EFFECTS OF NEAR-ULTRAVIOLET RADIATION AND CHOLESTEROL ON GROWTH AND SPORULATION OF CYTOSPORA CINCTA AND C. LEUCOSTOMA

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ABSTRACT

EFFECTS OF NEAR-ULTRAVIOLET RADIATION AND CHOLESTEROL ON GROWTH AND SPORULATION OF CYTOSPORA CINCTA AND C. LEUCOSTOMA

By

Richard E. Stuckey

Radiation significantly increased the number of pycnidia formed and sporulation of isolates of Cytospora cincta and C. leucostoma. Cultures were grown on potato maltose agar (PMA) and potato maltose broth (PMB) adjusted to pH 6 and held at 25 C in a growth chamber. Pycnidial numbers in cultures irradiated continuously with near-ultraviolet (UV) radiation at 2.0 to 7.0 X 10^2 ergs/cm^2sec were increased over those irradiated with cool-white flourescent lamps at an intensity of 1.0 to 1.3 X 10^4 ergs/cm^2sec. Cultures not receiving radiation failed to produce pycnidia. The numbers of pycnidia exuding spores from near-UV radiated cultures was increased from double to 130 fold depending on the isolate when compared to cultures not receiving near-UV radiation. Moreover, the percentage of sporulating pycnidia was increased nearly 2-fold in cultures irradiated with near-UV.

Near-UV radiation did not significantly decrease dry weight of cultures grown in PMB suggesting that its affect on sporulation may act in a stimulatory rather than injurious manner.

Addition of 100 ppm cholesterol to PMB increased dry weight but had little or no effect on sporulation. Cultures of one isolate (Ma 4) produced conidia in 4 days from conidiophores arising directly from the mycelium. This type of conidial formation appeared to be favored by addition of cholesterol.

Although large variations exist among isolates they can generally be grouped in either of two species based on cultural differences in optimum temperatures for growth, colony morphology, pigmentation, and fruiting structure formation and their distribution.

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Dedicated to

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Perennial canker has been of major economic importance to Michigan fruit growers ever since Taft (23) in 1898 called attention to a "gum disease" of peach (*Prunus persica* (L.) Batsch.). He attributed the branch swellings and gummosis associated with the disease to the effects of freezing and thawing, and not to microorganisms. Two years later, Steward et al. (22) discovered a fungus, later identified as *Cytospora leucostoma* (Pers.) Sacc., intimately associated with dead and dying peach trees. Later *C. cincta* Sacc. was also found to produce cankers on peach and other stone fruit crops (4). The *Cytospora* fungi have been reported to cause cankers in the fruit growing regions of the Eastern (22) and Western (17) United States, except for the Pacific Northwest; Ontario, Canada (7); Europe (21); South Africa (19); and Japan (24).

The severity of damage inflicted by perennial canker depends upon the location of the infection. The most critical locations are the trunk and scaffold branches. A peach tree generally has 3 or 4 scaffold branches, and complete girdling of one branch reduces the yield by approximately 30%. In addition, this canker provides a source of inoculum from which new infection can arise.

Pruning wounds and cold injury wound which occur on the trunk of the tree are critical infection courts since from this location the canker fungi can cause extensive girdling. The trees are not usually killed outright, but productivity and longevity are reduced as cankers enlarge (13).

The identification of the two Cytospora species has traditionally been based on cultural characteristics because the perfect stage is difficult to find in nature. The following differences predominate for the two species. C. cincta is a more virulent pathogen than C. leucostoma (13, 25). The pycnidia of C. leucostoma are black with dark red cirri, while those of C. cincta are brown with amber-pink cirri (4). C. leucostoma is hair brown in culture with small dark pycnidia exuding cirri when mature. C. cincta, on the other hand, is whitish to olive buff in culture and has large light-colored pycnidia containing, though rarely exuding, spores (25). Helton and Konicek (9) reported an average optimum temperature of 30 C and 25 C for C. cincta and C. leucostoma respectively. However, the optimum temperatures generally accepted are 21 C and 30 C for C. cincta and C. leucostoma respectively as found in Hildebrand (13).

Recently, Kern (14) split the traditional genus Valsa on the basis that if the fruitification has a conceptacle it belongs to the genus Leucostoma, if the

conceptacle is absent it is known as Valsa. Kern (14) in his taxonomic studies of perennial cankers in Michigan refers to the two species as Leucostoma persoonii (Nit.) v. H., imperfect stage Cytospora leucostoma Sacc.; and L. cincta (Fr.) v. H., imperfect stage C. cincta Sacc. He states that although Cytospora species show morphological differences, they can not be split up on the basis of morphological characteristics due to the range of variability. Lukezic, DeVay and English (18) further expressed this variability observing that monoasosporic isolates from a single ascus of L. persoonii represented colony types characteristic of both C. cincta and C. leucostoma.

The success of future laboratory and field studies, directed toward the control of these pathogens, may be dependent on the ability to produce inoculum. Techniques using sterilized split peach twigs (20) or a pearl barley honey-peptone mixture (5) are not practical when large amounts of inoculum are desired. However, sporulation of Cytospora isolates on common laboratory media is slow and some isolates produce only a few pycnidia even after several months. In other cases, pycnidia may be formed in abundance but never produce spores. Calpouzos and Stallknecht (1) working with Cercospora beticola and Leach (16) using many diverse species reported increased sporulation with near-ultraviolet radiation. Hendrix (12) reported that for some fungi, but not others, growth and

reproduction were increased by cholesterol. The purpose of this research was to determine if near-ultraviolet irradiation and/or cholesterol would enhance sporulation and growth of isolates cultured on ordinary laboratory media.

MATERIALS AND METHODS

A. Isolates

Four *Cytospora* isolates were selected as representative of the range of isolates found in Michigan orchards. Two cultures designated as Oc 6 and Ja 17 were isolated from apricot (*Prunnus armeniaca* L.) and peach respectively. Their cultural characteristics and temperature requirements (see appendix) corresponded to those of *C. cincta*. The other cultures, Ma 4 and In 5, were isolated from peach and resembled the characteristics of *C. leucostoma*. Single spore subcultures of each isolate were obtained by transferring spores from pycnidia to sterile distilled water and plating a dilution series of the spore suspension onto potato maltose agar. One to two days later germinated single spores were transferred to fresh potato maltose agar and maintained thereafter through mycelial plug transfers. All isolates were pathogenic to peach in greenhouse tests.

B. Preparation of Media

The procedure of Lacy and Bridgman (15) for preparing potato dextrose agar from dehydrated potatoes was followed except maltose was substituted for dextrose as

suggested by the studies of Helton and Konicek (10). The media, potato maltose agar (PMA) was adjusted to pH 6 (Beckman Zeromatic II pH meter) and sterilized. Final pH following sterilization was 5.8. Cholesterol was ground using a mortar and pestle with 0.5 ml distilled water and one drop of Triton B - 1956. It was added to the media at the rate of 100 mg/liter before autoclaving. Twenty to 25 ml of medium was poured into 9 cm plastic petri plates to reduce drying out of the cultures. Media for the growth studies were made using the same procedure except omitting the agar. The resulting potato maltose broth (PMB) was pipetted into each dish at the rate of 20 ml/petri plate. One plug (cork borer #3) from 5-7 day old cultures of mycelia was placed centrally and inverted in each PMA petri dish or upright in PMB dishes.

C. Conditions

Control cultures were grown under 1.0 to 1.3 X 10^4 ergs/cm^2sec of continuous cool-white fluorescent lamps (15 watt General Electric F15T8/CW lamps). Other treatments (see appendix) were: (a) addition of 100 ppm cholesterol to the media, (b) continuous irradiation with 2.0 to 7.0 X 10^2 ergs/cm^2sec near-ultraviolet (UV) lamps (15 watt General Electric F15T8/BLB lamps emitting from 320 nm to 460 nm, max. at 365 nm), and (c) a combination of treatments (a) and (b). Radiation intensity

measurements were conducted with a YSI Kettering model 65 radiometer. Desiccation of cultures was reduced by placing the petri dishes at a single depth in plastic bags and closing the bags with twistums. All experiments were carried out in two growth chambers maintained at 25 C $\pm$ 1. Temperatures within the media and inside and outside the plastic bags were checked periodically with a telethermometer and found to be equivalent for both cool-white fluorescent lamps and black lamps.

D. Sporulation, Pycnidial Production, and Colony Diameter

At days 7, 14, 21, 28, and 35 observations were made in two ways: all plates were (1) judged on a rating scale from 1-5 (see Figure 1) for diameter increase; and (2) viewed under a microscope to determine the development and number of pycnidia. Observations from a field of vision (16 mm^2 each) were selected at random in each plate quadrant and recorded. All studies were replicated nine times.

E. Dry Weight Measurements

Sampling dates were 3, 6, 9, 12, and 15 days after inoculation. At every time period 9 plates (replications) were sacrificed for each treatment--isolate combination. A table of random numbers was used to determine plates chosen. Samples were taken by filtering and

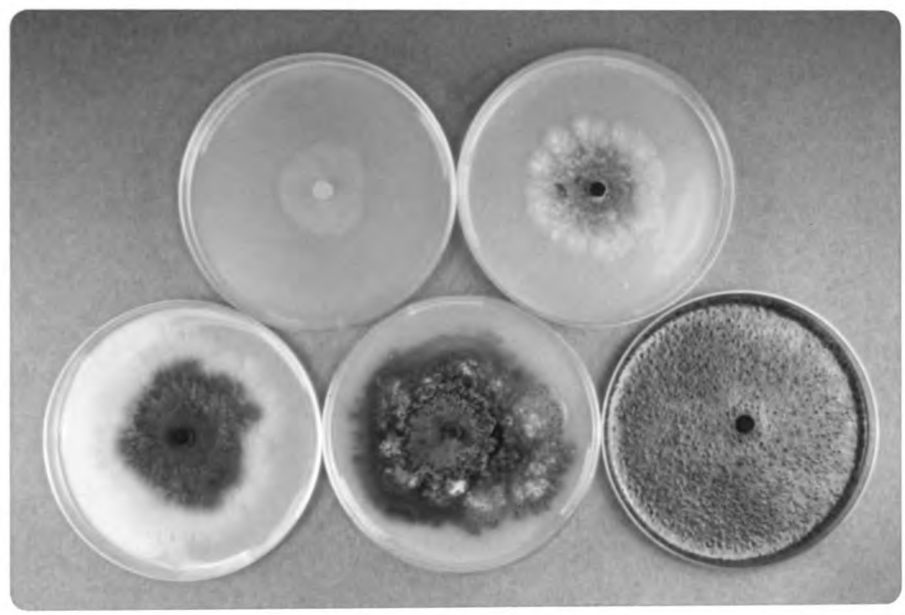

Figure 1. Colony diameter rating scale from 1 (smallest) to 5 for estimating growth of Cytospora.

rinsing the contents of each petri dish on pre-weighed filter papers using an Erlenmeyer filter flask, and placing the resulting residue in drying ovens at 65 C for 48 hours. Filter papers were then removed from ovens, allowed to cool and weighed.

F. Data Analysis

The experimental design was a split-split plot. The treatments, isolates, and sampling periods constituted the main unit, sub-unit, and sub-sub-unit respectively. Least significant differences (LSD) were computed using the appropriate mean square to compare means. Orthogonal contrasts using single degree of freedom comparisons and F-tests were used to compare combinations of treatments and isolates.

RESULTS

The diameter of cultures grown in total darkness for 35 days was comparable to cultures irradiated with either near-UV radiation or fluorescent radiation (Figure 2 and appendix). However, there was an obvious reduction in pigmentation and an absence of pycnidial formation for all isolates when radiation was excluded.

A. Pycnidial Production and Sporulation

Analysis of the data for isolates Oc 6, Ja 17, and In 5 indicate that cultures treated with near-UV radiation plus cholesterol produced more pycnidia than either of the non UV radiated treatments (Table 1). Pycnidial production of Ja 17 with the near-UV radiation plus cholesterol treatment contributed heavily in the significance of the treatment effects. Isolate In 5 produced more pycnidia than either Oc 6 or Ja 17. This was due largely to the near-UV radiation and fluorescent control treatments which were significant at the 1% and 5% level respectively. Further analysis of treatment effects show that a comparison of near-UV radiated treatments with those not receiving near-UV radiation indicate a highly significant difference in numbers of pycnidia

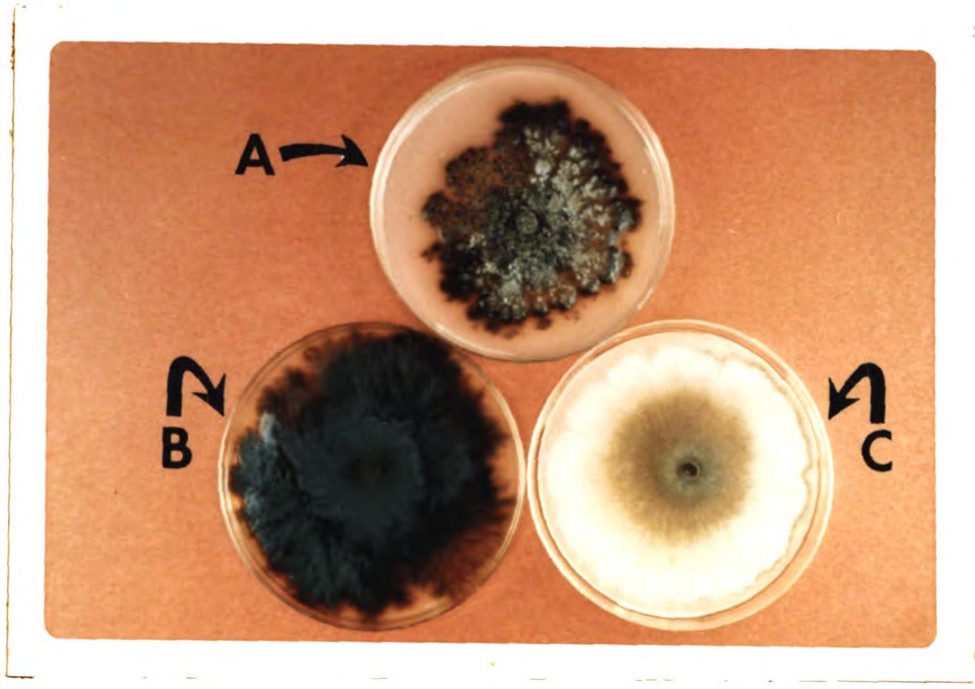

Figure 2. Cytospora cincta cultures grown for 35 days under continuous near-ultraviolet radiation (A), cool-white fluorescent radiation (B), and darkness (C).

Table 1. Effect of near-UV radiation and cholesterol on pycnidial production of three isolates of *Cytospora*.[a]

Treatment	Isolates			Main Effect Means
	Oc6	Ja17	In5	
Control[b]	1.13	0.00	5.47*	2.20
UV	2.33	1.00	8.42**	3.92
Cholesterol[b]	2.38	0.13	3.82	2.11
UV + Cholesterol	3.51	6.84**	5.44	5.27**
Main Effect Means	2.34	1.99	5.79**	

[a]Each value represents the mean number of pycnidia for 45 observations of 5 sampling periods.

[b]Irradiated with fluorescent light.

*LSD values significant at 5% and 1% levels for: Treatment 1.99, 2.70; isolates 1.95, 2.60; between treatments same isolate 3.44, 4.67; between isolates same treatment 3.91, 5.19 respectively.

produced between the two sets of treatments (Table 2). No differences were observed when cholesterol treatments were paired against non cholesterol treatments. The response of the near-UV radiated treatments did not appear to depend on the presence of cholesterol. An additional comparison of near-UV radiated treatments vs non UV radiated treatments shows increased pycnidial production

Table 2. Orthogonal contrast of treatment effects for pycnidial production of three isolates of Cytospora.

Anova Table			
Source of variation	df	Mean square	F ratio
Treatments	3	308.30	4.91**
UV vs non UV	1	801.79	12.76**
Cholesterol vs non Cholesterol	1	53.52	0.85ns
Interaction UV and Cholesterol	1	69.70	1.11ns
Error	24	62.82	

**Significant at 1% level.

by 50 and 65% for In 5 and Oc 6 respectively and by 60 fold for Ja 17.

Numbers of pycnidia for the main effect means increased throughout the time of the experiment. However, when comparing each time period to the preceding one, only days 14 and 35 were significant (Table 3). Increases of pycnidial numbers on day 14 are particularly true for Ja 17 and In 5 while on day 35, Oc 6 was markedly increased. Pycnidial numbers observed were greater for In 5 compared to Oc 6 on days 14 and 21, In 5 compared to Ja 17 on day 28, and both In 5 and Oc 6 compared to Ja 17 on day 35.

Table 3. The relationship of sampling time of three *Cytospora* isolates to pycnidial production.[a]

Time in Days	Numbers of Pycnidia			
	Isolates			Main Effect Means
	Oc 6	Ja 17	In 5	
7	0.14	0.00	2.86	1.00
14	0.81	3.08	5.25	3.05**
21	1.19	3.47	5.56	3.41
28	2.67	1.75	6.97	3.80
35	6.89	1.67	8.31	5.62**
Main Effect Means	2.34	1.99	5.79	

[a]Each value is the mean of 36 observations regardless of treatment.

**LSD values, significant at 5% and 1% levels for: Time 1.21, 1.60; same time between isolate 4.37, 5.81; same isolate between time 2.10, 2.77 respectively.

The effects of the near-UV radiated and cholesterol treatments on the number of pycnidia sporulating paralleled those for pycnidial production. The near-UV radiated and near-UV radiated plus cholesterol treatments had greater sporulation than the fluorescent irradiated control (Table 4). This was particularly true for In 5 under the near-UV radiation treatment and Ja 17 under the near-UV radiation plus cholesterol treatment. Sporulation with the cholesterol treatment had no detectable effect.

Table 4. Effect of near-UV radiation and cholesterol on numbers of pycnidia exuding spores and percentage of pycnidia sporulating for three isolates of Cytospora.[a]

Treatment	Number Pycnidia Sporulating				Percentage Pycnidia Sporulating			
	Isolate			Main Effect Mean	Isolate			Main Effect Mean
	Oc 6	Ja 17	In 5		Oc 6	Ja 17	In 5	
Control[b]	0.36	0.00	0.53	0.30	31.4	0.0	9.8	13.5
UV	0.91	0.31	2.29*	1.17*	39.1	31.1	27.1	29.8
Cholesterol[b]	0.60	0.02	0.51	0.38	25.2	16.7	13.4	17.9
UV + Cholesterol	1.18	2.33**	0.51	1.34*	33.5	34.1	9.4	25.5
Main Effect Means	0.76	0.67	0.96		32.5	33.8	16.6	

[a]Each value of the number of pycnidia sporulating represents the mean of 45 observations of 5 sampling periods.

[b]Irradiated with fluorescent light.

*LSD values significant at 5% and 1% levels for: Treatment 0.87, 1.19; isolate 0.83, 1.10; between treatments same isolate 1.51, 2.06; between isolates same treatment 1.65, 2.19 respectively.

Sporulation of cultures receiving near-UV radiation increased by 220 and 270% for Oc 6 and In 5 respectively and by 130 fold for Ja 17 when compared to similar non UV radiated cultures. The percentage of pycnidia sporulating was also doubled with near-UV radiated treatments compared to those not treated with near-UV radiation (Table 4).

Differences in the mean number of pycnidia sporulating between isolates were not significant. However, sporulation was greater with near-UV radiation alone for In 5 compared to Ja 17 while the reverse existed for the near-UV radiation plus cholesterol treatment (Table 4).

Although the mean number of pycnidia sporulating increased at each time period, only day 35 was significant when compared to each preceding time period (Table 5). This result is attributed to Oc 6 producing 90% of its total sporulation at day 35. On day 35, Oc 6 and In 5 produced greater sporulation numbers than Ja 17.

Cultural fruiting comparisons of isolates revealed pycnidia of In 5 were of uniform size, globose, black, submerged or superficial, and frequently covered with white mycelial growth. Conversely, Oc 6 and Ja 17 pycnidia were of two forms: large, globose, light-colored, superficial structures or small, embedded or erumpent structures within a dark stroma. Developing pycnidia

Table 5. The relationship of sampling time of three Cytospora isolates to number of pycnidia exuding spores.[a]

Time in Days	Number of Pycnidia Exuding Spores			
	Isolates			Main Effect Means
	Oc 6	Ja 17	In 5	
7	0.00	0.00	0.00	0.00
14	0.03	1.08	0.08	0.40
21	0.08	1.14	0.39	0.54
28	0.25	0.53	1.56	0.78
35	3.44	0.58	2.78	2.27**
Main Effect Means	0.76	0.67	0.96	

[a]Each value is the mean of 36 observations regardless of treatments.

**LSD values significant at 5% and 1% levels for: Time 0.73, 0.96; same time between isolates 1.85, 2.45; same isolate between time 1.26, 1.66 respectively.

not exuding spores were frequently found to contain spores when sliced or squashed.

The conidia were normally exuded from the pycnidia in a colored fluid (Figure 3). Generally pycnidia of Oc 6 and Ja 17 exuded cream colored spore masses in contrast to In 5 which produced an amber to orange exudate and upon drying formed a cirrus. However, In 5 occasionally produced cream colored exudate while Oc 6 and Ja 17 produced orange exudate, especially from stroma-formed

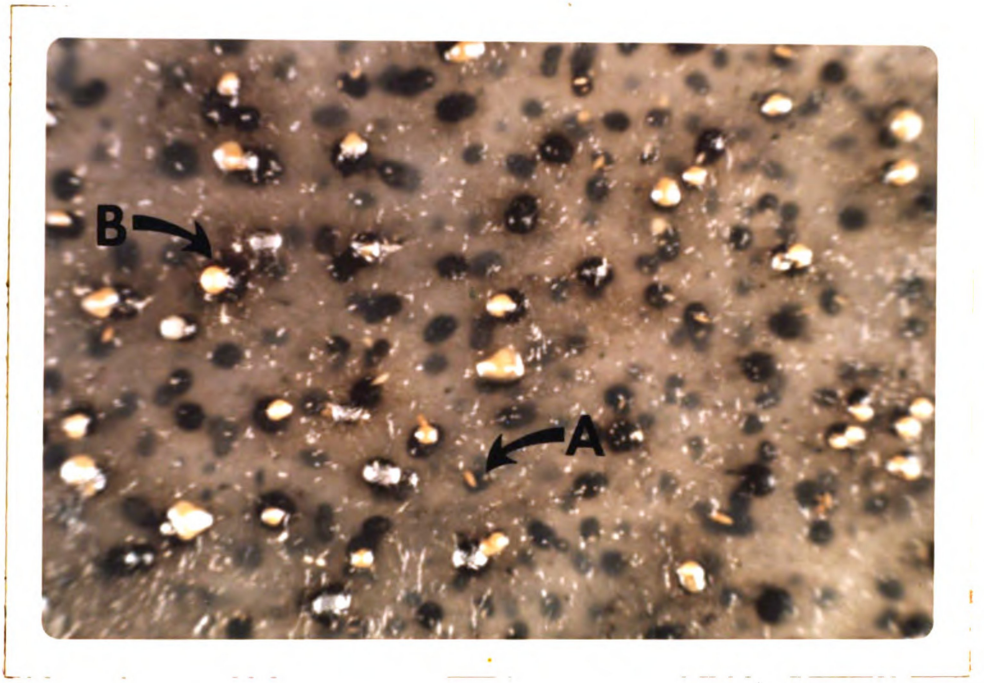

Figure 3. Exudation of orange spore masses from submerged (A) and superficial (B) pycnidia of isolate In 5 at 3 weeks.

pycnidia. Another isolate, Ma 4, exudated both cream colored and orange exudate (Figure 4).

Isolate Ma 4 not only produced pycnidia, but also formed conidia arising directly from the mycelium (Figure 4). These conidia were produced singly, or in some cases in chains, on short conidiophores (Figure 5). They appeared to be slightly larger than conidia produced in the usual manner. Spore production in Ma 4 was visible after about 4 days and increased rapidly thereafter. Plates with cholesterol appeared to have a higher number of spores than the other treatments.

Neither near-UV radiation nor cholesterol treatments appeared to affect spore germination. Spores of each isolate including Ma 4, germinated on PMA when held at room temperature for 24 hours.

B. Dry Weight and Colony Diameter

Dry weights of cultures treated with cholesterol and near-UV radiation plus cholesterol were significant at the 5% and 1% level respectively over treatments not containing cholesterol (Table 6). These differences are attributed to high dry weight increase of In 5 with cholesterol and near-UV radiation plus cholesterol treatments and dry weight increases of Oc 6 and Ma 4 with the cholesterol treatment. Only the dry weight of Oc 6 was decreased by near-UV radiation.

Figure 4. Sporulation of Cytospora leucostoma isolate Ma 4 at day 21: (A) orange and cream colored spore exudate from pycnidia; (B) spores arising directly from the mycelia.

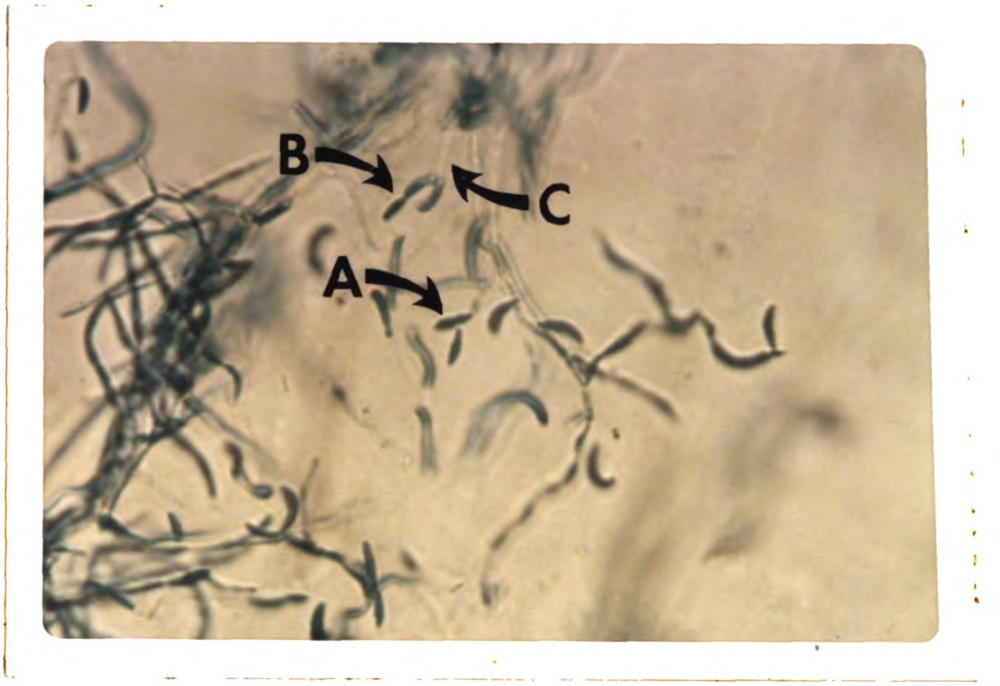

Figure 5. Microscopic view of conidia of Ma 4 at day 5. Conidia are borne singly (A) or in short chains (B) from conidiophores (C). 400X magnification, lactol phenol cotton blue stain.

Table 6. Effect of near-UV radiation and cholesterol on mycelial dry weight production by four isolates of Cytospora.[a]

Isolate	Treatments				
	Control[b]	UV	Cholesterol[b]	UV-Chol.	Main Effect Means
Oc 6	95.08	76.72	114.60	99.57	96.49
Ja 17	55.44	53.99	66.78	56.14	58.09**
In 5	133.88	138.26	162.60	161.44	149.05**
Ma 4	82.96	85.06	101.07	85.84	88.73
Main Effect Means	91.84	88.51	111.26**	100.75*	

[a]Each value represents an average dry weight (mg) of 45 observations of combined time recordings at 3, 6, 9, 12, and 15 days.

[b]Irradiation with fluorescent light.

*LSD values significant at 5% and 1% levels for: Treatment 8.81, 11.96; isolate 9.22, 12.20; same treatment different isolate 18.44, 24.41; same isolate different treatment 17.61, 23.91 respectively.

Analysis of isolates showed In 5 had the highest mean dry weight while Ja 17 had the lowest (Table 6). These deviations from Oc 6 and Ma 4 were significant at the 1% level and were found to be consistent regardless of treatment . The mean dry weight of In 5 was greater than 2.5 times that of Ja 17. Comparison of dry weight differences between presumed C. cincta isolates Oc 6 and

Ja 17, and C. leucostoma isolates In 5 and Ma 4 were highly significant (Table 7). Dry weight differences between Oc 6 and Ja 17 and between In 5 and Ma 4 also existed.

Table 7. Dry weight comparisons of four Cytospora isolates using a single degree of freedom orthogonal contrast test.

Anova Table

Source of Variation	df	Mean Square	F ratio
Isolates	3	0.25720	133.16**
Oc 6 and Ja 17 vs. In 5 and Ma 4	1	0.31146	161.25**
Oc 6 vs. Ja 17	1	0.13273	68.72**
In 5 vs. Ma 4	1	0.32742	169.51**
Error	96	0.00193	

**Significant at 1% level.

A highly significant dry weight mean difference at day 6 compared to day 3, existed as all isolates showed significant increases (Figure 6). Also, the mean increase of dry weight was increased at day 9 due to high increases of Oc 6, In 5 and Ma 4. The mean at day 12 and day 15 were not significant, part of which was due to a decrease of dry weight at day 12 for Oc 6 and In 5. The dry weight of In 5 was superior to other isolates at all times. Oc 6 was slightly less than Ma 4 and Ja 17 at day

Figure 6. Dry weights at days 3, 6, 9, 12, and 15 for four *Cytospora* isolates grown on potato maltose broth. Each value is the sum of 36 samples. The LSD (.01) for the mean of all isolates at different days is 0.42 gram.

3 but was much greater at day 6 and day 9. Ma 4 and Ja 17 separated sharply after day 6. Between day 9 and day 12 the dry weight of Ma 4 surpassed Oc 6.

Colony diameters of the near-UV radiation plus cholesterol treatment were significantly less than other treatments due to reduced colony diameter of Oc 6 and Ja 17 (Figure 1 and Table 8). The colony diameter of Ja 17 was also less with the cholesterol treatment. Isolates In 5 and Ma 4 were different from Oc 6 and Ja 17 and also different from each other. This phenomenon existed for all treatments. Colony diameters of In 5 and Ma 4 increased rapidly covering the entire plate while those of Oc 6 and Ja 17 were slow and had an average rating of 4 at the end of the experiment (Figure 1).

Table 8. Effect of near-UV radiation and cholesterol on colony diameter of four isolates of Cytospora.[a]

Isolate	Treatments				
	Control[b]	UV	Cholesterol[b]	UV-Cholesterol	Main Effect Means
Oc 6	3.13	3.18	3.18	2.84	3.08
Ja 17	3.33	3.16	2.91	2.93	3.08
In 5	4.80	4.69	4.80	4.71	4.75**
Ma 4	4.40	4.24	4.33	4.22	4.30**
Main Effect Means	3.92	3.82	3.81	3.68**	

[a]Each value represents the mean of 45 observations on a rating scale of 1-5.

[b]Irradiated with fluorescent light.

**LSD values significant at the 1% level for: Treatment .112, isolate .139, same treatment different isolate .277, same isolate different treatment .224.

DISCUSSION

The mechanisms involved in pycnidial formation were not examined but rather an attempt was made to determine if near-UV radiation or cholesterol affected pycnidial production and sporulation. The result that near-UV irradiation increased the number of pycnidia formed and their subsequent sporulation did not significantly decrease growth as measured by mycelial dry weight deserves attention. Perhaps the often proposed mechanism discussed by Cochrane (2) of injured or death of cells followed by a releasing of substances into the medium which acts on surviving cells, diverting them into new developmental pathways, namely that of favoring fruiting structure development, does not hold true for Cytospora. Favored rather is a contrary hypothesis (2) that sporulation of Cytospora requires specific positive stimuli. Near-UV radiation may initiate or stimulate the synthesis of factors for reproductive activity. It appears that this stimulus influences the number of pycnidia formed and the proportion which produce spores. The idea that near-UV radiation results in earlier sporulation is dismissed by the failure of treatments to affect differences in time. Failure of cholesterol to affect the

reproductive process via pycnidial formation suggests cholesterol acts independent of reproduction. The relationship between cholesterol visually producing more "free" conidia with Ma 4 and the results of other treatments on pycnidial sporulation are not fully understood. Perhaps increased growth of Ma 4 with cholesterol (Table 6) naturally led to increased production of conidia.

Growth studies suggest that cholesterol increased metabolic activity either by being incorporated or stimulating additional growth as measured by mycelial dry weight. Although cholesterol increased growth it did not adversely affect pycnidial formation. Also the consistent dry weight increase of 20-22% among isolates treated with cholesterol indicate that the effect of cholesterol is independent of the isolate. The absence of significant time differences after day 9 (Figure 6) suggest that recommended data recording be at closer intervals up to 10 days.

Colony diameter was less with the near-UV radiation plus cholesterol treatment, demonstrating that growth as measured by diameter and dry weight are not equivalent. Dry weight data are considered better measures of growth (2). Therefore, differences in colony diameter were only recognized at the 1% level. Failure of near-UV radiation to significantly inhibit dry weight may have been due to improper radiation dosage or intensity. This again

suggests that for Cytospora there is, and possibly for other fungi there may be, a critical dosage of near-UV radiation that is not inhibitory to growth yet stimulatory for reproduction.

Significant differences among replications occurred in several growth cases. This does not invalidate the significance of other tests but suggests there was an unknown factor involved. Perhaps the method of inoculating plates could have been improved to achieve more uniformity. Frequently mycelial plugs were slow or failed to initiate rapid growth resulting in large variations. Ellingboe (6) found germinated single spore inoculations quite suitable for growth studies of Phoma herbarum, a technique that may in the future alleviate significance among replications. Single spore inoculations were not feasible at the start of this study due to scarcity of inoculum.

Large differences between presumed species exist; however, isolates within species also differed supporting the literature (3, 8, 11, 14) and making interpretation of information difficult. Excessive variability among isolates lend some reservations as to whether these results are expressive of all Cytospora canker isolates. The four isolates can, however, be separated into two loosely defined species based on several trends where greater variations between groups than among isolates

existed. Temperature studies (appendix) found optimum growing temperatures at 21 C for Oc 6 and Ja 17 and 27-30 C for In 5 and Ma 4. Pigmentation of Oc 6 and Ja 17 ranged anywhere from white, yellow, or brown while In 5 and Ma 4 were black to olive green. In 5 and Ma 4 pycnidia were evenly distributed and sporulated uniformly on plates. Variations existed in density and distribution of reproductive structures among and within plates of Oc 6 and Ja 17. Oc 6 and Ja 17 had a higher sporulating percentage of pycnidia formed and produced many of their pycnidia within a stroma in a circular ring at varying distances from mycelial plugs. The number of "ring" observations greatly influenced the number of fruiting structures recorded. Only Ma 4 produced conidia and conidiophores arising directly from the mycelium, confirming results of Hildebrand (13) who noted this same striking characteristic for some of his C. leucostoma isolates. Colony diameters of In 5 and Ma 4 were much superior to Oc 6 and Ja 17. Growth as measured by mycelial dry weight was least with Ja 17 and greatest with In 5, however, a sharp distinction between species does not exist as dry weight of Oc 6 and Ma 4 were not different.

This study indicates that for the four isolates herein tested: (a) radiation is necessary for fruiting structure formation, (b) it is the near-UV radiation which is responsible for fruiting structure formation,

confirming studies by Leach (16), (c) near-UV radiation increases numbers of pycnidia, sporulation, and percentage of pycnidia sporulating but has no affect on the time of occurrence, (d) near-UV radiation does not significantly decrease dry weight, (e) cholesterol increases dry weight but not pycnidial formation and sporulation, and (f) large variations exist among isolates yet they can generally be grouped in either of two species.

For sporulation of stone fruit Cytospora isolates cultures should be grown on PMA, pH 5-6, at 25 C under continuous long wave UV irradiation. Isolates sporulating directly from the mycelium may be increased by addition of cholesterol to the media.

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APPENDIX

APPENDIX

Materials and Methods

Preliminary studies:

(a) Temperature: Cultures of isolates Oc 6, Ja 17, Ma 4, and In 5 were grown on potato maltose agar (PMA) in oven incubators with temperatures adjusted to 15, 18, 21, 24, 27, and 30 C. Colony diameters of the isolates were compared every 4 days for 3 weeks. The relative growth of the isolates was recorded.

(b) Sterols: Cultures subjected to sterols in several cases showed favorable growth and sporulation. Differing concentrations of two sterols, ergosterol and cholesterol, were tested using isolates Ja 17 and In 5. Concentrations tested were: cholesterol 100 ppm, ergosterol 100 ppm, and a 50:50 combination of cholesterol and ergosterol totaling 0, 50, 100, and 1000 ppm, of sterol per treatment, respectively. In a second test, cholesterol at 0, 50, 100, 250, and 500 ppm was tested using all four isolates. Three replications were used. Every 3 days for 3 weeks the colony diameters for each treatment were compared. Concentrations producing the largest colony diameters were selected for use in later studies.

(c) Photoperiodicity and thermoperiodicity: Many fungi require alternating periods of light and dark or of high and low temperatures for optimum growth and sporulation (2). All four isolates were treated with five combinations of light and temperature as follows: (1) Light 12 hours 26 C, dark 12 hours 26 C; (2) light 12 hours 26 C, dark 12 hours, 12 C; (3) dark 10 days 26 C, light 10 days 12 C, light 10 days 26 C; (4) light 24 hours 26 C; and (5) dark 24 hours 26 C. Three replicates were used. Observations of the number of pycnidia and the number of pycnidia exuding spores were taken periodically at 7 day intervals to day 35.

(d) Continuous and alternating near-ultraviolet (UV) radiation: Isolates of Oc 6, Ja 17, In 5 and Ma 4 were placed in incubators at 26 C with continuous near-UV radiation or alternating 12 hour periods of near-UV radiation and darkness. Colony diameter, number of pycnidia and number of pycnidia exuding spores were observed at 4 weeks.

(e) Media: Cultures were grown on chemically defined media (18) with the following ingredients: $MgSO_4 \cdot 7H_2O$ 3g, NH_4Cl 3g, KH_2PO_4 3g, Dextrose anhydrous 20g, $FeCl_3 \cdot 6H_2O$ 0.24 mg, $ZnCl_2$ 0.15 mg, H_3BO_3 0.06 mg, $CuCl_2 \cdot H_2O$ 0.05 mg, $MnCl_2 \cdot 2H_2O$ 0.04 mg, $NaMoO_4 \cdot H_2O$ 0.03 mg, Thiamin 100 ug, Biotin 0.05 ug, Choline 100 mg, and enough distilled H_2O to make one liter of media. Failure

of cultures to produce fruiting structures or any substantial growth led to a reversion to the PMA media. PMA at 2X, X, 1/2X, and 1/4X concentration was tested to determine if nutrient levels had an effect on growth and sporulation. Colony diameter and numbers of pycnidia and pycnidia sporulating were the criteria used to judge the suitability of the various substrate. Observations were made periodically at 4 day intervals for 4 weeks. In a third experiment concentrations of 0.0, 0.01, 0.1, 1.0 % NaCl were added to PMA plates. Numbers of pycnidia and pycnidia exuding spores were compared for each treatment after 4 weeks.

(f) Growth chamber studies: In some of the previous studies the fluorescent or UV lamps had been installed in cupboards where temperature could not be controlled nor outside light sources excluded. This resulted in considerable variation in the growth and sporulation observed within treatments. Two growth chambers were secured and additional experiments varying the relative humidity and radiation intensity were done to select optimum relative humidity and radiation intensities.

Results and Discussion

(a) Temperature: Isolates Oc 6 and Ja 17 grew best at 21 C, isolates Ma 4 and In 5 at 27 to 30 C. At 24 C

both species were capable of good growth, thus 25 ± 1 C was selected for growing cultures of both species.

(b) Sterols: Colony diameter, pycnidial number and sporulation of In 5 with 100 ppm cholesterol were visually superior to ergosterol at 100 ppm and to the combinations of cholesterol and ergosterol. Sporulating of Ja 17 was unaffected by concentrations of sterols; however, colony diameters were increased with 100 ppm cholesterol. Since Ja 17 and In 5 selectively preferred cholesterol to ergosterol, differing concentrations of cholesterol were tested using all four isolates. Variations among isolates for most favorable response to increased colony diameters and sporulation ranged from 50 ppm to 250 ppm with 100 ppm rated as the best overall.

(c) Photoperiodicity and thermoperiodicity: Although there were slight variations among isolates, continuous radiation at 26 C was considered best overall for future studies. No sporulating pycnidia occurred on any cultures subjected to continuous darkness at 26 C; however, a few pycnidia were observed on C. leucostoma isolates. Their appearance is believed to be due to brief weekly exposures to the radiation as subsequent 35 day dark treatments revealed no fruiting structures. Treatment 3 with 10 day photoperiods was less favorable than daily photoperiods (treatments 1, 2, 4). Continuous

radiation at 26 C was the only treatment resulting in sporulation on all cultures except Oc 6.

(d) Continuous and alternating near-UV radiation: Colony diameters of Ja 17 and Oc 6 were greater with alternating near-UV radiation than with continuous near-UV radiation. However, sporulation of Ja 17 and Oc 6 was much greater under continuous near-UV radiation than under alternating near-UV radiation. Ma 4 and In 5 sporulated and grew well in both conditions.

(e) Media: Cultures grown on chemically defined media had greatly inhibited colony diameters and failed to produce fruiting structures even under the influence of sterols or near-UV radiation. Colony diameters and pycnidial numbers were greatest with PMA concentrations of 2X and X for all isolates. In contrast to some fungi, lower concentrations of nutrients did not increase reproduction (2). Apparently stone fruit Cytospora isolates are not stimulated to sporulate by starvation. Addition of varying concentrations of 0.01 - 1.0 % NaCl on PMA did not increase pycnidial formation as suggested by Hamptom.[1]

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[1]M. C. Hampson, 1969. Valsa canker investigations. Part I. Pathogenesis by Cytospora leucostoma, and mechanism of wilt induction in peach. II. Effects of water stress on disease development and fungal behavior, and a role for wound gum. Ph.D. Thesis, Cornell University, 89 p.

(f) Growth chamber studies: Petri dishes had a tendency to dry out at relative humidities of 30 to 50%; therefore, dishes were placed at single depths in plastic bags (6 per bag) and tied with twistums to reduce moisture loss. Cool-white fluorescent lamps producing energy at a rate from 1.00 to 1.32 X 10^4 erg/cm^2sec were considered best for growth and sporulation of Cytospora isolates. The near-UV radiation used ranged from 2.0 to 7.0 X 10^2 ergs/cm^2sec.