# PIGMENTS ASSOCIATED WITH SOME STRAWBERRY LEAF DISEASES

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This is to certify that the

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ABSTRACT

PIGMENTS ASSOCIATED WITH SOME
STRAWBERRY LEAF DISEASES

by Maurice A. Veenstra

The reddish-purple pigments associated with some strawberry leaf diseases were investigated to 1) determine the chemical nature of the pigments involved, 2) ascertain whether the pigments were host or parasite products, 3) determine the function of the pigments in the disease picture.

Pigments were extracted from strawberry leaves diseased with leaf spot, leaf blight, and leaf scorch, caused by *Mycosphaerella fragariae*, *Dendrophoma obscurans*, and *Diplocarpon earliana*, respectively. Similar-appearing pigments were also extracted from cultures of *M. fragariae* for comparison with that found in diseased leaves. The pigments were purified chromatographically. The leaf pigments were identified by ascending paper chromatography of the whole molecules and their hydrolytic products along with authentic markers in several solvent systems. A suitable solvent system was not found for the chromatography of the *Mycosphaerella* pigments. Absorption spectra of the pigments were also compared with those of strawberry fruit anthocyanins.

The pigments from diseased leaves were identical to

each other and to one of the anthocyanins from strawberry fruit (cyanidin-3-monoglucoside). The fungal pigments were not identified, but several of their physical characteristics are described. No pigment corresponding to the fungal pigments was found in leaf spot-infected leaves. The pigment in diseased leaves is considered a host product.

A bioassay was conducted comparing the growth of M. fragariae with that of D. obscurans in media containing the pigments and their precursors and breakdown products. Although M. fragariae which causes a restricted lesion was inhibited by phloroglucinol, cinnamic acid, and somewhat by protocatechuic acid, and D. obscurans which causes a non-restricted lesion was stimulated by protocatechuic acid, results are not extensive enough to permit definite conclusions concerning the role these compounds might play in determination of lesion size in the diseased leaf.

# PIGMENTS ASSOCIATED WITH SOME STRAWBERRY LEAF DISEASES

By

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To my three affectionate children,

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# INTRODUCTION

Red or purple pigmentation is a part of the syndrome of many plant diseases. The pigmented halos surrounding the lesions of the leaf diseases of strawberry are common examples. Conceivably, this pigment could be produced by either host or parasite as a general or specific response to one another. While many facets of host-parasite relationships have been explored in detail, the study of the pigments associated with plant diseases, their nature, origin and function has been largely ignored. This investigation was initiated to 1) determine the chemical nature of the pigments involved in strawberry leaf spot primarily, but also in other strawberry leaf diseases; 2) ascertain whether the pigment is produced by host or parasite or both, and if it is the result of a specific or general response; 3) find some clue as to the role of the pigment in the disease picture.

The coloration of strawberry fruit was long ascribed to an orange-red anthocyanin, callistephin, (pelargonidin-3-monoglucoside) (81, 82, 88). More recently a second and minor anthocyanin was isolated and identified as chrysanthemin (cyanidin-3-monoglucoside)(63). Although strawberry fruit is now considered a standard source of both these

pigments for identification purposes (50), at the time this study was begun there was no report in the literature describing the pigments in strawberry leaves. Bate-Smith (13) described leuco-anthocyanins (colorless compounds closely related to anthocyanins) in Fragaria as part of a survey of these compounds in the plant kingdom.

Paper partition chromatography, after its introduction into the study of plant pigments by Bate-Smith (11, 12), became an almost indispensable tool in the separation and identification of water soluble pigments such as anthocyanins (47, 1). The characterization of an unknown anthocyanin involves not only comparing the whole molecule with standard markers, but also, after acid hydrolysis, identifying the aglycone and determining the number and kinds of sugars present. Paper chromato--graphy serves admirably for this purpose at each step in the process. Spectrophotometry is commonly used in conjunction with chromatography to assist in the characterization of anthocyanin pigments (62, 48, 68). By this technique the number and position of attachment of sugars can be determined, and whether the anthocyanin has adjacent hydroxyl groups in the "B" ring. Both of the above methods were used in this study.

Mycosphaerella fragariae (Tul.) Lindau, the causal organism of strawberry leaf spot produces pigments in culture which are very similar in color to the pigment found

on diseased strawberry leaves (Fig. 1). These pigments were thought to be an anthocyanin by Palchefsky & Allison (69). If so, this would be of great interest, for anthocyanins and other flavonoids are not known to occur in microorganisms. Although fungi synthesize flavonoid precursors, they do not possess the ability to combine these into $C_6$-$C_3$-$C_6$ compounds as do the higher plants. Only a few reliable reports exist of the occurrence of anthocyanins in lower plants, viz., those of Bendz et al (16, 17) concerning mosses of the genus Bryum. Peterson et al (72) extracted from a soil fungus a pigment which had some properties resembling those of anthocyanidins but also those of anthraquinones (49). Considering its solubility properties, it is not likely an anthocyanidin.

Kuyama and Tamura (59, 60, 61) isolated a deep red polycyclic hydroxyquinone from Cercosporina kikuchii M. et T., a fungus which causes the purple speck disease of soybean. This pigment, which they named cercosporin, was also identified in infected soybean tissue. In view of this finding, the question arose as to whether the M. fragariae pigment might also be at least partly responsible for the pigment associated with strawberry leaf spot. If so, the unknown pigments associated with other strawberry leaf diseases must be of different chemical nature.

The function of the water soluble pigments found in green plant tissues is still largely a matter of surmise. The pigments themselves cannot be involved in

establishment of disease, since they appear late in the process. The pigments or their precursors or breakdown products could possibly be involved in determining lesion size, however. Cunningham (28) found that wounded leaves of Fragaria virginiana Duch. formed a cicatrice around the edge of the wound. Mesophyll cells multiplied, enlarged and became suberized. None of this activity took place in leaves infected by M. fragariae, although in the "plesionecrotic" zone around the dead area the epidermal cells contained a substance which stained deeply with haemotoxylin. Parasitic hyphae were limited to the necrotic tissue of the spot.

Contrasting with leaf spot, the leaf blight lesion caused by Dendrophoma obscurans (E. and E.) H. W. Anderson spreads until it often kills the entire leaflet. Possibly the pigment in diseased leaves, or its precursors or breakdown products inhibits the growth of M. fragariae but not of D. obscurans. This would offer one explanation for the difference in size of the lesions caused by the two fungi. The bioassay in this study was conducted in the hope that it would shed some light on this subject.

Figure 1. Pigments of strawberry leaf spot and *Mycosphaerella fragariae*. Top, pigment extracted from infected leaves. Middle, infected leaves. Bottom, *M. fragariae* on potato dextrose agar with pigment diffusing into the medium.

# LITERATURE REVIEW
# OF
# STRAWBERRY LEAF SPOT, LEAF SCORCH, AND LEAF BLIGHT

## Leaf Spot

This disease is found wherever there are strawberries, either cultivated or wild. It is widespread in North America and Europe and has been reported from several countries in South America, Asia, and Australia (78). Anderson (9) considers it indigenous to America on the wild species. The Tulasne brothers (92) reported the disease in Europe as early as 1863, and mentioned that it was observed frequently every year in France. Reports of leaf spot in this country in the 1880's indicate that the disease had been present for many years. Trelease (91) mentioned that leaf spot had been destructive for several years. Garman (43) stated "Probably no other enemy of strawberry plants does so much injury year after year as the fungus which causes the familiar spot disease of the leaves". Earle (34) named leaf spot (known as rust, blight, and sunscald) the most important disease of strawberries and proposed the name "white rust".

Leaf spot is economically important wherever climatic conditions are favorable for disease and susceptible varieties are grown. As mentioned above, leaf spot has repeatedly been referred to, especially in the earlier literature, as the most important disease of strawberries.

When loss occurs, it consists of reduction in total yield, lower grade fruit, and weak runner plants, which in turn cause a reduced yield the next season. Spraying experiments conducted by Plakidas (73) in Louisiana in 1930, 1931 illustrated the destructiveness of this disease. In 1930 the plots sprayed with Bordeaux (4-4-50) gave a 64% higher yield than the unsprayed plots. In 1931 the difference was 169%. Leaf spot is more severe in the southern states than in the North. Louisiana growers must spray in order to produce a profitable crop (78). In most states, however, the disease is not an annual problem. In 1957 epiphytotics were reported from Arkansas (30) and Michigan (40). Rainy, cool weather prevailed during the early part of the season in both states. Total crop loss was experienced in some areas in Arkansas. In recent years leaf spot has become much less important than in the past because most of the new varieties carry some measure of resistance.

Lesions most commonly appear on the leaf blades, but also may occur on the petioles, fruit stems, calyxes, and fruit. When they first appear, the spots are small, round, and uniformly purplish in color. As the spots enlarge, the centers become necrotic and eventually turn white. This characteristic distinguishes leaf spot from other strawberry leaf diseases. The mature lesion consists of the white necrotic center surrounded by a narrow band of brown dead cells. Immediately adjacent to the brown

area the living cells are very dark purple and have lost their turgidity. Progressing outward the cells become less pigmented and more turgid until the purple color fades into the normal green. The white center is very thin and sometimes tears, leaving a hole. The spots usually get no larger than 1/8 - 1/4 in. in diameter. They tend to be circular except when they lie next to a large vein or the edge of the leaflet or when they coalesce. Lesions on petioles and fruit stems are elongate. Calyx infection results in drying and browning of this tissue. Infection of the fruit is thought by Demaree and Wilcox (32) to enter through the stigma. The affected achenes and surrounding tissue turn dark in color. This condition is known as "black seed".

The causal organism of strawberry leaf spot is Mycosphaerella fragariae (Tul.) Lindau. The asexual stage is known as Ramularia tulasnei Sacc. The Tulasne brothers named the perfect stage Stigmatea fragariae (92). Saccardo (83) renamed it Sphaerella fragariae. Lindau gave the fungus its present name of Mycosphaerella fragariae.

For many years the connection between the Mycosphaerella and Ramularia stages was based upon the fact that the Tulasne brothers had observed both stages in the same lesions. Dudley (33) added more circumstantial evidence when he observed conidial production on the surface of a perithecium. It was not until 1941 that cultural evidence for the connection of the two stages was pub-

lished. Plakidas (75) observed conidial production in cultures made from single ascospores.

Perithecia average about 100 $\mu$ in diameter (75). They are black, globose, erumpent, mostly epiphyllous. Dudley (33) declares them to be ostiolate and includes a drawing showing them as such, but Plakidas (78) claims that they have no ostiolum. The asci are clavate, fasciculate, eight-spored, and measure 10-15 x 30-45 $\mu$ (75). Ascospores are fusiform, hyaline, 2-celled, and 3-4 x 15 $\mu$ (83). They germinate in the ascus (33, 89, 38). Conidia range in size from 2-5.5 x 12-85 $\mu$ (20), but more commonly measure 3-4 x 20-40 $\mu$. They are hyaline, cylindrical, 1-3 celled.

A sclerotial stage has been reported as occuring in large numbers on infected dead leaves during the winter (33, 91). The sclerotia resemble perithecia in size and shape, but have no cavity. In the spring they germinate to form conidia.

A spermagonial stage may also occur, although this has not been demonstrated. Other species of Mycosphaerella are known to produce spermagonia (52, 53, 54).

Various races of the fungus have been described. Palchefsky & Allison (69) separated several types on the basis of colony color and ability to color the substrate. Bolton (20) found that, when isolating M. fragariae, each strawberry variety gave rise to a more or less distinct cultural type. The instability of the fungus was noted by

these authors. This writer also has repeatedly observed the marked proclivity of M. fragariae to form sectors in culture. Plakidas (77) found pathogenic variation among isolates from various areas in the United States. Bolton (21) grouped 49 isolates into six races according to their pathogenicity on nine strawberry varieties.

As far as is known, the hosts of M. fragariae are limited to the genera Fragaria and Potentilla. Fall (38) isolated from Potentilla anserina L. a leaf spot fungus which was morphologically similar to Mycosphaerella fragariae, but which would not attack strawberry. She found that P. monspeliensis L. was readily infected by M. fragariae, but P. reptans L. and P. canadensis L. were not infected by either fungus. If these two fungi are shown to be taxonomically identical, they will fit Ainsworth and Bisby's (2) criteria for physiologic races.

The leaf spot fungus overwinters in various ways. In northern latitudes it may survive in the mycelial form in strawberry leaves which have remained alive and green, or it may be found in the perithecial or sclerotial stage on dead leaves. In the South where no ascigerous or sclerotial stages occur, and where strawberry plants remain green year-round, infection by conidia is a continuous process. Both Plakidas (78) and Anderson (9) claim that primary infection can result from ascospores as well as from conidia. However, many authors, Anderson included, state that the ascospores germinate within the ascus (38, 33, 89). Dud-

ley (33), the only author to illustrate a perithecium with an ostiole also describes how the ascospores germinate while still in the perithecium. He includes an illustration showing germ tubes crowding out of the ostiole. Conidia were produced by the ascosporic mycelium. After sowing ascospores on strawberry leaves Dudley observed germ tubes ramifying over the epidermis, but no penetration occurred and no infection resulted. The spores were probably placed on the upper surface of the leaves, and this could account for the lack of infection (74). Plakidas (78), on the other hand, states that ascospores are forcibly ejected into the air, although he mentions no observation of this phenomenon. It seems likely that most, if not all, primary infection in the spring occurs from conidia produced by overwintered mycelium, germinating sclerotia, and ascosporic hyphae. The importance of the sexual stage lies in the production of new genetic types rather than in its being a source of infection.

According to Dudley (33) infection may occur through both upper and lower leaf surfaces, and the germ tube enters by direct penetration rather than through stomates. The findings of Plakidas (74) are at considerable variance with those of Dudley. Plakidas in a series of experiments involving 30 plants of the Klondike and Marshall varieties found that infection occurred almost entirely through the lower epidermis and that penetration was completely stomatal. His photographs show germ tubes enter-

ing stomates and curling around and between mesophyll and palisade cells. In the Klondike variety the number of stomates on the upper surface was 5.5 per $mm^2$ compared to 265.6 per $mm^2$ on the lower surface. Considering the number of stomates alone, the probability is about 50-1 that infection will occur on the underside of the leaf. In several other varieties examined no stomates were found on the upper leaf surface.

After entering a stomate the germ tube ramifies upward through the mesophyll and palisade tissue. The mycelium remains intercellular without haustoria (74). As the mycelium develops, the adjacent leaf cells die, dry up, and finally fill with air (33, 91). This last change is what imparts the characteristic whitish appearance to the center of a spot. This central area is only about 1/5 as thick as as the healthy portion of the leaf (33). Trelease (91), after microscopic examination of leaf spot lesions, stated that the purplish pigment surrounding the necrotic area occurred only in the epidermal cells. According to Cunningham (28), who made a detailed histological study of several leaf-spotting diseases, the cells at the outer edge of the necrotic center are filled with a brown tannin-like substance. Fungal hyphae did not extend beyond these cells. There was no evidence of anycicatrice formation. Although Anderson (9) states that conidia are produced in the area of the pigmented halo, observations of this writer, as well as those of Garman (43), Dudley (33), and Fall (38), show

that sporulation occurs only on the dead central area. These observations agree with Cunningham's statement.

Prolonged periods of cool, damp weather which usually occur in spring and fall are required for infection (30, 40). The optimum temperature for disease development is 65-72° F (73). Incubation period is 10-14 days. Conidia are produced in abundance and spread mostly by wind and splashing rain (73). Fall (38) discovered by a series of inoculation experiments with several varieties that only the middle-aged leaves are very susceptible, the young leaves being entirely resistant, and the older ones nearly so. In late fall in the northern regions sclerotia and perithecia develop in the old, dying leaves. Perithecia mature in March and April (33).

Strawberry leaf spot can be controlled in two ways: 1) fungicidal sprays, 2) resistant varieties. For years Bordeaux and the fixed coppers were recommended and used (73, 76, 90, 23). They generally give good control but they have the disadvantage of staining the fruit, and therefore cannot be used close to harvest. In recent years the organic fungicides have largely replaced the coppers. Cox and Winfree (25) found nabam (disodium ethylene bisdithiocarbamate), zineb (zinc ethylene bisdithiocarbamate), and dyrene (2,4-dichloro -6-O-chloroanilino -s- triazine) were very effective but slightly phytotoxic. Zineb, however, increased susceptibility of the fruit to gray mold. Horn (55) found that captan (N - trichloromethylthio -4- cyclo-

hexene -1,2- dicarboximide) controlled leaf spot as well as gray mold, without any phytotoxicity. Captan's tolerance permits its use even during harvest (95). The recommendations for Michigan in 1966 call for a mercury spray in fall or early spring, followed by captan or thylate (bisdimethythiocarbamoyl disulfide) beginning when blossom buds are visible in the crown and continued through harvest (95).

Many resistant varieties are now available, but their levels of resistance vary from one geographical area to another due to races of the pathogen (21, 77). In Michigan, Fulton (40) observed that Catskill, Premier, and Robinson were highly resistant, while Sparkle, Redglow, Paymaster, and Fairland were very susceptible. Evaluations at Purdue showed Aroma, British Sovereign, Catskill, Crimson Flash, Empire, Fairfax, and Midland to have lowest incidence of infection when both leaf spot and leaf scorch were considered. The newest list of varieties from Arkansas (67) has Aroma, Robinson, Surecrop, Catskill, Fairfax, Midland, and Premier as resistant, and Blakemore, Earlidawn, Sparkle, Midway, Redglow and Stelemaster as susceptible.

Besides spraying and the use of resistant varieties, plant disease control should always include good cultural practices. Among these are planting in well-drained soil, weed control, and care in spacing runner plants. Transplants should always have the old leaves re-

moved before setting. This practice alone may eliminate the disease during the first season (9).

Leaf Scorch

Leaf scorch, like leaf spot, is found wherever strawberries occur. It was first reported from Europe in 1832. In the U.S. it was collected from Illinois in 1883 (36) and from New York the following year (71), both collections having been made from wild strawberries. The disease was reported by Dudley in 1889 (33) as economically important on cultivated strawberries in New York, and later Clinton (24) mentioned that it had caused considerable damage in Connecticut plantings. Since then, leaf scorch has been recognized throughout the strawberry growing areas of the United States and Canada as a potentially important disease. Like leaf spot, it is more often damaging in the South than in the North.

Losses from leaf scorch result from reduced efficiency of diseased leaves, and from infection of calyxes and fruit stems which gives reduced yield and unsightly berries. It is difficult to place a dollar value on losses from this disease though, for several reasons. In the first place, leaf scorch usually occurs in conjunction with other foliage diseases. Further, where strawberries are grown as a perennial crop, a heavy infection in one year may, by reason of weakened plants, cause a reduced yield the next year. Also, weather conditions cause the disease level to vary greatly from year to year. Another

type of loss which is difficult to estimate occurs when a good variety is replaced, because of its susceptibility to leaf scorch, by a less desirable one which is resistant. For example the popular, but highly susceptible, Klondike variety was replaced in southern Illinois by Missionary, although the latter was not as well adapted to that area (9). When Klondike was the leading variety in Louisiana, Plakidas (78) estimated the annual reduction in yield at five to ten percent. Wolf (93, 94) reported an average loss of 25% in North Carolina in 1922.

Symptoms may appear on leaf blades, petioles, pedicels, and calyxes. Young leaf scorch lesions resemble those of leaf spot at an early stage. Small, purplish spots develop into irregularly circular blotches 1-5 mm in diameter. When mature, the lesions never exhibit the white centers typical of leaf spot, but instead black acervuli become apparent in the central area. Often the lesions are so numerous as to coalesce, giving the entire leaflet a purplish cast. When this happens the leaflet dries up, its edges curling upward, and takes on a burned appearance (9, 78). Hence the name leaf scorch. Infected calyxes wither and die, resulting in less attractive fruit.

The fungus causing leaf scorch is Diplocarpon earliana (E. & E.) Wolf. Ellis & Everhardt (36) described and named the ascigerous stage Peziza earliana. After some revision, Wolf, noting the similarity of the strawberry

fungus to the fungus causing black spot of rose, Diplocarpon rosae, transferred the strawberry fungus to the same genus under its present name (93). The imperfect stage is sometimes referred to in modern pathology literature (9, 78) as Marssonia fragariae, the name given by Saccardo in 1896. The correct name, however, is Marssonina fragariae (E. & E.) Wolf, since Magnus changed the genus Marssonia to Marssonina in 1906 (65). This transfer is recognized by mycologists (3, 18).

Host plants of the fungus are restricted to the genus Fragaria (93).

Conidia are produced in acervuli which occur most frequently on the upper surface of the leaves (74). The acervuli are visible as black glistening dots 0.1 - 0.2 mm in diameter. Conidia are two-celled, curved, the upper cell beaked, hyaline, constricted at the septa, guttulate, and measure 5-7 x 18-30 $\mu$ (93). Apothecia develop on the under surface of dead, overwintered leaves. Asci are oblong with apical papillae, 15-20 x 55-70 $\mu$ (93). The ascospores are hyaline, elliptical, unequally two-celled, and measure 4-4.5 x 20-22 $\mu$ (93). Spermatia have also been described by Wolf (93, 94) who considers them to be vestigial male cells.

In the spring as new strawberry leaves are unfolding, ascospores are discharged in great numbers. Plakidas (78) and Wolf (93) believe that ascospores are the chief source of primary infection of new foliage in the North.

In the South, however, strawberries maintain their growth throughout the winter and, as in the case of leaf spot, the perfect stage of the fungus does not occur. There conidia constitute the sole source of inoculum. Given favorable environment, ascospores germinate in about 24 hr and the germ tube penetrates directly through the epidermis, in contrast to the stomatal infection of Mycosphaerella fragariae (93). Wolf assumed that infection took place through the upper epidermis, but the more careful observations of Plakidas (74) showed that infection occurs predominantly, if not entirely, through the lower epidermis. The fungus grows intercellularly through the mesophyll and palisade cells and into the epidermal layer where it forms the subcuticular stroma which gives rise to the acervuli. Both Wolf (93) and Plakidas (74) report the occurrence of club-shaped haustoria. The entire process from infection to conidial production may take place within a two-week period (9). Conidia are produced throughout the growing season, and strawberry leaves are susceptible at any stage of growth (31). Repeated infections may occur whenever weather conditions are favorable. Optimum temperature for disease development is 72-80° F, somewhat higher than that for leaf spot (73). Spores are disseminated by wind and rain. Calyx infection may be brought about by pollinating insects (9).

Control measures are essentially the same as those for leaf spot (58, 66, 95).

Leaf Blight

In contrast to leaf spot and leaf scorch, most writers consider leaf blight a minor disease (78, 23, 29). For the most part it affects the older leaves in midsummer or later, after the fruit has been harvested. It may, however, destroy much of the foliage late in the season (9). This weakening of the plants must be reflected in yield the next year, but there are no published estimates of crop losses.

Leaf blight was first reported in 1895 by Ellis and Everhardt (37) who named *Phoma obscurans* as the causal organism. Their specimens came from West Virginia and New Jersey. Halstead (45) may have been speaking of leaf blight when he reported a new strawberry disease collected from New York and New Jersey in 1892. He regarded the fungus as a species of *Aposphaeria*. In 1920 Anderson (8) reported a rather severe outbreak of the disease in Illinois. He named the disease leaf blight and renamed the fungus *Dendrophoma obscurans*. Fall first reported leaf blight from Canada in 1951 (38). It is now widespread in North America (78), but no reports have been found of the disease occurring outside this continent.

The disease usually occurs on the leaves but sometimes is found on calyxes (9). Usually not more than five or six lesions occur per leaflet. However, they are much larger than those of leaf spot or leaf scorch. Young spots are uniformly purplish in color and are difficult

to distinguish from young leaf spot and leaf scorch lesions. Spots near veins become elliptical in shape. As the lesions mature they develop three zones: a central dark brown area 2-3 mm in diameter, a light brown intermediate area about 5 mm in width, and an outer purplish zone which fades into normal green (8). When a spot originates on a main vein, a wedge-shaped lesion is formed, with the necrotic tissue, or at least the purplish area, extending fan-like to the edge of the leaf. This writer observed on cultivated strawberries at East Lansing that often a single lesion would spread until the entire leaflet was killed. Black specks, the protruding necks of the pycnidia, dot the central area of the lesion.

The present name of the causal fungus is Dendrophoma obscurans (Ell. & Ev.) H.W. Anderson. No perfect stage is known. Alexopoulos and Cation (4) once isolated a fungus from strawberry fruit which produced Dendrophoma-like pycnidia as well as perithecia in culture and concluded that the perithecial stage was probably the perfect stage of D. obscurans. In a later report (5) they identified this fungus as Gnomonia fragariae, the perfect stage of Zythia fragariae. The pycnidia are 200-300 $\mu$ in diameter, deeply embedded in the leaf tissue with the neck breaking through the epidermis and extending above (8). The conidiophores are branched. Spores are hyaline, continuous, oblong, biguttulate, and measure 1.5-2 x 5-7 $\mu$. In humid conditions they are exuded in long, thin gelatinous

cirrhi.(8).

The only known hosts for this fungus are species and varieties of the genus Fragaria.

Our knowledge of the disease cycle and etiology of leaf blight is based entirely on the work of Anderson (8), which was limited. The fungus can overwinter as mycelium in lesions on green leaves or by means of spores in pycnidia. Anderson (8) found viable spores in pycnidia on leaves brought into the laboratory every month from September to May. Spores are spread during rainy periods when there is sufficient moisture to dissolve the exuded spore masses. Although the disease does not appear until midsummer, preharvest sprays gave some control, indicating that primary infection may occur early in the season. Maximum infection apparently occurs later, however. The mode of penetration by the fungus is not known. In infection experiments both Anderson (8) and Fall (38) obtained inconsistent results. Even painting both surfaces of leaves with a spore suspension failed to give extensive infection. These results are consistent with the fact that in the field very few lesions occur per leaflet. Fall found infection more severe at 25° C than at 15° or 20° C. This finding also agrees with the observation that leaf blight is a warm-weather disease.

Varieties differ in their susceptibility to leaf blight. Anderson (9) reports that of many varieties tested in Illinois, none were found to be highly resistant, but Dunlap, Robinson, Red Crop, and Premier were considered

very susceptible.

Anderson (8) and Plakidas (78) do not consider the disease important enough to warrant chemical control. Fulton and Cation (41) found that it can be controlled by a single application of an eradicant fungicide (organic mercury) in early spring as the plants are beginning to break dormancy.

## MATERIALS AND METHODS

Leaves:

Leaves were collected both from cultivated varieties of strawberry and from wild strawberry (*Fragaria virginiana*). Collections were made in Berrien, Kent, Manistee, and Ingham counties in Michigan. In the case of diseased leaves, only those showing typical symptoms of a single disease verified by isolation of the causal organism were used. Leaves were kept in separate lots according to disease, *viz.*, leaf spot, leaf scorch, and leaf blight, caused by *Mycosphaerella fragariae*, *Diplocarpon earliana* (E. & E.) Wolf, and *Dendrophoma obscurans*, respectively. The leaves were processed immediately or kept frozen until use.

Standard pigments:

Commercially frozen strawberries were used as the source of pelargonidin-3-monoglucoside (callistephin) and cyanidin-3-monoglucoside (chrysanthemin). Petals of greenhouse-grown red rose (*Rosa hybrida* Schleich. var. Yuletide) were used as the source of cyanidin-3,5-diglucoside (cyanin).

Fungus isolates:

Isolates of M. fragariae and D. obscurans were obtained from diseased leaves of *F. virginiana* in Ingham

county by transfer of surface-sterilized tissue sections from the edge of lesions to commercial potato dextrose agar (PDA). The isolates were sub-cultured and maintained on this medium.

Pigment extraction and purification:

Extraction methods varied as the study progressed. The following methods were used.

A. Strawberry leaves

1) Leaves were homogenized in a solution of methanol acidified with 1-2% hydrochloric acid in a Waring Blendor and centrifuged. The supernatant was washed several times with petroleum ether to remove chlorophyll (47), condensed *in vacuo* below 50° C, streaked on large sheets (18¼ x 22⅛ in.) of Whatman # 3 MM paper, and chromatographed at least three times using different solvent systems (usually *n*-butanol: acetic acid: water [4:1:5] and n-butanol: 2 N hydrochloric acid [1:1]). The pigment was eluted from the papers with methanol acidified with hydrochloric acid and concentrated by distillation at reduced pressure.

2) Leaves were homogenized in a Waring Blendor with 50% aqueous methanol acidified with hydrochloric acid and centrifuged. An aqueous solution of lead sub-acetate was added with rapid stirring to the supernatant (50)

until the pigment was completely precipitated as the green lead salt. After filtration the pigment was redissolved with 10% methanolic hydrochloric acid, concentrated *in vacuo* and purified by chromatography as in method 1.

3) Leaves were homogenized in water acidified with 1-2% hydrochloric acid in a Waring Blendor and centrifuged. The supernatant was added to a 2 x 32 in. cation exchange column containing two lb of Amberlite IRC-50 resin (carboxylic methacrylate), 20-50 mesh (42)(Fig. 2). The column was washed with water, and the pigment eluted with 1% methanolic hydrochloric acid, concentrated *in vacuo*, and purified by chromatography as in method 1.

B. Strawberry fruit

Commercially frozen strawberries were thawed, homogenized in a Waring Blendor and centrifuged. The supernatant was shaken with *n*-butanol acidified with hydrochloric acid in a separatory funnel. The pigment transferred to the butanol layer which was then shaken with a large volume of petroleum ether. This caused the partitioning of the anthocyanin pigment into the small amount of water which was released from the butanol. The aqueous solution was then purified by chromatography

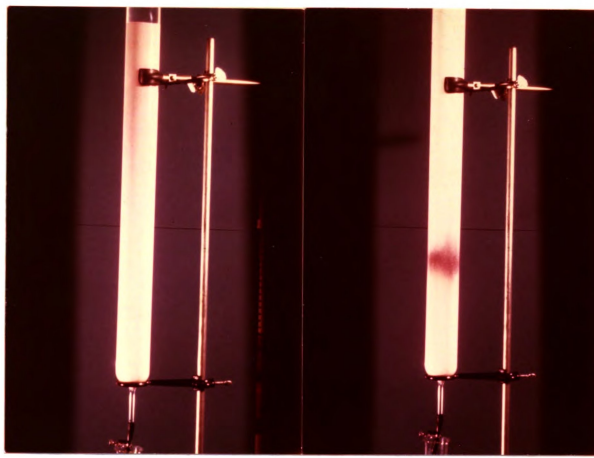


Figure 2. Column of cation exchange resin used for partial purification of anthocyanin pgiments. Left, column loaded with crude anthocyanin. Right, anthocyanin being eluted from column with 1% methanolic HCL.

in the same manner as the leaf pigments.

C. Flower petals

Petals of rose and *Chrysanthemum morifolium* Ramat. var. Woking Scarlet were placed in 2% methanolic hydrochloric acid overnight, after which the liquid was drained off, filtered, and purified by chromatography in the same manner as the leaf pigments.

All the plant pigments were stored in the freezer as concentrated acidified methanolic solutions.

D. Fungus

*M. fragariae* was grown in still culture in commercially prepared Czapek Dox Broth for about four weeks. The pigments were removed from the culture filtrate by shaking with acidified benzene.

Identification of pigments:

A. Chromatography and spectrophotometry of pigments

A large sheet of Whatman #1 chromatography paper was cut in half lengthwise. The pigments were spotted 2 cm apart on a line 2-3 cm from one edge of the paper so that the solvent would proceed against the mill direction of the paper. The papers were hung in a rectangular glass chromatog-

raphy jar in an insulated room. The temperature ranged from 24-26° C. Several hours were allowed for equilibration before the papers were placed in the solvent. It was found that more consistent results were obtained with ascending chromatography than with the descending method, and thereafter the former was used exclusively. Six or more $R_f$ values for each pigment were obtained for each solvent system for comparison. Since anthocyanins are stable only at acid pH, solvent systems containing acid must be used. If the solvents which do not contain hydrochloric acid are used, it is necessary that enough hydrochloric acid be present in the extract to keep it in the chloride form during chromatography (11). Solvent systems used in this study are listed in Table 1.

For spectrophotometry the purified strawberry and rose pigments were streaked on Whatman #3 MM paper and chromatographed in BAW (4:1:5). They were eluted with 0.1 N methanolic hydrochloric acid. The *M. fragariae* pigments in benzene were dried *in vacuo* and redissolved in 0.1 N methanolic hydrochloric acid. Optical measurements were made in a Cary 15 automatic recording spectrophotometer.

B. Hydrolysis of anthocyanins

The minor pigment from the fruit (chrysanthemin) and all the leaf pigments were hydrolyzed

according to the following method. Several drops of the various anthocyanin solutions were added to separate test tubes, each containing 1 ml of 10% hydrochloric acid. The test tubes were held in a boiling water bath for ½ hour. After cooling the samples were poured into small glass funnels, each containing a plug of glass wool in the stem and a small amount of carboxylic methacrylate cation exchange resin (Rexyn 102 H). The sugar moieties were washed through with a small amount of water, collected in beakers, and condensed by warming on a hot plate for several hours. The anthocyanidins were eluted from the resin with methanol acidified with hydrochloric acid.

C. Chromatography of hydrolysates

After hydrolysis the anthocyanidins were immediately spotted on Whatman #1 paper and chromatographed in the "Forestal" solvent (acetic acid: hydrochloric acid: water 30:3:10 v/v)(13). They were chromatographed ascendingly without vapor equilibration of the paper.

The sugars were chromatographed in two solvent systems, viz., ethyl acetate: pyridine: water (8:2:1 v/v) and acetone: n-butanol: water (7:2:1 v/v). The descending method was used, with glucose, galactose, and arabinose as standards. In

both cases the solvent was allowed to run off the serrated edge of the paper. Final disposition of the spots was measured in relation to glucose. In the ethyl acetate: pyridine: water solvent the chromatograms were allowed to develop for over 30 hr to give good separations.

Two methods were used to detect the sugars on developed chromatograms:

1) Silver nitrate dip (19)

One tenth ml of saturated aqueous silver nitrate was diluted with 20 ml of acetone. The precipitated silver nitrate was redissolved by dropwise addition of water. The dried chromatogram was quickly dipped into the reagent, redried and sprayed with 0.5 N sodium hydroxide in ethanol. The excess silver oxide background was removed by dipping the chromatogram in a solution of sodium thiosulfate.

2) Benzidine spray (19)

Dried chromatograms were sprayed with a reagent consisting of 0.5 g benzidine, 200 ml glacial acetic acid, and 80 ml absolute ethanol, and heated at 100-105° C for 15 minutes.

Bioassay of the pigments and their derivatives:

A. Paper disc method

Sterilized filter paper discs (Schleicher & Schuell ½ in. for antibiotic assay) were impreg-

nated with three and twelve drops of a 0.1 M ethanolic solution of the compounds listed in Table 2. Control discs were impregnated with twelve drops of 95% ethanol. All discs were allowed to dry.

Cultures used for inoculum were one week to ten days old. A culture of D. obscurans was homogenized in an autoclaved stainless steel Waring Blendor cup with 50 ml sterile distilled water. One ml of this suspension was added aseptically to sterile plastic Petri plates.

Twenty ml of distilled water were added to a Petri plate culture of M. fragariae. The surface of the colonies was rubbed lightly with a sterile transfer loop to dislodge spores. One ml of this suspension was added to sterile plastic Petri plates.

Twelve ml of PDA at 43° C were added aseptically to each of the inoculated Petri plates. The plates were swirled and the agar allowed to solidify. For each treatment, two discs of different concentrations of the same compound plus one control disc were placed on the agar surface of a plate. The cultures were incubated at room temperature for five days, after which measurements were taken. The experiment was replicated three times and the treatments were duplicated within

each replication.

B. Dry weight method

In the dry weight assay phloroglucinol and protocatechuic acid were used in concentrations of 0.05 M, 0.01 M, and 0.001 M, and cinnamic acid at 0.001 M. Appropriate quantities of the compounds were weighed into Czapek Dox Broth and potato dextrose broth, and held in a water bath at 45° C overnight to dissolve. The solutions were sterilized by passing through a bacteriological fritted glass filter. Sixteen ml of the solutions were aseptically transferred to sterile 125 ml flasks. One half ml of a mycelial suspension of *D. obscurans* or a spore suspension of *M. fragariae* was also added to the flasks which were then incubated on a shaker at 24-26° C. Each treatment was replicated three times, and the experiment was performed three times. After two weeks the cultures were filtered through weighed Whatman #3 filter paper, dried, and weighed.

Table 1. Solvent systems used for chromatography of anthocyanins and hydrolytic products.

| Symbol | Composition | Proportion (v/v) | Use |
|---|---|---|---|
| BAW | n-butanol: acetic acid: water | 4:1:5[a] | Anthocyanins |
| Bu HCl | n-butanol: 2 N hydrochloric acid | 1:1[a] | do |
| 1 N HAc | acetic acid: water | 57:943 | do |
| 30% HAc | acetic acid: water | 30:70 | do |
| 1% HCl | 12 N hydrochloric acid: water | 3:97 | do |
| HAc HCl W | acetic acid: hydrochloric acid: water | 3:1:8 | do |
| Forestal | acetic acid: hydrochloric acid: water | 30:3:10 | Aglycones |
| EA P W | ethyl acetate: pyridine: water | 8:2:1 | Sugars |
| ABW | acetone: n-butanol: water | 7:2:1 | do |

[a] Upper layer was used after aging for three days (12).

Table 2. Compounds used in bioassay.

| Compound | Grade | Source | Quantity/disc | |
|---|---|---|---|---|
| | | | 3 drops | 12 drops |
| | | | g | g |
| Cyanidin-3-monoglucoside | Chromatographically pure | Strawberry leaves | $3.41 \times 10^{-3}$ | $12.8 \times 10^{-3}$ |
| Cyanidin chloride | P(minimum 95% pure) | Mann Res. Labs., Inc. | $2.15 \times 10^{-3}$ | $8.08 \times 10^{-3}$ |
| Caffeic acid | CfP(chromatographically pure) | Calif. Corp. for Biochem. Res. | $1.2 \times 10^{-3}$ | $4.5 \times 10^{-3}$ |
| *trans*-Cinnamic acid | Eastman grade | Eastman Organic Chemicals | $.99 \times 10^{-3}$ | $3.7 \times 10^{-3}$ |
| Phloroglucinol | C.P. | Nutritional Biochemicals Corp. | $.84 \times 10^{-3}$ | $3.15 \times 10^{-3}$ |
| Protocatechuic acid | P(minimum 95% pure) | Mann Res. Labs., Inc. | $1.03 \times 10^{-3}$ | $3.85 \times 10^{-3}$ |

## EXPERIMENTAL RESULTS

Although all plant tissues of the same type, e.g., senescent leaves, were not extracted at the same time or in the same way, the isolated pigment was the same in every case. As the study progressed extraction techniques were varied to decrease the time involved, facilitate the procedure, or to increase the yield of pigment.

At one point in the study it was learned that the concentration of an anthocyanin solution must be done *in vacuo*. A portion of a fruit extract which was concentrated overnight under a stream of air was more bluish in color and had a different $R_f$ value than its counterpart concentrated *in vacuo*. Since anthocyanins have been shown to form bluish metal complexes with iron and aluminum (15), magnesium, potassium, calcium, and copper (51, 87), it is likely that the fruit extract chelated metal ions in the air stream.

An attempt was made to extract anthocyanin from mature, healthy, green strawberry leaves for comparison with the pigment from diseased leaves, but none was found. Similarly, it was thought desirable to obtain pigment from mechanically injured leaves. Leaves of strawberry plants growing in the field were slit with a knife and rubbed with carborundum in an attempt to induce pigmentation, but little or no pigment was produced at the wound sites.

During purification only one band of reddish pigment was evident on chromatograms of any of the leaf extracts. Since its $R_f$ value in BAW was within the range of .20 - .50, it was assumed to be an anthocyanin. Two bands appeared on the chromatograms of the strawberry fruit extract as expected, one of which was in very low concentration. More than 10 lb of frozen strawberries were necessary to produce enough of this pigment (chrysanthemin) for chromatographic analysis. It was almost entirely lacking in some packages of fruit.

The leaf pigments were chromatographed together with the fruit anthocyanins and cyanin, a diglucosidic anthocyanin from red roses (49). Chromatography was ascending. The solvent systems used were of two types, aqueous and alcoholic, to accentuate the differences in the pigments tested (Fig. 3). Solvents BAW, 30% HAc, and HAc-HCl-W gave elliptical spots from the centers of which the $R_f$ values were easily calculated. In Bu HCl the spots tailed to some extent. In solvents 1 N HAc and 1% HCl the spots tailed to such a degree that the centers could not be determined, so $R_f$ values were calculated from the leading edges which were very definite. The $R_f$ values given in Table 3 are averages of six measurements. It is obvious that the minor fruit pigment and the leaf pigments are different from the major fruit anthocyanin as well as that from rose. Since the $R_f$'s of the minor fruit pigment and the leaf pigments do not differ more than .02, it is likely that these compounds are the same. The $R_f$ values of these

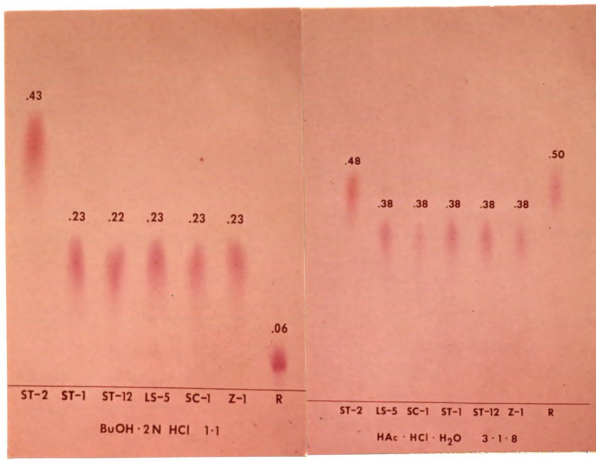



Figure 3. Paper chromatograms of anthocyanins from strawberry and rose. Left, with alcohol solvent. Right, with aqueous solvent. Note difference in positions of rose diglucoside. ST-1 = minor fruit pigment, ST-2 = major fruit pigment, ST-12 = senescent leaves, LS-5 = leaf spot, SC-1 = leaf blight, R = rose.

pigments range from .30 - .32 in BAW. Some $R_f$ values that have been reported for chrysanthemin in this solvent are .30 (70), .32 (63), .33 (14), .34 (64), .38 (47). Co-chromatography of the minor fruit pigment and the leaf pigments gave only one spot. The $R_f$'s of the rose diglucoside show the increased affinity for water over the monoglucosides and the reduced solubility in the alcohol solvents (Fig. 3).

After hydrolysis of the anthocyanins, the aglycones were immediately chromatographed ascendingly in the "Forestal" solvent. A strong mineral acid is necessary for the chromatography of anthocyanidins, since they are unstable at high or neutral pH and fade badly in some solvents. The $R_f$'s of the anthocyanidins from the strawberry leaves and minor fruit pigment agree well and are given in Table 3 and Figure 4.

The sugar moieties were chromatographed descendingly in ethyl acetate: pyridine: water (8:2:1) and acetone: n-butanol: water (7:2:1). The spots were measured in relation to glucose. The $R_g$ ($R_f$ in relation to glucose) values compare well and are much different from those of arabinose and galactose (See Table 3 and Fig. 4).

Spectrophotometric measurements were made of the purified plant pigments and the *M. fragariae* pigments in 0.1 N methanolic HCl. These data are given in Figure 5 and Table 4. In Figure 5 the leaf spot curve is representative of all the leaf pigments. The curve of the diglu-

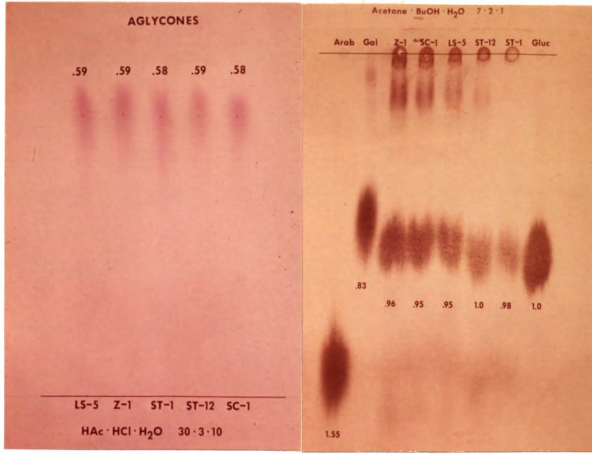



Figure 4. Paper chromatograms of hydrolytic products of strawberry anthocyanins. Left, anthocyanidins. Right, sugars. ST-1 = minor fruit pigment, ST-12 = senescent leaves, LS-5 = leaf spot, SC-1 = leaf scorch, Z-1 = leaf blight.

coside from rose does not appear very different from the curves of the strawberry leaf pigments and the minor fruit pigment. In Table 4, however, the similarities among the strawberry pigments, and the differences between them and the rose pigment are accentuated when the OD at 440 mμ is expressed as the percentage of the maximum OD (49). This is known as the extinction coefficient ratio. Upon addition of $AlCl_3$ the rose pigment, minor fruit pigment, and all the leaf pigments demonstrated a bathochromic shift of their maximum OD's of 9-20 mμ. This indicated the presence of adjacent -OH groups on the aromatic "B" ring (44). The structures of the compounds used in this study are diagrammed in Figure 6.

The fungal pigments are pH indicators which are deep red in acid solution and bright green in basic solution. The pK of the chromophore, or the pH at which the color change occurs, is 8.5 - 9.0.

Because of the way it partitions between acidified benzene and water, as well as its chromatographic behavior, the fungal pigment is believed to consist of three or more compounds all of the same color. When an aqueous solution of the "pigment" is shaken with slightly acidified benzene, part of the pigment transfers to the benzene layer. More highly acidified benzene removes more of the pigment, and still more highly acidified benzene removes all the remaining pigment from the water. For this reason the plural is used when speaking of the fungal "pigment".

The *M. fragariae* pigments are soluble in water, and acid solutions of acetone, iso-amyl alcohol, *n*-butanol, benzene, ether, ethanol, ethyl acetate, methanol.

Preliminary paper chromatography of the fungal pigments was done on Whatman #1 paper strips 2 x 15 cm hung in 125 ml Erlenmeyer flasks. Results are listed in Table 5. The EG W solvent showed promise, but when the pigments were chromatographed descendingly with this solvent on larger papers (9 1/8 x 22½ in.) for 18 hr, they streaked most of the distance to the solvent front. In this solvent as well as those containing diethylamine the streaks appeared to be composed of at least two fractions. Thus, of the solvent systems tested, none was found useful for chromatography of the *M. fragariae* pigments.

Since strawberry fruit is a poor source of chrysanthemin, this anthocyanin was extracted from petals of *Chrysanthemum morifolium* var. Woking Scarlet, purified, and identified by co-chromatography with authentic chrysanthemin. The *Chrysanthemum* pigment was used to augment that from strawberry in the bioassay.

The bioassay of the pigment from diseased leaves and some of its precursors and breakdown products was done in two ways: paper disc method and dry weight method. The average results of the paper disc assay are given in Figure 7. These results show that in some cases the growth of *M. fragariae* was inhibited to a slightly larger extent than that of *D. obscurans*. The anthocyanin and its agly-

cone had no noticeable effect upon M. fragariae, but stimulated aerial growth of D. obscurans which normally does not produce aerial mycelium on PDA. Protocatechuic acid, while causing slight inhibition of M. fragariae, simultaneously caused partial inhibition of the growth of D. obscurans and stimulated the production of aerial mycelium over the same area.

The results of the dry weight assay are given in Figure 8. Two media, one minimal (Czapek Dox), the other more complete (potato dextrose), were used in this test. Little growth was shown by either fungus in Czapek Dox broth, although M. fragariae was stimulated by protocatechuic acid at .01 M and .001 M. In potato dextrose broth D. obscurans still grew only slightly, except in the protocatechuic acid series, in which it was greatly stimulated, even at .05 M. M. fragariae was almost completely inhibited by all treatments except protocatechuic acid at .01 M, where it was somewhat inhibited, and at .001 M where it was comparable to the control.

Table 3. Chromatographic results of anthocyanins isolated from strawberry and rose, and their hydrolytic products.

| Chemical / Solvent | Source | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fruit Major | Fruit Minor | Senescent Leaves | Leaf Spot | Leaf Scorch | Leaf Blight | Rose | Arabinose | Galactose |
| Anthocyanins | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | | |
| BAW | .51 | .30 | .32 | .30 | .31 | .31 | .15 | -- | --- |
| 1 N HAc [a] | .37 | .26 | .26 | .26 | .24 | .25 | .49 | -- | -- |
| 30% HAc | .70 | .63 | .63 | .63 | .62 | .63 | .69 | -- | -- |
| 1% HCl [a] | .22 | .13 | .13 | .13 | .12 | .12 | .23 | -- | -- |
| HAc HCl W | .50 | .37 | .38 | .38 | .38 | .38 | .51 | -- | -- |
| Bu HCl | .39 | .20 | .20 | .20 | .20 | .20 | .05 | -- | -- |
| Anthocyanidins | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | $R_f$ | | |
| Forestal | -- | .58 | .58 | .59 | .59 | .59 | -- | -- | -- |
| Sugars | | $R_g$ | $R_g$ | $R_g$ | $R_g$ | $R_g$ | | $R_g$ | $R_g$ |
| EA P W | -- | 1.00 | 1.01 | 1.02 | 1.04 | 1.04 | -- | -- | .85 |
| A B W | -- | .99 | .97 | .97 | .98 | 1.00 | -- | 1.58 | .85 |

[a] In this solvent calculations were made from the leading edge of the spots.

Figure 5. Absorption spectra of pigments of strawberry, rose, and M. fragariae.

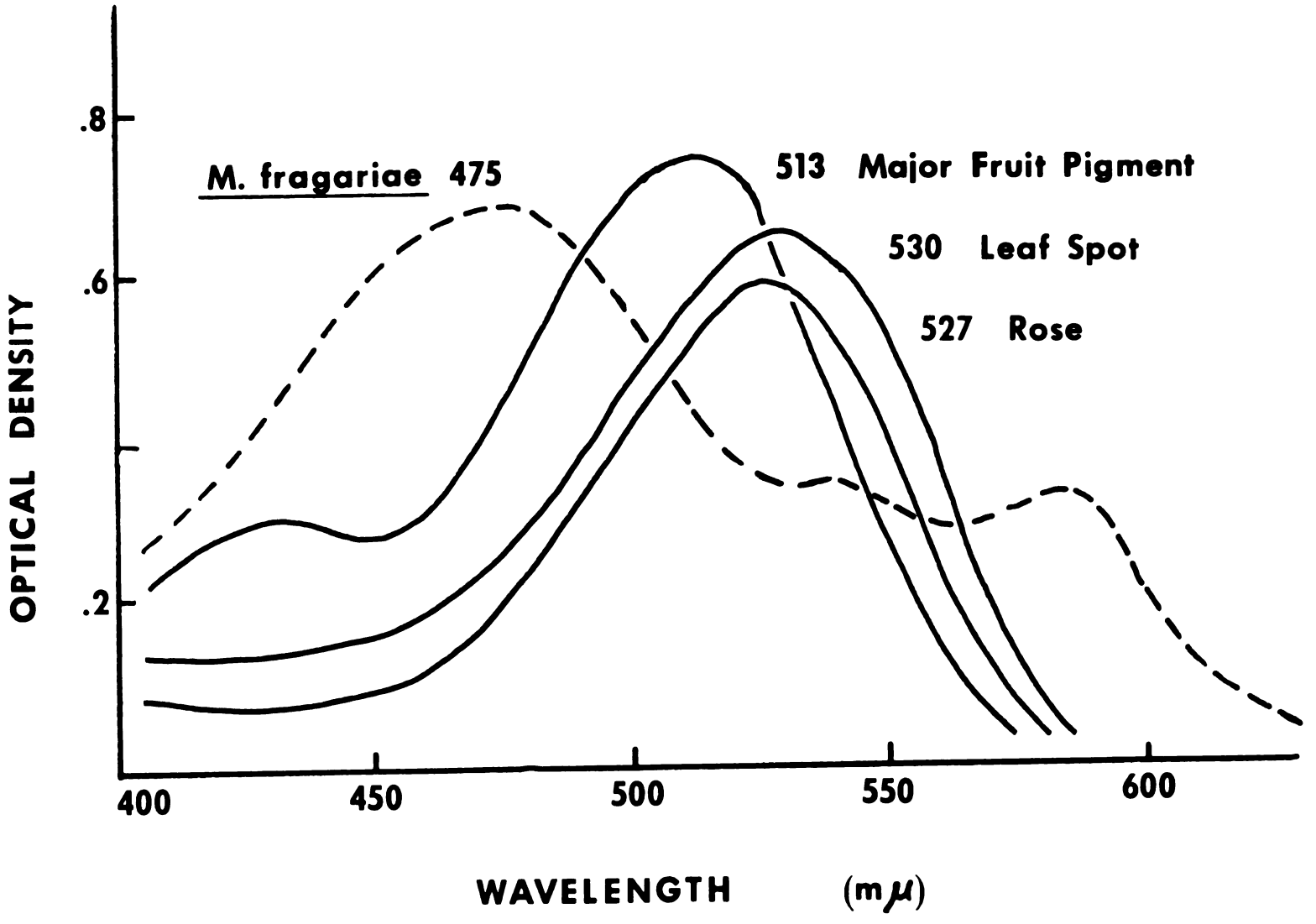
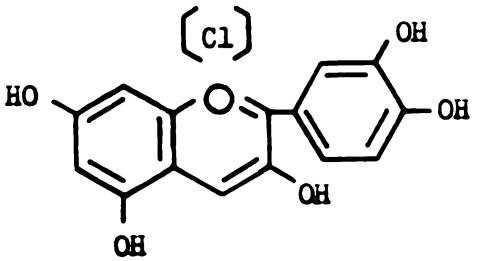


Table 4. Spectral maxima of pigments isolated from strawberry and *M. fragariae*.

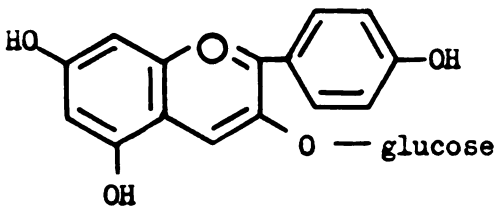
| Pigment Source | Max.[a] (m $\mu$) | $OD_{440}/OD_{max.}$ (as %) |
|---|---|---|
| Fruit (major) | 513 | 40 |
| Fruit (minor) | 529 | 26 |
| Leaf Spot | 530 | 26 |
| Leaf Blight | 530 | 26 |
| Leaf Scorch | 530 | 27 |
| Senescent Leaf | 530 | 26 |
| Rose | 527 | 14 |
| *M. fragariae* | 475 | 75 |

[a] Measured in 0.1 N methanolic HCl.

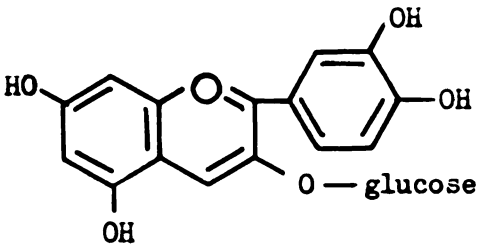
**Figure 6.** Structures of compounds used in this study.



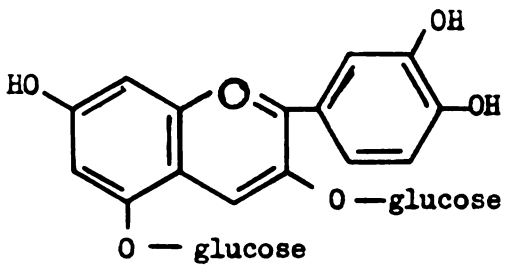

Cyanidin chloride



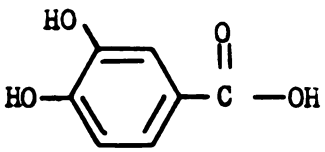

Pelargonidin-3-monoglucoside



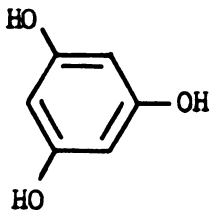

Cyanidin-3-monoglucoside



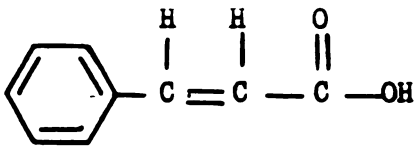

Cyanidin-3,5-diglucoside



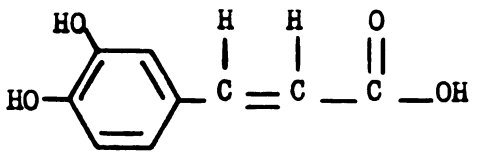

Protocatechuic acid




Phloroglucinol




Cinnamic acid




Caffeic acid

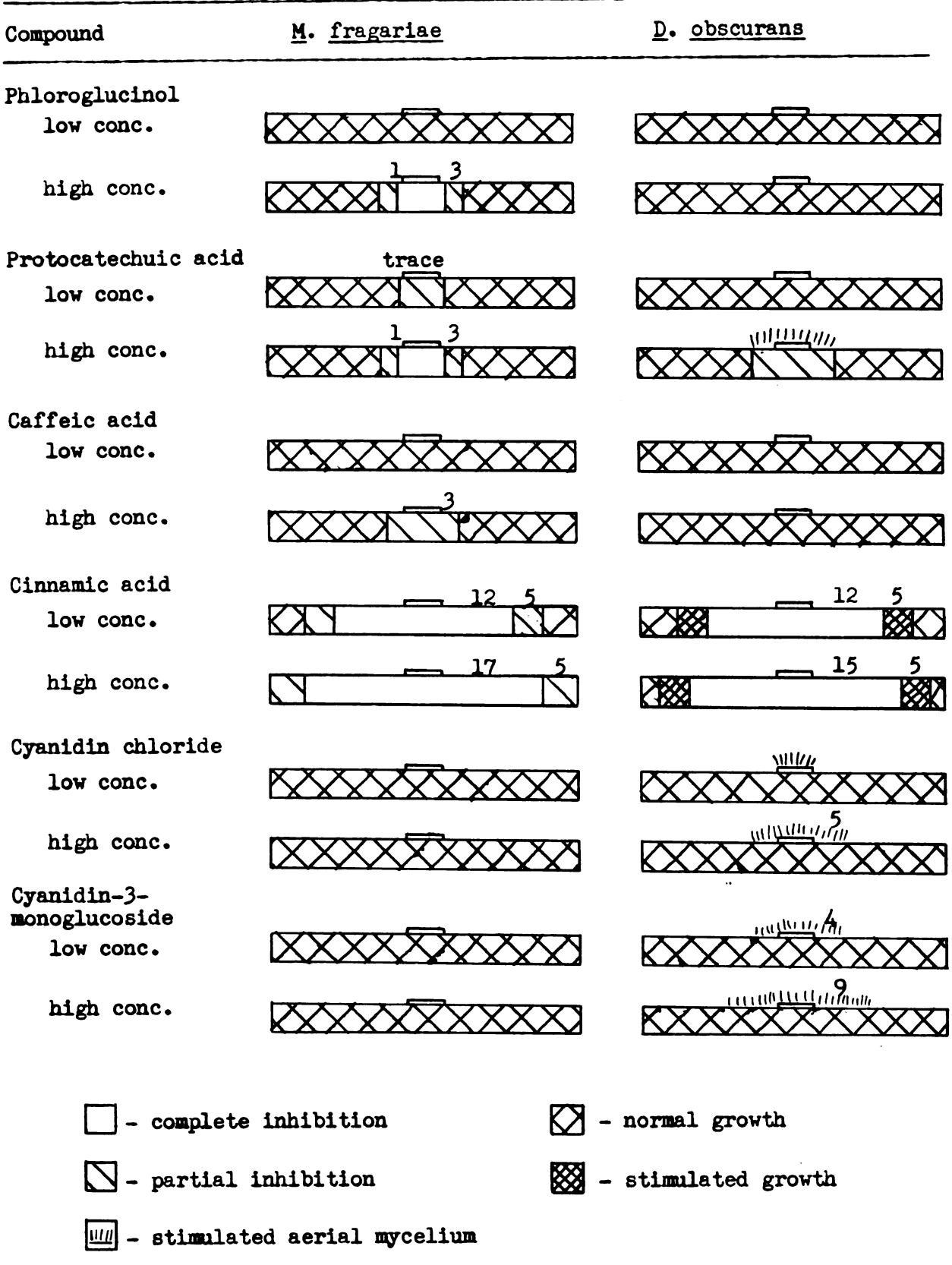
Table 5. Response of *M. fragariae* pigment in various solvent systems.

| Solvent [a] | Response |
|---|---|
| BAW (4:1:5) [b] | Pigment moved at solvent front |
| BAW (1:1:1) | do |
| Bu 2N HCl (1:1) | do |
| P HAc W (20:1:10) | do |
| B HAc H W (2:1:2:2) [b] | do |
| Bz HAc W (2:1:1) [b] | do |
| 1% HAc | Pigment remained at starting point |
| 1 N HAc | do |
| P AH W (8:1:3) | Pigment streaked from start to solvent front |
| B AH W (4:1:2) [b] | do |
| B DA W (10:1:5) | do |
| P DA W (10:1:5) | do |
| P DA W (5:1:4) | do |
| E DA W (10:1:4) | do |
| EG W (3:1) | Compact spot, $R_f$ approx. .75 |

[a] Abbreviations:
P HAc W — n-propanol: acetic acid:water
B HAc H W — n-butanol: acetic acid: hexane: water
Bz HAc W — benzene: acetic acid: water
P AH W — n-propanol: ammonium hydroxide: water
B AH W — n-butanol: ammonium hydroxide: water
B DA W — n-butanol: diethylamine: water
P DA W — n-propanol: diethylamine: water
E DA W — ethanol: diethylamine: water
EG W — ethylene glycol: water

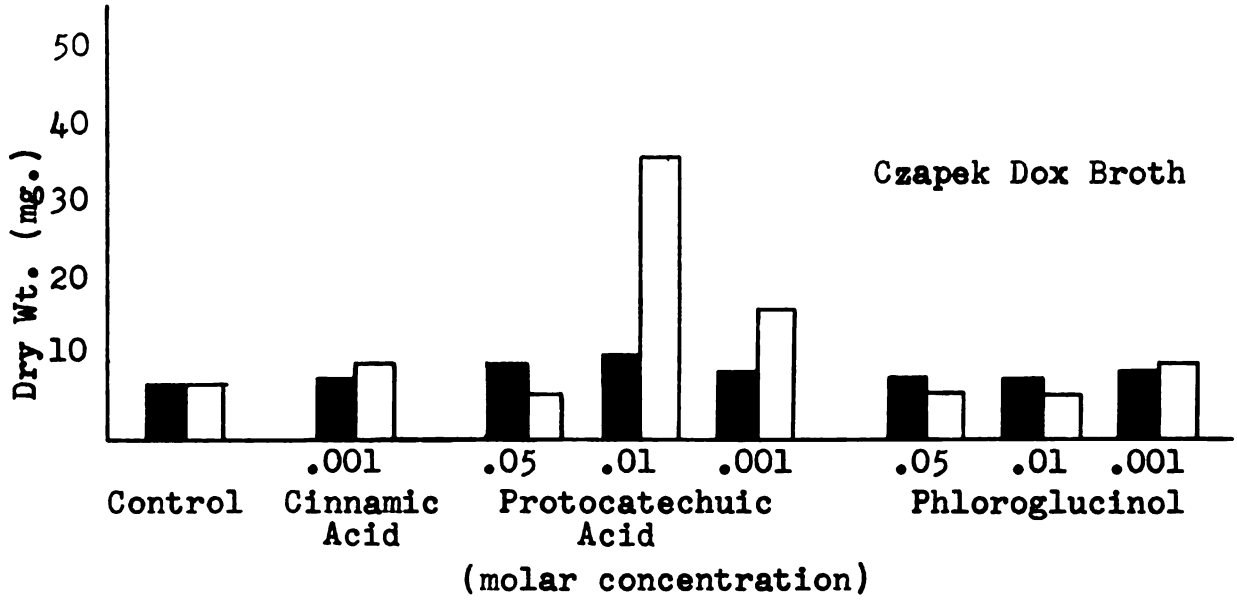
[b] Upper phase of the mixture was used.

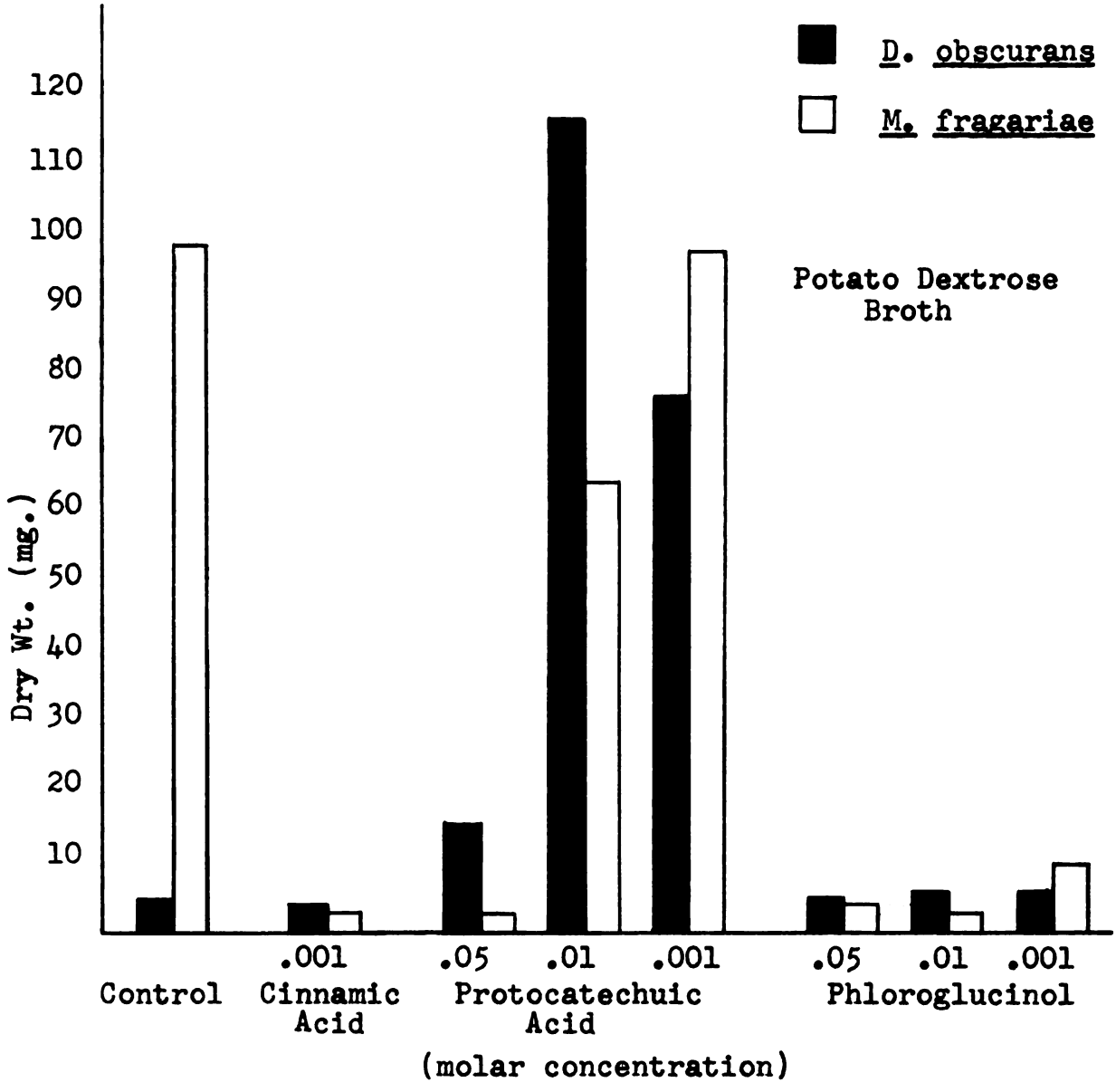
Figure 7. Results of bioassay by paper disc method.




Numerals refer to measurements in mm.

Figure 8. Results of bioassay by dry weight method.



## DISCUSSION

Results indicate that the same pigment is responsible for the reddish or purplish halos surrounding the lesions of all three strawberry leaf diseases studied. Furthermore, it is identical to that which occurs naturally in strawberry leaves in the fall of the year, and to one of the anthocyanins (cyanidin-3-monoglucoside) found in strawberry fruit. This is supported by data from paper chromatography of the whole pigments and their hydrolytic products and from spectrophotometry. These results agree with those of Creasy *et al* (26, 27) who published the first reports of anthocyanin in strawberry leaves. These workers, studying the physiology of anthocyanin in strawberry, extracted and identified cyanidin-3-monoglucoside from leaf discs which had been floated on a sucrose solution. The same anthocyanin was found in both cultivars tested, *viz*., *Fragaria vesca*, var. Alpine, and a commercial variety, Lassen.

The pigments produced by *Mycosphaerella fragariae* were not identified chemically, but sufficient characteristics were observed to establish that they were not anthocyanins, contrary to the opinion of Palchefsky and Allison (69). Like the anthocyanins, they are pH indicators, but rather than changing from red to blue, their color change

is from red to green. The color change occurs at a higher pH (8.5 - 9.0) than that of anthocyanins. The fungal pigments are soluble in non-polar solvents like acetone and benzene, but this is not true of anthocyanins. Furthermore, their spectral and chromatographic properties differ markedly from those of the anthocyanin group. In fact, a good solvent system was not found for the paper chromatography of the Mycosphaerella pigments.

Since the pigment associated with the strawberry leaf diseases studied here is 1) the same in every case, 2) an anthocyanin, 3) identical to the anthocyanin normally produced by strawberry fruit and leaf tissue, and since the fungal pigments are not anthocyanins, it is concluded that the pigment found in strawberry leaves infected by M. fragariae is a host product, not a parasite product. This conclusion is further supported by the fact that never was a pigment corresponding to the fungal pigments observed in any extract from leaf spot-infected leaves.

The conclusion can also be drawn from the above data that pigment production by strawberry leaf tissue is a general response to adverse conditions rather than a specific response to a peculiar stimulus, though the response is greater to an infection than to a wound. Anthocyanin production occurs in tissue where there is increased metabolic activity (35). Bopp (22) and Eberhardt (35) showed that wounding of leaves caused an increase in

respiration which directly paralleled anthocyanin synthesis at the wound edges. It is also generally recognized that one of the symptoms of infectious diseases of plants is a marked increase in respiration (6, 7). Shaw and Samborski (85) demonstrated the accumulation of various radioactive substances at infection sites of rust and mildew. Barnes and Williams (10), Hughes and Swain (56), Farkas and Kiraly (39), among others, have shown a build-up of phenolic compounds in plants in response to infection. Pigment production in diseased strawberry leaves can be explained, then, as a result of the increased respiration of this tissue due to parasitic invasion.

The anthocyanin pigments of grape and strawberry have been reported to be both inhibitory and stimulatory to certain bacteria (46, 79, 80, 86). Hulme and Edney (57) showed that germination of Gleosporium perennans was inhibited by the anthocyanidins delphinidin, pelargonidin, petunidin, and cyanidin. Cyanidin was the most toxic, inhibiting germination 95%.

The bioassay in this study was conducted to determine whether the difference in the size of the lesions caused by M. fragariae and D. obscurans could be correlated with a difference in growth of these fungi in the presence of the pigments or their precursors or breakdown products.

The filter paper disc assay was employed first to give a quick indication of where differential inhibition or stimulation might lie. Besides chrysanthemin itself,

its aglycone was used, as well as some likely precursors, cinnamic acid and caffeic acid, and breakdown products, phloroglucinol and protocatechuic acid. Caffeic acid had very little effect and was not used in the dry weight assay. Chrysanthemin and its aglycone likewise showed little activity, promoting only sparse aerial growth of D. obscurans. Phloroglucinol, protocatechuic acid, and cinnamic acid all showed differential effects. Cinnamic acid was especially interesting in that it caused dense growth of D. obscurans outside the zone of inhibition.

The dry weight assay was disappointing because neither medium supported much growth of D. obscurans in the control flasks. This made it impossible to detect any inhibitory effects the tested compounds might have had against this fungus. The same general pattern of growth is found in both media, although much suppressed in Czapek Dox broth. The stimulatory effect of protocatechuic acid may be because the fungi were able to metabolize this compound, or simply because of a pH difference. Unfortunately, the pH of the various solutions was not measured.

Apparently cinnamic acid was used in too high a concentration to show the stimulation of growth to D. obscurans that was visible in the paper disc test.

The possibility exists that the tremendous stimulatory effect of protocatechuic acid upon D. obscurans and the inhibition of M. fragariae by cinnamic acid and phloroglucinol could help to explain the difference in lesion

size on the diseased strawberry leaf. In order to make this statement meaningful, however, one would have to demonstrate the presence of these compounds in the diseased strawberry leaf in concentrations which were inhibitory or stimulatory to the respective fungi. Until this is done one can only speculate upon the role of the pigment associated with strawberry leaf spot, if indeed it plays a role at all.

The natural growth habit of the two fungi should be considered here. On a solid medium *M. fragariae* typically makes a rather small, compact, mounded colony, while *D. obscurans* spreads out much faster radially in a thin, flat weft. It is possible that in the strawberry leaf *D. obscurans*, by reason of its fast growth, outstrips the mobilization of potentially inhibitory phenolic compounds by the host, whereas *M. fragariae* does not.

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