determine antibody titre. Antigenic relationships were determined using Ouchterlony's agar-gel double diffusion technique, herein referred to as immunodiffusion tests (Crowle, 1961). The gel medium consisted of 1% Agar-Noble (Difco) in PBS with 0.02% NaN_3. Agar containers were glass or plastic 100 x 15 mm 4-compartment Petri plates with 2 to 2.5 ml agar in each sector. Glass plates were coated with Formvar (1% polyvinyl methylal resin in chloroform) (van Slogteren, 1954) and allowed to dry for a few minutes before pouring agar into them. Wells were charged once and the plates incubated at 10°C, in plastic bags to retain moisture. Precipitin lines were visible after 4 days, with intensity increasing up to 14 days. Intra-gel cross-absorption (van Regenmortel, 1967) was unsatisfactory. Antigens of the four "Canadian" races of P. helianthi, and 15B and 56 of P. graminis f.sp. tritici, were subsequently reacted with antisera to P. helianthi race 2 and P. graminis f.sp. tritici race 56 in equal proportions, in test tubes, on a shaker at about 30°C for 90 min and incubated overnight at 10°C. The precipitate was removed by centrifugation at 12,100 g for 20 min.

Immunoelectrophoresis (Grabar and Williams, 1953) was conducted with LKB 6800A immunoelectrophoresis equipment,

using a Gelman model 3800 power supply and electrophoresis chamber. Standard 25 x 75 mm microslides were coated with adhesive agar (0.1% Agar-Noble and 0.05% glycerine in distilled water) and then layered with 1.0% Agar-Noble in 0.25% NaCl-0.01 M K_2HPO_4-KH_2PO_4 with 0.02% NaN_3 (pH 7.2). Wells cut in the agar were filled with antigen and current applied for 2 h at 250 v/30 ma; using the same buffer (without NaN_3) as electrolyte, and a temperature of 5°C. Antiserum to race 2 was then placed in a trough between the wells and the slides incubated in a humid chamber at 10°C.

Electrophoresis was also performed on a sucrose density gradient using an ISCO model 210 density gradient apparatus (Brakke, Allington and Langille, 1968). Antigens of the four "Canadian" races of P. helianthi, and antigens of races 2 and 4 absorbed by antibody to race 2, were observed for mobility on a 5 to 20% sucrose gradient. Buffer was 0.0025 M K_2HPO_4-KH_2PO_4 with 0.0016 M KCl (pH 7.3). Temperature of the gradient was 5°C and current was applied at 3 ma; voltage fluctuated between 250 and 450 v.

The ISCO apparatus essentially comprises a central column consisting of a 10 x 550 mm Teflon tube containing the gradient and antigen sample, surrounded by a thermostatically controlled water jacket. The top and bottom of the gradient are connected to buffer chambers which in turn

are connected to positive and negative electrodes. Electrolyte in the buffer chambers is separated from the central column by dialysis membranes. Electrophoresis proceeds automatically for predetermined periods of 1 to 30 min. At completion, the gradient column is moved past a UV source (wavelength 254 nm) by a motor driven syringe pump. The UV absorption of the antigen sample in the gradient is recorded on an external strip chart recorder connected to an ISCO model UA-2 UV analyser. The pump automatically records a witness mark on the chart, providing a reference point for the position of the sample in the gradient.

Eight ml of a prefixed sucrose gradient in buffer were transferred to a buffered solution of 25% sucrose in the central column. Antigen in 0.08 ml buffer, containing sucrose at 25 mg/ml was floated on the gradient by micropipette. The column was lowered and an additional 16.8 ml buffer added. The upper chamber was filled with buffer; the lower with buffer containing the same concentration of sucrose (25%) as in the bottom of the column, preventing flow from osmotic differences on opposite sides of the dialysis membrane in the chamber.

Experimental Results

Antibody Titre of Antiserum to *Puccinia helianthi* Race 2

Antibody titre was low (Table 4). A titre of 1:4 was obtained at the first bleeding and was increased only slightly by a further injection. Antiserum from rabbit No. 213 showed a slightly higher titre than the others at the first bleeding but differences by the final bleeding were probably negligible. The extra injection administered to No. 210 at the outset appears to have had no effect. There is no indication that chloroform purification of the antigen affected titre. At the second bleeding, antigen of race 1 appeared to give a higher titre than that of race 2. This may be due to a loss of activity in race 2 antigen which had been frozen for 6 days before use. It indicates, however, a lack of specificity in race 2 antiserum.

Immunodiffusion Tests

Precipitin bands, obtained when antigens of the four "Canadian" races of P. helianthi were reacted against antiserum to race 2 (Figure 6), suggest the presence of a multiple antigen system. Lines were clearly seen but were too faint to be photographed. Races of P. helianthi were not distinguishable by their precipitin patterns with P. helianthi or P. graminis f.sp. tritici antisera (Figure 6, A-D). Cross-absorption of antigens by P. helianthi and P. graminis f.sp. tritici antisera revealed no differences between races of P.

Table 4. Antibody titre of antiserum to Puccinia helianthi race 2.

Antiserum	Antigen[1]	Bleeding Date	Titre[2] 1:1	1:2	1:4	1:8	1:16	1:64
210	Ag_2C	1	0	-	+	-	0	0
	Ag_2P	1	+	-	++	-	0	0
	Ag_2C	2	+	-	±	-	0	0
	Ag_2P	2	+++	-	++	-	0	0
	Ag_1C	2	+++	+++	+++	±	0	0
	Ag_1P	2	+++	+++	±	0	0	-
	Ag_2C	3	+	+	+	+	+	-
	Ag_2P	3	+++	++	+	0	0	-
211	Ag_2C	1	0	-	+	-	0	0
	Ag_2P	1	+	-	++	-	0	0
	Ag_2C	2	0	-	+	-	0	0
	Ag_2P	2	+++	-	++	-	0	0
	Ag_1C	2	+++	+++	+++	0	0	-
	Ag_1P	2	+++	+++	0	0	0	-
	Ag_2C	3	+++	+++	+++	+	+	-
	Ag_2P	3	+++	+++	+	+	+	-
212	Ag_2C	1	0	-	+++	-	±	0
	Ag_2P	1	0	-	++	-	0	0
	Ag_2C	2	0	-	+++	-	+	0
	Ag_2P	2	0	-	++	-	0	0
	Ag_1C	2	+++	+++	±	0	0	-
	Ag_1P	2	+	+	0	0	0	-
213	Ag_2C	1	+++	-	+++	-	+	0
	Ag_2C	2	+++	-	+++	-	±	0
	Ag_2P	2	+	-	+	-	±	0
	Ag_1C	2	+++	+++	±	±	±	0
	Ag_1P	2	±	±	±	±	±	0
	Ag_2C	3	+++	+++	++	+	+	-
	Ag_2P	3	+	+	+	+	+	-

[1] Ag_1 - Antigen of P. helianthi race 1; Ag_2 - Antigen of P. helianthi race 2; C - crude spore homogenate; P - chloroform purified preparation.

[2] + = positive reaction; 0 = negative reaction; - = no test.

Figure 6. Line drawings of immunodiffusion patterns.

A-D Reactions between races of *P. helianthi* and *P. graminis* f.sp. *tritici*. Central wells antiserum peripheral wells antigen.

E Antigens from *P. helianthi* and *P. graminis* f.sp. *tritici*, absorbed by antiserum to *P. helianthi* race 2 and *P. graminis* f.sp. *tritici* race 56, reacted with antiserum to *P. helianthi* race 2.

1-4 Denote corresponding races of *P. helianthi*; 15 and 56 denote races 15B and 56 of *P. graminis* f.sp. *tritici*, respectively; H4 represents a hybrid between races 1 x 3 and H7 a hybrid between races 1 x 2 of *P. helianthi*.

Dotted lines indicate less distinct precipitin reactions than solid lines.

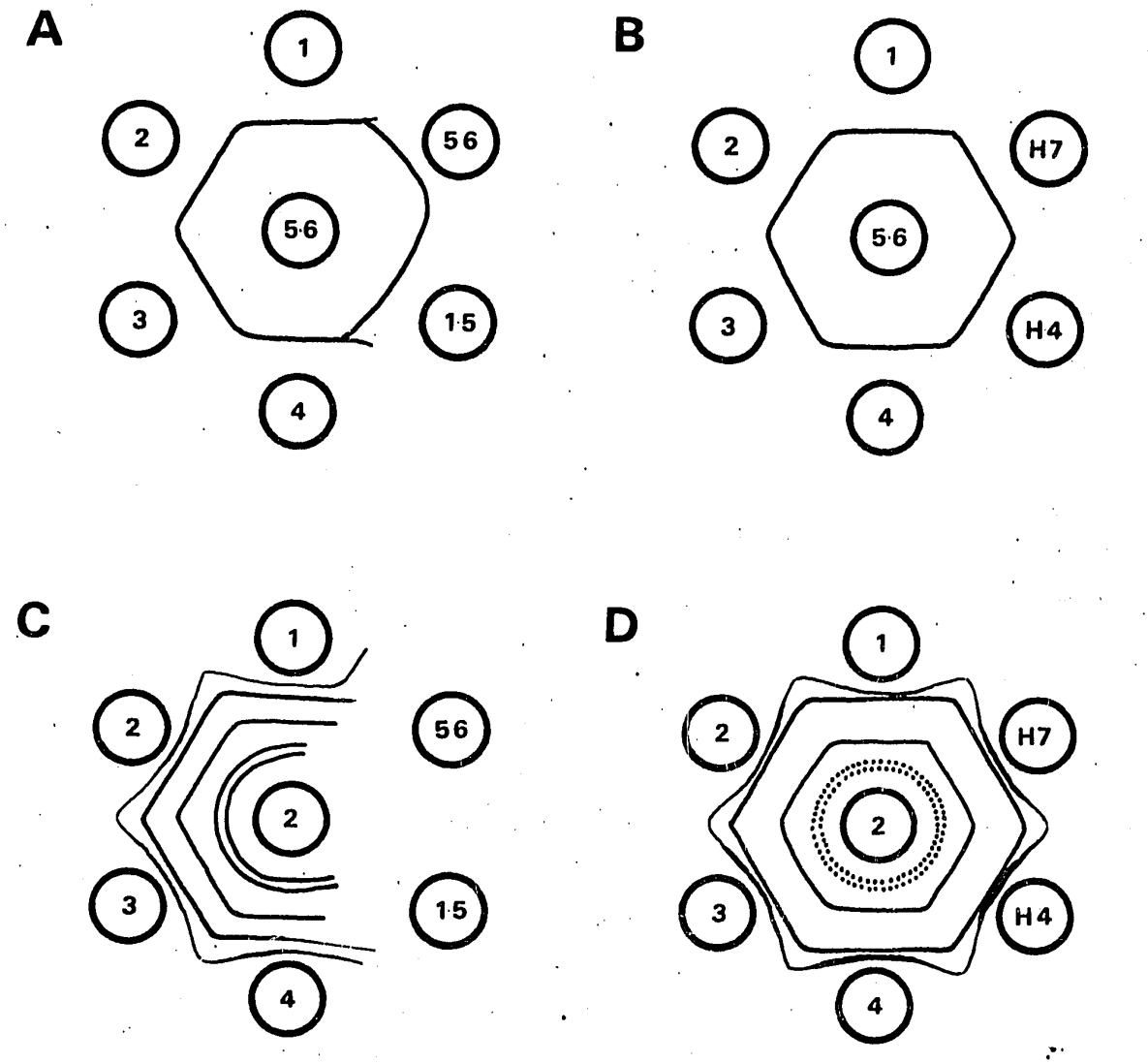

A
1
2
5·6
5·6
3
1·5
4
B
1
2
H7
5·6
3
H4
4
C
1
2
5·6
2
3
1·5
4
D
1
2
H7
2
3
H4
4
E
1
2
3
4
1·5
5·6
ANTIGENS ABSORBED X2
2
2
2
2
2
2
ANTISERUM 2
1
2
3
4
1·5
5·6
ANTIGENS ABSORBED X56

Figure 6

helianthi or between races 15B and 56 of P. graminis f.sp. tritici. Precipitin lines, formed when antigens absorbed by P. graminis f.sp. tritici race 56 antiserum were reacted against race 2 antiserum (Figure 6, E) were evidence of a difference between the species.

Antigens from P. graminis f.sp. tritici failed to react with P. helianthi antiserum (Figure 6, C). No difference was detectable between races 15B and 56 of P. graminis f.sp. tritici using antiserum against race 56 (Figure 6, A). Spurs between the antigens of P. helianthi and P. graminis f.sp. tritici (Figure 6, A) indicate that the antigens of these species are similar but not identical (Crowle, 1961).

There was no precipitin reaction between race 2 antiserum and antigens from the differential sunflower varieties.

The difference in pattern when P. helianthi antigens were reacted against P. helianthi antiserum (4-5 lines) and P. graminis f.sp. tritici antiserum (1 line) (Figure 6, A,B and C,D) may be the result of an antigenic difference between them, indicating at least one common antigen, or it may be due to low titre in the P. graminis f.sp. tritici antiserum. It is possible that the spurs in Figure 6, A are also due to a difference in antigen concentrations. The number of lines formed between P. helianthi antigens and P. helianthi antiserum was variable with different samples of antigen and

even between plates with the same antigen. The significance of the difference in number of lines in Figure 6 is therefore not clear.

Chloroform purification of antigen resulted in a reduction in the number of precipitin lines and simplification of patterns in some tests but results were inconsistent; differences between races could not be established, and the reactions have not been illustrated.

Electrophoresis

Antigen concentration was too low for precipitin bands to be seen in gel immunoelectrophoresis with LKB equipment. Some mobility of P. helianthi antigen was detected on a sucrose density gradient but the antigens of the four "Canadian" races showed no appreciable differences. An attempt to visualize the removal of antigen by running cross-absorbed antigen on the gradient was not satisfactory. Sufficient residual protein, possibly antibody, remained to be recorded and masked the effects of absorption (Figure 7). The removal of antigen by cross-absorption was verified by immunodiffusion. The irregular outlines to the patterns in Figure 7 (ii) indicate that the proteins are heterogeneous.

Figure 7. Scanning patterns of Puccinia helianthi antigen and antibody proteins showing absorbance at 254 nm after electrophoresis on a 5 to 20% sucrose density gradient.

A - Antigen of race 2.

B - Antibody to race 2.

C - Homologous antigen (race 2) absorbed by race 2 antiserum.

D - Heterologous antigen (race 4) absorbed by race 2 antiserum.

(i) Absorbance patterns at start of electrophoresis.

(ii) Absorbance patterns after electrophoresis for 1 h in the case of A and ½ h for B, C and D. Dotted arrow indicates direction of mobility.

x - Indicates witness mark.

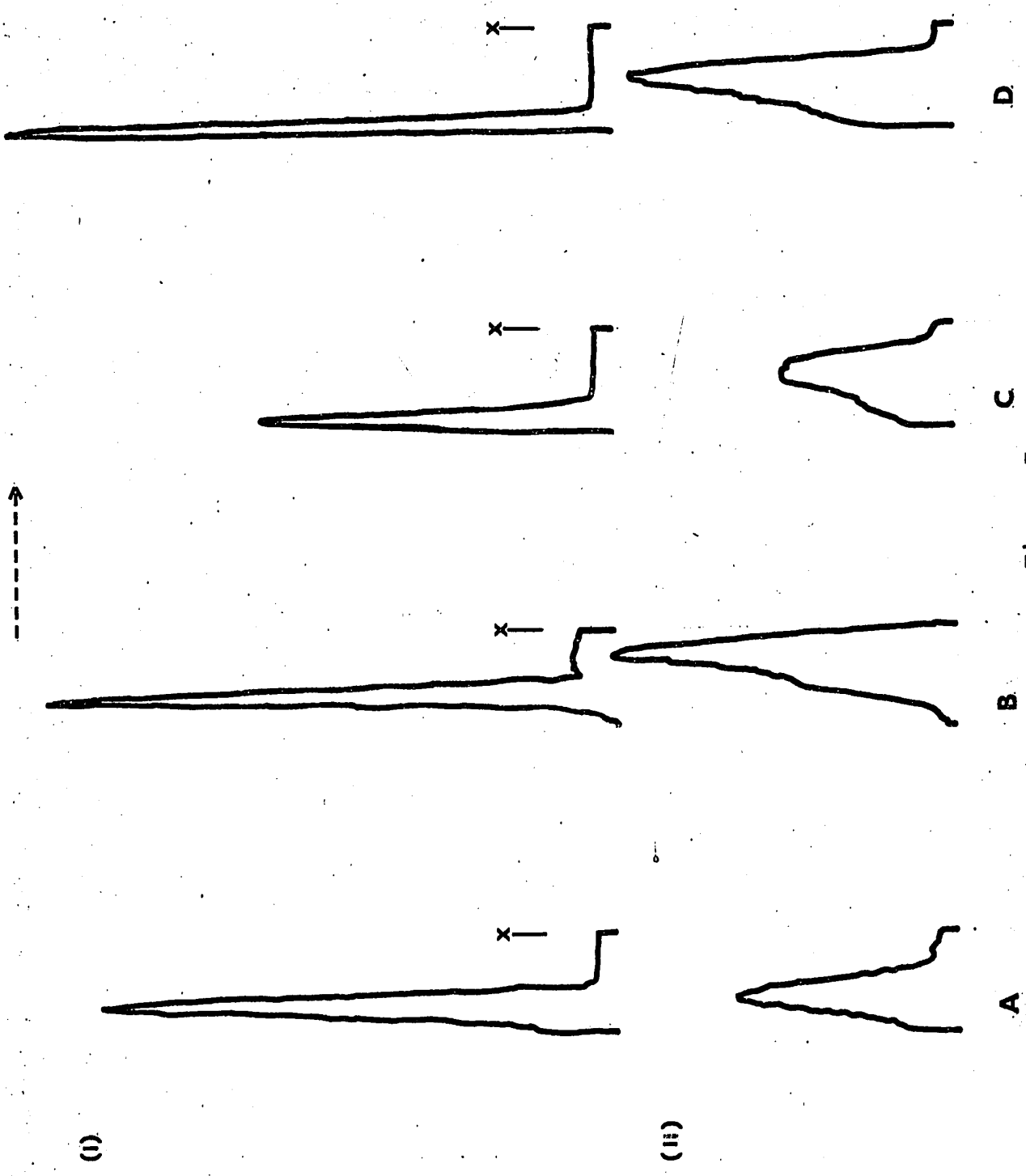


Figure 7

Discussion

Puccinia helianthi appears to be rather weakly antigenic. Nevertheless, multiple precipitin bands were discernible in immunodiffusion tests indicating the presence of several antigens interacting with antiserum to race 2. It was not possible to distinguish differences between races of P. helianthi or between races 15B and 56 of P. graminis f.sp. tritici. This result for P. graminis f.sp. tritici is in agreement with that of Bahn (1957) who showed a similarity between races 15B and 56 when antiserum to race 56 was tested against antigen of 15B. He considered the relationship "intermediate", i.e. between "similar" and "dissimilar", when antiserum to 15B was tested against 56 antigen. Antigen and antibody titres in this study were low for critical comparisons, which in any case, require antiserum to each antigen (Crowle, 1961).

An antigenic difference between P. helianthi and P. graminis f.sp. tritici was indicated but it was evident that there is also a similarity between them. False spurring may occur when concentrations of the antigens being compared are unequal and can be wrongly interpreted as showing the non-identity of identical antigens (Crowle, 1961). Evidence from cross-absorption indicates that these species are antigenically different. Nevertheless, this study is inconclusive

in view of the low titres and few antisera tested.

Bahn (1957) and Beno (1964) showed that most races of the cereal rusts constituted serological groups rather than distinct entities. Races of P. helianthi, identified by reactions on only two differentials, are less well defined. Evidence of several antigens in P. helianthi suggests that, with refined techniques for concentrating antigens (Gooding and Powers, 1965) and isolating macromolecular components (Gooding, 1966), further breeding work on both the fungus and its host could lead to separation of serological components. A thorough study of variation in P. helianthi requires a combination of serology and in vitro isolation of serological entities. Current work on P. graminis f.sp. tritici (Williams, Scott and Kuhl, 1966; Williams et al., 1967; Williams, 1971) indicates this may soon be possible.

This investigation was discontinued because of the difficulty in producing adequate quantities of uredospores. Puccinia helianthi normally sporulates profusely but in order to maintain genetic purity cultures were isolated by growing plants beneath plastic covers and, under these conditions, sporulation was inhibited. An alternative source of antigen may be homogenised rust-infected leaves, as used in the chromatographic study (Part IV).

No antigenic affinity was found between P. helianthi

antiserum and sunflower antigen. This may be due to a weakness of the sunflower antigen and requires specific sunflower antiserum for verification. It may be, however, that the relationship proposed by Doubly et al. (1960) is not generally applicable. The 1:1 complementary of fit between two genetic systems postulated by Flor (1956) does not require that homologous gene systems are interacting (Flangas and Dickson, 1961a) and need not lead to identical proteins in host and parasite.

IV. THIN-LAYER CHROMATOGRAPHY

Literature Review

Paper chromatography was developed in 1944 for protein analysis (Consden, Gordon and Martin, 1944) and has been widely used in animal and plant taxonomy. Thin-layer chromatography, first described by two Russian workers in 1938, owes its present popularity to Stahl's work in the late 1950's (Pelick, Bolliger and Mangold, 1966). It was applied to a biosystematic study in Lotus (Grant and Whetter, 1966) and has since been generally adopted for similar studies in other genera.

Phenolic compounds appear to be linked to carbohydrate metabolism in plants through acetate and shikimic acid precursors (Bogorad, 1958; Neish, 1964). Their occurrence is largely independent of environmental and/or nutritive influence (Scora, 1966) and is of taxonomic significance in higher plants (Bate-Smith, 1962). Genetic determination of phenolic constituents has been demonstrated by Nybom (1964), Fröst (1966), Moore et al. (1970) and many others.

Disease resistance in plants has for some time been ascribed to the action of phenolic compounds (Newton and

Anderson, 1929; Farkas and Király, 1962; Cruickshank and Perrin, 1964; Rohringer and Samborski, 1967; Olah and Sherwood, 1971) although the majority show little or no toxicity to parasitic organisms (Cruickshank and Perrin, 1964).

Phenolic compounds are widely distributed among the fungi (Miller, 1961) and have been implicated in symptom development in some diseases (Cruickshank and Perrin, 1964). A number of coumarins and phenolic acids have been identified, associated with germination, in the uredospores of Puccinia graminis f.sp. tritici (van Sumere et al., 1957). Differences between races have been described in terms of sugars (Broyles, 1956), amino acids (Broyles, 1956; McKillican, 1960) and proteins (Macko, Novacký and Stahmann, 1967). Analysis of the major components of rust uredospores, however, has so far failed to define the nature of physiological specialization (Eyal, Peterson and Cappellini, 1967). Eyal et al. (1967) and Shipton and Fleischmann (1969a) were unable to show protein differences between races and Shipton and Fleischmann (1969b) concluded that differentiation of physiologic races of the rusts from protein patterns in disc electrophoresis was not possible, but that the method had some taxonomic value at the formae speciales and species levels. Gill and Powell (1968) observed that polyacrylamide gel electrophoresis was less sensitive than immunoelectro-

phoresis.

This study was undertaken to establish whether differences between races of P. helianthi were detectable through the appearance of phenolic compounds or similar metabolites in methanol extracts of uredospores and rust-infected sunflower leaves. It was thought that products of host-parasite interaction might possibly be more specific than metabolites in the spores themselves. Spots appearing in chromatograms were compared with those from other obligate and non-obligate fungal pathogens of sunflower, and from several varieties and species of Helianthus. Varietal and species differences in the sunflower were not examined beyond the expression of metabolites in infected tissue. A chemotaxonomic study of the genus is being pursued by Dr. D. Seigler at the University of Illinois (personal communication).

Materials and Methods

Chromatographic analyses were made on healthy sunflower leaves, and leaves infected with P. helianthi, Erysiphe cichoracearum DC, Plasmopara halstedii (Farl.) Berl. et de T., Sclerotium bataticola Taub. and Verticillium dahliae Kleb. Puccinia helianthi infection was investigated on the differential varieties CM90RR and Morden Cr29, M62-2672-2-r1

and M62-2685-14-I, the "universal suscept", S37-388 and the wild species H. annuus, H. debilis ssp. silvestris and H. praecox ssp. praecox. Plasmopara halstedii was cultured on variety Sunrise and the other pathogens on S37-388. Comparison was also made with infections from P. graminis f.sp. tritici races 15B and 56 on wheat cultivars Little Club, Marquis, Arnautka and Mindum. Race 15B is virulent on all of these, race 56 is virulent on Little Club and Marquis, and avirulent on Arnautka and Mindum (Stakman, Stewart and Loegering, 1962).

With the exception of the wild species of Helianthus and plants infected with S. bataticola, plants were grown in controlled environment cabinets at 22 ± 2°C day and 20 ± 2°C night temperature, daylength of 16 h and light intensity at plant level of 13,200 ± 2200 lux (1200 ± 200 ft-c) from VHO cool white fluorescent tubes, supplemented with incandescent lamps. The wild species were grown at 25 ± 2°C day and 22 ± 2°C night temperature and S. bataticola infections were developed at 30 ± 2°C after inoculation. All plants were grown in a soil:sand:peat moss mixture in 100 mm pots.

Puccinia helianthi was inoculated by dusting uredospores on to moistened leaves of 14- to 21-day-old plants and incubating in a Percival dew chamber at about 15°C for

12 to 18 h. Leaves were collected at intervals during development of infection and after sporulation, 10 to 12 days after inoculation. Chromatographic analyses were made from whole leaves inoculated in this way and also segments of infected leaves, from single inoculation sites, in multiple inoculation experiments in the pathogenicity study reported in Part II.

Erysiphe cichoracearum, isolated from a naturally occurring infection on H. strumosus L., was cultured on detached S37-388 leaves in Petri plates. Infections for chromatography were obtained by dusting S37-388 plants with conidia in the manner described for P. helianthi. Leaves were harvested when sporulation was evident, 7 to 10 days after inoculation.

The inoculation procedure for P. halstedii was that of Viswanathan and Sackston (1968). Seeds were dehulled and surface sterilized in 1% sodium hypochlorite (diluted commercial Javex) for 5 min and germinated between sheets of moistened black paper in Petri plates for 48 h. The germinating seedlings were then immersed in a suspension of sporangia of P. halstedii for 4 to 5 h and planted. Sporulation of P. halstedii was induced on infected seedlings 10 to 15 days after planting by subjecting them to an elevated humidity beneath glass lantern chimneys for 24 to 48 h.

Two cultures of S. bataticola:E, avirulent on S37-388, isolated from Cucumis melo L. in Iran, and N, virulent on S37-388, isolated from cotton in North Carolina (Chan and Sackston, 1970) were grown in 50 ml potato-dextrose-broth (PDB), prepared with the decoction from 200 g potatoes, 20 g glucose and 7 g nutrient broth (Difco) made up to 1000 ml in distilled water, on a shaker for 4 days (Chan and Sackston, 1969). Mycelium from 4-day-old shake cultures was poured over PDB-impregnated cotton in Petri plates and allowed to grow in the dark at 30°C for 48 h. Plugs of cotton and mycelium were then applied to leaves of 14- to 21-day-old S37-388 plants. Inoculum was applied to several points on the lamina and petiole. Each plant was covered with a plastic bag after inoculation to maintain humidity. Necrotic lesions developed beneath the cotton about 48 h after inoculation (Chan and Sackston, 1969). Bags were then removed and representative leaves harvested. Plants were left uncovered and a second collection of leaves made 14 days later. Mycelium for chromatographic analysis was cultured in PDB on a shaker for 10 days.

Verticillium dahliae, culture V58, isolated from sunflowers at La Pocatière, Quebec (Devaux and Sackston, 1966) was grown on potato-dextrose-agar (PDA) and in PDB shake cultures. Inoculum was prepared by homogenizing 14-day-old

cultures from PDA. Plants (14- to 21-day-old S37-388) were inoculated by dipping the roots into the resulting mycelial suspensions and repotting. Necrosis developed about 10 days later and leaves were collected between 14 and 21 days after inoculation. Mycelium for chromatographic analysis was taken from 10-day-old PDB shake cultures.

The chromatographic procedure was essentially that of Grant and Whetter (1966). Samples of 0.08 g of fresh or dried leaves, and mycelium of S. bataticola and V. dahliae were extracted overnight in 0.5 ml of 1% HCl in methanol at room temperature in the dark. A greater number of spots appeared in chromatograms from dried material and after a few preliminary tests material was routinely dried in an oven at 70°C for 12 to 24 h (Grant, 1971) before extraction. In order to obtain sufficient material to identify fluorescent compounds several grams of dried leaves were extracted at a time; the proportion of leaves:HCl-methanol was maintained at 0.08:0.5.

Rust uredospores (200 to 400 mg) were germinated in calcium phosphate-potassium phosphate buffer (pH 7.0) containing 1×10^{-4} M nonyl alcohol and 0.01% Tween 20 (polyoxyethylene sorbitan mono-laurate) (Maheshwari and Sussman, 1970), washed with distilled water and homogenized in 1% HCl-methanol with carborundum in a pestle and mortar. Tests were

made with ungerminated, dried (70°C) spores and germinated spores without drying. In both cases the proportion of spores:HCl-methanol was 0.08:0.5 as for leaves and mycelium.

Shandon thin-layer chromatographic equipment was used throughout. Standard plates 200 x 200 mm were coated with silica gel G (E. Merck, Darnstadt, Germany) 250μ in thickness. Sufficient silica gel for 5 plates was prepared by weighing 30 g silica gel into a 500 ml conical flask, adding 60 ml distilled water, 3 ml 95% ethanol and shaking for 90 sec. Coated plates were left to set for 5 min and then dried at 100°C for a minimum of 20 min or overnight. Once dry, further heating does not affect the chromatographic properties of the layers. Coated plates were kept in a desiccator until required. If kept for more than one day they were reactivated by heating at 100°C for at least 10 min before use.

Cellulose plates have certain advantages over silica gel (Grant and Whetter, 1966) but were not found satisfactory for this study and were used only for a preliminary trial.

Extract was usually applied by micropipette at a rate of 10 to 15 μl per spot. Some spots were detectable with 5 μl; definition of others improved with up to 20 μl but at the higher rate, spots detectable with a lower volume were liable to become blurred. Some samples of uredospore extract required 100 μl before spots were visible. With a Shandon

template up to 9 spots, 20 mm apart, could be placed on one plate. For elution, a continuous band 160 mm long, utilizing 2 to 3 ml of leaf extract, was applied to the plates. The solvent front was run 145 to 150 mm from the origin. Ascending development was carried out at room temperature in filter paper lined chromatography tanks sealed with masking tape. The separation of spots was examined in long and short wave ultraviolet light by means of a Chromato-Vue Model C-3 viewer. Identifications were based on appearance under UV, reaction to ammonia vapour, diazotised -p-nitroaniline and diazotised sulfanilic acid sprays and with UV spectroscopy, the results of which were inconclusive. The empirical formula of Blue 1 was determined by mass spectroscopy which was arranged by Dr. C.J. Mirocha, University of Minnesota, and conducted by Shrader Analytical and Consulting Laboratories Inc., Detroit, Michigan.

Solvent systems used were cyclohexane:ethyl acetate (1:1) run twice; methanol:chloroform (3:7); chloroform: acetic acid (5:2); tert-butanol:acetic acid:water (3:1:1). Acetic acid (15%) was used as second solvent with tert-butanol:acetic acid:water in two-dimensional development; in one-dimensional development acetic acid (2%) was used. The best resolution of spots was obtained with two-dimensional development in cyclohexane:ethyl acetate (1:1) as

first solvent and chloroform:acetic acid (5:2) as second solvent. Blue 1 had a higher solubility in methanol: chloroform (3:7) than in cyclohexane:ethyl acetate (1:1). For purification and identification, Blue 1 was eluted and run several times through methanol:chloroform; Blue 2 was similarly eluted and run repeatedly through cyclohexane: ethyl acetate. Both compounds were, in addition, run once through chloroform:ethanol (97:3) prior to mass spectroscopic analysis. All R_f values and comparisons in the following section refer to mobility in cyclohexane:ethyl acetate. The other solvents were found to be less satisfactory and were not used beyond preliminary tests.

R_f values represent the means of various numbers of samples, as follows: Blue 1 and Blue 2 in P. helianthi infections, 70 samples; S. bataticola infections, 12 samples; V. dahliae infections, 18 samples; E. cichoracearum infections, 2 samples. Figures 8 to 10 and 12 to 14 are tracings of representative chromatograms, R_f values in them differ from the averages given in the text.

Experimental Results

Chromatographic Analysis of Sunflower Rust Infection

Chromatograms of leaf extract from rust-infected

sunflower leaves were characterised by 11 spots representing phenolic compounds, chlorophylls and carotenoids (Figure 8). Of particular interest were two spots, colourless in white light which fluoresced bright blue in UV. Blue 1 appeared at Rf 0.19, and Blue 2 at Rf 0.72. Blue 1 was sometimes detectable in healthy uninoculated leaves but was more prominent in inoculated leaves. It was particularly evident in hypersensitive necrotic tissue (Figure 9) and appeared to be a host metabolite associated with necrosis. It was found also in necrosis due to environmental factors and mite infestation. Blue 2 occurred in compatible rust reactions on the differential varieties and on wild H. annuus. It was not seen in extracts from other species (Figure 10). The spot did not appear during development of infection until after sporulation. Washing spores from the leaf surface prior to extraction did not affect its concentration in the extracts and it may represent a constituent of the rust mycelium. There were no apparent differences between races of P. helianthi in respect of Blue 2 in infected tissue. Both Blue 1 and Blue 2 tended to be better developed on Morden Cr29 than on the other varieties but there were no varietal differences in terms of R_f value. In tests with small samples, Blue 2 was detectable with as little as 3 mg of leaf tissue comprising about 6 pustules. It was

Figure 8. Tracing from two-dimensional chromatogram of leaf extract from sunflower var. S37-388 infected with Puccinia helianthi race 1. Solvents used in first direction, cyclohexane:ethyl acetate (1:1), in second direction, chloroform:acetic acid (5:2).

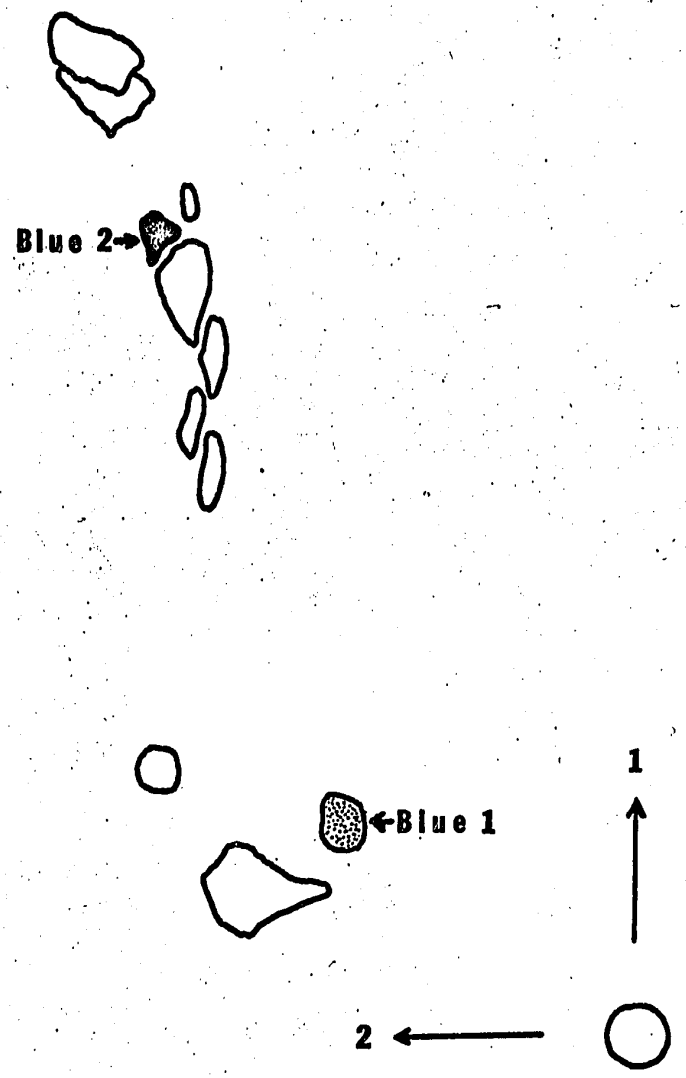


Figure 8

Figure 9. Chromatographic patterns showing presence of Blue 1 (stippled spot) and Blue 2 (black spot) in rust-infected sunflower leaves.

A. S37-388 uninoc.
B. S37-388 inoc. race 1 (susc.).
C. Morden Cr29 uninoc.
D. Morden Cr29 inoc. race 3 (res.).
E. Morden Cr29 inoc. race 2 (susc.).
F. CM90RR uninoc.
G. CM90RR inoc. race 1 (res.).
H. CM90RR inoc. race 3 (susc.).

Solvent used, cyclohexane:ethyl acetate (1:1).

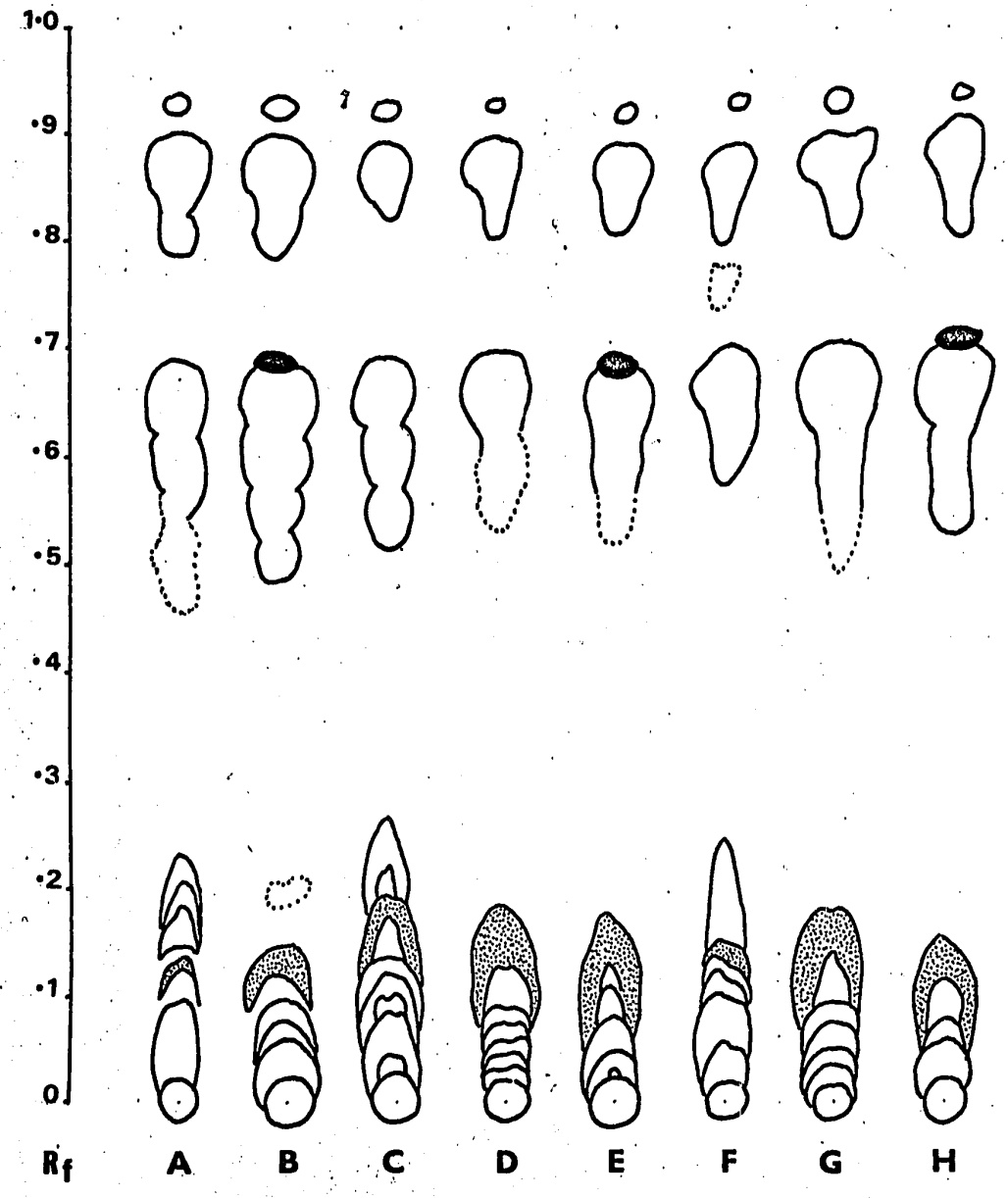


Figure 9

Figure 10. Chromatographic patterns showing the effect of host species on appearance of Blue 1 (stippled spot) and Blue 2 (black spot). Compatible rust infections on:

A. S37-388.
B. Wild H. annuus.
C. H. debilis ssp. silvestris.
D. H. praecox ssp. praecox.

Solvent used, cyclohexane:ethylacetate (1:1).

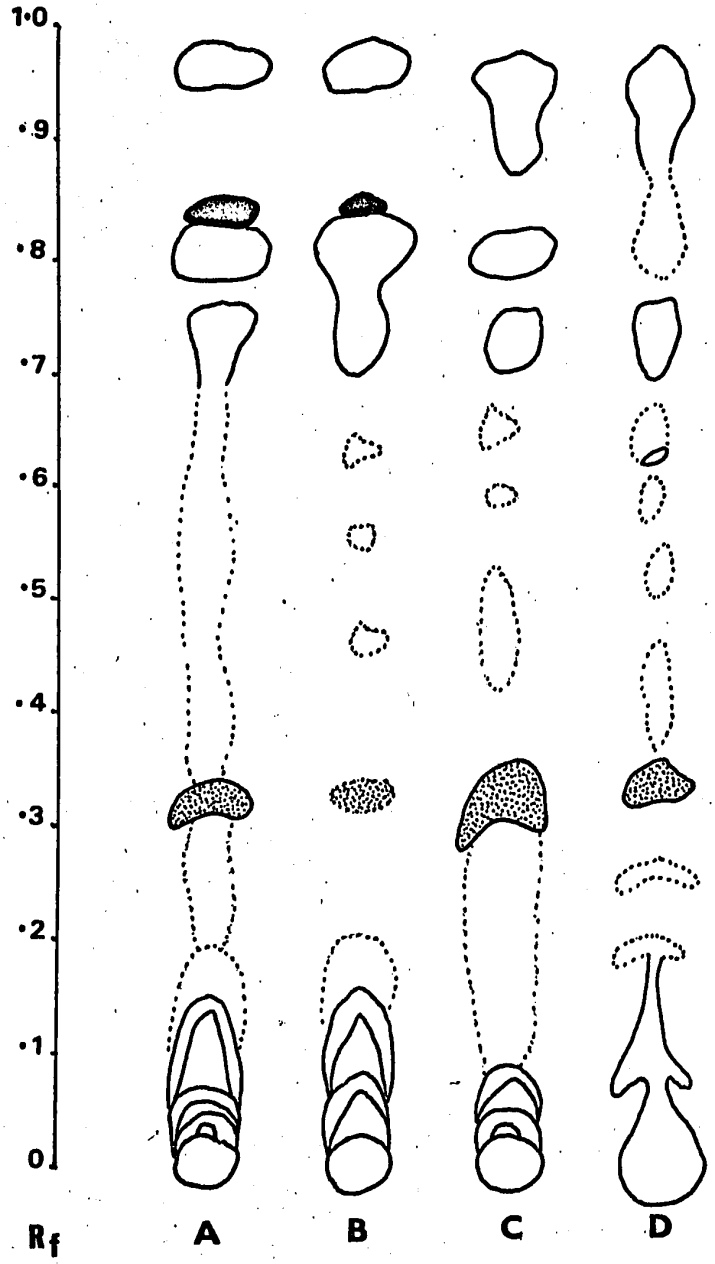


Figure 10

apparent only in the immediate vicinity of a pustule and was not found in uninfected areas of inoculated leaves.

The empirical formula of Blue 1, determined by mass spectroscopy, is $C_{15}H_{16}O_5N$ (mol wt 290). Mass spectroscopic analysis data showed two principle subsidiary fragments: one at m/e 149 having the empirical formula $C_8H_5O_3$, and another at m/e 263 with the formula $C_{14}H_{15}O_5$ (Figure 11 and Appendix Table IV). The base peak at m/e 149 usually indicates a dicarboxylic benzene ring, such as in phthalate esters. The compound described as Blue 1 may have included co-chromatographing non-fluorescing compounds and its purity is questionable.

Blue 2 proved to be unstable and it was not possible to resolve it into a single constituent for identification.

Extracts from ungerminated uredospores showed a few grey-blue spots below R_f 0.37 which were indistinct and appeared to be variable. Extracts of germinated uredospores contained compounds giving faint spots with R_f 0.16 and R_f 0.76. These may correspond to Blue 1 and Blue 2, respectively (Figure 12). There were no differences between races of P. helianthi. The spots were not seen in extracts of P. graminis f.sp. tritici uredospores (Figure 12).

Examination of P. graminis f.sp. tritici infection on wheat showed a spot comparable to Blue 1 in all varieties,

Figure 11. Mass spectrum of Blue 1. (Drawn from data in Appendix Table IV).

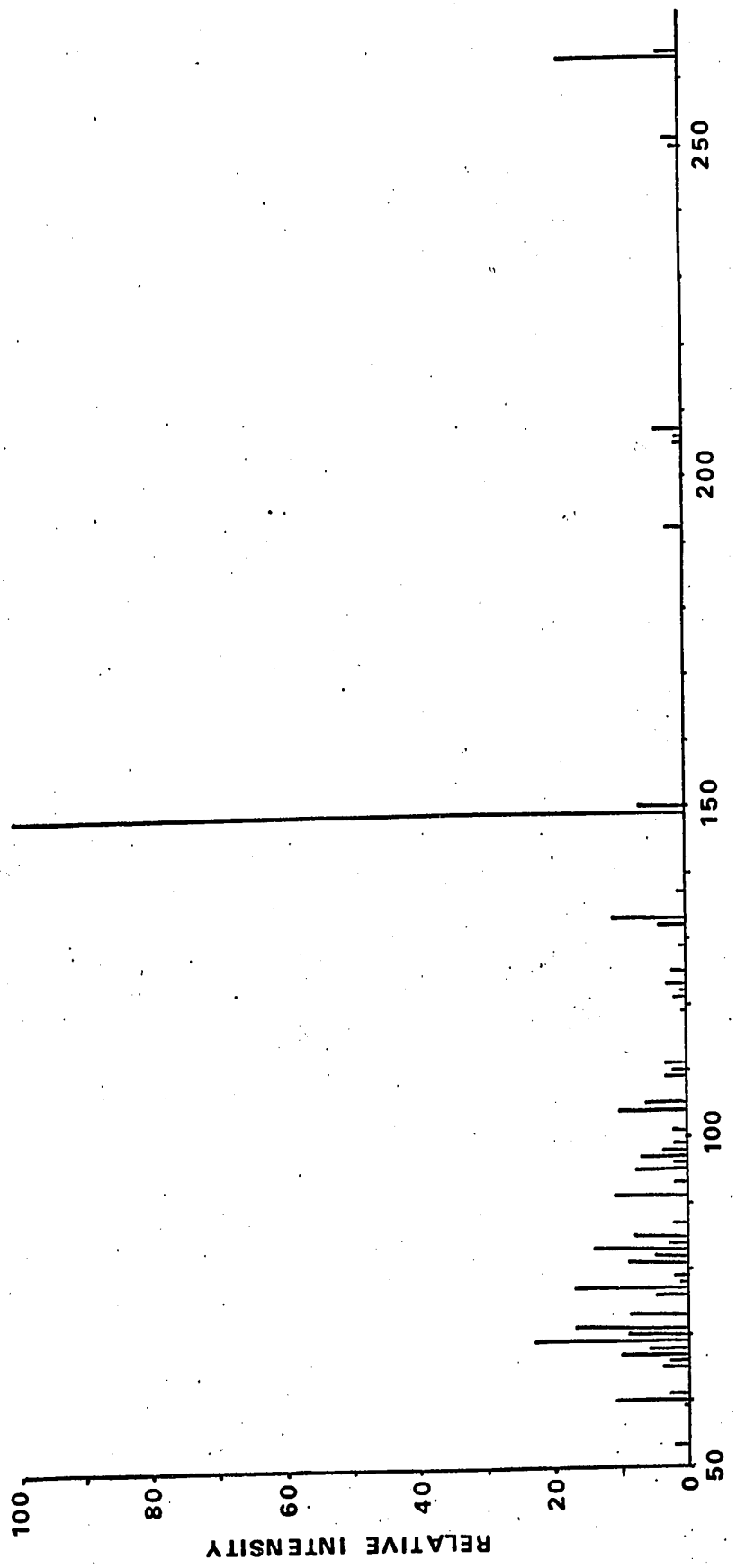

RELATIVE INTENSITY
100
80
60
40
20
0
50
100
150
200
250
MEASURED MASS (m/e)

Figure 11

Figure 12. Chromatographic patterns from extracts of uredospores and rust-infected leaf tissue.

A. *P. graminis* f.sp. *tritici* race 15B.

B. *P. graminis* f.sp. *tritici* race 56.

C. *P. helianthi* race 1.

D. *P. helianthi* race 2.

E. *P. helianthi* race 3.

F. *P. helianthi* race 4.

G. Morden Cr29 infected with race 2 (susc.).

Blue 1 -- stippled spot.

Blue 2 -- black spot.

Solvent used, cyclohexane:ethyl acetate (1:1).

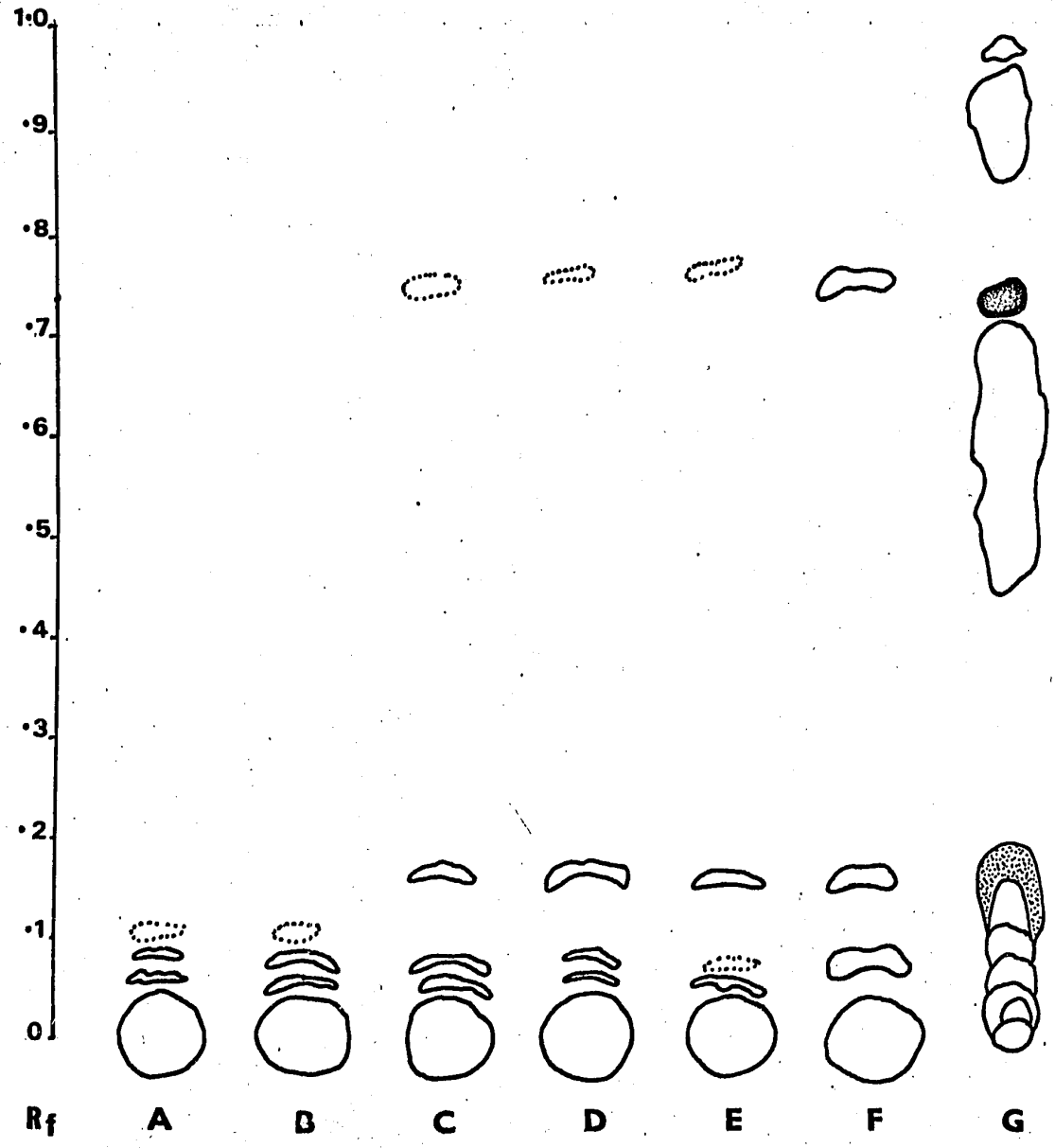


Figure 12

whether infected with rust or not, but Blue 2 was absent (Figure 13).

Chromatographic Analysis of Sclerotium bataticola Infection

Extracts of leaves infected with S. bataticola showed two characteristic blue fluorescent spots at R_f 0.61 and at R_f 0.77 (Figure 14). Both spots were evident only where necrotic symptoms had developed, which occurred only with isolate N.

Mycelial extracts of both isolates of S. bataticola showed a very bright blue spot at R_f 0.84 with a variable number of less distinct spots below. Isolate N was distinguishable from E by the presence of a pink spot at R_f 0.04.

Chromatographic Analysis of Verticillium dahliae Infection

Extracts of leaves infected with V. dahliae showed a faint blue fluorescent spot at R_f 0.60 (Figure 14). As with S. bataticola this was only evident when infection had progressed to the point of killing the host tissue. Extracts of V. dahliae mycelium showed a blue spot at R_f 0.86 and a fainter one at R_f 0.75.

Chromatographic Analysis of Erysiphe cichoracearum and Plasmopara halstedii Infections

Figure 13. Chromatographic patterns of rust-infected wheat and sunflower leaves.

A. S37-388 uninoc.

B. S37-388 inoc. race 1 (susc.).

C. Arnautka uninoc.

D. Arnautka inoc. race 56 (res.).

E. Arnautka inoc. race 15B (susc.).

F. Marquis uninoc.

G. Marquis inoc. race 56 (susc.).

H. Marquis inoc. race 15B (susc.).

I. Little Club inoc. race 15B (susc.).

Blue 1 -- stippled spots.

Blue 2 -- black spot.

Solvent used cyclohexane:ethyl acetate (1:1).

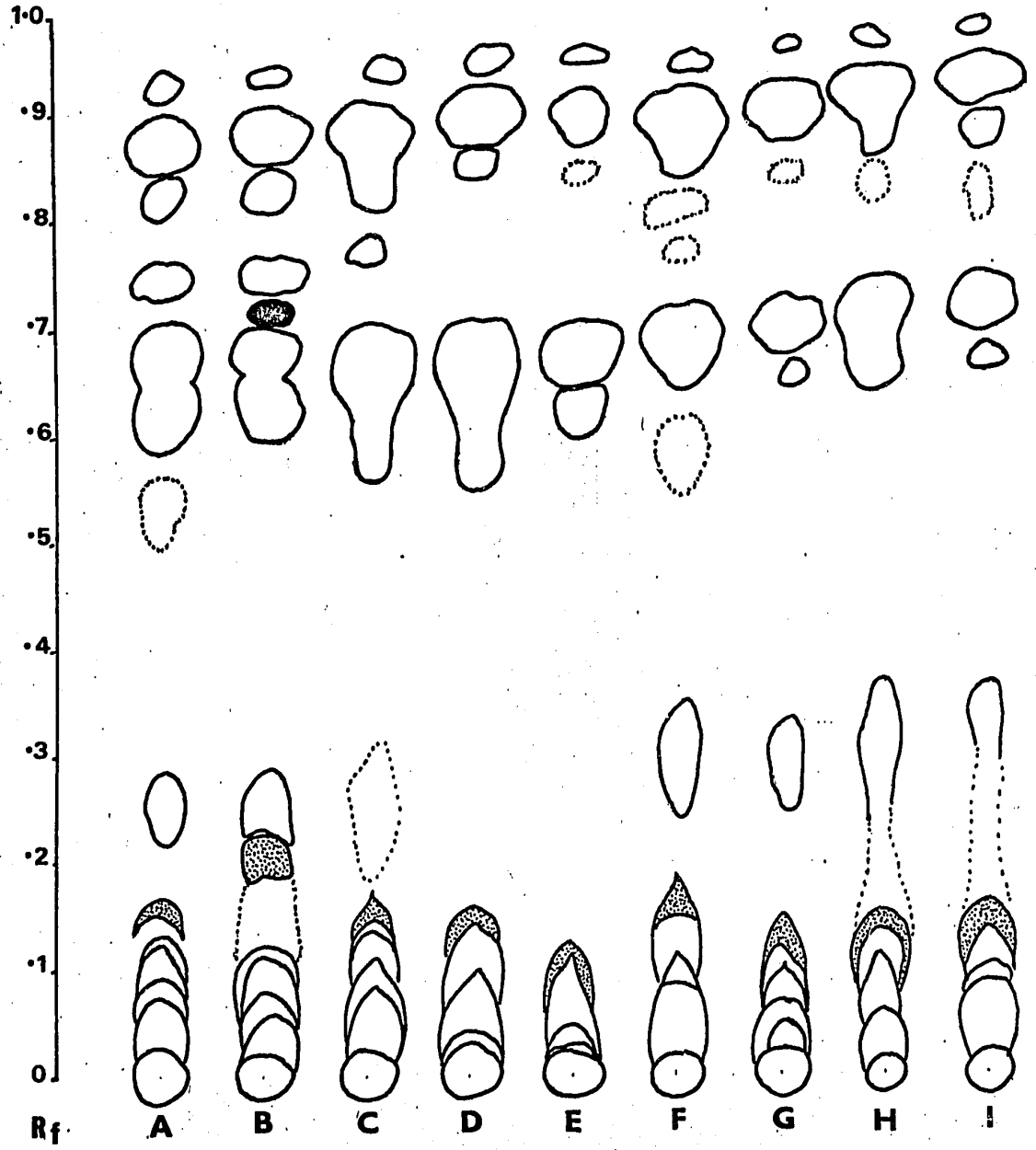


Figure 13

Figure 14. Chromatographic patterns.

A. Blue 2 purified by elution.

B. S37-388 inoc. V. dahliae (necrotic leaf).

C. S37-388 inoc. V. dahliae (dead leaf).

D. V. dahliae mycelial extract.

E. S37-388 inoc. E. cichoracearum.

F. S37-388 inoc. S. bataticola N.

G. S. bataticola E mycelial extract.

H. S. bataticola N mycelial extract.

I. S37-388 inoc. P. helianthi race 1.

Blue spots indicated: stippled -- Blue 1.
black -- Blue 2.
Diagonal lines, Verticillium. Vertical lines, Erysiphe. Horizontal lines, Sclerotium. Pink spot in S. bataticola N denoted by cross-hatching. Solvent used, cyclohexane: acetate (1:1).

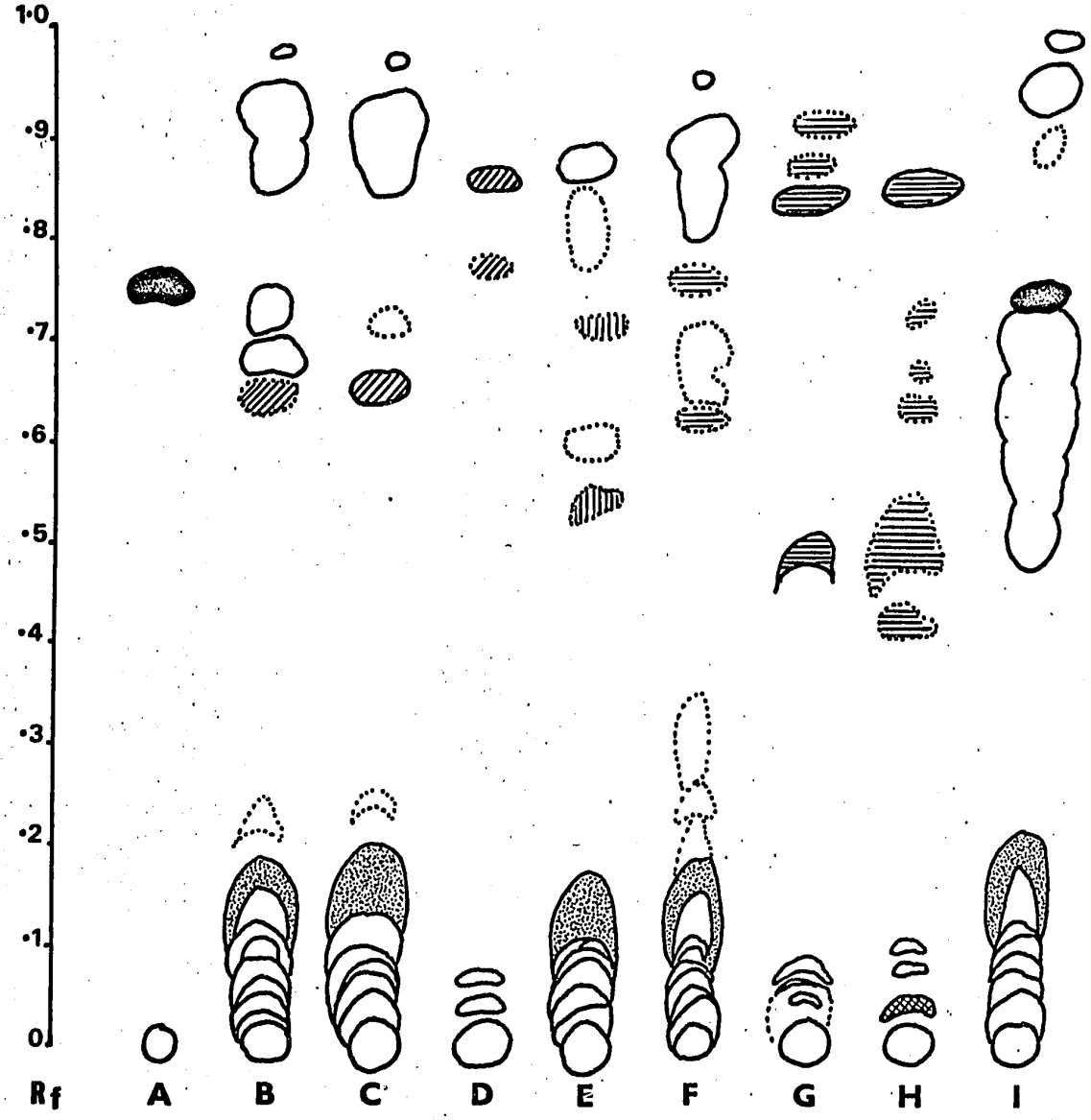


Figure 14

Chromatograms of E. cichoracearum-infected leaves showed faint blue fluorescent spots at R_f 0.56 and R_f 0.74 (Figure 14). Spot development was, however, weak and was seen in only 2 samples out of 4 tested.

No distinctive spots were found in extracts from P. halstedii infections. Blue 1 was seen, as it was in all infections studied.

Discussion

Two phenolic compounds present in rust-infected sunflower leaves were studied. Blue 1 was apparently a nonspecific metabolite of necrotic tissue, present in all infections examined and in abiotically induced necrosis in sunflowers. A compound which may have been identical to Blue 1 was also observed in rust-infected wheat leaves. Samborski and Rohringer (1970) discussed the synthesis of 2-hydroxyputrescine amides in rust-infected wheat plants. Blue 1 is not a putrescine but there are similarities in its occurrence which suggest it may have a similar metabolic function. A more complete identification of Blue 1 was beyond the scope of this investigation. Blue 2 appeared to be specific to compatible rust reactions. It was detected in trace amounts in extracts of germinated uredospores and could be observed in chromatograms from as little

as 3 mg of leaf tissue bearing about six pustules. It may be a fungal component but the data suggest synthesis during the infection process. This compound, however, proved to be unstable and could not be identified.

Phenolic compounds observed in infections of sunflower due to V. dahliae, S. bataticola and E. cichoracearum may also have been products of host-parasite interaction. Since R_f values are influenced by the other components in a mixture it is not possible to identify spots in extracts of infected tissue with particular spots in mycelial extracts (Figure 14). Time did not permit eluting each spot in the extracts and co-chromatographing them to check the identity of R_f values.

The purpose of this study was to determine whether phenolic compounds could be used as markers for investigations into physiological variation in P. helianthi. No differences between races were found. Two isolates of S. bataticola were distinguished by a single pink spot present in isolate N, which was lacking in isolate E. Failure to detect distinctive characters in P. helianthi may be attributed to the use of insufficient material for extraction. It may, however, be that races of P. helianthi are less clearly differentiated than isolates of S. bataticola. Differences in enzyme synthesis between these two isolates of S. bataticola

were reported by Chan and Sackston (1970).

Extracts of infected tissue are evidently not comparable to mycelial extracts in observations on fungal constituents. The pink spot characteristic of S. bataticola isolate N was not seen in extracts from infected leaves. Products of host-parasite interaction may be specific and indicate differences between pathogens. The similarity between spots in S. bataticola and E. cichoracearum (Figure 15) shows that such comparisons may have limited application.

This study has shown that thin-layer chromatography can be a useful adjunct to comparative investigations in some of the fungal pathogens of sunflowers, and probably in other host-parasite systems. Further work is necessary to determine the levels of affinity that can be detected.

The failure of this study to show a correlation between pathogenic specialization and secondary phenolic compounds may reflect a lack of specialization in this respect. Race groups in the cereal rusts have been shown to constitute serological entities. Races of P. helianthi could not be separated serologically (see Part III). Pathogenic race types of P. helianthi are but crudely defined on two differential varieties. The genus Helianthus is highly variable and P. helianthi has a fairly wide host range. Puccinia helianthi must be considered relatively unspecialized in

Figure 15. R_f values of blue-fluorescent compounds observed in sunflower leaves infected with:

A. P. helianthi.

B. S. bataticola.

C. V. dahliae.

D. E. cichoracearum.

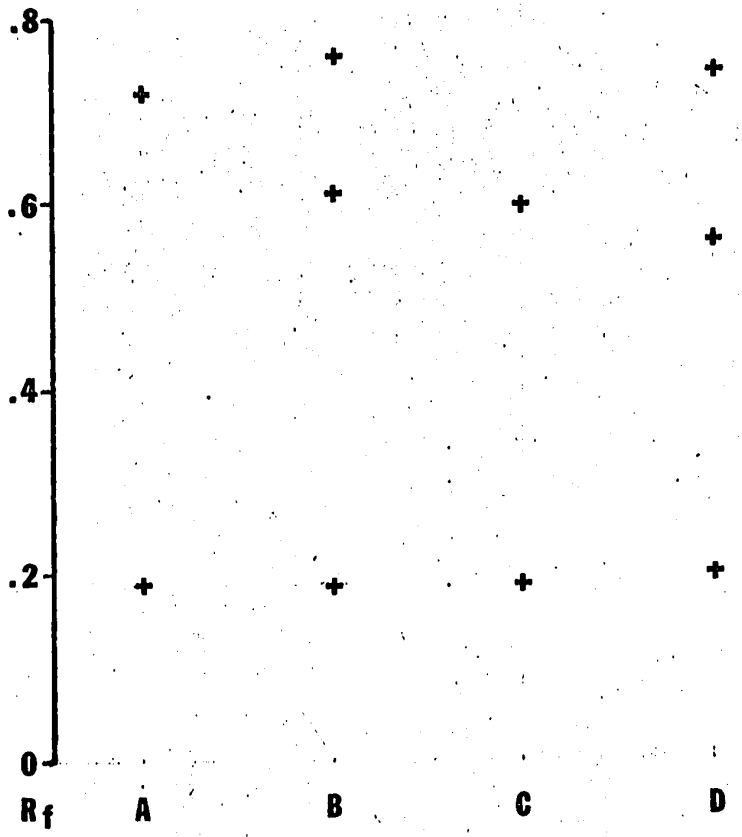


Figure 15

comparison with *P. graminis* and *P. sorghi*. It is possible that some correlation could be found with other compounds if larger samples were available for analysis and/or different tests applied. It appears, however, that studies of this kind require more critically defined races and must await the availability of better differentials.

V. INHERITANCE OF SPORE COLOUR

Literature Review

Spore colour mutations are a frequent occurrence in the cereal rusts. They have been reported in uredospores of P. graminis f.sp. tritici (Newton and Johnson, 1927; Waterhouse, 1929; Newton, Johnson and Brown, 1930b; Johnson and Newton, 1938; Nelson, 1956; Green, 1961;1964; Luig, 1967), P. graminis f.sp. avenae (Gordon, 1933; Johnson and Newton, 1940; Johnson, 1949; Green and Kirmani, 1969), P. graminis Pers. f.sp. secalis Erikss. and Henn. (Cotter and Levine, 1932; Green, 1964), P. recondita Rob. ex. Desm. f.sp. tritici Erikss. and Henn. (Vakili and Caldwell, 1957), P. triticina Erikss. (Johnston, 1930; Brown and Johnson, 1949), P. glumarum Schmidt f.sp. tritici Erikss. and Henn. (Gassner and Straib,1932 - in d'Oliveira,1939), P. anomala Rost. (d'Oliveira, 1939). Mutant pycnia and aecia have been reported in P. graminis (Cotter, 1934; Johnson and Newton, 1943; Garrett and Line, 1962; Green, 1961;1964).

Uredospore colour is derived from a carotenoid pigment in the cytoplasm and a brown pigment in the spore wall, each determined by a single independent gene (Johnson, Newton, and

Brown, 1934; Johnson, 1949). Green (1964) showed that white mutant pycnia, lacking pigment in the cytoplasmic granules of the pycniospores, gave rise to white aeciospores, which in turn, produced greyish-brown uredospores deficient in the carotenoid cytoplasmic pigment. Pigment in the pycniospores was determined by a single gene.

A mutation for spore colour in P. helianthi was reported by Brown (1940). It affected pycnia and aecia as well as uredia and telia. The genetic basis of the colour change was not established. Spore size, shape and echinulation, viability, host range and temperature tolerance were also affected and Brown suggested that more than one gene was probably involved.

Colour mutants provide valuable markers in genetic studies in the rusts and have been used to demonstrate asexual nuclear exchange (Nelson, 1956), somatic recombination (Vakili and Caldwell, 1957; Ellingboe, 1961; Watson and Luig, 1962) and somatic segregation (Green and Kirmani, 1969).

The life cycle of the rusts has been amply documented (Johnson and Newton, 1946; Buller, 1950; Olive, 1953) but there continues to be disagreement as to the unit of fertilization. It has long been realized that more than one phenotype may be recovered from a single aecial cup. Stakman,

Levine and Cotter (1930) and Newton, Johnson and Brown (1930a), in studies of P. graminis f.sp. tritici, considered such cases to be exceptions to the rule that all aeciospores within an aecium are identical, although aecia within a cluster may differ. In P. coronata Corda f.sp. avenae Fraser and Led., Murphy (1935) found only one physiological type per aecium and Zimmer, Schafer and Patterson (1965) concluded that the aecium commonly resulted from a single fertilization, the few exceptions possibly being explained by contamination. On the other hand, Cotter (1932) in P. graminis, d'Oliveira (1939) in P. anomala, Wahl et al. (1960) and Dinoor, Khair and Fleischmann (1968) in P. coronata f.sp. avenae, and Buller (1950) considered the aecium to be the product of multiple fertilizations. Cytological studies by Andrus (1933), Allen (1934), Craigie (1959) and Craigie and Green (1962) have elucidated the phenomenon of nuclear migration and dicaryotization of the protoaecial cells. The unit of fertilization, from these studies, appears to be the basal cell which gives rise mitotically to a chain, or chains, of dicaryotic aeciospores. The cytological evidence has, however, been invoked to support both the argument for single fertilization (Zimmer et al., 1965) and multiple fertilizations (Dinoor et al., 1968) in the ontogeny of the aecium.

A study on the mode of inheritance of spore colour in P. helianthi was undertaken in the hope that it might clarify this fundamental difficulty and facilitate further studies on the inheritance of pathogenicity in this and other rust fungi.

Materials and Methods

A yellow-spored mutant of P. helianthi arose spontaneously from a culture of "Canadian" race 2 on a single leaf in an otherwise normal detached leaf culture growing at 10°C. Two of the five pustules found were cultured (as Y1 and Y2) for further study. Uredospores from the others, when transferred to susceptible leaves, failed to induce infections.

Single spore isolates of these cultures were maintained on detached sunflower leaves on moist filter paper in Petri plates in controlled environment growth chambers at 22 ± 2°C day and 20 ± 2°C night temperature, daylength of 16 h and light intensity at plant level of 13200 ± 2200 lux (1200 ± 200 ft-c) from cool white VHO fluorescent tubes supplemented with incandescent lamps, giving approximately 4400 lux at leaf surface within the plates. Cultures were also grown on plants in the greenhouse (22 to 25°C) isolated beneath plastic covers.

Teleutospores were produced on detached leaves at

10°C as reported by Hennessy and Sackston (1970). When these were used to inoculate detached leaves pycnia and aecia developed, but the aecia dehisced too rapidly in plate cultures to permit collecting spores from individual aecia. Pycnia and aecia were therefore produced on attached leaves. The plants were protected from insects by gauze cages.

Reciprocal crosses Y1 x normal race 2 and Y2 x race 1, and back-crosses of the hybrids to the mutant parent, were made by exchanging nectar between pairs of isolated pycnia. F_1 progeny were selfed by collecting nectar from a number of pycnia with a micropipette, pooling it on a microscope slide, and applying the mixture to the pycnia from which it was taken.

Colour inheritance was determined from uredial development on detached leaves. Single aeciospore inoculations from excised aecia allowed to dehisce on dialysis tubing (Fleischmann, Khair and Dinoor, 1966), proved unsatisfactory due to poor viability or poor infectivity. In crosses H6 and H8, unopened aecia were transferred to detached leaves and were either allowed to dehisce or were crushed and their contents spread over several leaves. In cross H12, the massed aeciospores from entire clusters, or groups of clusters, were applied to a number of detached leaves. Colour of the resulting uredia was assessed 10 to 12 days after inoculation.

In the code used to describe cultures the hybrid number, e.g. H5, H6, H8, etc., is followed by (a) denoting progeny from the mutant maternal parent or (b) from the normal maternal parent. A second number indicates the aecium from which the original hybrid was isolated, and P the pustule number in a succeeding subculture.

Uredospores were measured and photographed in lactophenol (Kerr, 1959). In vitro germination was determined on agar in Petri plates at room temperature.

Experimental Results

Mutants Y1 and Y2 appeared to be identical from their origin, appearance under the microscope, and behaviour in crosses with normal cultures. The mutation in Y1 and Y2 results in a deficiency of spore wall pigment. The walls of uredospores and teleutospores appeared hyaline under the microscope (Figures 16 and 17). The colour of mutant uredia (Figure 18) based on Ridgway's colour standards (Ridgway, 1912) ranged from "maize yellow" and "warm buff" to "yellow ochre", "ochraceous orange", or "buckthorn brown", compared with "cinnamon brown", Prout's brown", or "mummy brown", for normal uredia of P. helianthi. Colour tended to be darker at higher temperatures.

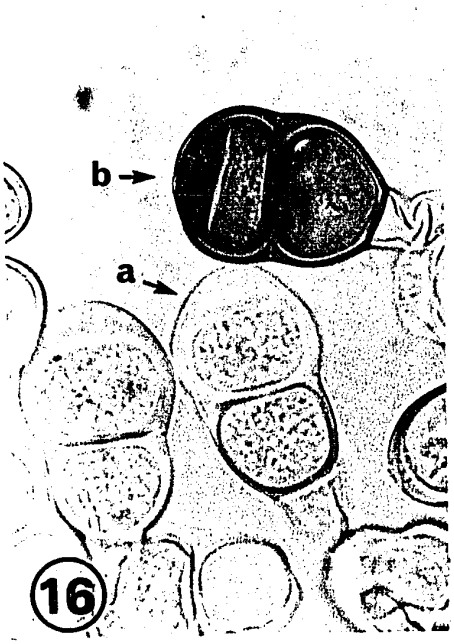
Mutant telia appeared white or hyaline. The mutation

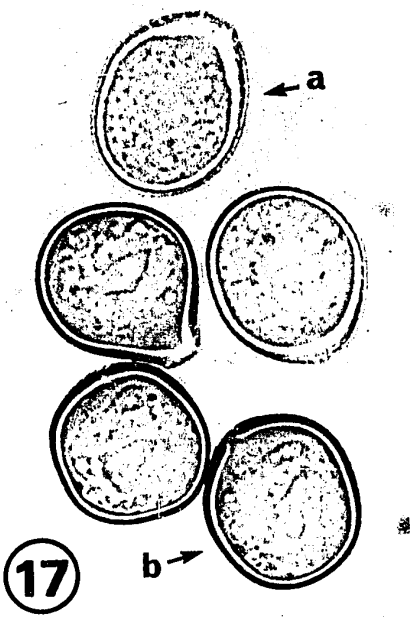
Figures 16 - 18. Mutant a and normal b sunflower rust.

Figure 16. Teleutospores (x 1500).

Figure 17. Uredospores (x 1500).

Figure 18. Segregating uredial pustules (x 7).


b
a
16


a
b
17


b

was not detectable in aeciospores, the walls of which are normally colourless, or in pycnia. Spore size was not significantly affected, mean measurements of 100 uredospores of each strain were 22 x 27 μ and 22 x 28 μ for normal and mutant spores, respectively, with P values (c test) of 0.7 for the comparison of spore width and 0.1 for spore length. There was no accompanying change in echinulation or pathogenicity, but mutant cultures showed a lower level of germination at room temperature (Table 5) and a reduced longevity in storage. Mutant uredospores failed to induce infection after being stored in a freezer (-15°C) for six months, whereas normal spores stored for the same period produced copious infections. The non-infectious, stored, mutant cultures showed a low level of germination *in vitro*. A more critical study of the longevity of mutant uredospores may be desirable but was not the objective of this work.

A culture of Y1 was grown in the greenhouse for 19 uredial generations with no indication of reversion to normal type. Selfing of a culture of Y1 yielded 953 "yellow ochre", 45 "buckthorn brown" and 32 "maize yellow" uredial pustules from 22 aecial clusters. The variation in colour was attributed to differences in moisture on the leaf surfaces since "buckthorn brown" and "maize yellow" reverted to "yellow ochre" in succeeding uredial generations.

Table 5. Comparison of germination between freshly collected uredospores of mutant Y1 and normal race 1 of Puccinia helianthi on agar.

Germination Time (hours)	Percentage Germination	
	Mutant	Normal
3	36.9	94.9
6	48.7	98.5
12	56.4	96.5
24	50.7	96.5
Mean	48.2	96.6

Three reciprocal crosses Y1 x normal race 2, and one Y2 x race 1 gave uniformly normal F_1 progeny. One cross Y1 x normal race 2 (H5) yielded a mixture of yellow and brown F_1 pustules from the maternal side (Table 6). This may indicate cytoplasmic influence but in the absence of a similar effect in the other crosses it is probably the result of multiple fertilizations involving a compound yellow pycnium.

The F_1 progeny from three crosses, H6, H8, H12, subcultured through successive uredial generations, showed somatic segregation for colour up to the fourth or fifth asexual generation. Brown cultures isolated from single

Table 6. First generation progeny from a reciprocal cross Y1 x normal race 2 (H5) of *Puccinia helianthi*.

Y1 Maternal Parent	Aecium	Number of Uredial Pustules[1] B	Mixed	Y	Normal Maternal Parent	Aecium	Number of Uredial Pustules B	Mixed	Y
H5a[2]	1	7	0	0	H5b	1	22	0	0
	2	9	1	0		2	2	0	0
	3	4	0	0		3	9	0	0
	4	16	3	11		4	40	0	0
	5	7	2	2		5	14	0	0
	6	9	1	1		6	3	0	0
	7	12	0	1		7	1	0	0
	8	5	0	0		8	3	0	0
	9	1	0	0		9	10	0	0
	10	2	0	1		10	3	0	0
	11	8	1	1					
	12	1	0	0					
	Total	81	8	17		Total	107	0	0

[1]B = brown or normal pustules; Y = yellow, mutant, pustules; Mixed = pustules comprising both yellow and brown spores.

[2]a = mutant as maternal parent; b = normal as maternal parent.

spores, or from single uredial pustules, produced occasional yellow pustules. Yellow cultures were never observed to produce brown pustules. A fourth cross (H13), which was not subcultured beyond the second uredial generation, showed segregation within uredial pustules developing directly from aeciospore inoculation. All F_1 pustules from this cross were initially normal brown in appearance. When placed at 10°C for two weeks for telia to develop a few pustules produced a mixture of brown and yellow uredospores. One pustule produced a ring of secondary uredia, five of which were normal and one was yellow ("light buff"). When telia formed, the uredospores were washed off to determine telial colour. One leaf was found to have a preponderance of mutant telia, 54 yellow:36 brown.

Segregation in F_2 populations was irregular (Table 7). The F_2 progeny from the first cross (H5) was predominantly mutant. In the second (H6) the ratio normal:mutant was 2:1 but there was significant heterogeneity between F_1 parental cultures. Values for the total progeny of one F_1 culture (H12b1P2) from the third cross were in agreement with a 3:1 segregation ratio. However, three series of pycnia from this culture which were selfed separately yielded ratios of 13:3 ($\underline{P} = 0.9$), 1:1 ($\underline{P} = 0.5$), and 3:1 ($\underline{P} = 0.001$). Another

Table 7. Segregation in F_2 progeny from crosses between mutant and normal strains of Puccinia helianthi.

Cross	Parental Culture	Number of Pycnia	Uredospore Colour: Brown	Uredospore Colour: Yellow	Ratio	P value (X^2)
1 (Y1x2)	H5b4[1]	9	15[2]	846		
2 (Y1x2)	H6a1	35	687	319	2:1	0.3
	H6a3	3	140	16		
Family Total			827[3]	335	2:1	0.001
	H6b2	7	730	430	2:1	0.01
	H6b3	43	345	172	2:1	0.9
Family Total			1075	602	2:1	0.02
Total all H6			1902	937	2:1	0.7
3 (Y1x2)	H12b1P2	21	2383	546	13:3	0.9
		22	563	541	1:1	0.5
		30	6422	1938	3:1	0.001
Family Total			9368	3025	3:1	0.1
	H12b1P3	12	328	0		
		6	74	0		
Family Total			402	0		
4 (Y2x1)	H8b2	10	136	0		
	H8b4	3	100	0		
Family Total			236	0		
Overall Total			11923	4808	3:1	0.001
Total (excluding Cross 1)			11908	3962	3:1	0.9

[1] a = F_1 progeny from mutant maternal parent, b = from normal maternal parent.

[2] Number of uredial pustules developing from inoculations with aeciospores.

[3] Total counts for each cross, and overall totals, underlined.

culture from the same cross (H12b1P3) was apparently homozygous for normal pigment. Both cultures from the fourth cross (H8) also appeared to be homozygous normal.

Hybrid culture H5b4 was back-crossed to Y1 and Y2 but both these crosses were unsatisfactory; few progeny were obtained and the pustules that developed were all yellow.

Discussion

Previous workers (Johnson et al., 1934; Johnson, 1949; Green, 1964) showed a simple, single factor, Mendelian inheritance for spore colour in P. graminis, while Green and Kirmani (1969) showed that cytoplasmic factors may also be involved. The results presented here for a recessive mutation for loss of wall pigment in uredospores and teleutospores of P. helianthi are inconclusive. Cytoplasmic influence was rejected because evidence for it occurred in only one cross out of five (Table 6) and this can be explained as multiple fertilizations involving a compound yellow pycnium. Segregation data from F_2 populations (Table 7) fitted a number of ratios. A ratio of 2:1 indicates differential viability, the dominant allele being lethal in the homozygous condition, which is not possible in this case. A ratio of 13:3 suggests the influence of two genes with one epistatic over the other. The evidence for this is less

convincing than that for one operative gene. Both these ratios, and the 1:1 ratio, may be attributed to sampling error.

A 3:1 ratio, for monofactorial inheritance, was significant only for the total progeny from culture H12b1P2 and the overall total for all crosses, except cross 1. The latter is no doubt an unjustified manipulation, for one could as easily omit the non-segregating cultures H12b1P3 and H8 as well and obtain a different ratio. More work is required before a mechanism of inheritance can be postulated.

A confusing range of colours was observed in the mutant cultures. Mutant spores with colourless walls and dark cytoplasm could be distinguished from spores with normal wall pigmentation and pale cytoplasm ("ochraceous tawny") only under the microscope. "Maize yellow" uredia, with colourless spore walls and pale cytoplasm, could not be maintained in culture; they gave rise to darker forms ("yellow ochre" and "ochraceous orange") in succeeding uredial generations.

The observed variation in colour and irregular segregation ratios may be caused by factors determining pigment in the cytoplasm. No evidence of genetic control of cytoplasm colour was found. An explanation of these results may be that cytoplasm pigmentation is unstable and subject to

environmental influence.

Uredospore colour in P. helianthi is affected by the metabolic state of the host; spores produced on chlorotic leaves were paler than normal (Hennessy, 1967). Colour in this investigation appeared to be influenced also by temperature and moisture.

D'Oliveira (1939) discussed the induction of colour mutations in uredospores (of P. anomala) by low temperature. The mutations he described tended to revert to normal. Temperature does not seem to have been a factor in the loss of wall pigment in this study. Although the mutation occurred at 10°C, it was observed only once in hundreds of cultures grown at the same low temperature.

Genetic studies in the rusts are difficult, with many sources of error. Much of the inconsistency of these data may be ascribed to sampling error. Miah (1968) discussed sampling errors in rust genetics. Assuming the aecium to be the unit of fertilization, he proposed a means of establishing genetic ratios from segregation within and between aecial clusters. The method was inapplicable in the present study because it was not possible to obtain representative numbers of aecia from an adequate number of clusters.

These data show segregation to occur within the aecium (Table 6). The argument of Zimmer et al. (1965) that this

may be the result of contamination is difficult to disprove. The frequency with which it was observed in all crosses from which F_2 populations were obtained, a total of 44 aecia from 40 clusters, suggests strongly that multiple fertilizations do occur in the developing aecium. The fact that multiple fertilizations may occur in some aecia and not in others makes genetic analysis of rust populations more difficult. The contents of a single aecium will not yield true segregation ratios because the basal cells, which are the units of fertilization, may produce more than one chain of aeciospores (Craigie and Green, 1962) and all spores derived from one basal cell will be identical. Confirmation of these observations requires, as Dinoor et al. (1968) have said, single spore analysis of groups of aeciospore chains, a marker for aeciospore colour or isotopic tracer studies. Single spore analysis did not provide satisfactory data in this investigation.

The ratios from cross 2 (Table 7), obtained by summing the products of individual aecia, may reflect irregular segregation within the aecium. More meaningful results were obtained from the massed contents of entire clusters and groups of clusters (cross 3, Table 7) but even with large populations, sampling error is presumably responsible for the ratios 1:1 and 13:3. The solution to this problem is

difficult to find. It appears to lie in sampling large numbers of clusters; testing large populations seems unavoidable.

Somatic segregation presented another problem in this study. Yellow cultures were recovered at regular but low frequency from F_1 populations up to the fourth or fifth asexual generation. The apparent homozygosity of F_1 cultures H8 and H12b1P3 (Table 7) is attributed to somatic segregation.

Somatic variation in the rusts was reviewed by Christensen (1961), Watson and Luig (1962), and Hartley and Williams (1971). Plasmagenes (Green and Kirmani, 1969), reassortment of haploid nuclei (Nelson, 1956; Flor, 1964), pseudomiosis (Ellingboe, 1961), and parasexualism (Watson and Luig, 1958) have been suggested as mechanisms by which it may arise. As somatic segregation in this instance occurred in F_1 progenies but was not observed in F_2 populations, it seems to be explained adequately by nuclear reassortment. The practical significance of somatic variation as a means of generating new pathogenic genotypes has already been mentioned (Watson and Luig, 1958; Christensen, 1961). Its importance in rust genetic studies lies in the consequence that the genetic constitution of hybrid cultures cannot be predicted. Changes during vegetative culture which have hitherto been attributed to mutations may, more

correctly, be interpreted as the result of somatic segregation.

VI. SUMMARY

A survey of physiological races of P. helianthi from wild species of Helianthus in Texas conducted in 1969 showed a measure of host specificity. Races 1 and 2 predominated on H. annuus and H. debilis, race 2 on H. praecox and race 4 on H. petiolaris.

Rust from H. annuus was equally pathogenic on H. argophyllus, H. debilis and H. praecox ssp. praecox. Cultures from H. debilis attacked H. argophyllus and H. praecox ssp. praecox, and cultures from H. praecox ssp. praecox attacked H. argophyllus and H. debilis but infectivity of cultures from these species on H. annuus was doubtful. Rust from H. praecox ssp. hirtus, identified as race 2, was virulent on all annual species except H. petiolaris. Rust from H. petiolaris was virulent on all the differentials but, on the wild species, was pathogenic only on H. annuus, H. argophyllus, H. debilis ssp. silvestris and H. praecox ssp. runyonii.

Collections of H. annuus, H. argophyllus, H. debilis and H. praecox ssp. praecox tested in the greenhouse were heterozygous for susceptibility to most rust cultures. Helianthus praecox ssp. hirtus and H. petiolaris both

appeared resistant to all cultures except their own.

Local specialization may occur in P. helianthi in some areas. It is also likely that inoculum spreads northwards annually with the prevailing wind, resulting in selection of new pathogenic biotypes each year. It was not possible to say which of these processes is the more important in the epidemiology of sunflower rust in Texas. It was obvious that P. helianthi has a considerable potential for variation and adaptation.

A serological investigation showed no antigenic differences between the four "Canadian" races of P. helianthi or between races 15B and 56 of P. graminis f.sp. tritici. There was both a similarity and a difference between antigens from P. helianthi and P. graminis f.sp. tritici. No antigenic affinity was detected between P. helianthi antiserum and antigens from the differential varieties of cultivated sunflower.

Thin-layer chromatograms from methanol extracts of uredospores and rust-infected sunflower leaves were characterized by two phenolic compounds which fluoresced bright blue in UV. Blue 1 had the empirical formula $C_{15}H_{16}O_5N$ (mol wt 290) and was considered to be a non-specific metabolite of necrotic tissue. Blue 2 was a product of host-parasite interaction in compatible rust infections on H.

annuus and appeared to be present in trace amounts in uredospores. This compound was unstable and could not be identified. Fluorescent spots were also observed in extracts of sunflower leaves infected with E. cichoracearum, S. bataticola and V. dahliae which may be specific to these pathogens. Races of P. helianthi were not distinguishable by their phenolic constituents. A single spot difference separated virulent isolate N of S. bataticola from avirulent isolate E.

A mutation for loss of wall pigment in uredospores and teleutospores of P. helianthi is described. The mechanism of colour inheritance could not be established precisely. Wall pigment appeared to be determined by one, or a small number of genes. Pigment in the cytoplasm proved to be unstable and influenced by environmental factors.

Evidence for multiple fertilizations in single aecial cups supported earlier reports that the basal cells should be considered the units of fertilization. The contents of individual aecial cups gave unreliable genetic ratios.

Somatic segregation was observed in colour hybrids. Its occurrence was explained by nuclear migration. Somatic segregation was considered responsible for the apparent homozygosity of some F_1 cultures and may offer an alternative explanation for reports in the literature of mutations in the rusts.

VII. CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

This is the first report of the distribution of physiological races of *P. helianthi*, as defined by standard differentials, on the putative ancestral species of the cultivated sunflower in its centre of origin. It was shown that *P. helianthi* has a considerable capacity for variation and a relatively wide host range. Resistance genes from wild *Helianthus* are important in commercial breeding programmes but resistance from a restricted source appears unlikely to provide lasting protection.

This was the first application of serology and thin-layer chromatography to a study of variation in *P. helianthi*. Physiological races were not distinguishable by their antigenic properties or their phenolic components. Absence of specialization in these characters suggests that there may be no correlation between them and pathogenicity. In view of the degree of pathogenic variation in natural populations, however, these results may simply be a consequence of the heterogeneity of the species and indicate that races are at present inadequately categorized.

A single fluorescent spot difference between isolates

E and N of S. bataticola, which has not previously been described, demonstrated the usefulness of thin-layer chromatography in comparative studies on other fungal pathogens of sunflowers.

There has been only one previous report of a colour mutation in P. helianthi (Brown, 1940) and this is the first attempt to explain colour inheritance in this species. Spore wall pigment appeared to be determined by one or a small number of genes, while pigment in the cytoplasm was unstable and subject to environmental influence.

Aecial cups were found to contain the products of multiple fertilizations, in agreement with Dinoor et al. (1968). A consequence of this is that progeny from individual aecia do not yield meaningful genetic ratios and sampling in inheritance studies requires a large number of aecial clusters.

Somatic segregation was observed in colour hybrids. It was shown that this may result in homozygosity in F_1 cultures, making genetic analysis of rust populations more difficult. It was suggested that some reports of mutations in the rusts may be open to re-interpretation as somatic segregation.

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IX. APPENDIX TABLES

Appendix Table I. Reactions and race types of sunflower rust collections from wild Helianthus species tested on Canadian differentials.

Host	Site	S37-388	Differ. Var. Cm90RR	Morden Cr29	M62-2672-2-r1	M62-2685 14-I	Race Type
H. annuus							
Texas	Artesia Wells 1	+[1]	-	-	-	-	1(a)[2]
	Artesia Wells 2	+	-(int)	+(Int)	-	-	2
	Batesville 1(a)	+	-	-(Int)	0	0	1
	Batesville 2	+	-	-	0	0	1
	Belton 1	+	-	-	0	-	1
	Belton 2	+	-	+	0	-	2
	Belton 3	+	-	+	0	-	2
	Bluff Springs(a)	+	-	+	0	-	2
	Bluff Springs(b)	+	-	+	0	0	2
	Bluff Springs(c)	+	-	+	-	-	2
	Carrizo Springs 3	+	-	-	0	0	1
	Commerce	+	+	-	-	-	3
	Cone	+	-	-	-	+	1(b)
	Cotulla	+	-	+(Int)	-	-	2
	Denton	+	-	+	-	-	2
	Driscoll	+	-	-	0	0	1
	Encinal 1	+	+	+	-	-	4
	Encinal 2	+	-	-	-	-	1(a)
	Encinal 3	+	-	+	-	-	2
	Gainsville	+	+	+	-	-	4
	La Pryor	+	-	-	0	0	1
	Laredo 1	+	-	+	-	-	2
	Laredo 2	+	-	+	-	-	2
	Leander	+	-	+	0	0	2
	Leonard	+	+	-	-	-	3
	Loraine(a)	+	-	-	-	+	1(b)
	Loraine(b)	+	-	-	-	+	1(b)
	Los Angeles	+	-	-	-	-	1(a)
	McKinney(a)	+	+	+	-	-	4
	McKinney(b)	+	+	+	-	-	4

Appendix Table I (cont'd)

	Moody(a)	+	-	+	0	-	2
	Moulton	+	-	-	0	-	1
	New Braunfels	+	-	-	0	-	1
	Nolanville	+	-	+	0	0	2
	Normangee(b)	+	-	+	-	-	2
	Padre Island	+	-	-	0	0	1
	Refugio*	+	+	-(Int)	0	-	3
	Renner	+	+	-(Int)	0	-	3
	Rockdale	+	-	-	0	0	1
	Rogers	+	+	+	-	-	4
	San Antonio 1	+	-	-	-	+	1(b)
	San Antonio 2	+	-	-	0	+	1
	San Antonio Rd	+	-	+	0	-	2
	San Marcos(a)	+	-	+	0	-	2
	San Marcos(b)	+	-	-	-	-	1(a)
	Sequin	+	+	+	0	-	4
	Shiner	+	-	-	0	-	1
	Stephenville	+	+	+	-	-	4
	Stoneburg	+	-	-	-	+	1(b)
	Taylor	+	-	-	0	-	1
	Terrell	+	+	-	-	-	3
	Tolar	+	+	+	-	-	4
	Tom Bean	+	-	+	-	-	2
Kansas	Claflin(a)	+	+	-	-	-	3
	Claflin(b)*	+	+	-	-	-	3
	Dodge	+	-	-	-	-	1(a)
	Englewood	+	-	+	0	-	2
	Pawnee Rock	+	-	-	0	+	1
Oklahoma	Shattuck	+	-	-	-	+(Int)	1(b)
	Rosston(a)	+	-	-	0	+	1
Nebraska	Fairbury(a)	+	-	-	-	-	1(a)
	Fairbury(b)	+	-	-	-	-	1(a)
	† Red Oak*	+	+	+(Int)	+	+	4(l)
	Tecumseh(a)	+	-	-	-(Int)	-	1
H. debilis ssp. cucumerifolius	College Sta. (a)	+	-	-	0	0	1
	College Sta. (b)	+	+	+	+	-	4(k)

† Should read: Red Oak, Iowa.

Appendix Table I (cont'd)

	Pearsall 3	+	-	-	0	-(Int)	1
	Yoakum	+	-	-	0	0	1
ssp. silvestris							
	Caldwell	+	-	+(Int)	0	-	2
	Jewett	+	-	+	0	0	2
	Neches River	+	-	+	-	+	2(d)
	Oakwood	+	-	+	0	0	2
H. petiolaris							
	Baird(b)	+	+	-	+	+	3(i)
	Baird(d)	+	+	-	+	+	3(i)
	Glazier	+	+	+	+	+	4(l)
	Higgins*	+	+	+	+(Int)	+(Int)	4(l)
	Wellington	+	+	+	0	0	4
	Wheeler(a)	+	+	+	+	+	4(l)
	Wheeler(b)	+	+	+	+	+	4(l)
Kansas	Ellsworth	+	+	+	+	+	4(l)
Oklahoma	Red River	+	+	+	+	+	4(l)
	Rosston(b)	+	+	+	+	+	4(l)
H. praecox ssp. praecox							
	Gilchrist 1*	+	-	+	-	-	2
	Gilchrist 2*	+	-	+	-	-	2
	ssp. hirtus						
	Carrizo Springs 4	+	-	+	+	-(Int)	2(e)
	ssp. runyonii						
	Falfurrias*	+	+	-	-	-	3
H. giganteus							
Indiana	Nashville	+	-	-	-	-	1
H. grosseserratus							
Iowa	Mt. Pleasant	+	-	-	+(Int)	-	1
H. tuberosus							
Nebraska	Tecumseh(b)	+	+	-	-(Int)	+(Int)	3(h)

Appendix Table I (cont'd)

Cultivated Sunflowers						
College Sta. Nursery						
(Hybrid)	+	+	-	0	0	3
McGregor (var. Peredovic)	+	-	+	0	-	2

[1]+ = susceptible reactions; - = resistant reactions; Int = intermediate or ambiguous reactions; 0 = no test data available.

[2]Supplementary classification on Miah's differentials.

*Reactions differed in different tests; see also Appendix Table III.

Appendix Table II. Host species and rust race distributions in ecological zones of Texas (including adjacent parts of Oklahoma).

A. North: Rolling Plains and High Plains.

Site	Species	Race Type
Cone	H. annuus	1
Loraine (a) and (b)	H. annuus	1
Rosston (a)	H. annuus	1
Shattuck	H. annuus	1
Baird (b) and (d)	H. petiolaris	3
Glazier	H. petiolaris	4
Higgins	H. petiolaris	4
Wellington	H. petiolaris	4
Wheeler (a) and (b)	H. petiolaris	4
Rosston (b)	H. petiolaris	4

Distribution of Rust Races and Host Species

Race	Number of Sites	Percentage	Species	Number of Sites	Percentage
1	4	40	H. annuus	4	40
2	0	0	H. petiolaris	6	60
3	1	10			
4	5	50			
Total	10	100		10	100

Distribution of Rust Races on H. annuus

Race	Number of Sites	Percentage
1	4	100

Appendix Table II B. East: Pineywoods, Blackland Prairies and Cross Timbers and Prairies.

Site	Species	Race Type
Commerce	H. annuus	3
Denton	H. annuus	2
Gainsville	H. annuus	4
Leonard	H. annuus	3
McKinney (a) and (b)	H. annuus	4
Renner	H. annuus	3
Stephenville	H. annuus	4
Stoneburg	H. annuus	1
Terrell	H. annuus	3
Tolar	H. annuus	4
Tom Bean	H. annuus	2
Neches River	H. debilis silvestris	2
Oakwood	H. debilis silvestris	2
Red River	H. petiolaris	4

Distribution of Rust Races and Host Species

Race	Number of Sites	Percentage	Species	Number of Sites	Percentage
1	1	7	H. annuus	11	79
2	4	29	H. debilis silvestris	2	14
3	4	29	H. petiolaris	1	7
4	5	35			
Total	14	100		14	100

Distribution of Rust Races on H. annuus

Race	Number of Sites	Percentage
1	1	9
2	2	19
3	4	36
4	4	36

Appendix Table II C. Central: Post Oak Savannah, Blackland Prairies and Gulf Prairies and Marshes.

Site	Species	Race Type
Belton 1	H. annuus	1
Belton 2	H. annuus	2
Belton 3	H. annuus	2
Bluff Springs	H. annuus	2
Leander	H. annuus	2
Moody	H. annuus	2
Moulton	H. annuus	1
New Braunfels	H. annuus	1
Nolanville	H. annuus	2
Normangee	H. annuus	2
Rockdale	H. annuus	1
Rogers	H. annuus	4
San Antonio 2	H. annuus	1
San Antonio Rd.	H. annuus	2
San Marcos (a)	H. annuus	2
San Marcos (b)	H. annuus	1
Sequin	H. annuus	4
Shiner	H. annuus	1
Taylor	H. annuus	1
College Station (a)	H. debilis cucumerifolius	1
College Station (b)	H. debilis cucumerifolius	4
Yoakum	H. debilis cucumerifolius	1
Caldwell	H. debilis silvestris	2
Jewett	H. debilis silvestris	2
Gilchrist 1	H. praecox praecox	2
Gilchrist 2	H. praecox praecox	2

Distribution of Rust Races and Host Species

Race	Number of Sites	Percentage	Species	Number of Sites	Percentage
1	10	38	H. annuus	19	73
2	13	50	H. debilis	5	19
3	0	0	H. praecox	2	8
4	3	12			
Total	26	100		26	100

Appendix Table II C (cont'd)

Distribution of Rust Races on H. annuus

Race	Site Number	Percentage
1	8	42
2	9	47
3	0	0
4	2	11
Total	19	100

Appendix Table II D. South: South Texas Plains.

Site	Species	Race Type
Artesia Wells 1	H. annuus	1
Artesia Wells 2	H. annuus	2
Batesville 1	H. annuus	1
Batesville 2	H. annuus	1
Carrizo Springs 3	H. annuus	1
Cotulla	H. annuus	2
Encinal 1	H. annuus	4
Encinal 2	H. annuus	1
Encinal 3	H. annuus	2
La Pryor	H. annuus	1
Laredo 1	H. annuus	2
Laredo 2	H. annuus	2
Los Angeles	H. annuus	1
Refugio	H. annuus	3
San Antonio 1	H. annuus	1
Driscoll	H. annuus	1
Padre Island	H. annuus	1
Pearsall 3	H. debilis cucumerifolius	1
Carrizo Springs 4	H. praecox hirtus	2
Falfurrias	H. praecox runyonii	3

Distribution of Rust Races and Host Species

Race	Number of Sites	Percentage	Species	Number of Sites	Percentage
1	11	55	H. annuus	17	85
2	6	30	H. debilis	1	5
3	2	10	H. praecox	2	10
4	1	5			
Total	20	100		20	100

Distribution of Rust Races on H. annuus

Race	Site Number	Percentage
1	10	59
2	5	29
3	1	6
4	1	6
Total	17	100

Appendix Table III A. Infectivity of *Puccinia helianthi* on wild species of sunflower.[1]

Rust Cultures Isolated From	Race Type	HOST SPECIES: *H. annuus* Artesia Wells 2 (A)	*H. annuus* Carrizo Springs 1 (B)	*H. annuus* Laredo 1 (C)	*H. annuus* Pearsall 1 (D)	*H. annuus* San Antonio 1 (E)	*H. debilis cucumerifolius* College Sta. (a) (F)	*H. debilis cucumerifolius* Yoakum (G)	*H. debilis silvestris* Caldwell (H)	*H. debilis silvestris* Flynn (J)	*H. debilis silvestris* Jewett (K)	*H. debilis silvestris* Neches River (a) (L)	*H. debilis silvestris* Oakwood (M)
Canadian Race 1		+(5)		+(1)	+(1)	+(1)	+(1)		+(4)	+(5)	+(1)	+(4)	+(3)
		-(2)	-(7)	-(4)	-(3)	-(5)	-(2)	-(2)	-(10)	-(5)		-(3)	
									Int(1)				
	2	+(3)		+(1)		+(1)		+(1)	+(3)	+(3)	+(1)	+(3)	+(3)
		-(5)	-(7)	-(3)	-(4)	-(5)	-(1)	-(1)	-(11)	-(6)		-(3)	
									Int(1)			Int(1)	
	3	+(2)			+(4)	+(3)		+(1)	+(3)	+(5)	+(1)	+(1)	+(2)
		-(5)	-(7)	-(2)		-(3)	-(2)		-(11)	-(6)		-(6)	
									Int(3)				
	4	+(5)			+(4)				+(2)	+(4)	+(1)	+(1)	+(3)
		-(3)	-(7)	-(4)		-(6)	-(3)		-(12)	-(6)		-(4)	
									Int(4)			Int(2)	
Annual Species													
H. annuus													
Artesia Wells 1	1		+(7)							+(11)			
										-(1)			
Belton 1	1	+(4)				+(2)							
		-(4)				-(4)							

Appendix Table III A (cont'd)

		A	B	C	D	E	F	G	H	J	K	L	M
Belton 2	2					+(4) -(4)			+(2) -(6)				
Bluff Springs(c)	2		+(4)							+(4) -(1)			
Carrizo Springs	1	+(2)											
	3	-(1) Int(1)		-(4) Int(2)									
Claflin(b)*	1	+(5) -(3)				+(2) -(4)							
Commerce	3	+(4) -(4)				+(2) -(4)							
Cotulla	2		+(3) -(2)		+(3) -(1)					+(1) -(4)			
Denton	2	+(7)				+(1) -(5)							
Dilley		+(4)		+(2) -(4)									
Encinal 2	1					+(3) -(6)			+(2) -(6)				
Encinal 3	2					-(5)			+(2) -(4)			-(6)	
La Pryor		+(4)		+(2) -(4)									
Laredo 2	2		+(5) -(1)		-(2)					+(4) -(3)			
Moody	2		-(7)							+(3) -(2) Int(2)			
Moulton	1					+(4) -(5)			+(1) -(6) Int(1)				

Appendix Table III A (cont'd)

		A	B	C	D	E	F	G	H	J	K	L	M
Padre Island	1						+(3)						
			-(3)										
Red Oak*	1					+(4)			+(1)				
						-(5)			-(7)				
Refugio*	2		+(1)		+(1)					+(4)			
			-(5)		-(3)					-(1)			
Rosston (a)	1					+(4)			+(2)				
						-(5)			-(6)				
H. debilis cucumerifolius													
College Sta. (b)	4					+(1)			+(5)			+(6)	+(1)
						-(4)		-(1)	-(2)				
									Int(1)				
Pearsall 3	1					+(4)		+(1)	+(6)		+(1)	+(2)	+(3)
				-(4)		-(5)		-(1)	-(7)				
Yoakum								+(1)	+(2)		+(1)	+(1)	
			-(1)	-(3)									
H. debilis silvestris													
Caldwell	2									+(3)			
			-(3)										
Jewett	2										+(1)	+(2)	+(2)
		-(3)		-(10)				-(2)	-(4)				
		Int(1)											
Neches River (b)	2							+(1)	+(4)		+(1)	+(1)	
				-(4)				-(1)					
Oakwood	2								+(2)		+(1)	+(1)	
		-(3)		-(8)				-(1)					
		Int(1)											

Appendix Table III A (cont'd)

		A	B	C	D	E	F	G	H	J	K	L	M
H. petiolaris													
Ellsworth	4	+(7)				+(2)							
						-(4)							
Higgins*	3		+(4)										
										Int(5)			
Red River	4					-(5)			-(6)			-(6)	
Wellington	4												+(1)
								-(1)	-(2)				-(2)
				Int(1)									
Wheeler	4				+(1)					+(5)			
			-(4)		-(3)								
			Int(2)										
H. praecox hirtus													
Carrizo Springs	4 2					+(1)			+(5)			+(5)	
						-(4)							
									Int(1)				
H. praecox praecox													
Gilchrist 1*	1								+(5)	+(5)		+(6)	
			-(6)			-(4)			-(12)				
			Int(1)			Int(1)			Int(1)	Int(3)			
										+(1)			
Gilchrist 2*	1												
			-(4)										
										Int(4)			
H. praecox runyonii													
Falfurrias*	2			-(3)					+(1)		+(1)	+(1)	
								-(2)			-(1)		-(3)

Appendix Table III A (cont'd)

	A	B	C	D	E	F	G	H	J	K	L	M
Perennial Species												
H. giganteus												
Nashville	1											
								-(2)			+(1)	
H. grosseserratus												
Mt. Pleasant	1										+(1)	+(2)
								-(2)				
H. tuberosus												
Tecumseh (b)	3										+(1)	
								-(2)				
Cultivated Sunflowers												
College Sta.	3	+(1)										
Nursery		-(3)		-(6)								

Appendix Table III B. Infectivity of Puccinia helianthi on wild species of sunflower.[1]

Rust Cultures Isolated From	Race Type	HOST SPECIES						
		H. petiolaris Quitaque (A)	H. praecox hirtus Carrizo Springs 4 (B)	H. praecox praecox Gilchrist 1 (C)	H. praecox runyonii Gilchrist 2 (D)	H. praecox runyonii Falfurrias (E)	H. argophyllus ex Kinman (F)	H. grosseserratus ex Heiser (G)
Canadian Race 1		-(2)	-(20)	+(1) -(6) Int(2)	+(1) -(10)	-(3)	+(1) -(4)	-(6)
	2	-(2)	-(20)	-(9)	-(11)	-(3)	+(1) -(3)	-(6)
	3	-(2)	-(20)	+(3) -(5)	+(1) -(10)	-(3)	+(1) -(4)	-(6)
	4	-(2)	-(20)	+(1) -(6)	+(1) -(10)	-(3)	+(1) -(5)	-(6)
Annual Species								
H. annuus								
Artesia Wells 1	1		-(12)		-(11)			
Belton 1	1		-(4)					
Belton 2	2		-(8)					

Appendix Table III B (cont'd)

		A	B	C	D	E	F	G
Bluff Springs (c)	2		-(8)		-(8)			
Carrizo Springs 3	1			+(1)			+(1)	
				-(1)			-(2)	-(1)
				Int(1)				
Claflin (b)*	1		-(4)					
Commerce	3		-(4)					
Cotulla	2					-(3)		
Denton	2							
Dilley				+(3)			+(1)	
				-(1)			-(2)	-(1)
Encinal 2	1		-(8)					
Encinal 3	2	-(2)	-(5)	-(5)				
La Pryor				+(1)			+(1)	
				-(2)			-(3)	-(1)
				Int(2)				
Laredo 2	2							
Moody	2		-(8)		-(11)			
Moulton	1		-(8)					-(2)
Padre Island	1							
Red Oak*	1		-(7)					
			Int(1)					
Refugio*	2					-(3)		
Rosston (a)	1		-(8)					
H. debilis cucumerifolius								
College Sta. (b)	4			+(5)			+(1)	
		-(2)	-(4)					
			Int(1)					

Appendix Table III B (cont'd)

		A	B	C	D	E	F	G
Pearsall 3	1						+(2)	
			-(10)					-(3)
Yoakum							+(1)	
H. debilis silvestris								
Caldwell	2				+(2)			
Jewett	2			+(3)			+(3)	
			-(2)				-(3)	-(3)
				Int(1)				
Neches River	2						+(1)	
							-(1)	-(3)
Oakwood	2			+(2)			+(2)	
							-(3)	-(2)
				Int(2)				
H. petiolaris								
Ellsworth	4		-(4)					
Higgins*	3		-(5)		-(6)			
			Int(3)		Int(2)			
Red River	4	+(1)						
		-(1)	-(5)	-(7)				
Wellington	4						+(1)	
								-(2)
Wheeler	4					+(1)		
						-(2)		
H. praecox hirtus								
Carrizo Springs 4	2		+(3)	+(4)				
		-(2)	-(3)	-(1)				
H. praecox praecox								
Gilchrist 1*	1				+(6)			
		-(2)	-(12)		-(2)			
			Int(1)		Int(3)			

Appendix Table III B (cont'd)

		A	B	C	D	E	F	G
Gilchrist 2*	1		-(5) Int(3)		+(8)			
H. praecox runyonii Falfurrias*	2						+(1)	-(3)
Perennial Species								
H. giganteus Nashville	1		-(1)					
H. grosseserratus Mt. Pleasant	1		-(1)					-(4)
H. tuberosus Tecumseh (b)	3		-(1)					-(1)
Cultivated Sunflowers								
College Sta. Nursery	3			+(1) -(2)			+(1) -(2)	-(1)

[1] + = susceptible reactions; - = resistant reactions; Int = intermediate or ambiguous reactions.

Number of plants tested shown in parenthesis.

*Reactions differed in different tests, see also Appendix Table I.

Appendix Table IV. High resolution mass spectrum of Blue 1[1]

Measured Mass (m/e)	Relative Intensity	Atomic Constituents Cl2/13	H	O	N
290[2]	0.2	15/0	16	5	1
264	2.7	9/0	16	7	2
263	17.9	14/0	15	5	0
251	2.3	14/0	7	3	2
250	1.3	11/0	8	6	1
207	3.9	10/0	7	5	0
206	1.1	7/0	14	5	2
205	1.1	15/0	11	0	1
192	2.1	10/0	8	4	0
150	7.1	3/0	6	5	2
149	100.0	8/0	5	3	0
137	1.4	10/0	17	0	0
133	1.5	10/0	13	0	0
133	10.6	6/0	13	3	0
132	4.4	8/0	4	2	0
129	1.3	7/0	13	2	0
125	1.6	9/0	17	0	0
123	2.5	9/0	15	0	0
122	1.3	7/0	6	2	0
121	1.3	9/0	13	0	0
121	1.7	7/0	5	2	0
119	1.2	9/0	11	0	0
111	2.9	8/0	15	0	0
110	1.7	8/0	14	0	0
109	3.3	8/0	13	0	0
105	1.4	8/0	9	0	0
105	6.2	7/0	5	1	0
104	10.4	7/0	4	1	0
101	1.5	5/0	9	2	0
99	1.9	7/0	15	0	0
98	4.4	6/0	10	1	0
97	7.1	7/0	13	0	0
97	1.3	6/0	9	1	0
96	2.3	7/0	12	0	0
95	8.4	7/0	11	0	0
93	2.1	7/0	9	0	0
91	11.3	7/0	7	0	0
87	1.5	4/0	7	2	0
85	7.9	6/0	13	0	0
84	2.9	6/0	12	0	0

Appendix Table IV (cont'd)

Measured Mass (m/e)	Relative Intensity	Atomic Constituents Cl2/13	H	O	N
84	2.3	5/0	8	1	0
83	14.3	6/0	11	0	0
82	5.2	6/0	10	0	0
81	9.2	6/0	9	0	0
79	1.5	6/0	7	0	0
78	1.2	6/0	6	0	0
77	5.1	6/0	5	0	0
77	16.7	2/0	5	3	0
76	4.6	6/0	4	0	0
73	8.9	3/0	5	2	0
71	16.7	5/0	11	0	0
71	1.8	4/0	7	1	0
70	8.8	5/0	10	0	0
69	22.8	5/0	9	0	0
68	6.0	5/0	8	0	0
67	9.5	5/0	7	0	0
66	2.7	4/0	2	1	0
65	3.8	5/0	5	0	0
61	2.7	2/0	5	2	0
60	3.1	1/0	4	1	2
60	11.0	2/0	4	2	0
59	1.4	1/0	5	0	3
53	2.4	3/0	3	0	1

[1]Analysis by Shrader Analytical and Consulting Laboratories, Inc., Detroit, Michigan.

[2]Parent ion. Other fragments with a Relative Intensity of less than 1.0 have been omitted.