ROLE OF ASSOCIATED FUNGI IN THE PREVENTION OF GRAY MOLD (*BOTRYTIS CINEREA* PERS.) OF STRAWBERRIES

by

DIBYA DEO BHATT

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APPROVED:

[redacted]

Professor of Plant Pathology

In Charge of Major

[redacted]

Chairman of Department of Botany

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

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ROLE OF ASSOCIATED FUNGI IN THE PREVENTION OF GRAY MOLD (*BOTRYTIS CINEREA* PERS.) OF STRAWBERRIES

INTRODUCTION

Botrytis cinerea Pers. ex Fr. (gray mold) is the principal rot organism of strawberries in the field in the Pacific Northwest. Losses from this disease vary from year to year; if cool moist weather prevails during blossoming and harvest, 25 to 50 per cent of the crop may be lost. According to an estimate made by the Oregon Department of Agriculture, in 1959 $1,365,980 was lost due to this disease alone (47).

Past studies have shown that infection by gray mold in strawberry usually starts at the stem end of the fruit and progresses downward until the entire fruit is rotted. In ripe strawberries most of the microorganisms are concentrated at the calyx end of the fruit. It has also been established that *Botrytis cinerea* is frequently associated with other microorganisms, many of which have little or no pectolytic activity.

The fact that *Botrytis cinerea* becomes established on dead or senile floral parts before it invades living tissue, enables study of the interaction of this fungus and associated microflora and the extent of natural protection that is provided by the latter against the

Botrytis.

As a general principle when two organisms become associated, they may either mutually help each other (a truly symbiotic relationship) or one may be parasitic or antagonistic towards the other. Between these extremes, are conditions where the two associated organisms have no influence on each other ("co-existence"). The incidence and severity of a disease may be influenced by its relationship to the associated microflora as well as by weather and condition of the host. In case of weak parasites such as Botrytis cinerea the associated saprophytic microflora may entirely prevent infection because the pathogen has to become established saprophytically before it begins to utilize nutrients from uninjured tissue.

The principal purpose of the studies reported herein was to determine quantitative and qualitative composition of microflora associated with strawberry flowers and fruits and their influence on incidence of Botrytis rot.

In strawberry culture, preharvest (bloom) application of fungicides is now a recognized control practice for gray mold. Fungicides change microbial equilibrium in any ecological situation and in doing so, they may destroy whatever natural antibiotic protection is present. It also is quite possible that by acting selectively upon a microbial population, fungicides may help build up

antagonistic, saprophytic populations, which provide additional protection to the plant. Some support for this theory has been found in the present work.

During recent years, considerable interest has been shown in determining the effect of associated microorganisms in the initiation and subsequent course of disease development by a pathogenic organism. Soil borne diseases e.g. take-all of wheat, cotton root rot and potato scab are examples of diseases sometimes successfully controlled by exploiting antibiosis. Only limited success has been achieved in controlling plant diseases affecting aerial parts of the plant by this method. Of the many factors that make biological control of these diseases infeasable, fluctuating environmental conditions are preeminent. They make it difficult for saprophytic antagonists to become established ahead of the pathogen and therefore, prevention of disease affecting aerial parts of the plant is not assured.

The investigations reported here were conducted to (1) determine the kinds of fungi associated with strawberry fruits throughout growth from bloom to the ripe fruit stage, (2) study recolonization and changes in fungal populations of fruits following fungicidal sprays, (3) study interactions between fungi isolated from strawberry and Botrytis cinerea in vitro and to determine

the nature of antibiosis, (4) evaluate control of Botrytis cinerea by the saprophytic antagonists Cladosporium herbarum, Pullularia pullulans and Penicillium sp. (5) determine relative effectiveness of a fungicide and saprophytic antagonist (Cladosporium herbarum) in prevention of fruit rot of strawberries in the field.

LITERATURE REVIEW

A number of fungi are known to cause rot in strawberries. In the Pacific Northwest Botrytis cinerea Pers. ex Fr. is by far the most common organism causing decay in the field. Losses up to fifty per cent have been reported (8, p.4).

In the midwestern states stem-end rot caused by Dendrophoma obscurans is quite serious (1, p.704-9). In Oregon, although Dendrophoma is frequently isolated from stolons and sepals, typical stem-end rot symptoms are not observed. Similarly leather rot (Phytophthora cactorum Leb.) is not common. Rhizopus rot or "Leak" is of consequence only in storage and transit; it is occasionally found on over ripe berries in the field (48, p.4).

Beneke et al (6, p.254) in Michigan isolated a large number of fungi from the surface and sub-surface of green and ripe fruits. Fungi reported on the surface (in order of decreasing frequency) were: Hormodendron sp., yeasts, Rhizopus nigricans, Alternaria sp., Pullularia pullulans, Penicillium sp., Botrytis cinerea, Pezizella lythri, Aspergillus sp., Trichoderma sp., Mucor sp., Monilia sp., and Epicoccum species. However, when inoculated into fresh strawberry fruits, strains of Botrytis cinerea, Rhizopus nigricans and Aspergillus sp. were shown to produce macroscopically greater degradation than Pullularia

pullulans, Trichoderma, Penicillium, Hormodendron and Alternaria.

Of molds isolated from black raspberries, Alternaria and Cladosporium accounted for approximately seventy per cent of the total (70, p.243). In fact, the organisms that normally occur on or within small fruits of all kinds consist largely of saprophytes which do not form pectolytic enzymes.

Alexopoulos and Cation (1, p.704) described a stem-end rot of strawberries caused by Dendrophoma obscurans. The perithecial stage at that time was described erroneously as Gnomonia. The latter was later identified as a distinct organism. Other diseases of fruit reported from Michigan were Black seed disease (Mycosphaerella fragariae) and soft rot (Sphaeronemella fragariae), the latter fungus closely resembling D. obscurans.

Lowings (37, p.85), reporting on fungal contamination of Kentish strawberry fruits, brought out the fact that high mold counts were due to Sphaerotheca humuli, an obligate parasite. Of the other fungi isolated from whole fruits, Mucor pyriformis and Botrytis cinerea (ninety to one hundred per cent and forty-five to seventy per cent respectively) were predominant. He concludes that the "Howard mold count" is a quite unsatisfactory basis for the certification of berries for canning or freezing.

Principal rot organisms and the symptoms they produce:

Botrytis cinerea Pers. ex Fr. (gray mold) is important mostly in the field. Weather conditions greatly influence disease severity. Cloudy weather with abundant rainfall or high relative humidity and low temperature favor development of epiphytotics. Berries affected by Botrytis are soft and watery at first but later become firm and retain their shape even after abundant spores are produced on the surface. They finally become shrivelled, hard and grayish-brown. The disease is characterized in the early stage by the appearance of a reddish spot, which later enlarges covering a large area of the fruit. If moist weather continues during the bloom and post bloom period, at which time the fruit is very susceptible to infection, 25 to 50 per cent of the fruit may be lost due to gray mold (40, p.646-648). Blossom blight and rotting of green berries is not uncommon under favorable climatic conditions. In wet blossoming periods, 20 per cent blossom blasting was observed in Oregon and Washington (48, p.40).

Botrytis cinerea is an imperfact fungus of which many forms have been described (34, p.274). In gross morphology, it is distinguished by the gray color of the mycelium. The conidiophores are slender, constricted at the septa, gregarious, simple or sparsely branched, erect and cinerous (27, p.40). Occasionally sclerotia develop in cultures.

There is great variation in isolates from strawberry fruits, with respect to sporulation, mycelial growth, color, texture and type of conidial production. Variation becomes more apparent when a single spore isolate on further culturing shows further variations (28, p.957).

Dendrophoma obscurans E. E. Stem-end rot (1, p.699) A brown discoloration accompanying a general dry rot beginning at the stem end is the usual symptom. Pycnidia of the fungus are found at first on the calyx but later may develop over the entire fruit. This fungus also causes leaf blight. Softening of the fruit starts at the calyx end and generally spreads to the whole fruit giving rise to a general soft rot followed by gradual shrivelling.

Gnomonia fragariae Klebahn. Leaf blotch (8, p.541) This fungus is associated with Dendrophoma obscurans and causes stem-end rot of strawberries. Gnomonia has been known to cause fruit rot of strawberries and is frequently isolated from calyces and pedicels.

Phytophthora cactorum Leb. and Cob. Leather rot (20, p.644) As the name suggests, this fungus causes a dry leathery rot in strawberries. Infection starts at the point of contact of the fruit with the soil. The first symptom is the appearance of tan spots with a clear line of demarcation between healthy and diseased tissue. The disease is also characterized by a marked vascular

discoloration. Affected berries turn brown to black (22, p.841).

Pezizella lythri (Desm.) Shear and Dodge. Light tan rot Infected tissue in the form of a tan spot is sunken and a corky layer separates the diseased from the healthy tissue.

Rhizoctonia Solani Kuhn. Rhizoctonia rot While the symptoms of this disease are very similar to those of Phytophthora rot, a distinctive feature is that soil particles are held to the diseased tissue by the fungus.

Place of infection:

It has been observed that a fresh wound is rarely a site of infection by Botrytis cinerea. Isolations made from necrotic and non-necrotic petals showed that the frequency of Botrytis isolation was 96/120 from necrotic with 4/90 from non-necrotic petals (48, p.37). While saprophytic fungi such as Cladosporium and Penicillium were frequently isolated from non-necrotic tissue, Botrytis was present only in necrotic floral parts. Green fruit is very resistant to puncture (29, p.888), while dead petals, sepals and stamens are very susceptible to infection. It is commonly observed that rot starts at the stem end of the fruit. When healthy and diseased fruits are in contact, the former shows rot symptoms first at the point of contact

with the latter. As the harvesting season progresses, inoculum builds up and, with suitable infection sites such as are always present in dense matted rows and in the micro-environment beneath calyces, new foci of infections are formed. This build up may be the reason for increased rot with successive pickings of strawberries.

Lipton and Harvey (36, p.838) observed decay of artichoke bracts by Botrytis cinerea. They found that infection always starts at the tip of the bract and rarely at the point of abscission from the fleshy base. Bracts with injured tips decayed more rapidly than those with uninjured tips. Large wounds at the point of abscission of the bract from the receptacle were rarely sites of infection.

Although Botrytis infection is initiated at the moribund capstem area of the strawberry fruit, in the latter part of the season many of the fruits become infected from spores germinating in the persistent water drops which contain products of ex-osmosis from the fleshy fruit (71, p.53-55). Under such conditions a direct penetration of the skin of fruit takes place. The products of ex-osmosis have been identified as sugars and organic acids such as citric and malic acid (71, p.54-55). However, direct infection of ripe fruit by air borne spores during harvest is of minor importance (48, p.58).

The severity of gray mold in the field is to a great

extent determined by prevailing weather conditions. Nelson (44, p.859-864) reported that in grapes at high relative humidities, 95 per cent of the infection was through skin and five per cent through the capstem area. Free water is not essential for infection by Botrytis cinerea.

There seems to be a close relation between relative humidity and temperature and the incubation period required for Botrytis spores to germinate. At 92 per cent or higher relative humidity Botrytis spores germinate on glass slides or on grapes in 48 hours at 12°C; however, at 90 per cent relative humidity only one spore on the grape skin had germinated (44, p.863). He further showed that at the lower humidity (e.g. 90 per cent) a longer incubation period of five days brings about improved germination (53 per cent). Berries that are apparently healthy but carry latent infection show gray mold symptoms after incubation in a saturated atmosphere. Hence, disease expression is dependent both upon external conditions of the environment and upon the internal (physiological) condition of the fruit.

The pathogenicity of Botrytis cinerea has been studied in great detail (12, 13, 24, 41, 73). The fungus produces pectolytic enzymes which bring about softening and breakdown of the fruit. The middle lamella, which is made

up of salts of calcium and magnesium pectate and some cellulose is hydrolysed by protopectinase (73, p.299-322). During the ripening of fruit, cell walls become quite thin and this change is attributed to decomposition of the insoluble protopectin into soluble pectinic acids. The firmer the berry, the higher is its pectic content (33, p.191).

The susceptibility of parenchymatous tissue to attack by soft rot organisms is increased by increasing water content of the tissue (73, p.132). Increased turgidity of the tissue leads to greater production of pectolytic enzymes. Botrytis cinerea produces a typical soft rot in potato tubers whose water content is artificially raised (41, p.338), while it can not parasitize normal potato tissue. The only explanation that is offered for this phenomenon is that in the absence of water diffusion of enzymes is delayed (41, p.338-340). But as Brown (13, p.325-421) points out, the amount of carbohydrate in the host tissue is an important factor in the production of tissue macerating enzymes. Poor quality of strawberries during or immediately after rainy periods is due more to low sugar rather than high water content. This may have direct bearing on production of pectolytic enzymes and increased severity of disease. A low concentration of sugar in senescent tissue (calyx) of flowers favors

infection by Botrytis (4, p.145-155).

Sensitivity of enzymes to changes in pH of the substrate may also have some effect on disease development. The pH of ripe strawberry juice varies with variety and presence of soluble materials in the fruit. In ripe Marshall berries, it is about 3.6 which is near optimum for growth of Botrytis (66, p.283-341). However, the pH for maximum enzymatic activity was found by Fushtey (24, p.273) to be around 5.5.

Biological control of plant pathogenic organisms:

The control of a plant pathogenic organism through the activity of other living organisms has received some attention in the recent years. Fawcett (23, p.545-550) in his Presidential address to the American Phytopathological Society stressed the need for studying mutualistic behavior of microorganisms in soil, especially those which could be used in the control of plant diseases. In a few cases one organism parasitises the other. Trichoderma lignorum coils around Rhizoctonia solani and kills the latter (67, p.837-845). In other cases, the relationship varies between symbiosis and antagonism. Elarosi (21, p.555) found from growth studies of fungi in culture filtrates, that a synergistic relationship exists between Rhizoctonia solani and Fusarium solani.

In the majority of cases biological control is of an

indirect type, accomplished by: a) production of staling products, b) creation of a nutritional deficiency, c) changes in pH of the substrate, d) production of an antibiotic substance or e) an increase in saprophytic population (64). While Wood and Tveit (74, p.422) use the term antibiosis and antagonism interchangeably, Waksman (64, p.48) makes a differentiation between these two terms. He uses the first term to describe a condition where an organism producing a toxic chemical substance has a harmful effect upon another, while the term antagonism is restricted to a condition where one living organism unfavorably effects the other. The metabolic products of an organism having harmful effects upon another have been given various names, such as antagonistic agents, antibiotics or simply toxins.

Control of soil borne diseases, such as cotton root rot, take-all of wheat, potato scab and citrus root rot are some of the striking examples where biological control has been successful. It is largely through the efforts of Garret in England, Sanford in Canada and Fawcett and his co-workers in America that we have been able to get a better picture of soil microflora and more particularly, of the influence of antibiosis in the incidence and course of a soil borne disease. It is now accepted that antagonism among microorganisms is a natural phenomenon that sometimes can be augmented artificially within a particular

environment. The indirect or passive antagonism may be seen when a change in an environment is made by green manuring, changing soil reaction, fumigation or production of toxic or enzymatic substances, as a result of which growth and multiplication of a plant pathogenic organism is checked. But in nature, "direct" or "true" antagonism is also sometimes observed; Weindling (68, p.475-496) found that Rhizoctonia solani parasitizes Pythium debaryanum and Rhizopus sp. by penetrating their hyphae and completely destroys them. As most of the work with saprophytic antagonism has been carried out in sterile soil or in vitro, the results obtained are difficult to reproduce under natural conditions. From the biological control standpoint, "indirect" or "passive" antagonism is of more importance than "direct" or "true" antagonism (69, p.247-260). Recolonization of a habitat by microorganisms after treatment with steam or chemical has an important effect on the incidence of a plant pathogenic fungus. Bliss (7, p.680) reported that even after complete sterilization, certain organisms increase and colonize the soil ahead of their competitors. Trichoderma viride overruns and kills Armillaria mellea in soils that have been fumigated with sub-lethal doses of CS_2. Newhook's work (which will be referred to again later) can be examined in this light. He obtained experimental evidence that saprophytic

antagonists, such as Penicillium and Cladosporium, survive after chemicals have been sprayed on tomato plants at the bloom stage for the control of gray mold (46, p.473-481). In the absence of competing organisms they rapidly build their population to a level which inhibits growth of Botrytis.

In an early work, Brooks (11, p.479-487) reported that spores of Botrytis cinerea were unable to infect healthy green lettuce leaves if sown in water suspension but caused infection when sown in a nutrient solution (grape juice). Brown and Montgomery (14, p.161-180) observed that lettuce growing in the hollows of the field is relatively free from gray mold infection while that on raised beds is very susceptible and they suggested that better survival may be due to increased microbiological activity. The studies of Wood (72, p.203-216) and Newhook (45, p.185-202) clearly showed that when older leaves die, they provide a favorable habitat for the establishment of saprophytes antagonistic to Botrytis cinerea and infection of young leaves by Botrytis is prevented.

Wood (72, p.203-216) and Newhook (45, p.185-202; 46, p.473-481), from their studies on microbiological control of lettuce and tomato gray mold, reported that a number of organisms present in dead leaves or senile floral parts have an inhibitory effect upon Botrytis cinerea. The

organisms reported by them include: Streptomyces 3 sp., Penicillium, Trichoderma viride, Fusarium, Trichothecium roseum, Cephalosporium acremonium, Phoma, Cladosporium, Cylindrocarpon, Stemphylium, Epicoccom and Geotrichum. Of these Penicillium, Fusarium, Streptomyces, Trichothecium roseum (syn. Cephalothecium) and Cephalosporium acremonium were reported to control growth and spread of Botrytis in vitro even when the two species were inoculated simultaneously.

Increased sporulation of Botrytis cinerea was frequently observed when it was grown in dual cultures with Trichoderma viride, Penicillium or Fusarium sp. (72, p.214). It was also found that most of the antagonistic organisms were very active at 25°C and this activity decreased sharply at 15°C. At 5°C, with the exœption of Penicillium none of the organisms inhibited growth of Botrytis. However, the results obtained in these experiments either in vitro or under controlled conditions could not be repeated under field conditions where fluctuating environmental conditions and competition from non-antagonistic saprophytes renders natural control of a disease ineffective. Three weekly applications of suspensions of Cephalosporium sp., Fusarium flocciferum and Phoma eupyrena failed to give a significant increase in the final survival of lettuce in the field (45, p.185).

Newhook (46, p.473-481) successfully controlled gray mold on greenhouse grown tomatoes by first establishing Cladosporium herbarum and Penicillium sp. on dead petals remaining attached to the fruit, followed by inoculating the latter with a heavy suspension of Botrytis cinerea spores.

In regard to the nature of antagonism between Cladosporium herbarum and Botrytis cinerea, it is suggested by Newhook (46, p.480) that this is not due to lack of moisture, nutrients or growth factors in the substrate because Botrytis spores germinate readily in distilled water and have their own reserves of these substances. The pH factor is discounted because inhibition also occurs in buffered media. It is suggested that inhibition could be due either to production of a toxic substance or simply to competition for space between the two organisms. It is apparent that this question has not been answered adequately.

Baigent and Ogawa (5, p.82) reported that Pullularia pullulans (de Bary) Berkh. produces an antibiotic substance that strongly inhibits Botrytis cinerea. As Pullularia is one of the common organisms associated with developing strawberry fruits, it may play a role in preventing infection by the gray mold fungus.

Effect of metabolic products of competing fungi upon growth of fungi:

A culture is said to become stale when the rate of growth of an organism growing in the medium diminishes gradually and finally ceases. This phenomenon occurs independently of any reduction in the amount of nutrient present in the media and is caused by the metabolic activities of the organism per se. In Pratt's opinion (52, p.567), staling products are autotoxic metabolites. The real cause of staling, according to her, is a drastic change in the pH with either carbonate or ammonia being responsible for increased alkalinity. Richard's medium (54, p.30) in which most of the studies on staling products were carried out, is used very slowly by the organisms and staling is, therefore, comparatively slow (10, p.133). In contrast to the utilization of ammonia by some fungi, Cladosporium causes a build up of free ammonia which is toxic to the fungus. A change in reaction of the medium invariably takes place in case of Cladosporium, whether the source of nitrogen is nitrate or ammonia. This change of reaction is due to utilization by the fungus of NO_3^- or NH_4^+ ions from the medium. Disappearance of these ions results in the production of an equivalent amount of OH- or H+ ions, as a result of which the medium becomes alkaline or acidic (18). Growth and sporulation of Cladosporium

is best in potassium nitrate medium with glucose as the main carbon source. As the colony ages, a variety of inhibitory products are elaborated which apart from being autotoxic are also antagonistic towards other organisms. The loss of germinative capacity of Botrytis cinerea spores in stale medium of Fusarium has been attributed to potassium bicarbonate (53, p.614). The spores of Botrytis cinerea are known to be tolerent to hydrogen ion concentrations over the range of pH 3.1-7.0 (10, p.133). Exhaustion of food is not of primary importance as is shown by Botrytis growth in dilutions of stale and fresh medium. The staling substance produced in Cladosporium herbarum cultures is reported to be enzmatic in nature because boiling the stale medium restores the germinative capacity of Botrytis.

METHODS AND MATERIALS

In preliminary investigations, flowers and fruits were collected from the Plant Pathology farm near Corvallis and from plantings near Woodburn and Stayton, Oregon. Sepals, petals, stamens and styles were removed individually and plated in Streptomycin PDA of the following composition:

potato decoction	200 gms
dextrose	20 gms
agar	20 gms
streptomycin nitrate (100 ppm)	0.5 ml

Tap water to make one liter.

This medium was also used for determining total number of fungi associated with strawberries at various stages of growth.

Botrytis cinerea was isolated from a diseased berry and a single spore isolate was obtained. Throughout the investigations, this particular strain of Botrytis was used; however, even in single spore isolates, considerable variation was observed in the amount of mycelium, density of spore mass and sclerotial production. Stock cultures of fungi isolated from strawberry were maintained on PDA and periodically were transferred to keep them in vigorous condition. A single spore isolate of Cladosporium herbarum was used in cultural studies as well as in greenhouse and

field investigations.

To determine qualitative and quantitative changes in the total fungal population associated with strawberries at various stages of growth, both before and after being sprayed with various fungicides, flowers or fruits were picked with sterile forceps and put in new paper bags. Forty flowers from each treatment were homogenized in 90 mls. of sterile distilled water for two minutes in a Waring blendor and a 10 ml. aliquot of this homogenized mass was diluted with 90 mls. of sterile distilled water. From the last dilution, a 5 ml. aliquot was mixed with 45 mls. of PDA cooled to 45°C and five plates poured. Figures given in tables 5 and 6 represent total numbers of fungal colonies on all five plates. Green and ripe fruits were assayed in similar manner for fungal contamination but only 24 and 12 berries were used to make "stock" solution for green and fully ripe berries respectively.

From the standpoint of antibiosis, surface microflora is as important as the sub-surface microflora; therefore, no attempt was made to surface sterilize flowers and green fruits. In the case of ripe fruits, however, fungal counts were made both after surface sterilization and without such treatment.

For cultural studies of Cladosporium, Czapek's and Richards' media (54, p.30) were used and pH determinations

made with a Beckman Zeromatic pH meter.

For studying interaction between Botrytis and associated fungi, both solid and liquid media were employed. Solid media used were potato dextrose agar, yeast extract agar (26, p.817) and strawberry extract agar. The last medium was made in the following manner; 200 gms. of fresh ripe strawberries were autoclaved for 20 minutes in 500 mls. of water and the pulped mass pressed through several layers of cheese cloth. To the juice thus obtained, 20 gms. of agar melted in 500 mls. of water was added. Some difficulty was experienced in getting the medium solidified if pH was not adjusted, so a few drops of N/10 NaOH was added to the medium to bring its pH to about six. It was then autoclaved for 15 minutes at 15 lbs. pressure and plates were poured.

Liquid media for cultural studies of Pullularia and Dendrophoma were prepared by using the same ingredients as in solid media except agar was left out.

Growth studies of Botrytis in culture filtrates of Pullularia and Dendrophoma were carried out by inoculating sterile culture filtrates of these fungi with a spore suspension of Botrytis and incubating it for about a week at 20°C. At the end of this period, mycelium was filtered through a Buchner funnel on #1 Whatman filter paper and oven dried for 24 hours at 90°-100°C. Dry weight of fungus

was determined. The sterile filtrates obtained from Cladosporium, Pullularia and Dendrophoma cultures did not show any inhibitive effect against Botrytis when tested by filter paper discs (Schleicher and Schuell, 740 E, 1/2 inch). Therefore, amount of growth of Botrytis in sterile filtrates of these fungi was used as a criterion for determining their antagonistic activity against Botrytis.

Sterile filtrates of Cladosporium and Dendrophoma cultures obtained by coarse filtering through #1 Whatman filter paper, followed by Seitz filtering were used for determining their inhibitive capacities against Botrytis spores and/or effect on total growth of this fungus.

Cultures of Pullularia were too viscous and could not be filtered through even a coarse filter paper. Therefore, culture was first centrifuged for 20 minutes at 5000 rpm and the decant was filtered through a Seitz filter.

To determine effect of culture filtrates on growth of germ tubes of Botrytis, a 4-5 day old culture of the latter was used. In general the procedure used was the same as in evaluation of fungicides by spore germination on slides (58, p.354-356). A spore suspension in sterile distilled water was centrifuged for five minutes at low speed to concentrate spores. Density of Botrytis spores when mixed with sterile filtrates of antagonists was adjusted

roughly to 40,000 to 50,000 spores per ml. and three drops of suspension were placed in the cavity of depression slides. All glassware used was sterile and slides were chemically cleaned (32, p.523), and thoroughly washed in running tap water and sterile water before being used in germination tests.

EXPERIMENTAL

Time and place of Botrytis infection:

Previous studies (49, p.494) indicated that Botrytis cinerea is a weakly parasitic organism which becomes established initially on senile floral organs from which infection spreads to fruits. In many instances, infection takes place at the point of contact of the green or ripe berries with a dead stamen or petal that has become trapped beneath the calyx (Figure 1). Floral infection is characterized in the present work as primary infection and is differentiated from secondary infection, which can originate at any point on the strawberry.

In order to determine whether fresh wounds on ripe strawberries are as susceptible to Botrytis infection as moribund tissue, fresh strawberries were washed in running water, dipped in a 20 per cent solution of commercial Clorox for five minutes and rinsed in sterile distilled water. A necrotic spot was artificially created on the surface of berries by contact with a small piece of solid CO_2 in an area ringed with sterile vaseline. Fresh wounds were made by cross shape incisions with a sterile blade. Checks consisted of uninjured berries. Both checks and treated berries were inoculated with Botrytis and incubated in plastic trays lined with moist paper towels. Visible growth of Botrytis on the surface of berries was taken

Figure 1. Early stage of decay by Botrytis cinerea at the stem end of strawberry. Note infection has started from a dead petal that is trapped between calyx and fruit.

as an indication of successful infection. It was found (Table 1) that gray mold infection of ripe strawberries can take place through uninjured, necrotic or non-necrotic tissue.

Table 1. Frequency of infection by Botrytis cinerea on necrotic and freshly wounded ripe strawberry tissues.

Treatment	Number of berries treated	Number of berries infected by Botrytis	Per Cent of infection
Necrotic spot (CO_2 treated)	36	27	75
Fresh wound	36	35	97.2
Check	24	12	50

In preliminary investigations during 1959 to determine the kinds of fungi associated with strawberry, floral parts, sepals, petals, stamens and styles, were plated on PDA both after sterilization with 20 per cent Clorox and without such treatment. Cladosporium herbarum, Pullularia pullulans, Dendrophoma obscurans, Fusarium, Melanconium and Botrytis cinerea appeared with highest frequency (Table 2).

Table 2. Fungi isolated from unsterilized floral organs at late bloom stage.*

Organism	Sepal	Petal	Stamen	Total
Cladosporium	8	12	11	31
Botrytis	1	0	1	2
Pullularia	0	0	2	2
Dendrophoma	1	0	0	1
Fusarium	2	4	0	6
Melanconium	3	0	2	5
Alternaria	1	0	0	1
Stemphylium	1	0	0	1
Unidentified				15
Total				64

*Total from 64 isolation attempts.

Even in these early studies Cladosporium appeared as the dominant component of fungal microflora associated with strawberry at the bloom and green fruit stage (Table 3). However, sterilized flowers yielded a large number of isolates of Gnomonia and Dendrophoma (Table 4).

Table 3. Frequency of isolation of different fungi from unsterilized green fruits.*

Organism	Sepal	Stamen	Total
Cladosporium	2	11	13
Botrytis	4	11	15
Dendrophoma	1	0	1
Fusarium	0	1	1
Melanconium	0	2	2
Total			64

*Total from 32 isolation attempts.

Table 4. Frequency of isolation of fungi from strawberry flowers (early bloom) after surface sterilization.*

Organism	Sepal	Petal	Stamen	Style
Gnomonia	37	0	0	0
Dendrophoma	20	0	0	0
Pestalotia	2	0	0	0
Cladosporium	1	0	0	0
Melanconium	0	0	1	1
Alternaria	1	0	0	0

*From a total of 60 isolations.

All senescent floral parts are equally susceptible to infection by these common fungi. The stigmatic surface has been thought to be the place where Botrytis spores germinate most readily (2, p.501); however, out of 60 isolations from withered styles, only four of the colonies from unsterilized flowers and one from sterilized green fruits proved to be Botrytis.

In the spring of 1960 further experiments were carried out to determine qualitative and quantitative changes in the microbial population in developing strawberry fruits throughout the growth period. Periodic isolations from strawberries from plots in the Plant Pathology farm were made starting at the full bloom stage and were continued to the ripe fruit stage. These plots had been sprayed with different fungicides on April 25, 30 and May 7 and 14. Rain followed within 24 hours of spraying on May 7 and 14. In the early stages of the experiment it became evident that there is a distinct difference in the number of fungi on dilution plates from sprayed and unsprayed berries. Initially there is nearly 100 per cent kill of the microorganisms, the residual effect of the chemical lasting about a week following application but decreasing gradually as the fruits approach maturity. Differences in the number of fungi obtained from berries from plots sprayed with captan and non-sprayed plots persisted until the end of harvest.

However, the number of fungi in berries from plots treated with Phygon was considerably higher than from non-sprayed plots at harvest (Figure 2, Tables 5 and 6). These results agree with those obtained by Vaughan (62, p.43) who obtained maximum increase in marketable berries and fruit rot inhibition with captan, the percentage of rotted berries being 19.52, 2.94 and 18.95 in check, captan and dichlone sprayed plots respectively. The number of fungi obtained from non-treated berries was greatest at the beginning and the end of harvest season. Weather was rainy at the first picking, cloudy with slight drizzle at the second and third pickings and warm and sunny when the last two pickings were made. It has been ascertained in earlier studies (6, p.253-258) that the length of interval between pickings has less effect on mold counts than did the rainfall. However, here the time factor was observed to be the determining factor. To determine whether the fungi are present only on the surface or invade the tissues, berries were sterilized in a 20 per cent solution of commercial Clorox, washed well in sterile distilled water, homogenized in a Waring blendor for two minutes and plated in Strep PDA. Fungi were not only present on the surface but were obtained from the sub-surface of the fruit as well (Table 5, Figures 3A and 3B).

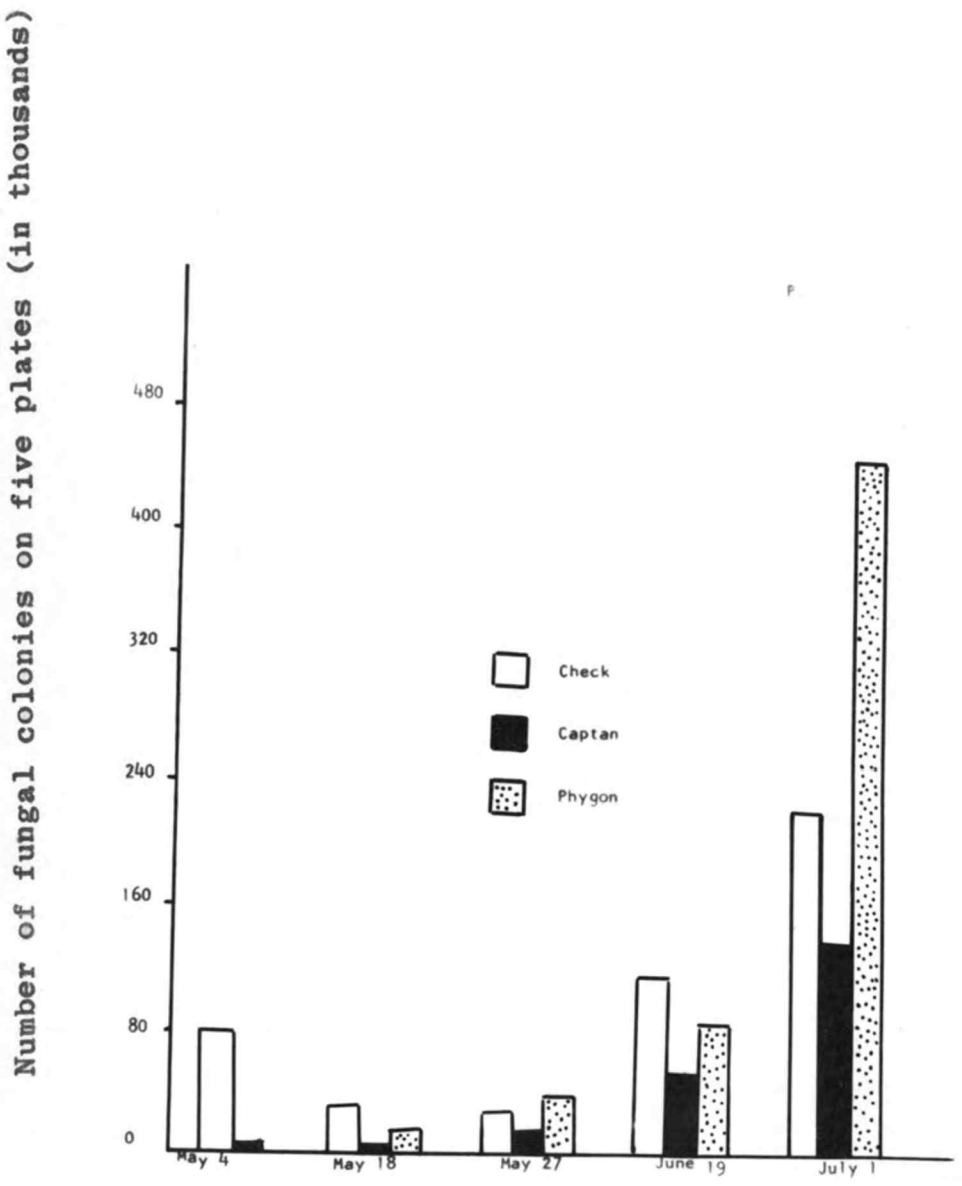

Figure 2. Recolonization and changes in the fungal population associated with strawberries at different stages of maturity.

Table 5. Frequency of isolation of fungi from the surface and sub-surface of ripe, Northwest variety strawberries, June 19, 1960.*

Fungi isolated	Field Spray Treatment					
	Check		Captan		Phygon	
	Surface	Sub-Surface	Surface	Sub-Surface	Surface	Sub-Surface
Botrytis	6	2	2	0	2	6
Cladosporium	84	1	5	9	0	7
Pullularia	3	0	5	0	0	1
Dendrophoma	1	0	2	0	12	0
Sphaceloma	1	0	0	0	5	1
Aspergillus	1	0	0	0	0	0
Fusarium	1	0	0	0	4	4
Penicillium	4	0	1	0	1	0
Rhizopus	1	0	0	0	0	0
Phoma	1	0	7	0	0	0
Cephalosporium	1	0	0	0	0	0
Trichoderma	0	0	1	0	1	0
Alternaria	0	0	0	0	1	0
Candida	1	0	0	0	0	1
Unidentified	6	8	23	2	55	17
Totals	111	11	46	11	81	37

*Count represents total of 5 plates from 12 berries at a dilution of 1:1000.

Table 6. Frequency of fungi isolated from Northwest variety strawberries (late harvest).* Date of isolation 7/1/1960.

Fungi isolated	Field Spray Treatment					
	Check		Captan		Phygon	
	Surface	Sub-Surface	Surface	Sub-Surface	Surface	Sub-Surface
Cladosporium	93	64	19	11	94	48
Pullularia	3	1	5	0	0	0
Dendrophoma	1	0	2	0	6	0
Penicillium	4	4	0	1	0	1
Botrytis	6	0	3	0	2	6
Sphaceloma	1	1	0	1	5	9
Aspergillus	1	0	0	0	0	0
Fusarium	1	0	0	0	0	0
Stemphylium	0	0	0	0	0	1
Rhizopus	0	0	1	0	0	0
Gnomonia	1	0	2	0	4	0
Pestalotia	0	0	1	0	0	0
Yeast	0	0	1	0	0	0
Coniothyrium	0	2	9	0	0	0
Unidentified	107	296	64	102	333	81
Totals	218	368	107	115	444	146

*Count represents number of colonies on 5 plates from 12 berries at a dilution of 1:1000

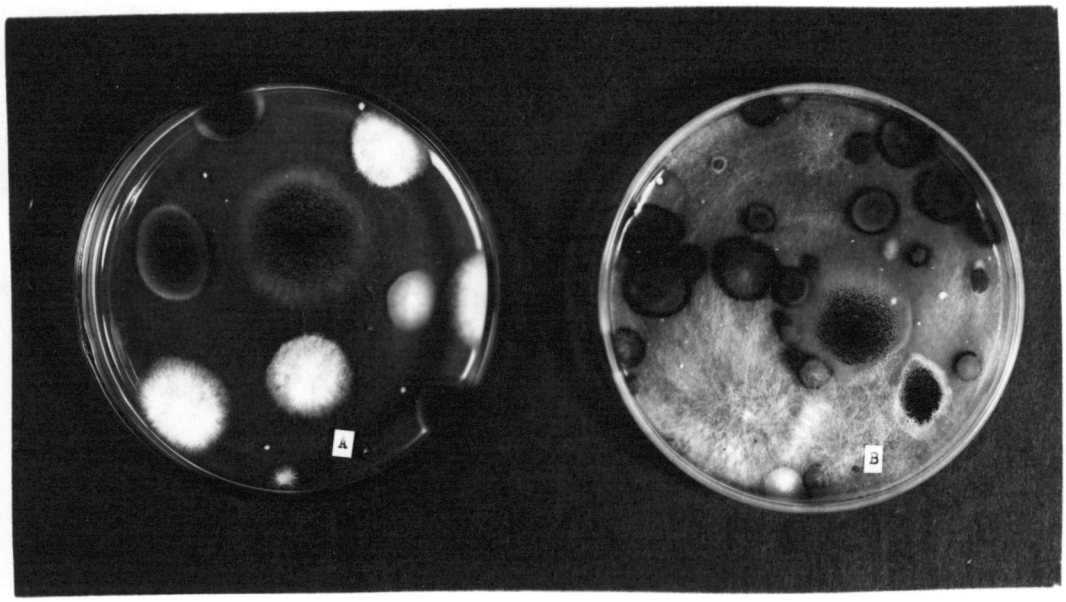

Figure 3. Typical dilution plates on PDA: (A) surface sterilized, (B) unsterilized ripe strawberry fruits. Colonies of Cladosporium, Botrytis, Pullularia and Aspergillus are easily identifiable.

Numbers of colonies were determined after 48 hours and identifications were made when the fungi produced fruiting structures. In cases of doubt, transfers were made and the cultures retained for later identification. After the first few attempts it was easy to identify many of the fungi. The frequency and range of fungi isolated from ripe strawberries was found to be similar to those reported by workers from other parts of the country (6, 253-258; 57, 1319-1331) from fresh and frozen pack materials. It should be recognized however, that the kind and number of fungi reported represent only a fraction of the total microbial population. Limitations are imposed by a) fluctuating weather conditions at different pickings, b) selectivity of the medium used in dilution plates, c) rapidity of growth of certain species and finally d) failure of obligate parasites such as Sphaerotheca humuli to grow on PDA. Moreover, not all fungi could be identified and frequency tables give only a general picture of the types of organisms associated with strawberries. There is a build up of microbial population as the harvesting season progresses, which becomes apparent when counts of fungi are made from berries at the end of the harvest period (Table 6, Figure 2).

Fungicides used to control fruit rot of strawberries are applied as bloom sprays (first bloom to petal fall

stage), the last usually being applied two weeks before the crop is harvested. Protection by fungicides is provided until about three weeks after the last application. The fungi that recolonize after application of a fungicide are primarily non-rot-causing (Tables 5 and 6). Therefore, *in vitro* studies were carried out to determine the relationship of these organisms with *Botrytis*.

Interaction between Botrytis and associated fungi:

Interaction among fungi has been characterized differently by different workers (51, p.168-188; 21, p.555-571). When two or more organisms are grown together or in close proximity, their growth may be altered. The effect of one organism on another varies from a strong antagonistic action to practically no interaction. The criterion of inhibition is a flattening of the *Botrytis* colony on either side of the competing fungus, stoppage of growth of both the fungi or a distinct mycelium-free zone between the competing fungi.

The type of interaction between *Botrytis* and associated saprophytic antagonists varied considerably with the type of medium used. The interaction was studied on yeast extract agar, potato dextrose agar, malt agar and strawberry juice agar. In all cases the saprophytic antagonist was inoculated 5-6 days before discs of *Botrytis* were placed

on either side at a fixed distance.

In another experiment, saprophytic antagonists and Botrytis were inoculated side by side in the same Petri plate and incubated at 5°, 10°, 20°, 25° and 30°C. Growth measurements of the antagonist and Botrytis were made after 4 and 6 days. It was found that all the ten genera of fungi tested grew best between 20° and 25°C, which is also optimum for Botrytis. None of them grew appreciably at 5°C, while at 30°C only Phoma, Dendrophoma, Pestalotia and Penicillium grew well compared to practically no growth of Botrytis. Therefore, interaction between ten selected fungi and Botrytis was studied on solid media at 20° C only. The results of interaction on yeast extract agar and strawberry agar are given in tables 7 and 8.

Ten genera of fungi were tested and all inhibited growth of Botrytis either at a distance or on contact with the latter. In order of decreasing zones of inhibition these are: Dendrophoma, Geotrichum, Chaetomium, Penicillium, Fusarium, Pullularia, Pestalotia, Phoma and Cladosporium. Gnomonia forms a wide inhibition zone on yeast extract agar (Figure 5, plate 1) only, while Dendrophoma strongly inhibits growth of Botrytis on strawberry agar (Figure 6). Trichoderma quickly overruns Botrytis and kills it within 4-5 days (Figure 4, plate A). Another fungus isolated from strawberry flowers was Coryneum sp.,

Table 7. Inhibition of Botrytis cinerea by antagonists on yeast extract agar.

Saprophyte	width of mycelium free zone, in cms*	final pH of the medium#
Chaetomium	0.50	6.8
Gnomonia	0.45	6.4
Penicillium	0.35	6.0
Pullularia	0.10	6.8
Dendrophoma	0.10	6.0
Cladosporium	-	8.0
Fusarium	-	8.0
Pestalotia	-	7.2
Phoma	-	8.0
Geotrichum	-	8.0

*Average of 3 plates measured at the end of 7 days.
#Initial pH of medium 6.2

Table 8. Inhibition of Botrytis cinerea by antagonists on strawberry agar.

Saprophyte	width of mycelium free zone, in cms*	final pH of the medium#
Dendrophoma	0.52	6.4
Geotrichum	0.45	8.0
Chaetomium	0.40	6.8
Penicillium	0.15	6.0
Fusarium	0.15	8.0
Pullularia	0.10	6.0
Pestalotia	-	6.0
Gnomonia	-	6.4
Phoma	-	8.0
Cladosporium	-	7.6

*Figures represent average of two plates measured at the end of 7 days.

#Initial pH of the medium 6.0

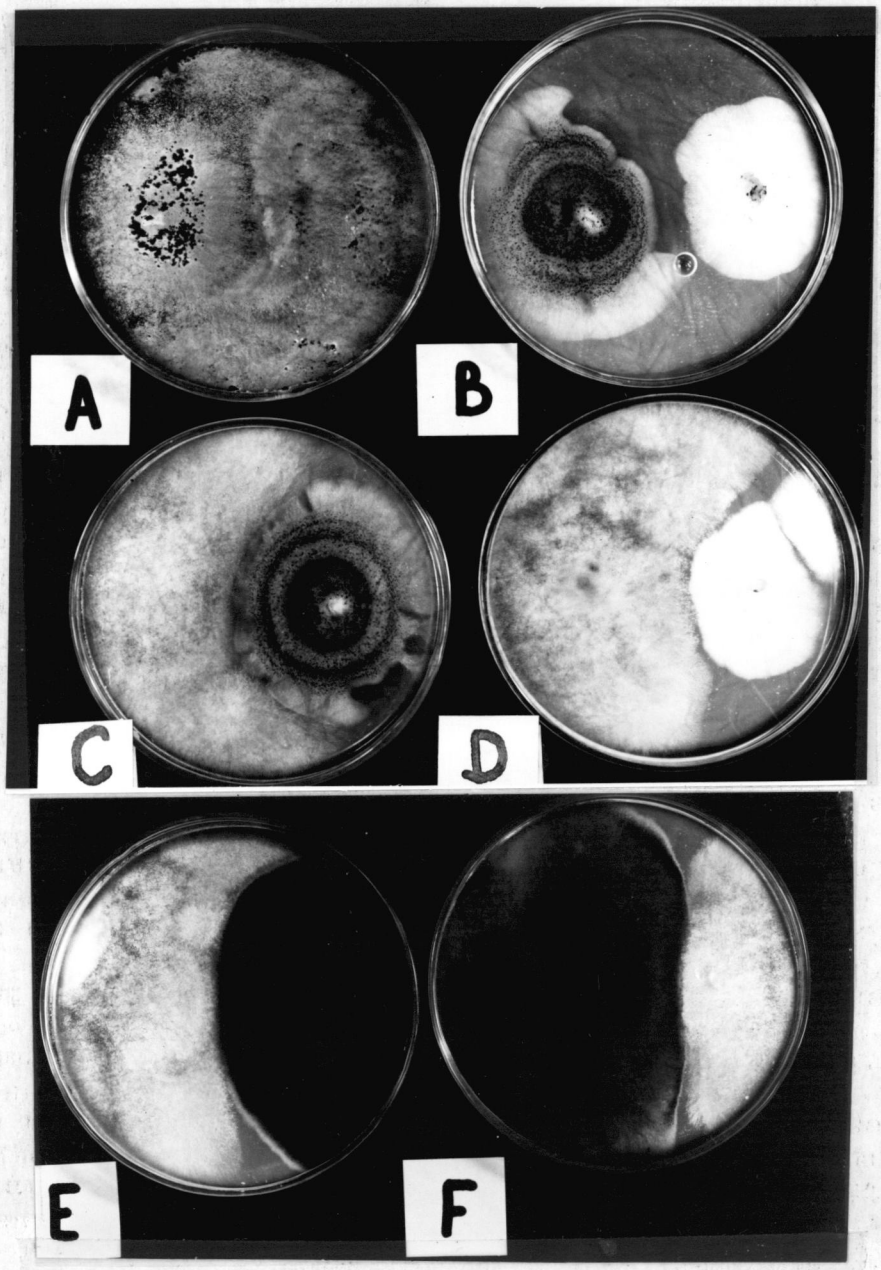

Figure 4. Interaction on potato dextrose agar of fungi isolated from strawberries. A. Trichoderma and Botrytis : B. Dendrophoma (left) and Pullularia (right) : C. Dendrophoma and Botrytis : D. Pullularia and Botrytis : E. Alternaria (right) and Botrytis : F. Stemphylium (left) and Botrytis.

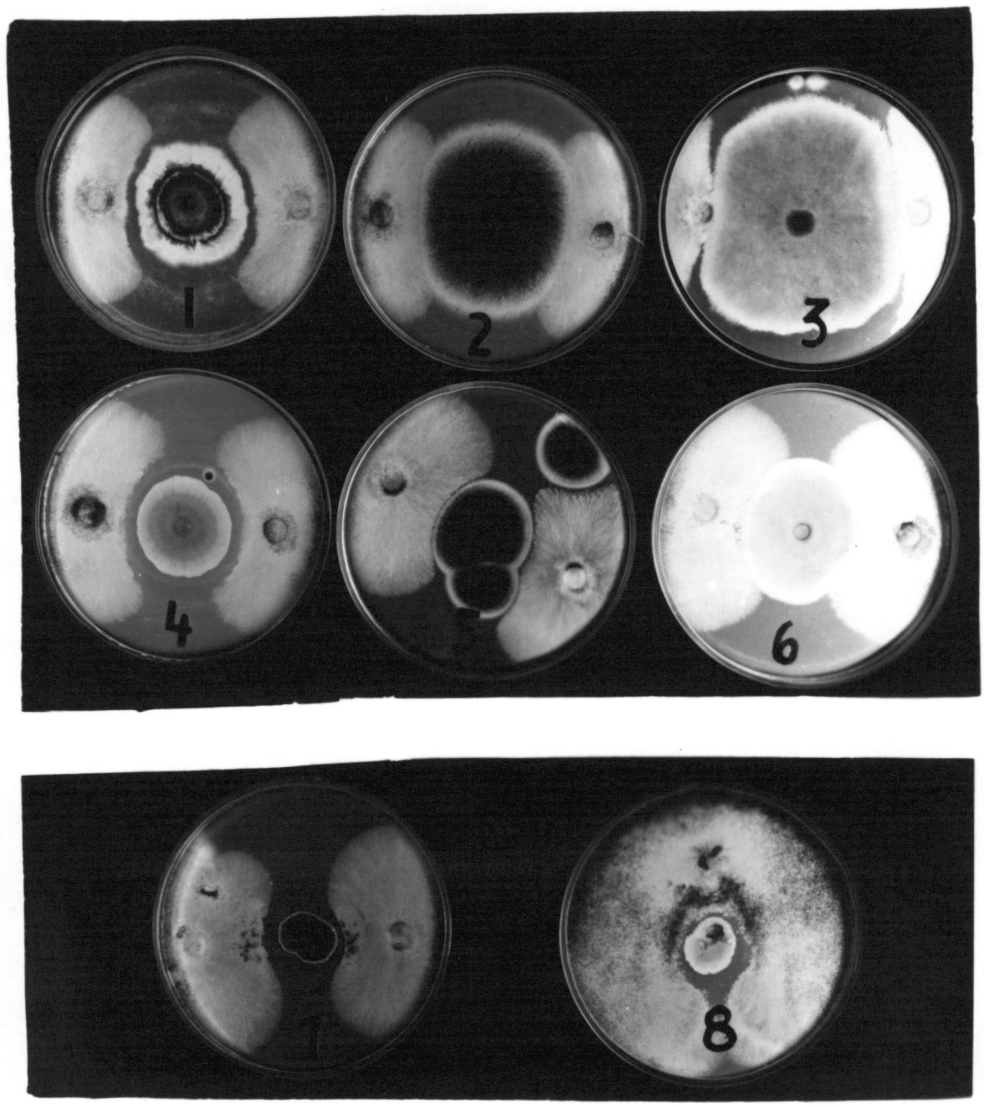

Figure 5. Interaction of fungi isolated from strawberries with Botrytis on yeast extract agar. Cultures are one week old. Botrytis inoculated on either side of the antagonists.

1. Gnomonia 2. Pestalotia 3. Fusarium 4. Chaetomium 5. Cladosporium 6. Phoma 7. Penicillium 8. Coryneum

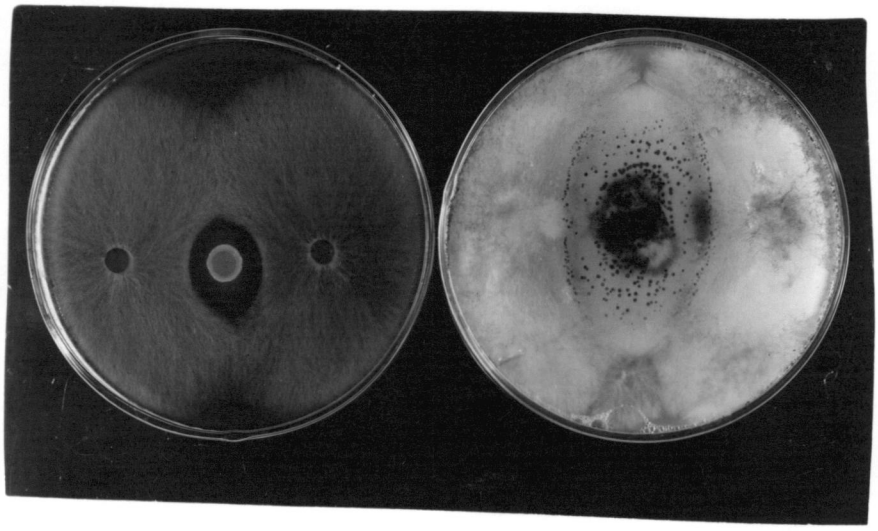

Figure 6. Inhibition of growth of Botrytis by Dendrophoma on strawberry agar (left) and on malt agar (right). Note that a wide zone of inhibition is formed only on strawberry agar.

which is inhibitory to Botrytis (Figure 5, plate 8) and to Phytophthora cinnamomi, P. lateralis, Cladosporium, Sclerotinia, Pestalotia and Rhizoctonia. As far as could be ascertained, Coryneum has not previously been reported on strawberries. Inasmuch as this fungus was isolated only once, its association with strawberries remains doubtful.

The interaction characterized below between Botrytis and associated fungi is based primarily on dual culture studies on yeast extract agar and strawberry agar.

Type A: A distinct mycelium-free zone between competing fungi indicates the production of a diffusible metabolic product by antagonist. This type of interaction occurs between Penicillium and Botrytis (Figure 5, plate 7), Dendrophoma and Cladosporium, Dendrophoma and Botrytis (Figure 4, plate C and Figure 6), Chaetomium and Botrytis (Figure 5, plate 4) and Gnomonia and Botrytis (Figure 5, plate 1).

Type B: There is no distinct mycelium-free zone between the two competing fungi. Botrytis grows to the edge of the antagonist but its further growth is stopped. There is no intermingling of hyphae of the two fungi. Botrytis which is a fast growing fungus grows around the other. This type of interaction is seen between Cladosporium and Botrytis (Figure 5, plate 5) and Fusarium and

Botrytis (Figure 5, plate 3).

Type C: There is no mycelium-free zone between the competing fungi and there is free intermingling of hyphae. However, the antagonist grows faster than Botrytis and in some instances grows over the latter. Pestalotia and Phoma (Figure 5, plates 2 and 6) show this type of interaction with Botrytis.

Nature of antibiosis between Cladosporium herbarum, Pullularia pullulans, Dendrophoma obscurans and Botrytis cinerea:

Three antagonistic saprophytes, Cladosporium herbarum, Pullularia pullulans and Dendrophoma obscurans were selected for intensive study in the laboratory because 1) they are always associated with healthy and diseased strawberries, 2) they can be established on senile floral organs before Botrytis can infect green and ripe fruits and 3) they grow and sporulate readily on PDA or other culture media. All three antagonistic organisms, Cladosporium, Pullularia and Dendrophoma, were grown in liquid media; potato broth with two per cent galactose was used for Pullularia; Richards' and Czapek for Cladosporium; and yeast extract media for Dendrophoma. The cultures were either incubated for varying lengths of time at fixed temperatures or were shaken continuously on a Burrell shaker at room temperature.

Sterile filtrates from cultures of these fungi were prepared by Seitz filtering. The inhibitory capacity of the sterile filtrates of cultures of Cladosporium, Pullularia and Dendrophoma against Botrytis were determined in three different ways: (1) suppression of spore germination (2) relative lengths of germ tubes in used vs. fresh media and (3) total growth as measured by dry weight.

Relationship between Cladosporium and Botrytis:

In early experiments, the influence of temperature on growth of Cladosporium herbarum and the resulting pH changes of the medium were determined. A forty ml. aliquot of Richards' medium was inoculated with Cladosporium. Four flasks (replicates) were used for each incubation temperature i.e. 5°, 20°, 25° and 30°C. After 22 days growth was measured by the dry weight method and the pH of the medium was determined (Table 9).

The experiment indicated that 20°C is the optimum temperature for growth of Cladosporium and at conditions favorable to its growth greatest shift towards alkalinity also takes place. At 30°C there was no growth and changes in pH were negligible.

In the next experiment period of active growth of Cladosporium with accompanying changes in pH were determined.

Table 9. Growth of Cladosporium herbarum at 5°, 20°, 25° and 30°C at the end of 22 days in Richards' medium.

Temperature in centigrade	growth/ gm	final pH*
5	0.2755	6.7
20	0.3212	7.0
25	0.3044	7.3
30	0.0074	4.0

*pH of the check medium 4.6

Cladosporium herbarum was grown in Czapek medium at 20°C and dry weights of mycelium and changes in pH of the culture were determined periodically (Figure 7).

It is apparent that the period of active growth of Cladosporium in Czapek medium comes to an end some time in the third week (Figure 7). There is a correlation between age of the culture and change in pH; when growth of the fungus had stopped, the pH of the culture continued to rise. The alkalinity of the stale medium was due to the production of potassium bicarbonate, which was identified by the $Ba(OH)_2$ test. Potassium nitrate is the nitrogen source in Czapek and Richards' media; as nitrate ions are used up by the fungus, potassium ions are set free in the medium forming potassium bicarbonate. From this experiment it is also indicated that staling products accumulate in a culture of Cladosporium when it is in the declining phase

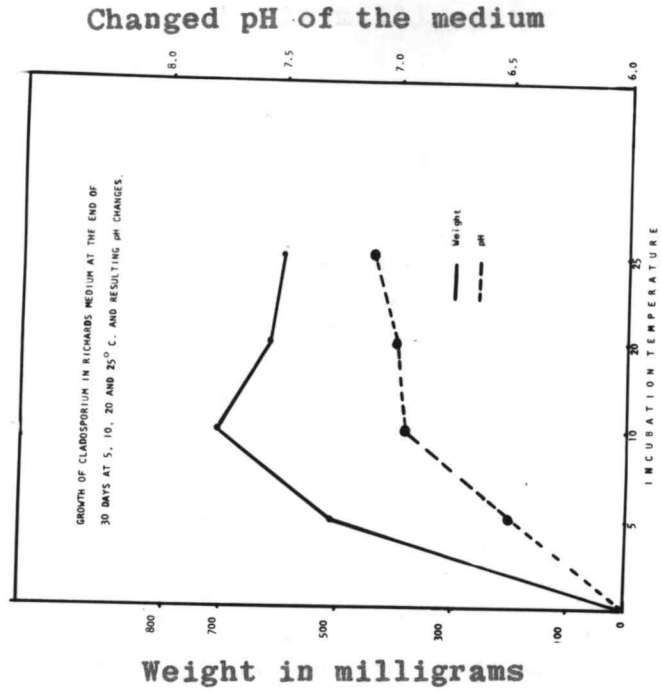

Figure 8.

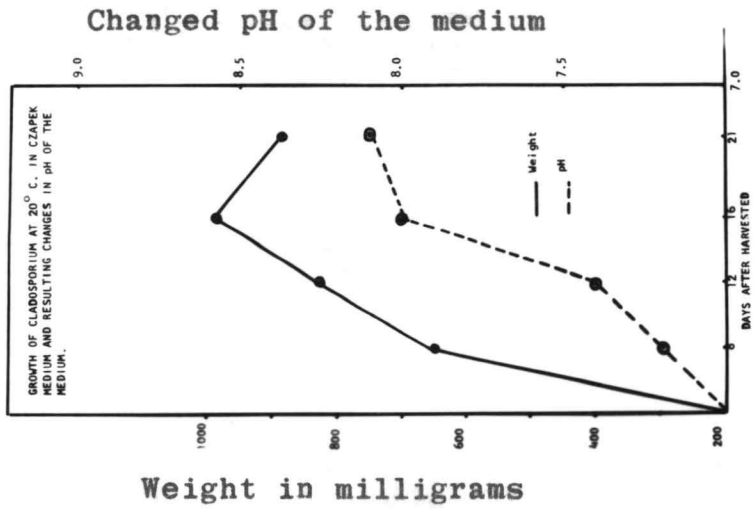

Figure 7.

of growth.

To determine the nature of antibiosis between Botrytis and Cladosporium cultural studies of the latter were undertaken. In 250 ml. flasks containing 40 ml. of the Richards' medium Cladosporium was grown at 5°, 10°, 20° and 25°C. Four flasks (replicates) were kept at each temperature and after 30 days dry weight and pH measurements of the cultures were determined (Figure 8). It is seen from figure 8 that less growth was present after 30 days in the cultures incubated at 20°C and 25°C than in the cultures incubated at 10°C. This may be explained by the fact Cladosporium grows best at temperatures of 20°C - 25°C and after 30 days the cultures at these temperatures were in the declining phase of growth.

Sterile filtrates of the medium on which Cladosporium had been grown were divided into three portions of 20 ml. each. One portion was brought to the pH of fresh Richards' medium (4.6) by addition of N/10 acetic acid, buffered with a phosphate buffer and autoclaved for 15 minutes. The second lot was autoclaved without the addition of buffer and the last portion was used without autoclaving or buffering. Each set was replicated four times and the media was inoculated with 0.5 ml. of suspension of Botrytis spores. All seeded cultures were incubated at 20°C for 7 days. Results of this experiment are given in table 10.

Table 10. Inhibitory effect of filtrates from 30 day old cultures of Cladosporium grown at 5°, 10°, 20° and 25°C on growth of Botrytis.*

Type of medium used	Incubation temperature of original culture of Cladosporium			
	5	10	20	25
Sterile filtrate	++++	+++	0	#
Sterile buffered and autoclaved filtrate	+++	++	0	#
Sterile autoclaved filtrate	++++	++++	#	0

*growth based upon visible and microscopic examination, maximum growth (in check, fresh Richards' medium) +++++.
0 no growth
trace growth

In filtrates from Cladosporium cultures grown at 20°C and 25°C (which are optimum for growth of Cladosporium), growth of Botrytis was negligible. At 5° and 10°C Cladosporium grows slowly and hence the inhibitory substance(s) is lacking, which is reflected in profuse growth of Botrytis in culture filtrates of Cladosporium obtained at these temperatures. The inhibitory action is thus most pronounced in filtrates of Cladosporium in which the conditions for growth of Cladosporium had been optimum and

the production of the inhibitive substance is a direct function of the amount of growth of Cladosporium.

This contention was further substantiated when spores of Botrytis were germinated in sterile filtrates of 23 day old Cladosporium cultures grown at 5°, 15°, 20°, 25° and 30°C and germ tube lengths measured after about 16 hours (Figure 9).

From these results it is evident that pH influences the germinative capacity of Botrytis spores to a certain degree but it is not the sole cause for inhibition because growth is restricted in pH adjusted sterile filtrates of Cladosporium cultures (Figures 7, 9, 10).

A gradual accumulation of inhibitory substance takes place in the medium supporting growth of Cladosporium with a corresponding increase in the pH of the medium. It was found that in filtrates from 7 and 28 day old cultures of Cladosporium that is 100 per cent germination of Botrytis spores; however, growth of germ tubes in the older filtrate was considerably restricted (Table 11, figures 10 and 11).

Sterile filtrates of 7 and 28 day old cultures of Cladosporium were further concentrated by drying at 90°C for 24 hours in an oven and redissolving the dry mass in 1/4 original volume of sterile distilled water. When Botrytis spores were germinated in these filtrates, no appreciable difference in percentage germination was obtained in 7 day

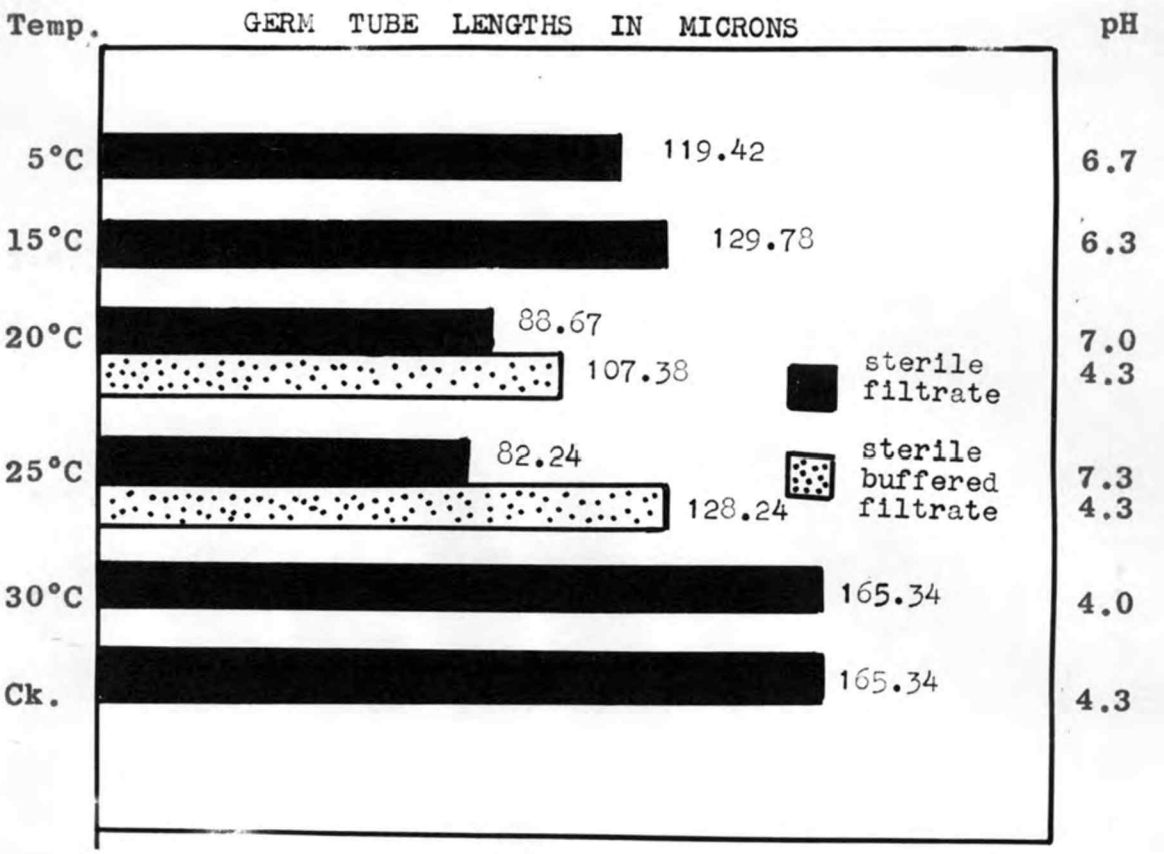

Figure 9. Inhibition of growth of Botrytis germ tubes by sterile filtrates of 23 day old culture of Cladosporium herbarum in Richards' medium incubated at 5°, 15°, 20°, 25° and 30° centigrade.

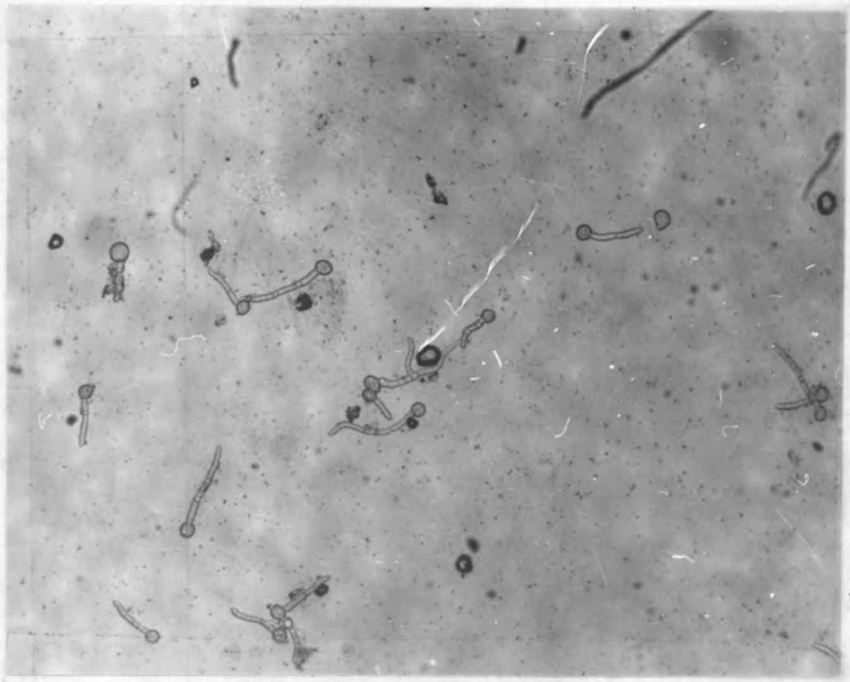

Figure 10. Inhibitory effect of filtrate from a 30 day old culture of Cladosporium herbarum on germ tube lengths of Botrytis cinerea, growth after 20 hours.

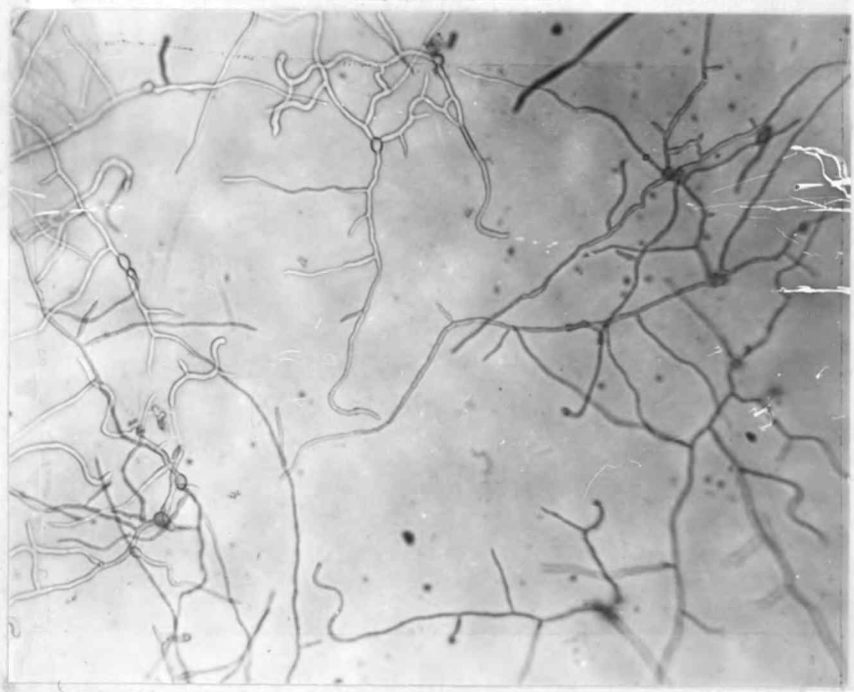

Figure 11. Growth of Botrytis spores in Richards' medium after 20 hours.

Table 11. Comparative effect of filtrates from 7 and 28 day old cultures of Cladosporium on growth of Botrytis germ tubes.

Age of culture filtrate	Growth of germ tubes in microns after 20 hrs.*	pH of the filtrate
7 day	515.2	6.3
28 day	50.4	7.9

*mean length of 30 germ tubes on each of 3 different slides

old oven dried filtrate and fresh Richards' medium but a concentrated 28 day filtrate suppressed Botrytis spore germination (Table 12).

Table 12. Comparative effect on germination of Botrytis spores of concentrated filtrates from 7 and 28 day old Cladosporium cultures.

Type of medium	Per cent germination of spores after 20 hours in:	
	filtrate from 7 day old culture	filtrate from 28 day old culture
Sterile filtrate	100	100
1/4 conc. filtrate	100	0
1/2 conc. filtrate	100	11.8
Check	100	100

Because the inhibitory effect of the filtrate on Botrytis spores was not destroyed by oven drying or autoclaving, it is obvious that the inhibitory substance is thermostable and therefore non-enzymatic in nature.

Effect of pH on germination of Botrytis spores:

In earlier studies (66, p.283-341) it has been found that spores of Botrytis germinate within a pH range of 2.8 - 7.4. It is also reported that growth usually stops at pH 3 and on the alkaline side between pH 8 and 9, the optimum for mycelial growth being 2.5 - 4.0. In the alkaline medium germ tubes become short and malformed (Figures 10 and 11).

In one experiment the effect of pH on germinative capacity of Botrytis spores was determined. A sterile filtrate of 30 day old Cladosporium culture was divided into five aliquots and the pH was adjusted with 2N HCl or N/10 NaOH to 4.4, 5.0, 7.0, 7.9 and 9.0 and buffered at these points with a phosphate buffer. Spore density was adjusted to 50,000 spores per ml. with a hemacytometer after mixing with the filtrates of Cladosporium cultures at the various pH levels. Percentage germination of spores and germ tube lengths were recorded after 20 hours (Table 13).

Table 13. Effect of pH of sterile filtrate from Cladosporium culture on germination and length of germ tube of Botrytis spores.

Adjusted pH of filtrate	Per Cent germination after 30 hrs.	Germ tube lengths in microns after 20 hrs.*
4.4	100	347.62
5.0	100	206.92
7.0	100	148.96
7.9	98	88.48
9.0	30	70.00

*average of 30 spores

The effect of pH in suppressing germ tube lengths becomes evident as the pH of the sterile filtrate is raised but the percentage of spores germinated is virtually unchanged between pH 4.4 and 7.9. However, at pH 9 only a third of the spores germinated.

Antagonism between Pullularia pullulans and Botrytis cinerea:

Pullularia pullulans produces an antibiotic substance in potato dextrose broth at pH 3.7, 6.6, 7.2 and 8.6 which stops growth of Botrytis cinerea in dual culture. The antibiotic substance is thermostable and can be kept for some time without losing its properties (5, p.82). Since Pullularia is frequently isolated from flowers and fruits of strawberries, studies were undertaken to explain the cause of antibiosis between it and Botrytis.

The isolate of Pullularia obtained from strawberry did not form a wide zone of inhibition on PDA, yeast extract agar or strawberry agar. Furthermore, when the isolate of Pullularia obtained from Baigent and Ogawa (5, p.82) was grown on PDA with Botrytis in dual culture, it did not form a wide zone of inhibition as reported by these workers.

The methods employed to study antibiosis between these two organisms were similar to those employed in the study of Cladosporium. Pullularia cultures in potato broth were shaken continuously for 7 days and a sterile filtrate of the culture obtained by Seitz filtering. Twenty ml. of filtrate was seeded with a heavy spore suspension of Botrytis. The checks consisted of fresh potato dextrose broth. Cultures were incubated at 20°C for a week, when growth of the fungus was determined by dry weights. In the same experiment, growth of Botrytis in sterile filtrates of Pullularia cultures was determined by autoclaving one portion of the latter for 15 minutes and adding fresh potato broth to the other part. All three sets were replicated.

As with Cladosporium, there was no inhibition of spore germination when the filtrate was tested against Botrytis on depression slides but fungistatic effect was clearly evident (Figure 12).

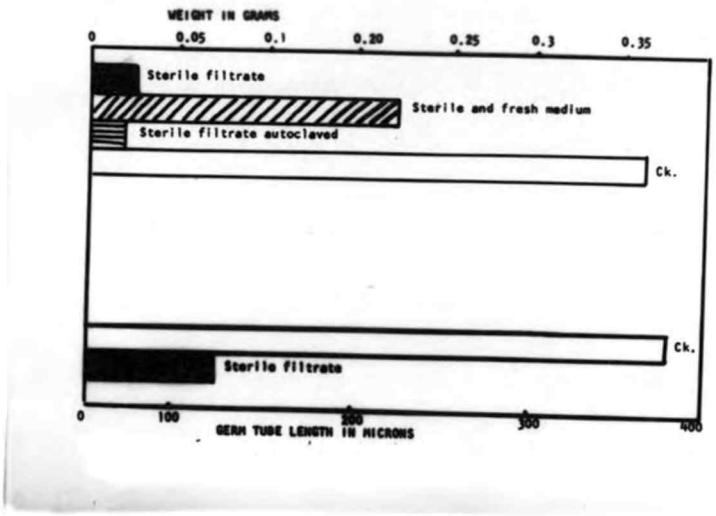

Figure 12. Effect of metabolic products of Pullularia pullulans on growth and germ tube lengths of Botrytis cinerea.

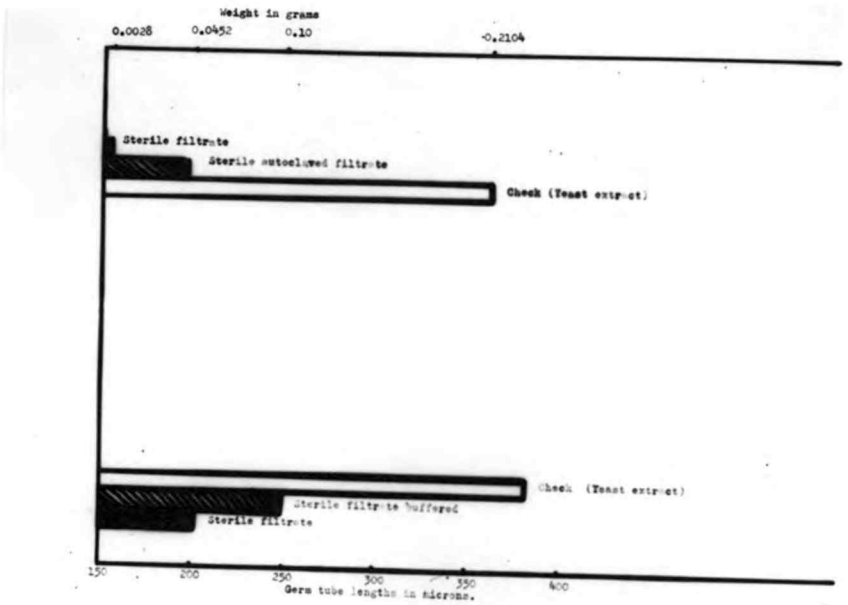

Figure 13. Effect of metabolic products of Dendrophoma obscurans on growth and germ tube lengths of Botrytis cinerea.

Addition of fresh medium to the sterile filtrate increased growth (gm.) of Botrytis cinerea by approximately a factor of ten but was still 45 per cent lower than the check. It would thus appear that the marked inhibition of growth of Botrytis observed is not the result of a lack of nutrients in the filtrate. Similar inhibition of Botrytis was noted when the lengths of germ tubes were used as the criterion of growth (Figure 12).

As the pH values of the filtrate were not considerably different from that of the check, the inhibition observed can not be ascribed to changes in hydrogen ion concentration.

The effect of toxic metabolites of Pullularia upon Dendrophoma was also investigated. When Dendrophoma spores were sown in the filtrate of 14 day old Pullularia cultures in potato broth, germination of Dendrophoma spores was suppressed, while in the check medium 100 per cent germination was observed. In dual cultures on potato dextrose agar a distinct mycelium-free zone is produced (Figure 4, plate B).

In the knowledge of this author, antagonism of Dendrophoma by Pullularia has not been previously reported. It is possible that suppression of Dendrophoma rot on strawberries in the Pacific Northwest may be due to this antagonism between Pullularia and Dendrophoma. This aspect might be worthy of further investigation.

Antagonism between Dendrophoma and Botrytis:

Gray mold and leaf blight or stem-end rot are both serious diseases of strawberries. However, in the Pacific Northwest, only gray mold is of serious consequence in the field. In the midwestern states, Dendrophoma is the principal rot causing organism (1, p.698-706; 9, p.7). These two diseases are rarely present together under Oregon conditions, although Dendrophoma is frequently isolated from petioles and sepals (Table 2 and 6). Therefore, an attempt was made to determine the type of relationship that exists between these two organisms.

It has been mentioned that the type of interaction between two organisms is influenced by the culture medium. In medium incorporating strawberry juice, Dendrophoma produces a wide zone of inhibition against Botrytis (Figure 6); this is observed also when the medium has been buffered to the pH optimum for growth of Botrytis. On yeast extract medium, the zone of inhibition between Dendrophoma and Botrytis was 0.10 cm, approximately one-fifth of that observed on the strawberry agar (Tables 7 and 8).

Cultural studies similar to those with Pullularia were undertaken to determine the nature of antibiosis between Dendrophoma and Botrytis. Eight 25 ml. aliquots of Dendrophoma culture filtrate, four of which had previously been autoclaved for 15 minutes were inoculated with 0.5 ml.

of a Botrytis spore suspension and incubated at 20°C. Checks consisted of yeast extract medium only. At the end of one week growth of Botrytis was determined by dry weight method. The results showed that Botrytis failed to grow in the medium in which Dendrophoma had been growing previously (Figure 13).

Because these studies indicated that the metabolic products of Dendrophoma are strongly inhibitory to growth of Botrytis, an experiment was carried out to determine the extent to which Botrytis rot can be prevented by Dendrophoma on green and ripe strawberries.

Fully ripe and green strawberries were surface sterilized with 20 per cent Clorox and rinsed with sterile distilled water. Holes were punched in the berries with a cork borer and 0.2 ml. spore suspensions of Dendrophoma and Botrytis were pipetted into these wells. Checks consisted of berries inoculated with either Dendrophoma or Botrytis. Berries were incubated in plastic trays lined with moist paper towels. Absence of mycelial growth and sporulation by Botrytis on dual inoculated berries was considered as an indication of successful prevention of Botrytis rot by Dendrophoma (Figure 14).

Two point four per cent of the green fruits and 35.3 per cent of the ripe berries showed Botrytis rot when they were inoculated simultaneously with Dendrophoma and Botrytis, compared to 80.4 per cent and 89.6 per cent in

Figure 14. Prevention of gray mold (Botrytis cinerea) on green strawberries by Dendrophoma. A. fruits inoculated with Dendrophoma only, B. fruits simultaneously inoculated with Botrytis and Dendrophoma, and C. fruits inoculated with Botrytis only. Note profuse mycelial development in fruits inoculated with Botrytis only.

checks (Tables 14 and 15).

Table 14. Prevention of Botrytis rot in ripe strawberries by Dendrophoma.

Treatment	Number of berries treated	Number with Botrytis infection	Per Cent infection
Dendrophoma plus Botrytis	102	36	35.3
Botrytis	51	41	80.4

Table 15. Prevention of Botrytis rot in green strawberries by Dendrophoma.

Treatment	Number of berries treated	Number with Botrytis infection	Per Cent infection
Dendrophoma plus Botrytis	84	2	2.4
Botrytis	48	43	89.6

Inhibition of Botrytis by the metabolic products of Dendrophoma, in the knowledge of present writer, has not previously been demonstrated. In spite of the reports of Dendrophoma rot in strawberries in certain parts of the

United States (1, p.698-706), it is of minor importance in the Pacific Northwest (48, p.27). As Botrytis rot and Dendrophoma (stem-end rot) are not usually found on the same fruit, it is possible that in parts of country where Dendrophoma is the predominant organism, Botrytis rot is suppressed by the former.

Control of Botrytis cinerea on strawberries in the greenhouse by Cladosporium herbarum, Pullularia pullulans and Penicillium sp.:

Strawberries were grown in the greenhouse either in 6" pots or in #10 cans during the winters of 1959 and 1960. By regulating the greenhouse temperature and providing extra illumination, fruits were produced in the second week of February. Spore suspensions of Cladosporium herbarum, Pullularia pullulans or Penicillium sp. in 1% glucose solution were sprayed on fruits at late petal fall or early green fruit stage and again after an interval of about one week. Check flowers and fruits on the same plants were sprayed with 1% glucose solution (Pullularia and Penicillium checks) or with a spore suspension of Botrytis (Cladosporium checks only) (Figure 15). In order to avoid other organisms becoming established on senile floral parts, treated flowers or fruits were caged or placed inside a small humidity chamber. At this stage humidity was kept low to discourage infection of fruits by Botrytis.

Figure 15. Control of Botrytis cinerea by Cladosporium herbarum. Fruit at left inoculated with Cladosporium and one at extreme right (check) with Botrytis only on same day. After three weeks, all fruits were inoculated with Botrytis. Note development of gray mold on green (check) fruit.

When the fruits had reached late green or whitening stage, a heavy suspension of Botrytis spores was sprayed on treated and check fruits. The plants were then removed to a mist chamber where humidity was kept high to provide optimum conditions for Botrytis infection. Plants were kept in a humidity chamber for a period of 8-10 days before final observations were made on fruit rot development (Table 16).

Table 16. Control of Botrytis cinerea by prior inoculation of Cladosporium herbarum on strawberry flowers at late bloom stage.

Treatment	Total number of treated	Number of fruits infected by Botrytis	Per Cent of rot	Per Cent inhibition
Cladosporium plus Botrytis	163	64	39	42
Botrytis	92	62	67	-

In the above experiment, fruits that showed cat facing or that were imperfectly developed were discarded and only those counted that showed normal development.

In another experiment, Cladosporium was sprayed at early green fruit stage instead of at late bloom stage. Percentage inhibition of rot in this case was only 2% as compared to 42% in the previous experiment (Table 17).

Table 17. Control of *Botrytis cinerea* by prior inoculation of *Cladosporium herbarum* on green strawberries.

Treatment	Number of fruits treated	Number of fruits infected by *Botrytis*	Per Cent inhibition of rot
Check (*Botrytis* only)	58	43	-
Cladosporium plus *Botrytis*	58	42	2

From these experiments it is quite obvious that if microbiological control of gray mold in strawberries is attempted, treatment with antagonistic saprophytes should be done at the right stage of fruit development and humidity should be kept at a minimum to discourage infection by *Botrytis* which also competes for the infection sites along with other associated microflora.

Two other fungi, *Pullularia pullulans* and *Penicillium* were also used to control *Botrytis* rot in strawberries in the greenhouse. Of these *Penicillium* produced a wide zone of inhibition against *Botrytis* on Petri plates and *Pullularia pullulans* although not so antagonistic as the former, did retard growth of *Botrytis*, as was also found by the

sterile filtrate studies. It was found that Penicillium was ineffective (Table 18) in preventing Botrytis rot in the greenhouse, while a significant amount of control was obtained with Pullularia.

Table 18. Control of Botrytis cinerea by prior inoculation of Penicillium and Pullularia pullulans on strawberry flowers at late petal fall stage.

Treatment	Number of fruits treated	Number infected by Botrytis	Per Cent of rot	Per Cent inhibition of rot
Penicillium	81	57	70	4
Check	81	60	74	-
Pullularia	127	56	44	31
Check	127	80	64	-

In these experiments no attempt was made to sterilize flowers or green fruits before inoculating them with antagonists; thus the possibility of infection of these parts by Botrytis at the time of application of antagonists is not precluded. Moreover, as the plants were kept after treatment with Botrytis under a very humid atmosphere, secondary infection of fruits due to germination and subsequent infection by air-borne Botrytis spores may

have taken place, thus nullifying the effect of the saprophytic antagonists.

Of the three fungi used to prevent infection of strawberries by Botrytis, Cladosporium is most successful. This fungus is eminently suited for use in artificial biological control of gray mold organism because of these considerations: 1) It can produce spores in large numbers in a short time and can become established and grow under dry conditions. 2) It is non-toxic and non-pathogenic to strawberries. 3) It is self propagating to some extent because its spores colonize readily infection sites that would otherwise be occupied by Botrytis.

Prevention of gray mold by Cladosporium in the field:

In 1961 a field experiment was carried out in cooperation with Dr. E. K. Vaughan to determine the effectiveness of Cladosporium in prevention of gray mold infection as determined by percentage of rot at harvest and the total yield of marketable strawberries. A heavy suspension of Cladosporium in one per cent glucose solution with a few drops of X-77 spreader activator* was sprayed on strawberries at the Plant Pathology farm at 50 per cent bloom stage, using the same equipment used to apply test fungicides. While fungicides were sprayed on April 24, May 2,

*Product of Colloidal Products Corporation, Sausalito, California.

11, 19 and 27, the fungus was sprayed only on May 2, 11 and 19. At regular intervals flowers and fruits were picked, homogenized in a Waring blendor and plated out in Strep PDA. The results of this experiment are summarized below (Table 19).

These data, in general, substantiate the results of a similar experiment carried out in 1960 to determine qualitative and quantitative changes in the fungal population throughout the development of strawberry fruits following application of fungicides. It was found that Dyrene sharply reduced the number of fungi in berries from treated plots and the difference between treated and untreated berries is maintained until the end of harvest. Although recolonization of berries by fungi on treated plots could not be prevented, most of these were non-rot-causing (Table 19). Cladosporium was the dominant species in isolations from all of the three treatments.

Fungi were found to occur mostly as surface microflora, only a few of the species invade the tissue. However, in the case of flowers and green fruits, isolations were made without surface sterilization and itis possible that some contaminants may have been included in total count. It is interesting to note that total mold count inside ripe strawberries (surface sterilized) from Cladosporium treated plots were lowest of all the treatments

Table 19. Frequency of isolation of fungi from Northwest strawberries at various stages of growth following application of Dyrene and Cladosporium as pre-harvest sprays.[1/]

Fungi	TREATMENT											
	Dyrene				Cladosporium				Check			
	5/16	5/30	6/7 A	6/7 B	5/16	5/30	6/7 A	6/7 B	5/16	5/30	6/7 A	6/7 B
Cladosporium	7	102	97	5	459	200	290	5	20	182	310	16
Pullularia	-	3	-	21	20	2	22	3	13	-	43	15
Botrytis	2	4	7	1	12	2	23	-	-	14	33	1
Dendrophoma	-	-	-	-	9	-	66	-	3	-	9	-
Penicillium	1	2	-	-	3	-	-	-	-	1	-	-
Fusarium	-	-	-	-	2	-	-	-	2	-	5	-
Rhizopus	-	-	3	-	-	-	-	-	-	5	-	-
Alternaria	-	1	-	-	-	-	-	-	5	2	-	-
Trichoderma	-	-	-	-	-	-	1	-	-	-	-	-
Aspergillus	-	-	-	-	1	-	-	-	-	-	-	-
Stemphyllium	-	-	-	-	-	-	-	-	-	1	-	-
Pestalotia	-	1	-	-	-	-	-	-	-	-	-	-
Gnomonia	-	-	-	2	-	-	-	1	-	-	-	-
Melanconium?	-	-	-	-	-	-	-	1	-	-	-	-
Total (identified)	10	113	107	29	506	204	402	10	43	205	400	32
Total (unidentified)	56	11	165	4	1938	2796	981	8	509	413	1318	29
TOTAL	66	124	272	33	2444	3000	1383	18	552	618	1718	61

[1/] Total of 5 plates with three replications per 50 flowers and green fruits at a dilution of 1:1000.

A Only 25 ripe berries (unsterilized)

B Only 25 ripe berries (surface sterilized)

(Table 19). This indicates that a high concentration of Cladosporium on moribund floral organs prevented other rot and non-rot-causing organisms from being established on or invading uninjured strawberry tissue.

To determine the effect of a fungicide (Dyrene) and Cladosporium on Botrytis rot and on yield of marketable strawberries, all the berries from 15' test rows (replicated five times for each treatment) were picked on seven different dates and rotted and marketable berries were counted on each picking (Tables 20 and 21; figure 16).

Table 20. Percentage of rot in strawberries from check, Cladosporium and Dyrene treated plots. Figures represent average of five replications.

Date picked	Weather	TREATMENTS		
		Cladosporium	Dyrene	Check
June 1	sunny	0	0	0
7	rainy	0.6	0.9	1.9
12 13	fair	2.5	2.6	4.3
16 17	hot sunny	8.4	5.3	8.4
21 22	sunny	4.9	3.8	4.0
26 27	sunny	10.2	6.1	7.6
30	sunny	8.3	1.2	6.7
Season		4.99	2.84	4.81

Table 21. Yield of marketable strawberries (in ounces) from check, Cladosporium and Dyrene treated plots. Figures represent average of five replications.

Date picked	Weather	TREATMENTS		
		Cladosporium	Dyrene	Check
June 1	sunny	8.0	8.8	7.6
7	rainy	37.6	39.2	39.2
12	fair	53.6	49.6	48.4
13				
16	hot	92.8	84.8	70.4
17	sunny			
21	sunny	61.6	56.0	46.4
22	sunny			
26	sunny	56.0	28.0	22.4
27				
30	sunny	13.6	4.8	9.6
Total		323.2	271.2	244.0

The incidence of fruit rot was highest in the middle of the picking season and low in the beginning and end (Figure 16). High temperatures prevailed during most of the picking period (61, p.55-108). There was very little precipitation in June which may be the reason for low per cent of rot in the field as compared to 1960 (62, p.43). During the 1961 strawberry bloom period (April - May), precipitation was above average; total rainfall was 2.23" and 2.71" during these two months (Figure 16; 61, p.55-108).

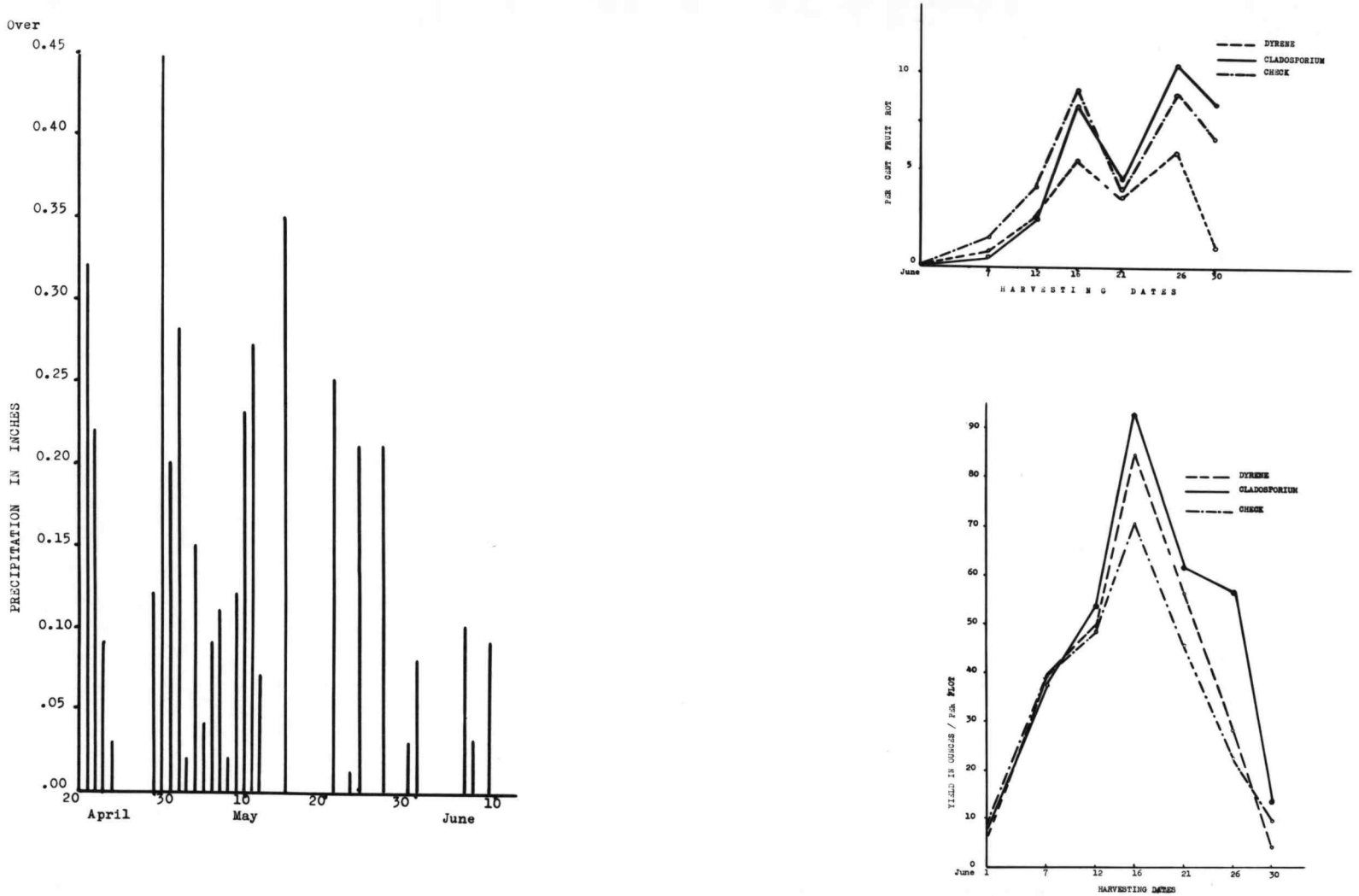

Figure 16. The relationship between daily precipitation and incidence of fruit rot and yield of marketable strawberries from Dyrene and Cladosporium sprayed plots.

Although conditions were very favorable for blossom blight and green fruit rot development, yield of marketable strawberries from Cladosporium treated plots was higher than from Dyrene and check plots (Table 21; figure 16).

Both Cladosporium and Dyrene reduced considerably primary infections of strawberries at late bloom stage which resulted in higher yields at harvest time. Cladosporium treated plots consistently yielded more marketable berries, although per cent rot was also highest in strawberries from these plots. This suggested that in Cladosporium treated plots blossom blight was greatly reduced, resulting in "setting" of more berries. Low yields from Dyrene treated plots may be due to foliage injury caused by this chemical.

It has been shown that apparently sound strawberries harbor latent infection of Botrytis (47, p.46 and 63; 657-658). The amount of latent infection was ascertained in strawberries from check, Cladosporium and Dyrene treated plots. Ripe strawberries were picked from these plots and surface sterilized in ten per cent Clorox for two minutes. After washing in sterile distilled water berries were placed in sterile egg cartons, which were kept in plastic containers lined with moist paper towels. After 72 hours of incubation at room temperature rotted berries were counted (Table 22).

Table 22. Frequency of latent Botrytis infection in marketable strawberries from checks, Cladosporium and Dyrene treated plots after incubation at high humidity.

Treatments	Number of berries incubated	Number showing rot symptoms	Percentage of rot
Cladosporium	48	5	10
Dyrene	72	6	8
Check	96	17	18

It was found that apparently healthy strawberries showed a high percentage of rot from untreated plots (18 per cent) as compared to Dyrene and Cladosporium ones (8 per cent and 10 per cent).

DISCUSSION

Biological control of a plant pathogen may be accomplished one of the three ways: (1) by occupation of available infection sites by saprophytic antagonists, creating a preventive barrier around susceptible host organs, (2) production of a toxic metabolite or antibiotic and (3) direct parasitization of the pathogen.

Biological control of plant pathogenic diseases has met with varying degrees of success. Control of some soilborne diseases has been made possible through stimulating antibiosis by changing cultural methods, such as green manuring. According to Sanford (55, p.525-547), this is brought about by increasing the population of saprophytic organisms which compete for food with pathogenic organisms. The latter, being poor competitors, are reduced in number and effectiveness.

In their natural environment microorganisms do not exist alone, pure cultures represent an artificial situation. The results obtained from pure culture studies in the laboratory often cannot be reproduced in the field. Infection and subsequent course of disease development are influenced by associated microflora. This is of importance when a pathogen which is non-specific in its food requirements has to compete for nutrition and space with many other heterotrophs.

Botrytis cinerea is a weakly parasitic organism that becomes established initially on necrotic or senile plant tissue before invading living cells. Infection of strawberry fruit by Botrytis generally occurs at late bloom stage. Powelson (49, p.491-494) showed that strawberries grown in the greenhouse under conditions favoring severe disease development were attacked less frequently by Botrytis if petals, stamens and calyces were removed after fertilization. This suggests that dead floral organs are the main sources of invading hyphae, while direct infection of ripe fruits by air-borne spores is of minor importance. However, the same worker also pointed out that "the amount and rate of rot development was influenced more by prolonged wet periods during harvest than during the bloom period" (48, p.76).

According to him, "the obvious factor for severe epiphytotics of Botrytis rot is the occurrence of prolonged periods of rainfall and high humidities during harvest" (48, p.76). This indicates that under certain conditions, direct infection of fruit must be considered of importance.

Botrytis spores germinate and send germ tubes into ripe fruit a few hours after being placed on the surface (29, p.864-888). Ex-osmosis of nutritive substances enhances germination. In the latter part of the season much of the fruit becomes infected from spores germinating in

persistent water drops which contain at least three sugars and citric and malic acid (71, p.55). If high humidity and low temperatures prevail, Botrytis becomes an active parasite and invades uninjured tissue of the ripe fruit. The importance of the role played by humidity is evidenced by the fact that apparently healthy strawberries show symptoms of rot when incubated in a very humid atmosphere.

The relative importance of fungicides and saprophytic antagonists in the prevention of gray mold disease of strawberry has not been extensively investigated. Fungicides may destroy all or a high percentage of the microbial population and, therefore, whatever natural protection is provided by the saprophytic antagonists is lost (46, p.473-481). From the present studies it was concluded that fungicides employed as pre-harvest sprays provide protection against gray mold on strawberries for only about two to three weeks following application of the last spray. As the fungicides disappear there is a rapid build up of fungi, most of which are saprophytic and non-rot-causing. It was also found that the fungi are present mostly as surface microflora, only a fraction of the species being present within the fruits.

From dual culture studies on various media, it has been shown that at least ten genera inhibit Botrytis at a distance or on contact. Cultural filtrates of Cladosporium

herbarum, Pullularia pullulans and Dendrophoma obscurans, all of which are commonly isolated from strawberries, contained thermostable substances which inhibited growth of Botrytis.

Of all the genera, Cladosporium was isolated with the highest frequency at all stages of development, both from sprayed and unsprayed berries. The relationships between Cladosporium and Botrytis have been studied. It has been suggested that the inhibitory mechanism in this case is less specific than that of the normally recognized antibiotics and that the inhibition is not the result of a pH change because it also occurs in buffered media (45, p.185-202). In the present studies, it was found that buffering does lessen the inhibitory capacity of culture filtrates of Cladosporium against Botrytis but the germinative capacity of the latter is not entirely restored in the buffered filtrates.

It was found that the pH of the medium in which Cladosporium is growing rises to seven or more at the end of a week (check 4.6), and continues to rise even after four weeks of growth. When spores of Botrytis are sown in sterile filtrates of seven and twenty eight day old cultures of Cladosporium, there is 100 per cent germination of the spores but the germ tubes become stunted and malformed in the older filtrate. The fact that a high pH has

a definite inhibitory effect on the growth of Botrytis was demonstrated by comparing the growth of Botrytis spores in sterile filtrates of a twenty eight day old Cladosporium culture adjusted to a pH range of 4.4 to 9.0. While the percentage of spores germinated was virtually constant between pH 4.4 and 7.9, at pH 9.0 about 75 per cent of the Botrytis spores remained ungerminated. Spores contain a limited food reserve and can germinate in a medium that is inhibitory to mycelial growth. Contrary to the findings of Webb (66, p.283-341) who reported that 7.4 was the upper pH limit for germination of Botrytis spores, in the present investigations it was found that even at a pH of 7.9, 98 per cent of Botrytis spores germinated in spite of the fact that the medium contained substances inhibitory to mycelial growth.

An earlier investigator reported that within wide limits, the pH value of a staled medium did not appear to be a limiting factor for growth of Botrytis (10, p.132-133). In sharp contrast to this finding, the present studies demonstrated that growth of Botrytis cinerea, as measured by germ tube lengths, was depressed by changing pH of the medium from 4.4 to 9.0.

Botrytis cinerea requires a pH of 6.5 on potato decoction medium for optimum pectolytic activity (24, p.273-280) and when conditions become acid there is little

enzymatic activity despite good growth of the mold, for which the optimum pH has been considered between 2.5 and 4.0 (65, p.201-222). It was found that 20° and 25°C are the most favorable temperatures for the growth of Cladosporium and that the greatest shift in the pH of the medium towards alkaline side takes place in cultures at these temperatures. Sterile filtrates obtained from Cladosporium cultures at these temperatures inhibited growth of Botrytis. As the inhibitory factor is thermostable and is accumulated as the cultures grow older, it is suggested that this substance is an auto-toxic metabolite (staling product) (35, p.30). The inhibitory effect of staling products on growth of fungi has been recorded in earlier studies (52, 53, p.563-595 and p.599-615).

Organisms like Cladosporium, which are poor producers of antibiotics, inhibit growth of Botrytis by more than one means. In dual cultures almost no mycelium-free zone occurs between Cladosporium and Botrytis and the culture filtrate from a seven day old culture has little inhibitory effect as compared to older filtrates. Although Newhook (46, p.480), disregards the effect of pH in antagonism between Botrytis and Cladosporium, other investigators consider it the most important factor in the pathogenecity of Botrytis cinerea (59, p.24). The production of pectolytic enzymes is conditioned by the pH of the substrate. At pH 8

or above, the extra-cellular enzyme obtained from Botrytis cinerea is unable to macerate living plant cells (59, p.351-358). Most pectolytic activity is obtained between pH 3.5 and 6.0. Apparently healthy strawberries carrying latent infection of Botrytis cinerea do not show rot symptoms because of unfavorable pH of the strawberry tissues.

In the opinion of the writer, the mechanism of prevention of gray mold (Botrytis cinerea) by Cladosporium on strawberries is through the following factors. (1) Increased pH of the substrate as a result of the metabolic activity of Cladosporium provides conditions unfavorable for the production of pectolytic enzyme by Botrytis which is essential for the production of rot in the strawberries. (2) Cladosporium, through production of toxic metabolites (which may be staling products) inhibits mycelial growth of Botrytis. (3) Because of the ability of Cladosporium to become established under relatively dry conditions on dead or senile floral organs of strawberry it can rapidly colonize the available infection sites thus excluding the pathogen.

Cultural studies of Pullularia pullulans and Dendrophoma obscurans showed that these organisms produce a substance inhibitory to the growth of Botrytis. This inhibitory effect was observed even in very young cultures and

thus can not be attributed to accumulation of staling products. No appreciable change in pH was observed in the media in which these two antagonists were grown and the inhibitory substance was demonstrated to be thermostable. In dual cultures with Botrytis cinerea, both antagonists formed a distinct zone of inhibition in strawberry agar. It is indicated that the inhibition is due to the production of a very specific substance, such as an antibiotic. No attempts have been made to characterize this substance chemically.

A great deal of similarity is seen between the behavior of fungi that live as saprophytes in the soil and those that are either saprophytic or weakly parasitic on aerial parts of the plant. Fungi which colonize first are always weak parasites or obligate saprophytes (25, p.35). Successful establishment and rapid multiplication on a substrate depends on the rate of growth of these fungi. This, however, can be compensated in slow growing forms by production of an ontibiotic substance that reduces competition from other organisms (25, p.34).

On moribund or senile strawberry tissue concentrations of antibiotics may be produced by antagonistic fungi such as Pullularia and Dendrophoma, which would restrict or inhibit growth of Botrytis.

Of the three fungi tested Penicillium forms the widest

zone of inhibition against Botrytis on Petri plates but is the least effective in preventing Botrytis rot of strawberries under greenhouse conditions. An organism showing strong antagonistic activity against another in vitro, does not necessarily behave similarly under field conditions. Penicillium failed to become established under the experimental conditions provided in the greenhouse because of the absence of right conditions for its growth. Moreover, as flowers were not surface sterilized before treatment with a saprophytic antagonist, competition from other species already present on the moribund tissue may have led to poor survival of Penicillium. The effectiveness of Cladosporium and Pullularia in preventing Botrytis rot in greenhouse berries is partly due to the fact that they are able to grow under much drier conditions than Botrytis and to compete successfully with other organisms present in the substrate.

A pathogen can become established only when the inoculum potential is high enough to overcome the resistance of the host and other competing organisms. Concentration of Botrytis spores rapidly increases in strawberry fields as the harvesting season progresses, paralleling a similar increase in total mold content of the fruit. When ripe, the fruit wall is least resistant to puncture and, under suitable conditions of temperature and humidity, a sizeable

portion of the crop may be lost due to secondary infection.

In the present investigations it has been demonstrated that artificial biological control has some chance of success when the pathogen is weakly parasitic and has to compete with other saprophytic microflora on wounded or moribund tissue before it is able to invade healthy tissue. Direct use of one organism against another (e.g. by spraying a spore suspension on susceptible plant organs) has definite limitations in the control of fungus diseases. Success of this method depends upon providing favorable growth conditions for the antagonist, which in many instances also are favorable to the pathogen. Moreover, it requires several days for the population of an antagonist to build up to a level where it is able to suppress a pathogen; a fungicide is effective as soon as it is applied.

SUMMARY

1. A number of fungi are found associated with strawberries at bloom, green and ripe fruit stages of development. They occur both as surface and sub-surface microflora, a high percentage of which are non-rot-causing.

2. Cladosporium, Pullularia, Dendrophoma, Botrytis, Gnomonia, Penicillium, Fusarium, Phoma and Pestalotia were most frequently isolated from strawberries.

3. The residual effect of pre-harvest sprays on strawberries lasts not more than two to three weeks after the last spray. Fungi rapidly recolonize sprayed strawberries. Effectiveness of fungicides lies in providing protection to strawberries against gray mold infection at the bloom and late bloom stages of development.

4. In dual cultures, ten genera of fungi showed various degrees of antagonism against Botrytis. Three types of interactions were noted: 1) formation of a distinct mycelium-free zone between the competing fungi, 2) mutual inhibition at the point of contact between the two colonies and 3) growth of one species over all or part of the other.

5. The pH of cultures of some of the antagonistic fungi rises after a period of growth. Sterile filtrates of these cultures, though unable to prevent Botrytis spores from germinating, restricted their growth. Acidity is a critical factor in the production of pectolytic enzymes by

Botrytis cinerea and an increase in pH of the substrate caused by the metabolic activity of saprophytic antagonists may prevent fruit rot in strawberries.

6. Inhibition of growth of Botrytis by Cladosporium was not due to the production of a specific substance such as an antibiotic.

7. The inhibitory factor in the case of Pullularia and Dendrophoma was found to be thermostable and non-enzymatic in nature.

8. In the greenhouse, prior inoculation of strawberries at late bloom stage with three non-rot-causing fungi i.e. Cladosporium, Pullularia and Penicillium resulted in 42, 31 and four per cent control of Botrytis rot respectively.

9. Success of microbiological control of Botrytis cinerea on strawberries depends upon the ability of a saprophytic antagonist to grow under relatively dry conditions and to utilize available infection sites on moribund tissue before Botrytis has a chance to become established.

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