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CHARACTERIZATION OF A HYPOVIRULENT ISOLATE OF *LEUCOSTOMA PERSOONII* AND ITS ASSOCIATED VIRUS-LIKE PARTICLE

BY

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ABSTRACT

CHARACTERIZATION OF A HYPOVIRULENT ISOLATE OF *LEUCOSTOMA PERSOONII* AND ITS ASSOCIATED VIRUS-LIKE PARTICLE

By

Carolyn J. P. Jensen

The lack of chemical control for Cytospora canker on peach caused by *Leucostoma persoonii* and *L. cincta* initiated the search for hypovirulent isolates. One isolate, 14.4A, contained 7 bands of dsRNA and had reduced virulence. Attempts to completely eliminate bands of dsRNA found in this isolate have proven ineffective, but a partially cured isolate, HT, derived by hyphal tipping of 14.4A, contains only two bands of dsRNA. The continued study of hypovirulent isolates included a comparison of their nitrogen metabolism with that of virulent isolates and the characterization of the virus-like particles of 14.4A.

In the nitrogen metabolism study, two hypovirulent isolates were compared with two normally virulent isolates of *L. cincta* (ATCC 62190) and *L. persoonii* (MI 11.13). Data from this study suggested that the two hypovirulent and the *L. cincta* isolate had alterations in nitrite reductase enzyme complex. There were distinctive differences between

the two virulent isolates in ability to use different nitrogen sources. The *L. cincta* isolate had a reduced range of usable nitrogen sources including an inability to utilize branched aliphatic amino acids, possibly a species specific character. Both hypovirulent isolates were able to utilize even fewer nitrogen sources, suggesting a possible link between altered nitrogen metabolism and reduced virulence.

Purification of 14.4A VLPs through PEG precipitation and differential and sucrose density gradient centrifugation yielded two classes of particles not found in HT. Capsid protein isolated from the two particle classes comigrated in SDS-PAGE with a common molecular weight of 32,000. Particle density differences in both sucrose and CsCl gradients reflected differences in nucleic acid content. The nucleic acid of these particles was dsRNA. The denser fraction contained a band of dsRNA which comigrated with a band present in HT. Compared to previously reported mycoviruses, this virus appeared to be a unique virus based on its ability to package more than one band of dsRNA in a single virion and on the small size of its capsid protein. There was evidence from probing northern blots with labeled cDNA probes reverse transcribed from specific bands of dsRNA that the bands associated with 14.4A but not HT shared little homology, while those that remain in HT may share some common sequence.

DEDICATION

This thesis is dedicated to my parents and to Dr Lori M. "not Lulubelle" Carris who, after she grew out of satin disco pants and Candies shoes, encouraged me to continue and is one hell of a mycologist.

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NITROGEN METABOLISM OF *LEUCOSTOMA PERSOONII* AND *L. CINCTA* IN VIRULENT AND HYPOVIRULENT ISOLATES

ABSTRACT

The nitrogen utilization pattern of two species of *Leucostoma* was examined using a normally virulent isolate of *L. persoonii* (MI 11.13), a normally virulent isolate of *L. cincta* (ATCC 62190) and two hypovirulent isolates of *L. persoonii* (NC 14.4A and HT). The normally virulent *L. persoonii* isolate was able to utilize all nitrogen sources tested except hydroxylamine, cysteine, anthranilic acid, indole, urocanic acid, methylated purines, pyrimidines and lysine. The *L. cincta* isolate was also unable to use these compounds. In addition, this isolate could not utilize nitrate, nitrite, and branched aliphatic amino acids. The inability to utilize branched aliphatic amino acids but not nitrate and nitrite may be a species specific character. The two hypovirulent *L. persoonii* isolates had a further restricted list of usable nitrogen sources. The sources of nitrogen that these two isolates could not grow on included hydroxylamine, nitrate, nitrite, cysteine, anthranilic acid, indole, urocanic acid, methylated purines, pyrimidines , lysine and the branched aliphatic side chain amino acids, isoleucine, leucine and valine. Growth of the two hypovirulent isolates on glutamic acid and aspartic acid, as well as aromatic amino acids was poor, though statistically, growth on these compounds were usually significantly greater than controls. The pattern of inorganic nitrogen utilization for the four isolates was determined and

discussed in reference to the known gene loci found in other fungi that control this process. The possible role of pattern of nitrogen utilization in virulence was also addressed.

INTRODUCTION:

Cytospora canker of peach caused by *Leucostoma cincta* (Fr.:Fr.) Höhn. (anamorph = *Leucocytospora cincta* (Sacc.) Höhn) and *Leucostoma persoonii* Höhn (anamorph *Leucocytospora leucostoma* (Pers.) Höhn) is a highly destructive disease on peach in Michigan. This disease is wide spread in orchards of the northern United States. It is often the limiting factor in peach production (Dhanvantari, 1982 and Biggs, 1989). In young trees, dieback of young branch tips can greatly reduce vigor and in older trees, large perennial cankers can often lead to death (Hildebrand, 1947 and Rozsnyay, 1977).

As fungicides have proven to be ineffective in controlling this disease (Helton and Rohrbach, 1967; Chandler, 1974 and Royse and Reis, 1978), a biological control agent that could reduce the impact of this disease would be highly desirable. A diseased isolate of *Leucostoma persoonii* (NC 14.4A) has been isolated from peach in North Carolina. Isolate NC 14.4A is a hypovirulent nonsporulating isolate that contains many virus like particles (VLP) in its cytoplasm when viewed in the electron microscope (Snyder *et al.*, 1989). The strain contains 9 segments of dsRNA. Isolate HT was derived from isolate NC 14.4A by successive hyphal tipping and has lost the viral particle and 7 of the 9 bands of dsRNA (Hammar *et al.*, 1989). Along with its

reduced virulence, there is evidence that isolate NC 14.4A and its derivative HT have an altered nitrogen metabolism.

To date, there are only very limited data on the basic nitrogen metabolism of these pathogens (Helton and Konicek, 1961 b). More detailed information on the basic nitrogen metabolism of this organism would be useful. Pathogenicity in fungi is known to be profoundly altered by both quantity and quality of the nutrient nitrogen (Weinhold *et al.*, 1969; Covey, 1967; Weinhold and Garraway, 1966, Stretch and Cappellini, 1965; for review see Van Andel 1966 and Huber and Watson, 1974). Specific amino acids can have inhibitory or stimulatory effects on pathogenicity. Within a plant host, levels of free amino acids are known to vary in response to infection (Stretch and Cappellini, 1965). Detailed information on the basic nitrogen metabolism comparing virulent and avirulent or hypovirulent strains of *L. persoonii* might elucidate the physiological basis of this hypovirulence.

An understanding of nitrogen metabolism might have applications such as species identification. The use of differential nitrogen sources to distinguish the two species could facilitate species identification which is often difficult due to the lack of anamorph characters, the inability to induce formation of the sexual state in culture and the relative scarcity of the teleomorph in nature. The information derived from studies on differential nitrogen sources could be used to develop a simple selective media. A com-

parison of the nitrogen metabolism of these two perennial canker pathogens of woody plants to the metabolism of a seed and stem rotting pathogen of herbaceous annuals [*Gibberella zeae* (Schw.) Petch, anamorph = *Fusarium roseum* (Link emed.) Snyder and Hansen 'Graminearum'] will add to our understanding of nitrogen metabolism in fungal pathogens.

The objective of this study was to characterize the nitrogen metabolism of the hypovirulent isolates and compare them with virulent wild type isolates of *L. persoonii* and *L. cincta*. An in depth comparison was made between four representative isolates to investigate the effect of the presence of dsRNA on nitrogen metabolism including utilization of a variety of organic and inorganic nitrogen sources. In addition, a comparison was made to determine differences in the pattern of nitrogen metabolism in *L. persoonii* and *L. cincta*. Selected nitrogen sources that differentiate the two species were used to compare representatives of five proposed phenetic groups of *L. persoonii* and *L. cincta* previously delineated by isozyme and RFLP analysis (Surve-Iyer *et al.*, 1992 a and b). The nitrogen metabolism of the *Leucostoma* species is discussed in reference to that of *G. zeae* and other fungi.

MATERIALS AND METHODS:

Fungal isolates:

The origin, host, source, isozyme phenetic group and virulence for each isolate in used in this study have been described (Adams *et al.*, 1989; Surve-Iyer *et al.*, 1992 a; and Surve-Iyer *et al.*, 1992 b). Twenty isolates from Michigan were used in the preliminary screening for nitrate nonutilization. Four isolates were used in the studies on inorganic and organic nitrogen metabolism, including the hypovirulent nitrate nonutilizing NC 14.4A, a derivative isolate HT, a standard nitrate utilizing isolate of *L. persoonii* MI 11.13 and a nitrate nonutilizing isolate of *L. cincta* ATCC 62190. Nine isolates were used to compare the two species (taxa) and the six isozyme phenetic groups. *L. persoonii* and *L. cincta* have been misidentifications in the past of by researchers; therefore, older references to these fungi are discussed in this manuscript under the generic epithet *Leucostoma*.

The studies in this manuscript arose with the discovery that the hypovirulent *L. persoonii* were defective in nitrate utilization during the following preliminary screen. Initially, twenty isolates of *Leucostoma* were placed on Leonian's malt agar (Leonian, 1921) amended with 1.5% potassium chlorate to screen the isolates for nitrate nonutilization. Growth on such media suggested an alteration in nitrogen metabolism; in particular, an inability to utilize

nitrate as a sole source of nitrogen (Aberg, 1947 as referenced in Cove, 1976; and Lewis and Fincham, 1970). In the same preliminary study, a known *L. cincta* isolate was also screened.

Nitrogen Metabolism:

The medium used in this study was a modified *Neurospora* synthetic crossing media as described by Leslie (1986) with two modifications: maltose, the preferred source of carbon for *Leucostoma*, was substituted for sucrose at a rate of 360 mM carbon equivalents (1% w:v) (Helton and Konicek, 1961 a). Additionally, it was necessary to supplement the medium with 1 ml of a vitamin stock, as *Leucostoma* requires a complex of vitamins for growth (Lukezic *et al.*, 1965).

Initial experiments using MI 11.13, the standard *L. persoonii* isolate, were carried out to determine the rate at which nitrogen was growth limiting. A spore suspension (10^6 spore/ml, 1 ml) was used to inoculate flasks with 0.0, 1.0, 5.0 and 10.0 mM nitrogen equivalents in the form of $NaNO_3$ or $(NH_4)_2SO_4$. Data from these preliminary experiments indicate that the rate at which nitrogen was growth limiting was 5.0 mM for *Leucostoma* (Fig. 1), similar to that of *Gibberella zeae* (Leslie, 1986).

Forty five nitrogen sources were tested at a rate of 5.0 mM nitrogen equivalents. The acidity of the medium was adjusted to pH 5.8 prior to autoclaving. Aliquots (100 ml) of each nitrogen media was added to a 500 ml flask and auto-

claved. Inoculum for each flask consisted of 1 ml of blended mycelium that had been grown for 7 days in *Endothia* complete broth (Day *et al.*., 1977). The substitution of mycelium for spores was necessary as hypovirulent isolates NC 14.4A and HT do not sporulate. After inoculation, the flasks were placed on a shaker and the mycelium was allowed to grow for 12 to 14 days at room temperature (25 ^{o}C). Mycelium was harvested by vacuum filtration through preweighed Whatman #4 filter paper, dried for 5 to 7 days at 50 ^{o}C with desiccant and weighed on an analytical balance. Each nitrogen source was tested a minimum of three times with two flasks per experimental unit. The means were compared to control growth levels using a Students t test.

To further characterize nitrogen metabolism of these isolates, the nitrite excretion test as outlined by Cove was performed (Cove, 1976). This test can be used to determine the activity of nitrate reductase. Each isolate was cultured on minimal medium with 1 M urea as a sole nitrogen source. After 3 days plates were flooded with 3 M sodium nitrate and incubated at 20 ^{o}C for three hours. The nitrate solution was removed and nitrite excretion was determined using sulfanilamide and N-(1-napthyl)- ethylenediamine hydrochloride (Sigma Chemical Company, St. Louis, Missouri). First, 1 ml of 1% (w/v) solution of sulfanilamide in 3:1, water:37% HCl, was added to the plate, followed by 1 ml of a 0.02% (w/v) aqueous solution of N-(1-napthyl) ethylenedi-

amine hydrochloride. A positive test was characterized by a bright pink halo around the culture, indicating a functional nitrate reductase enzyme in the absence of nitrite reductase activity (Cove, 1976).

To compare the nitrogen metabolism pattern of the *L. cincta* and *L. persoonii* isolates, 9 isolates were grown on solid minimal media as described above with different nitrogen sources suspected to be discriminatory between the two species. The nitrogen sources tested included $(NH_4)_2SO_4$, hypoxanthine, lysine, serine, leucine, isoleucine, valine, and tyrosine. Mycelial plugs (0.5 cm diameter) were placed in the center of 10 cm agar plates. Measurements of colony diameter were taken after 7 and 14 days of incubation at 27 ^{o}C. Isolates were also grown on 1.5% perchlorate media to determine if this character was species specific.

Virulence of all isolates was tested using the apple lesion test as previously described (Hammar *et al.*, 1989 and Fulbright, 1984). To elucidate the effect of nitrogen source on pathogenicity, three media were used to grow plugs for inoculation. The three media used were minimal media described above supplemented with leucine or serine and Leonian's malt agar (Leonian, 1921). Cultures were grown for 5 days on one of these media. Mycelial plugs (0.5 cm in diameter) were inoculated into unripe Granny Smith apples Inoculations were performed using plugs of mycelium pregrown

on Leonian's malt agar, pregrown on serine, pregrown leucine, and pregrown on leucine supplemented with a plug of serine medium.

RESULTS:

Inorganic Nitrogen Assimilation:

Of the twenty isolates screened in the initial study, only the two hypovirulent isolates and the *L. cincta* isolates were able to grow on perchlorate medium without a spontaneous mutation occurring. The four isolates tested represented three distinct groups based on inorganic nitrogen metabolism (Table 1 and 2). The *L. persoonii* isolate, 11.13, the other 16 Michigan *L. persoonii* and the 9 other isolates of *Leucostoma* have normal wild type nitrogen metabolism. They were readily able to use $NaNO_2$, $NaNO_3$, $(NH_4)_2SO_4$ and hypoxanthine. Three isolates, ATCC 62190, NC 14.4A and HT, had abnormalities in their assimilation of inorganic nitrogen. The *L. cincta* isolate, ATCC 62190, apparently has nonfunctional or nonexistent nitrite reductase enzyme. Though it was able to grow on $(NH_4)_2SO_4$ and hypoxanthine, ATCC 62190 could not use $NaNO_2$ or $NaNO_3$ as sole nitrogen sources. It also strongly excretes nitrite, implying a functional nitrate reductase. The two hypovirulent isolates appeared to have anomalous inorganic nitrogen assimilation, possibly a reflection of a mutation in a regulatory locus. These two isolates were readily able to grow on $(NH_4)_2SO_4$ and hypoxanthine, but unable to grow on $NaNO_3$ and $NaNO_2$ and do not excrete nitrite. Their inability to utilize both $NaNO_3$ and $NaNO_2$ coupled with a lack of

nitrite excretion suggests that both nitrate and nitrite reductases are not functioning.

Growth on different nitrogen sources:

All four isolates were able to utilize a wide variety of nitrogen sources. *L. persoonii* isolate MI 11.13 was able to use the most diverse sources of nitrogen. It was readily able to grow on all of the sources proposed to be involved in inorganic nitrogen assimilation except for hydroxylamine, which appeared to be toxic to all isolates. Of the 45 nitrogen sources tested, 33 sources of nitrogen encouraged growth for MI 11.13. The ten best nitrogen sources were allantoin, asparagine, inosine, serine, alanine, allantoic acid, guanine, xanthine, uric acid and glutamic acid. This isolate was unable to grow on hydroxylamine, cysteine, anthranilic acid, indole, urocanic acid, methylated purines (caffeine and theophylline), pyrimidines (cytosine, thymine, orotic acid and uracil) and lysine. In addition to hydroxylamine, cysteine, anthranilic acid and indole were also inhibitory to growth.

L. cincta isolate ATCC 62190 had a slightly more restricted list of usable nitrogen sources. Of the 45 nitrogen sources tested, 27 sources of nitrogen encouraged growth for ATCC 62190. The ten best nitrogen sources included serine, histidine, glycine, glutamic acid, alanine, adenine, aspartic acid, guanine, allantoin and tryptophan. There were many nitrogen sources which did not support growth in-

cluding hydroxylamine, nitrate, nitrite, cysteine, anthranilic acid, indole, urocanic acid, methylated purines (caffeine and theophylline), pyrimidines (cytosine, thymine, orotic acid and uracil), lysine and the branched aliphatic side chain amino acids, isoleucine, leucine and valine. As with MI 11.13, some nitrogen sources appeared to be inhibitory to growth of ATCC 62190; these included nitrite, hydroxylamine, cysteine, lysine, anthranilic acid and indole.

Hypovirulent isolate HT and NC 14.4A had a further restricted range of usable nitrogen sources. Of the 45 nitrogen sources tested, 24 supported growth. The best nitrogen sources were not identical for the two isolates but they did share many in common. The ten best nitrogen sources for HT were alanine, inosine, hypoxanthine, arginine, $(NH_4)_2SO_4$, adenine, urea, serine, uric acid and glycine. The ten best for NC 14.4A were arginine, adenine, serine, glycine, hypoxanthine, inosine, alanine, citrulline, asparagine, and histidine. The hypovirulent isolates were also unable to use hydroxylamine, nitrate, nitrite, cysteine, anthranilic acid, indole, urocanic acid, methylated purines (caffeine and theophylline), pyrimidines (cytosine, thymine, orotic acid and uracil), lysine and the branched aliphatic side chain amino acids, isoleucine, leucine and valine. Growth of the two hypovirulent isolates on glutamic acid and aspartic acid, as well as aromatic amino acids was poor, though

statistically, growth on these compounds were usually significantly greater than controls. The compounds that were inhibitory to the virulent isolates were also inhibitory to these isolates including hydroxylamine, cysteine, indole and lysine.

Comparison of *L. persoonii* and *L. cincta* nitrogen utilization:

Ability to grow on perchlorate media was apparently not a species specific character (Table 3). However, growth on the branched aliphatic side chain amino acids was always slower in *L. cincta* isolates (Table 3). Regardless of phenetic group, all *L. persoonii* isolates could readily grow on branched aliphatic amino acids as sole nitrogen sources. Growth of *L. cincta* isolates was in general poor on branched aliphatic amino acids.

Influence of nitrogen source on virulence of *L. persoonii* and *L. cincta* :

Virulence data from apple fruit lesion assays suggested that *L. cincta* isolates tested were less virulent (Table 4). On average, inoculation with a *L. cincta* isolate produced a lesion of 18.7 mm, whereas *L. persoonii* inoculations produced a lesion of 43.0 mm in diameter (Table 4). Though initial nitrogen source did not significantly effect virulence, additional supplementation of nitrogen did appear to influence lesion size, on average producing the largest lesions (Table 4).

DISCUSSION:

Inorganic Nitrogen Assimilation:

Some fundamental information on nitrogen catabolism can be deduced by examining growth on a few nitrogen sources and the nitrite excretion test (Marzluf, 1981). All the major genes involved in basic nitrogen catabolism are functional in the *L. persoonii* isolate MI 11.13 as indicated by growth pattern. The *L. cincta* isolate, ATCC 62190, and the two hypovirulent isolates appeared to have a mutation in a key step in the pathway of inorganic nitrogen assimilation. Isolate ATCC 62190 of *L. cincta* had a growth pattern that suggested it was a *nit 2* mutant with a block in the nitrite reductase enzyme (Correll, *et al.*, 1987). The lack of growth on $NaNO_2$ and $NaNO_3$ combined with nitrite excretion can be attributed to a nonfunctional nitrite reductase, coded for by a single gene in other fungi (Correll *et al.*, 1987). Its ability to use hypoxanthine ruled out the possibility of a block in the molybdenum cofactor locus. These feature of this isolate appears not to be a species specific character as it is unique to this phenetic group.

The two hypovirulent isolates appear to have nonfunctional nitrite and nitrate reductases. Data from other genetic studies indicate that this is most likely due to a malfunction of a major regulatory loci rather than a double structural locus mutation (Correll, *et al.*, 1987). The possible significance of this will be discussed later.

Growth of *Leucostoma* on different nitrogen sources:

It was apparent that in the virulent *L. persoonii* and *L. cincta*, nitrogen utilization is normal and complex. In many aspects of its nitrogen metabolism, *Leucostoma* shared many similarities with that of *Gibberella zeae* and with other fungal species where nitrogen utilization has been studied closely (Leslie, 1986; for review see Garraway and Evans, 1984). Isolates of these species can use a wide variety of nitrogen sources. The inability to use hydroxylamine was not surprising as it has been long hypothesized that reduction of nitrite occurs in a one step process and that this compound is not an intermediate in the reduction of nitrite to ammonia (Garraway and Evans, 1984). Toxicity of this compound has been observed in other species of fungi (Pateman and Kinghorn, 1976). Toxic effects of cysteine and lysine have also been noted in other fungal species including *Gibberella zeae, Bipolaris maydis, Neurospora crassa, Tolypocladium niveum* and several species of yeasts (Leslie, 1986, Evans and Black, 1981; Adiga and Sarma, 1970; LaRue and Spencer, 1967 b; and Kal'vish, 1990). The inability of *Leucostoma* to grow on cysteine was first observed by Helton (Helton and Konicek, 1962 b). Toxicity of cysteine is a complex process that affects many biochemical functions in the cell (Turner, 1959 and Adiga and Sarma, 1970 and 1971). Lysine toxicity is not uncommon in fungi and may relate to an inhibitory effect due to high

levels in the cell or the accumulation of a toxic intermediate (Leslie, 1986 and Garraway and Evans, 1984).

The utilization of compounds that contain nitrogen in ring structures is a complex process (Tarver, 1958). Pyrimidines were not used as sole nitrogen sources, suggesting an inability to open the pyrimidine ring. A pyrimidine salvage pathway, found in *Neurospora crassa* and some species of yeast, bacteria and animals, can be used for providing many of the nutrients for growth (LaRue and Spencer, 1967 b and Hartman, 1970); however, in other fungi, this pathway is apparently absent (Leslie, 1986). Other ring opening systems are not functioning in *Leucostoma*. Inability to use indole denotes an inability to open the indole ring. The amino group associated with anthranilic acid is also an unsuitable nitrogen source. Apparently, the ability to use tryptophan is through deamination rather than ring opening. Histidine use is also through deamination. A potential intermediate in the degradation of histidine, urocanic acid, was not utilized efficiently by this fungus. Degradation of the imidazole ring of urocanic acid is a complex process that is rarely observed in nature (Tarver, 1958 and LaRue and Spencer, 1967 a).

Other key pathways in nitrogen utilization were functioning in *Leucostoma*. All the intermediates in the urea cycle were efficiently used by *Leucostoma*, implying a functional cycle. The well characterized complex pathway of

purine degradation was completely intact, as all intermediates are utilized as sole nitrogen sources (LaRue and Spencer, 1968 and Marzluf, 1981). The inability to utilize methylated purines as sole nitrogen sources may relate to their inherent toxicity and the inability of *Leucostoma* isolate to detoxify them. The inability to utilize these compound has been noted in other species (Cochrane, 1958).

Comparison of *Leucostoma* and *Gibberella zeae* nitrogen utilization:

There were no major differences in nitrogen metabolism of *G. zeae* when compared with *Leucostoma*. In *G. zeae*, a wide variety of inorganic and organic nitrogen sources were reported as suitable substrates for growth (Leslie, 1986). The major exceptions to this were lysine and the sulfur containing amino acids and their analogues. As mentioned previously, *Leucostoma* was also unable to utilize lysine and cysteine, though it was able to utilize methionine effectively for growth. Some of the unique features of the nitrogen utilization profile of *G. zeae* include an ability to utilize histidine, phenylalanine and tyrosine as sole nitrogen sources as well as its efficient use of proline, asparagine and putrescine which may relate to its pathogenicity (Leslie, 1986). Of the aforementioned compounds, all but putrescine were tested in this study. *Leucostoma* was readily able to utilize all of these compounds. Thus, there appeared to be little difference between the stem-

rotting pathogen of herbaceous plants and the canker-forming pathogen of trees in regards to nitrogen metabolism.

Comparison of *L. persoonii* and *L. cincta* nitrogen utilization:

There were some differences between the two species of *Leucostoma*. In particular, the inability to use the branched aliphatic side chain amino acids leucine, isoleucine and valine. In liquid culture, the *L. cincta* isolate ATCC 62190 grew poorly with branched aliphatic compounds as a sole nitrogen source, whereas the *L. persoonii* isolate MI 11.13 grew normally on these compounds. Inefficient use of the branched aliphatic amino acids, especially leucine, has been observed in *Bipolaris maydis* (Evans and Black, 1981) and other fungi (Malca *et al.*, 1966 Weinhold and Garraway, 1966). This was probably due to inefficient uptake or the accumulation of toxic products during degradation of the carbon skeleton as the utilization of the nitrogen in these compound is through a general transamination system that appears to be functioning for other amino acids. There is evidence in other fungal species for specific amino acid uptake systems; however, general amino acid permeases are known to function in these species as well (Whitaker, 1976; and Garraway and Evans, 1984). Isolates representing the three phenetic groups in the taxon *L. persoonii* grew well on solid media supplemented with branched aliphatic amino acids as a nitrogen source

while isolates representing the three phenetic groups of *L. cincta* grew only sparingly on this same media; therefore, it appeared as though the inability to use these compounds is a species specific characteristic.

Influence of dsRNA on nitrogen metabolism of *L. persoonii*:

Alteration in general metabolism in dsRNA containing isolates of phytopathogenic fungi have been reported (Psarros and Lindberg, 1962; Detroy, *et al.*, 1973; Rogers *et al.*, 1986; Rigling *et al.*, 1988; and Mahanti *et al.*, 1991). In the case of *Ceratocystis ulmi* and *Cryphonectria parasitica*, increases in alternate oxidase activity is associated with the mitochondria. This inability to utilize nitrate was probably not linked with mitochondrial malfunction as this enzyme is a soluble cytoplasmic enzyme not localized in a specific organelle (Dunn-Coleman *et al.*, 1984). The inefficient utilization of glutamic and aspartic acid could be traced to the highly distorted mitochondria of isolate NC 14.4A (Snyder *et al.*, 1989), as some of the enzymes associated with transamination of these two amino acids are localized in the mitochondria. A malfunction in the enzymes normally associated with this organelle could be a reflection of the morphological distortion of the mitochondrial membrane.

The significance of poor growth of these isolates on aromatic amino acids is unclear. Aromatic compounds can be linked with plant disease resistance. Inability to effi-

ciently degrade these compound may contribute to the reduced virulence of these isolates. There are links between enzymes involved in phenolic detoxification, specifically laccase or phenol oxidase activity, in virulence in *Cryphonectria parasitica* (Rigling *et al.*, 1988). This enzyme is involved in polymerization of aromatic compounds and is found at reduced levels in European hypovirulent strains. A similar reduction in activity of enzymes involved in degradation of aromatic amino acids of *L. persoonii* may occur, though laccase activity in these isolates is normal (data not shown).

The hypovirulent isolates had a remarkably restricted range of usable nitrogen sources. The reduced efficiency of nitrogen metabolism may be correlated with reduced virulence but not necessarily dsRNA. Another dsRNA containing isolate, NC 9.2, is normally virulent and is unable to grow on perchlorate media. When tested on solid media, this isolate was readily able to use all nitrogen source. Nitrogen form may have a significant impact on plant disease (Huber and Watson, 1974; and Schoenweiss, 1975). In particular, supplementation of plant with specific amino acids have been reported to alter pathogenicity though no general pattern can be discerned (Van Andel, 1965). Much of this work has been focused on soil pathogens. The levels of free amino acids in tissue can change when a plant is under stress (Lyons, 1973 and Stewart and Larher, 1980). Perhaps the

cold stress that predisposes peach trees to infection with *Leucostoma* (Chang *et al.*, 1989) may be reflected in a difference in the level of nitrogen source availability in the sap. If nitrate, nitrite, or branched aliphatic amino acid level is raised in frozen tissue, this could play a role in the reduced pathogenicity of the two dsRNA containing isolates by restricting growth of the fungus. In addition, both glutamic and aspartic acid are found in relatively high concentration in peach xylem fluid (Gould *et al.*, 1991). As both hypovirulent isolates can exhibit reduced growth on these compound in culture, this situation may be mirrored in the plant tissue.

Influence of nitrogen source on virulence of *L. persoonii* and *L. cincta* :

Growth on solid media suggested some interesting trends in regards to species specific nitrogen metabolism. In some pathogens, the quantity or quality of antecedent nitrogen influences pathogenicity (Weinhold *et al.*, 1969 and Williams, 1965). Though *L. cincta* is generally considered to be a more aggressive pathogen than *L. persoonii* (Willison, 1936), variability in pathogenicity in both species has been observed (Wysong and Dickens, 1962 and Surve-Iyer, *et al.*, 1992). In Michigan, *L. persoonii* is the more prevalent species (Hildebrand, 1947 and Adams *et al.*, 1989). Inefficient use of the branched aliphatic side chain amino acids by the *L. cincta* isolates examined in this study

may relate to their pathogenicity in a similar fashion to the hypovirulent isolates as both the *L. cincta* isolates and the hypovirulent isolates formed smaller and slower developing lesions on apple.

Conclusions and Perspectives for Future Work:

The nitrogen metabolism of *L. persoonii* and *L. cincta* is varied and complex. There is apparently a link between pattern of nitrogen utilization and virulence. Field tests including the known *L. persoonii* and *L. cincta* isolates along with the hypovirulent isolates would provide more correlative evidence to support this hypothesis. An examination of the nitrogen content of peach bark, comparing frozen and unfrozen tissue, might add to the understanding of the virulence of these pathogens.

Perhaps more promising is the apparent differences between *L. persoonii* and *L. cincta* in ability to utilize certain nitrogen source. Testing more isolates on branched aliphatic amino acids and comparing these results with isozyme and RFLP analysis might lead to the development of a selective medium for distinguishing these two closely related species. A selective medium for the two pathogens could provide a rapid method of species identification and reduce confusion between the two.

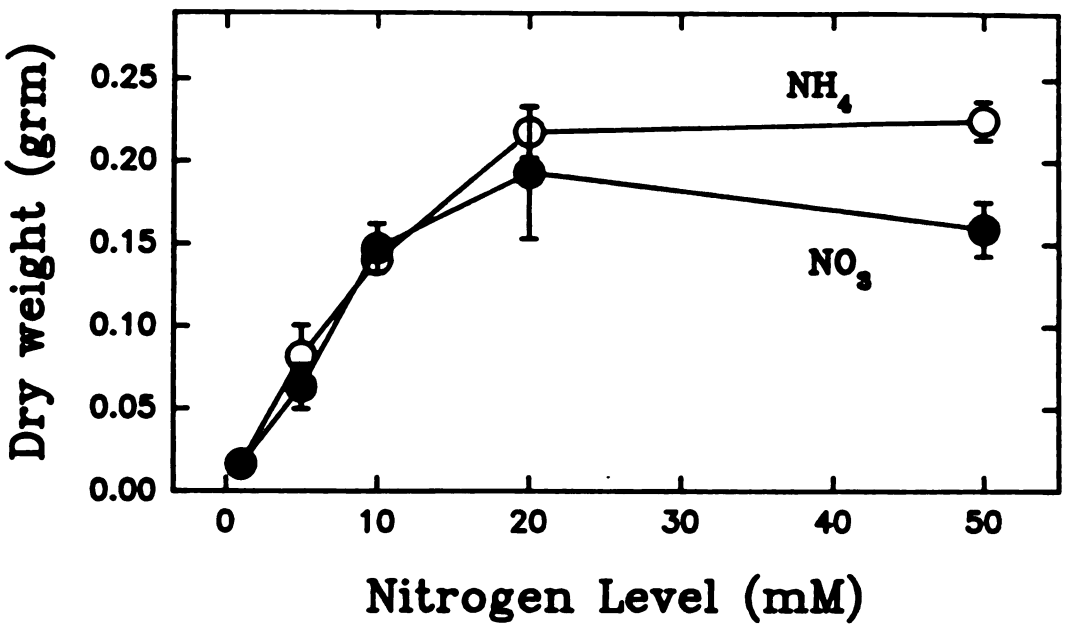

Figure 1. Dry weight of mycelium after 14 days of growth of *L. persoonii* (MI 11.13) in liquid minimal media with 1% maltose as the carbon source and 0.0, 5.0, 10.0, 20.0 or 50 mM nitrogen equivalents of $(NH_4)_2SO_4$ (open circles) or $NaNO_3$ (closed circles) as the nitrogen source.

Table 1. Growth on 45 nitrogen sources (mg dry weight) after 14 days of a standard isolate of *L. persoonii* (MI 11.13), a unique isolate of *L. cincta* (ATCC 62190) and two dsRNA containing isolates of *Leucostoma* sp. (NC 14.4A and HT).

Nitrogen source	Isolate and Species			
	-dsRNA		+dsRNA	
	L.persoonii	*L.cincta*	*L.persoonii*	
(5.0 mM Nitrogen equivs.)	11.13	62190	HT	14.4A
Control	67.2	70.3	57.2	35.8
Inorganic Nitrogen Assimilation Compounds				
$NaNO_3$	166.7*	101.3	68.3	40.2
$NaNO_2$	169.8*	15.3	25.9	23.5
$(NH_4)_2SO_4$	229.1*	206.3*	282.5*	138.2*
Hydroxylamine	28.2	29.2	22.6	9.20
Hypoxanthine	243.0*	190.3*	296.9*	187.5*
Amino acids and related compounds				
Alanine	333.5*	321.1*	322.4*	172.1*
Arginine	264.2*	211.7*	286.1*	224.8*
Asparagine	339.9*	226.4*	222.6*	170.8*
Aspartic acid	221.6*	298.3*	70.3	74.4
Cysteine	92.3	13.8	26.5	17.4
Glutamic acid	273.0*	335.8*	98.0*	93.5*
Glutamine	226.4*	168.1*	157.1*	122.8*
Glycine	248.2*	337.6*	245.2*	193.2*
Histidine	270.5*	349.1*	182.1*	159.0*
Urocanic acid	80.0	93.7	62.6	40.1
Isoleucine	270.5*	111.9	43.2	49.7
Leucine	237.4*	100.9	71.1	47.6
Lysine	102.2	56.5	36.5	32.1
Methionine	233.9*	159.2*	165.0*	123.5*
Phenylalanine	256.7*	215.9*	164.4*	92.9*
Proline	250.2*	229.1*	179.8*	115.3*
Serine	334.1*	403.6*	254.7*	203.2*

Nitrogen source	Isolate			
	-dsRNA			+dsRNA
(5.0 mM Nitrogen equivs.)	11.13	62190	HT	14.4A
Threonine	202.2*	162.9*	135.4*	90.3*
Tryptophan	136.3*	243.0*	93.6*	50.0
Indole	8.3	12.6	9.0	8.3
Anthranilic acid	42.5	43.4	73.3	53.9
Tyrosine	168.4*	121.1*	104.6*	84.5
Valine	193.0*	116.2	49.1	46.4
Purines and related compounds				
Adenine	214.9*	303.2*	269.3*	218.2*
Guanine	291.9*	285.5*	214.8*	100.0*
Hypoxanthine	243.0*	190.3*	296.9*	187.5*
Inosine	338.4*	183.9*	303.8*	184.5*
Xanthine	276.4*	135.8*	191.7*	90.7*
Allantoic acid	293.2*	155.4*	267.0*	100.0*
Allantoin	353.9*	272.6*	206.2*	118.7*
Uric acid	276.4*	220.3*	252.7*	117.0*
Caffeine	109.8	78.0	72.5	45.4
Theophylline	103.2	99.1	65.8	54.4
Pyrimidines and related compounds				
Cytosine	118.0	99.5	87.8	64.5
Thymine	103.0	107.9	77.0	45.4
Uracil	113.0	110.6	76.2	47.4
Orotic acid	97.8	72.6	48.5	46.6
Urea Cycle Compounds				
Arginine	264.2*	211.7*	286.1*	224.8*
Urea	272.2*	164.7*	261.2*	136.2*
Citrulline	227.0*	118.0*	231.6*	152.3*
Ornithine	228.7*	222.3*	236.3*	92.5*

Table 2. Inorganic nitrogen assimilation by *Leucostoma persoonii* MI 11.13, *L. cincta* ATCC 62190, and the hypovirulent *L. persoonii* NC 14.4A and HT with correlations to the classes of nitrate nonutilizing mutants as proposed by Correll *et. al* (1987).

	Growth on nitrogen sources[a]				
Blockage Site	Nitrate	Nitrite	Ammonium	Hypo-xanthine	Nitrite[b] Excretion
None Wild Type *L. persoonii* MI 11.13.	+	+	+	+	slight
Nitrate reductase structural locus *nit 1*	-	+	+	+	NT
Nitrite reductase structural locus *nit 2* *L. cincta* ATCC 62190.	-	-	+	+	+
Pathway specific regulatory locus *nit 3* *L. persoonii* NC 14.4A and HT.	-	-	+	+	-
Molybdenum cofactor locus *Nit M*	-	+	+	-	NT

a.) Significantly different increase in dry weight of mycelium after growth on minimal media with compound as sole nitrogen source. + = growth, - = no growth.

b.) Assay for enzymatic activity of nitrate reductase as described by Cove, 1976. + = positive (pink halo), - = negative (no color change), slight = normal wild type activity (slight pink halo), NT = not tested.

Table 3. Diameter in mm of *L. cincta* and *L. persoonii* colonies after 14 days growth on minimal media with 5 mM nitrogen equivalents of selective nitrogen sources suspected to be discriminatory between species.

Species Isolate (phenetic group)	Leucine	Iso-leucine	Valine	NH_4	Serine	Lysine
L. cincta						
LP39 (pg4)	45.0	23.7	28.3	80.0	80.0	19.0
LP47 (pg5)	55.0	37.7	43.3	76.6	80.0	31.6
F1H (pg5)	19.6	16.3	33.0	76.6	65.0	ng[a]
A48 (pg6)	25.3	25.0	45.7	20.0	76.7	37.6
62190 (pg4)	61.0	63.3	30.0	80.0	80.0	38.3
L. persoonii						
T28.1 (pg2)	43.7	58.3	58.7	58.0	73.3	45.6
LCN (pg3)	80.0	69.0	76.6	40.3	74.3	75.0
11.13 (pg1)	70.0	75.0	76.0	80.0	80.0	46.7
11.9 (pg1)	65.0	62.7	64.6	70.0	80.0	61.3

Table 4. Assay of virulence of isolates of *L. cincta* and *L. persoonii* on apple fruit after pregrowth on defined media or Leonian's malt agar.

Isolate (phenetic group)	Mean lesion width (mm) after pregrowth on: Leonians	Leucine	Serine	Leucine + Serine	total mean
L. cincta					
LP 39 (pg4)	10.0	24.0	16.0	24.3	18.6
LP 47 (pg5)	10.0	18.0	19.7	23.6	17.8
F 1 H (pg5)	21.3	25.3	19.0	26.3	23.0
A 48 (pg6)	12.3	13.7	12.0	16.3	13.6
62190 (pg4)	15.3	24.0	19.0	24.0	20.6
				mean	18.7
L. persoonii					
T28.1 (pg2)	31.3	44.6	36.3	40.3	38.0
LCN (pg3)	36.7	41.3	44.7	45.7	42.1
11.13 (pg1)	36.0	37.0	40.0	47.7	40.1
11.9 (pg1)	48.7	43.3	54.0	61.0	51.7
				mean	43.0
L. persoonii (+ dsRNA)					
14.4A	13.0	17.0	11.0	22.0	15.8
HT	15.0	27.0	18.0	28.0	22.0
				mean	18.4

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EXPERIMENTAL II:

PURIFICATION AND CHARACTERIZATION OF A VIRUS-LIKE PARTICLE OF *LEUCOSTOMA PERSOONII*.

ABSTRACT

Abundant isometric virus-like particles were visible in the hyphae of isolate NC 14.4a, a hypovirulent isolate of Leucostoma persoonii. Our objective was to purify and characterize these particles. The particles were purified by concentration via polyethylene glycol precipitated and clarified by differential centrifugation. Spectrophotometric analysis of sucrose and CsCl density gradients revealed two peaks with an absorbance at 254 nm. The peaks contained isometric particles with diameters of 40 nm. The particles had a buoyant density in CsCl of 1.313 g/cm3 and 1.339 g/cm3 and had a sedimentation coefficient of 105 s and 151 s as determined from linear-log sucrose gradients. Extraction of protein from the peaks yielded one major coat protein in SDS-PAGE with a molecular weight of 32,000. The top component contained five bands of dsRNA that ranged in size from 0.7 kb to 4.2 k . The bottom component contained the same five bands of dsRNA found in the top component plus an additional band of dsRNA with a size of 7.9 kb. Many of the features of this virus like particle were atypical of a fungal virus. The relationship as determined by northern blot analysis between the bands of dsRNA found in the tissue of NC 14.4A and the partially cured isolate HT suggested that the bands of dsRNA normally associated with virus infection do not share significant homology whereas the two bands that remain when virus infection is lost may be related.

INTRODUCTION:

Cytospora canker caused by *Leucostoma cincta* (Fr.:Fr.) Höhn. (anamorph = *Leucocytospora cincta* (Sacc.) Höhn.) and *Leucostoma persoonii* Höhn. (anamorph *Leucocytospora leucostoma* (Pers.) Höhn.) is often the limiting factor in peach production in the northern United States. In this region, this disease is a major contributor to decline of peach orchards (Biggs, 1989). Large perennial cankers which are characteristic of this disease can cause splitting of the major scaffold limbs often resulting in the death of the tree (Hildebrand, 1947). Fungicides have proven to be ineffective in controlling this disease especially in established orchards (Helton and Rohrbach, 1967 and Royse and Ries, 1978). A biological control agent that could reduce the impact of this disease would be highly desirable. During investigations to identify a hypovirulent isolate of this pathogen, an isolate was recovered that contained double stranded RNA (dsRNA) and exhibited low virulence (Hammar *et al.*, 1989). Electron microscopy of the hyphae of this isolate revealed abundant virus like particles (Snyder *et al.*, 1989).

The presence of virus-like particles (VLPs) has been detected in many species of both phytopathogenic and non-phytopathogenic fungi (Buck *et al.*, 1984). The history of fungal viruses is relatively brief. The first fungal virus

was described in the early 1960's in *Agaricus bisporus*, followed by one in *Penicillium funiculosum* (Hollings, 1962; and Banks *et al.*, 1968). Interest in these viruses was focused on their pathogenicity in the commercial mushroom and their capacity for interferon stimulation in mammalian cells by fungal extracts (Hollings, 1965; Planterose *et al.*, 1970; and Banks *et al.*, 1970).

Following these initial discoveries, numerous fungal viruses have been characterized (Buck *et al.*, 1984 and Nuss and Koltin, 1990). A typical fungal virus is an isometric particle with a single major capsid protein and dsRNA as its nucleic acid component (Lemke and Nash, 1974). In 1984, six groups of fungal viruses were proposed by Buck (Buck *et al.*, 1984). The groups were based primarily on the available information including particle size, capsid protein, sedimentation coefficient and dsRNA components number and encapsidation. Though these particles have many of the characteristics of a true virus, the lack of demonstrated cell free transmission has lead to the convention of referring to these viruses as virus-like particles or VLPs.

Further interest in mycoviruses has been stimulated by their potential use as biocontrol agents for phytopathogenic fungi. There are many examples of dsRNA and VLPs in fungi that cause significant plant disease (Anagnostakis, 1982; Buck *et al.*, 1981; Hoch *et al.*, 1985; Castanho *et al.*, 1978; Rogers *et al.*, 1986; and more; for review see Nuss and

Koltin, 1990). In addition, there is good evidence that dsRNA is involved in a killer phenomena in two systems, *Saccharomyces cerevisiae* (Wickner, 1986) and *Ustilago maydis* (Ganesa *et al.*, 1989; and Nuss and Koltin, 1990).

Evidence is increasing that mycovirus may be related to viruses found in other kingdoms including double stranded RNA viruses of animals (bi- and tri-picoviridae), the cryptic viruses of plant and the viruses of protista (Wang and Wang, 1986a and 1986b; Bocaardo *et al.*, 1987; Pereira *et al.*, 1988; Miller, Wang and Wang, 1988; Leite *et al.*, 1990; and Pereira, 1991). Furthermore, newly described and previously characterized fungal viruses that do not fit in the 6 proposed mycovirus groups appear related to other viruses that infect hosts in the plant, animal and bacterial kingdoms (Kazama and Schornstein, 1973; Tavantzis *et al.*, 1980; Pryor and Boelen, 1987; Dickinson and Pryor, 1989; Goodin *et al.*, 1992; and Enebek *et al.*, 1991).

Despite the increased understanding of mycoviruses, characterization of fungal viruses is often incomplete. The purpose of this study was to characterize the VLP associated with a hypovirulent isolate of *L. persoonii* and attempt to classify it in relation to known dsRNA virus groups. The relationship between the 7 bands of dsRNA in the virus-containing isolate were compared with each other and with those of the partially cured isolate that had lost the virus like particle and all but two bands of dsRNA.

MATERIALS AND METHODS:

Fungal Isolates:

All the isolates of *Leucostoma persoonii* used in this study were initially recovered from cankers on peach trees. The hypovirulent isolate NC 14.4A was provided by E. Endert-Kirkpatrik and D. F. Ritchie and recovered from peach in North Carolina. Isolate HT was derived from isolate NC 14.4A by successive hyphal tipping (Hammar *et al.*, 1989). A normally virulent dsRNA-free isolates of *L. persoonii*, isolate MI 11.13, was recovered from peach in Michigan. The cultures were maintained on Leonian's malt agar (LMA) (Leonian, 1921).

Virus-Like Particle Isolation and Purification:

Mycelium was grown in *Endothia* complete broth for 2 to 3 weeks at room temperature (Day *et al.*, 1977). Mycelium was harvested by vacuum filtration, pressed dry and used fresh for virus purification or stored at -20 ^{o}C for future use. Mycelial mats (10 - 50 grams) were homogenized by grinding with 10 g glass beads (0.12-0.18 mm, Thomas Scientific, New Jersey) in liquid nitrogen and resuspended in 10 volumes of 0.1 M potassium phosphate buffer, pH 7. The suspension was disrupted further by blending with glass beads for two minutes in a blender. The cellular debris was removed by low speed centrifugation (9,000 G) for 15 minutes and filtration of the supernate through miracloth (Calbiochem Corporation, California). The VLP's were

precipitated by adding 8% (weight:volume) polyethylene glycol (PEG, MW 6000) and 1% (weight:volume) NaCl and stirring for 4 hours at 4 ^{o}C. VLPs were recovered by low speed centrifugation (16,000 G) for 30 minutes and the resulting pellet was resuspended at 4 ^{o}C overnight in 0.1 original volumes of 0.05 M potassium phosphate buffer, pH 7.0. After resuspension, the mixture was subject to low speed centrifugation (7000 G) for 15 minutes to remove the PEG and remaining cellular debris. The suspension was layered onto a 30% sucrose pad and centrifuged in a Beckman 55.2 rotor at 130,000 G for 4 hours at 4 ^{o}C. The final pellet was resuspended overnight at 4 ^{o}C in 1 ml of 0.05 M potassium phosphate buffer and layered onto 10% to 30% linear log sucrose gradients and centrifuged in a Beckman SW 41 rotor at 245,000 G for 90 minutes. Gradients were fractionated using an Isco density gradient fractionater equipped with a U. V. analyzer (Isco Co., Lincoln, NE). Appropriate fractions were diluted with buffer and centrifuged in a Beckman 55.2 rotor at 130,000 G for 4 hours. The resulting pellets were resuspended in 0.05 M potassium phosphate buffer, pH 7.0.

Physical properties determination:

Density was determined by loading resuspended pellets onto a solution of CsCl (initial density = 1.367 g/cm^3) and centrifuging in a Beckman SW 55 rotor at 190,000 G at 20 ^{o}C for 20 hours. Cesium gradients were fractionated as

described above and the density of the virus containing fractions was determined with a refractometer using the formula, density = 10.4091 $\eta\delta$ - 12.8812, where $\eta\delta$ = refractive index (Bozarth, Wood and Mandelbrot, 1971).

Sedimentation coefficients were determined using linear-log gradients with Sowbane Mosaic Virus (SMV) ($S_{w,20}$ = 104s), Tobacco Ringspot Virus (TRSV) ($S_{w,20}$ = 53 s-Top component, 91 s-Middle component and 126 s-Bottom component) and Tobacco Mosaic Virus (TMV) ($S_{w,20}$ = 196 s) as size standards (kindly provided by J. Gillett and R. Allison).

The extinction coefficient was determined by measuring the absorbance of a sample at 260 nm, placing 100 μl aliquots into dried preweighed weigh-bottles, vacuum desiccating for 5 days and weighing the dried sample to determine the mass of the VLPs. The extinction coefficient was derived from data of three replications of this procedure using the Beer Lambert equation, E = A x [C] where E = extinction coefficient, A = absorbance at 260 nm/ 1 cm path and [C] = concentration.

Isolation of Protein and Nucleic Acid Component:

Proteins were extracted and denatured by heating VLPs to 100 ^{o}C in 125 mM Tris-HCl, pH 6.8 with 4% SDS, 10% 2-mercaptoethanol 20% glycerol as described by Laemmli (1970). The protein was separated by electrophoresis in a 10% SDS-polyacrylamide gel with a 4% stacking gel at 30 Amps for 3

hours. The gel was fixed in 5:1 methanol: acetic acid, stained with 0.1% Coomassie blue and destained in several changes of 5:1:4 methanol: acetic acid: water. Molecular weight was determined using the following size standards: Bovine albumin (MW = 66,000), Egg albumin (MW = 45,000), Glyceraldehyde-3-phosphate dehydrogenase (MW = 36,000), Carbonic anhydrase (MW = 29,000), Trypsinogen (MW = 24,000), Soybean trypsin inhibitor (MW = 20,100), and α-Lactalbumin (MW = 14,200) (Sigma Chemical Company, St. Louis, MO).

Nucleic acid was extracted from the VLPs using a two phase phenol extraction system (Diener and Schneider, 1968). Purified particles were vortexed with 10% SDS, followed by extraction in STE-saturated phenol : chloroform : isoamyl alcohol (25:24:1) (STE buffer = 50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 1 mM EDTA). This extraction was repeated twice and nucleic acids from the resulting aqueous phase were precipitated in 2.5 volumes of 95% ethanol with 10% sodium acetate at -20 ^{o}C for 4 hours. Total dsRNA was isolated and purified from fungal tissue using a modification of CF11-cellulose column technique of Morris and Dodds (1979). Nucleic acid components were separated by 5% polyacrylamide gel electrophoresis as previously described (Hammar *et al.*, 1989) or by agarose gel electrophoresis (1.2% agarose gels). The gel buffer for both systems was TAE (40 mM Tris-HCl, pH 7.8, 20 mM sodium acetate and 1 mM EDTA). To determine the nature of the nucleic acid, sensitivity to nucleases was

tested using RNAse free DNAse I, and RNAse in both high (0.3 M NaCl) and low (0.015 M NaCl) salt (Rigling *et al.*, 1989). Lambda-HindIII DNA fragments and dsRNA extracted from NC 14.4A tissue were used as controls. To confirm the strandedness of the nucleic acids, the thermal melting profile was determined using a Gilford model 2400 spectrophotometer equipped with a thermoprogrammer (Miura *et al.*, 1966). Viral RNA was gradually heated from 20 ^{o}C to 100 ^{o}C and its absorbance at 260 nm was recorded. Double stranded RNA from the bacteriophage ϕ 6 (kindly provided by S. Demler) and single stranded RNA from yeast (kindly provided by J. Scott-Craig) were used as controls. Size of the bands was determined by the method outlined by Bozarth and Harley (1976) using dsRNA from a VLP (Hm9) of *Helminosporium maydis* (size = 8.3 kb) from a reovirus, serotype 3 (size = 3.9, 3.6, 3.3, 2.3, 2.0, 1.9, 1.3 and 1.2 kb).

Electron Microscopy:

Grids coated with 0.15% formvar and carbon were sprayed with a 1:1 mixture of purified virus preparation and 1% ammonium molybdate and examined in a JEOL 100 CX transmission electron microscope.

Antiserum Production:

Antiserum to the VLP was prepared in a rabbit by subcutaneous injection with purified top component.

Purified material (0.5 ml, 2 AU/ml) was mixed 1:1 with Freunds complete adjuvant for the first injection and with incomplete adjuvant for subsequent injections. Serum collection began one week after the third injection. Ouchterlony double diffusion tests to determine titer and specificity were carried out in petri plates in 0.8% agarose containing 0.85% sodium chloride and 0.1% sodium azide. Twofold serial dilutions of sera in phosphate buffered saline (0.01 M phosphate buffer, pH 7 and 0.85% NaCl) were used to determine titer.

Northern Blotting :

All glassware, plasticware and solutions used in the northern blotting procedures were treated to remove RNAse. Glassware was baked at 280 ^{o}C for 12 hours to destroy RNAse. Plastics were purchased new and sterile or soaked in SDS solutions and rinsed with autoclaved diethyl pyrocarbonate (DEPC) treated millipore water to destroy RNAse. Solutions were made in autoclaved DEPC treated millipore water with RNAse-free reagents.

Total dsRNA purified from fungal tissue or from VLPs was separated in a 1.2% agarose gels. The dsRNA was then denatured in the gel (Shapira, *et al.*, 1991). Denaturation was carried out after electrophoresis by treating the gel in 40% formamide and 17% formaldehyde in 1 X TAE buffer at 60 ^{o}C for 1 hour. The gel was cooled and subject to an alkali

treatment (50 mM NaOH and 100 mM NaCl) for 30 minutes, followed by two 20 minute washes in 20 X SSC (1 X SSC = 15 mM sodium acetate and 150 mM NaCl). The RNA was transferred to a nylon membrane (S&S Nytran, Schleicher and Schuell, Keene, New Hampshire) by capillary action and fixed to the membrane by baking at 80 ^{o}C for 45 minutes. Filters were prehybridized for 2 to 8 hours in 1.5 X SSC, 1 X Denhardt's, and 50% formamide with denatured fish sperm as a blocking agent. The radiolabled cDNA probes (see below) were boiled, quenched on ice and added to the prehybridization solution at a concentration of 50,000 cpm/ml. The membranes were hybridized with the probe at 42 ^{o}C for 24 hours. Filters were washed in 1 X SSC and 10% SDS at room temperature followed by a wash in 0.5 X SSC and 10% SDS at 42 ^{o}C. The membranes were dried and exposed to X-ray film for 12 to 72 hours at room temperature (without intensifying screen) or at -80 ^{o}C (with intensifying screen).

Probe Production:

Individual bands of dsRNA were isolated from low melting point agarose. Agarose gels were run as described above, using BRL low melting point agarose. Individual bands detected by ethidium bromide staining were excised and melted at 65 ^{o}C in microcentrifuge tubes. After 1:1 dilution of the melted agarose with preheated 1 X TE buffer (50 mM Tris-HCl, pH 8.0 and 1 mM EDTA), the solution was extracted three times with STE-saturated phenol and twice

with ether. After the residual ether had been removed by gently blowing N_2 over the sample the dsRNA was precipitated in 2.5 volumes of 95% ethanol with 10% sodium acetate. The dsRNA concentrated by ethanol precipitation was resuspended in water, boiled for 5 minutes and quenched on ice. The denatured nucleic acid was added to a microcentrifuge tube containing the reaction mixture (0.2 mM dATP, dTTP and dGTP, 20 μCi 32 α P dCTP, 0.2 mM random hexanucleotide primer, 40 mM DTT, 0.2 M Tris, pH 8, and 0.2 M KCl). Fifty units of AMV (avian myeloblastosis virus) reverse transcriptase (Boeringher-Mannheim) were added to this mixture and incubated for 1 hour at 37 ^{o}C. At this time 2 μl unlabeled dCTP was added to the tube and the mixture was incubated for an additional 15 minutes. The reaction was stopped by the addition of 2 μl 0.5 M EDTA and the RNA template was destroyed by the addition of 5 M NaOH and heating to 60 ^{o}C for 40 minutes. The mixture was neutralized with 1 N HCl and the cDNA probes were purified by sephadex column centrifugation (Maniatis *et al.*, 1982).

RESULTS:

Purification Properties of *Leucostoma* virus-like particles (Lp-V):

Though several schemes of purification were tried including low pH buffers and the addition of antioxidants to the buffer system, the use of differential centrifugation coupled with PEG precipitation consistently produced high yields of *Leucostoma persoonii* virus (Lp-V). Sucrose density gradients yielded two peaks in the A_{254} absorbance profile (Fig. 1). These peaks were never observed in extracts from healthy (MI 11.13) or partially cured (HT) isolates of *L. persoonii* (Fig. 1). Occasionally, the A_{254} absorbance profile revealed a large peak at the top of the gradients loaded with extracts from healthy isolates of *L. persoonii* (MI 11.13) but this peak contained no VLPS or the associated nucleic acid or protein. When the UV absorbing peaks of hypovirulent NC 14.4A extracts were pelleted and examined in electron microscopy, isometric virus-like particles with a diameter of 40 nm were observed (Fig. 2). Both peaks had an absorbance profile typical of nucleoproteins and a 260/280 ratio of 1.2 (Table 1). The 260/280 ratio was quite low for an isometric particle. After pooling and concentrating by centrifugation, the UV absorbing fractions were run in cesium chloride gradients. Two UV absorbing peaks were detected in these gradients. Density for the two components was 1.313 gm/cm^3 and 1.339 gm/cm^3 respectively (Table 1). When examined for protein

and nucleic acid, the CsCl peaks contained the same components as found in the sucrose gradient peaks (see below). Linear-log gradients were used to determine the sedimentation coefficient. The sedimentation coefficients of the two components were 107 s and 151 s respectively (Fig. 3). When each component was examined for the presence of protein, SDS polyacrylamide gels revealed that both the top and bottom components contained one major band of protein with a molecular weight of 32000 (Fig. 4). No protein was ever detected in the same region in the gradients of the extracts from healthy or partially cured isolates of *L. persoonii*. Nucleic acids were extracted from the UV absorbing peaks. Acrylamide gels revealed 5 and 6 bands of nucleic acid. The top component contained five bands of dsRNA that ranged in size from 0.7 kb to 4.2 kb (Fig. 5). The bottom component contained the same five bands of dsRNA found in the top component plus an additional band of dsRNA with a size of 7.9 kb. Nuclease digestion indicated the nucleic acid was dsRNA, as the nucleic acid from Lp-V particle were insensitive to DNAse and RNAse in high salt but sensitive to RNAse in low salt (data not shown). The thermal melting profile confirmed the double stranded nature of the nucleic acid. The melting profile of Lp-V nucleic acid had a characteristic hypochromatic shift characteristic of double stranded nucleic acid molecules

(Fig. 6). A summary of the physical and biochemical properties of both component is included in Table 1.

Antiserum was produced to the top component only. This antiserum cross reacted with both purified top and bottom component to a dilution of 1 in 512 (Fig. 7). Mycelial extracts from NC 14.4A reacted with the antiserum as well. Mycelial extracts from healthy and partial cured isolates did not react with the antiserum, showing the specificity of the antibodies and confirming the absence of Lp-V in HT.

Northern blot analysis of dsRNA:

Individual probes were made for each of the bands of dsRNA from isolate NC 14.4A and HT. End labeled probes proved to be inconsistent so the cDNA technique described above was used for these tests. For most of the individual bands, hybridization was evident only with themselves and those bands with identical molecular weight (Fig. 8, A, B, C, D, E, F and G). The 7.9 kb band from HT was homologous with that of NC 14.4A and the reciprocal hybridization was true (Fig. 8 A and B). The 2.3 kb band hybridizes with itself and weakly with the 7.9 kb band. It is possible that the 2.3 kb band is a deletion product of the 7.9 kb band. Neither of the bands associated with HT hybridize with the 5 bands of NC 14.4A that were lost during hyphal tipping. The 3.0, 2.8 and 2.6 kb bands (the triplet) were distinct and shared little homology with each other (Fig. 8 D, E and F). The 0.7 kb band does show homology with the 2.6 kb band

(Fig. 8 G). The 0.7 kb band may be a deletion product of the 2.6 kb band.

DISCUSSION:

The data presented provide evidence for a unique virus-like particle of *L. persoonii* (Lp-V). A number of viruses have been described for a wide variety of fungi, though none for this particular species. Lp-V has some unique features that prevent classification into known fungal virus groups (Buck *et al.*, 1984) or into other described groups of dsRNA virus (Pereira, 1991) (Table 2). The gross morphology, sedimentation coefficient and type of nucleic acid is typical of a fungal virus; however, this VLP is much less dense than typical fungal viruses though other mycoviruses have been recently described with similar low buoyant densities (Dickinson and Pryor, 1989; and Tavantzis, 1988). This VLP also has a unique nucleic acid profile. Most fungal viruses with multiple segments of dsRNA separately encapsidate each segment (Buck *et al.*, 1984). A fungal virus of *Rhizoctonia solani*, Rhs-717, has been found that appears to encapsidate 2 segments of dsRNA in one particle (Tavantzis and Bandy, 1988). Rhs-717 has a low density similar to that of NC 14.4A, and a similar sedimentation coefficient though these two viruses have very different size capsid proteins (Tavantzis and Bandy, 1988). Analysis of proteins associated with Rhs-717 showed molecular weights ranging from 16 KD to 80 KD, with major protein bands having molecular weights of 71 KD and 77 KD. The capsid protein of Lp-V is 32 KD, quite small for a fungal virus. In addition,

genomes of most multipartite fungal viruses consist of only two or three segments that are individually packaged and often represent deletion products, common in fungal viruses, (Shapira *et al.*, 1991; Nuss and Koltin, 1991; and Tartaglia *et al.* 1986), not 5 or 6 segments packaged in one particle as in the case of Lp-V. Recent reports of fungal reo-like viruses in two widely divergent species of fungi, *Cryphonectria parasitica* and *Agaricus bisporus*, suggest that reoviruses might be widespread in the fungal kingdom (Goodin, *et al.*, 1991; and Enebek, *et al.*, Hillman, *et al.*, 1991). Some features of Lp-V are similar to reoviruses in that the triplet bands appear to be mostly unique (Nuss and Dall, 1990; Joklik, 1981; and Silverstein, *et al.*, 1976). However, other features of Lp-V do not fit characteristics of this group of viruses as it does not have the complex coat typical of reoviruses nor does it have the 10 to 12 bands of dsRNA characteristic of reoviruses (Nuss and Dall, 1990; Joklik, 1981; and Silverstein, *et al.*, 1976). This does not rule out the possibility that Lp-V is not related to the reoviruses and this relationship can be investigated with antibodies or nucleic acid sequence data. Ribonucleotide sequence data could help elucidate the true relationship between these bands and may also aid in classifying Lp-V into a known virus groups (Gould and Symons, 1983).

The presence of both encapsidated and naked dsRNA segments is not unusual in fungi as there is evidence of encap-

sidated and unencapsidated dsRNA in several genera of fungi including *Melampsora, Agrocybe, Agaricus, Ustilago* and *Cryphonectria* (Dickinson and Pryor, 1989; Barrosa and Labarere, 1990; Goodin, *et al.*,1992; Enebek, *et al.*, 1991; and Seroussi, *et al.*, 1989). It is possible to partially cure isolate NC 14.4A of dsRNA and this process yields an isolate, HT, that has lost the VLP but retained two bands of dsRNA. The difficulties in obtaining a cured isolate may be related to the presence of the two distinct forms of dsRNA, one of which is easily lost while the other is highly persistent. The presence of encapsidated and unencapsidated dsRNA may be a common occurrence in fungi. The stable maintenance of both forms is not surprising due to the nature of serial transmission of fungal viruses (Lecoq *et al.*, 1979). Reports of numerous deletion mutations in *Cryphonectria* that are stably maintained after their appearance lends some support to this hypothesis (Shapira *et al.*, 1991).

Thus, Lp-V is a unique fungal virus with some unusual properties. The next step in determining its place in a taxonomic group is to examine its relationship with similar fungal virus through antibody tests. It would also be worthwhile to exam the VLPs for the presence of polymerase activity, a common feature of dsRNA viruses (Buck *et al.*, 1984; and Pereira, 1991). Sequence data from the dsRNA of Lp-V could also be of use in its classification and also in

clarifying the apparent complex relationship between the segments. Finally, transmission of Lp-V to a healthy isolate would confirm the viral nature of this virus-like particle. The completion of Kochs postulates would prove the 'pathogenicity' of the VLPs on *Leucostoma*.

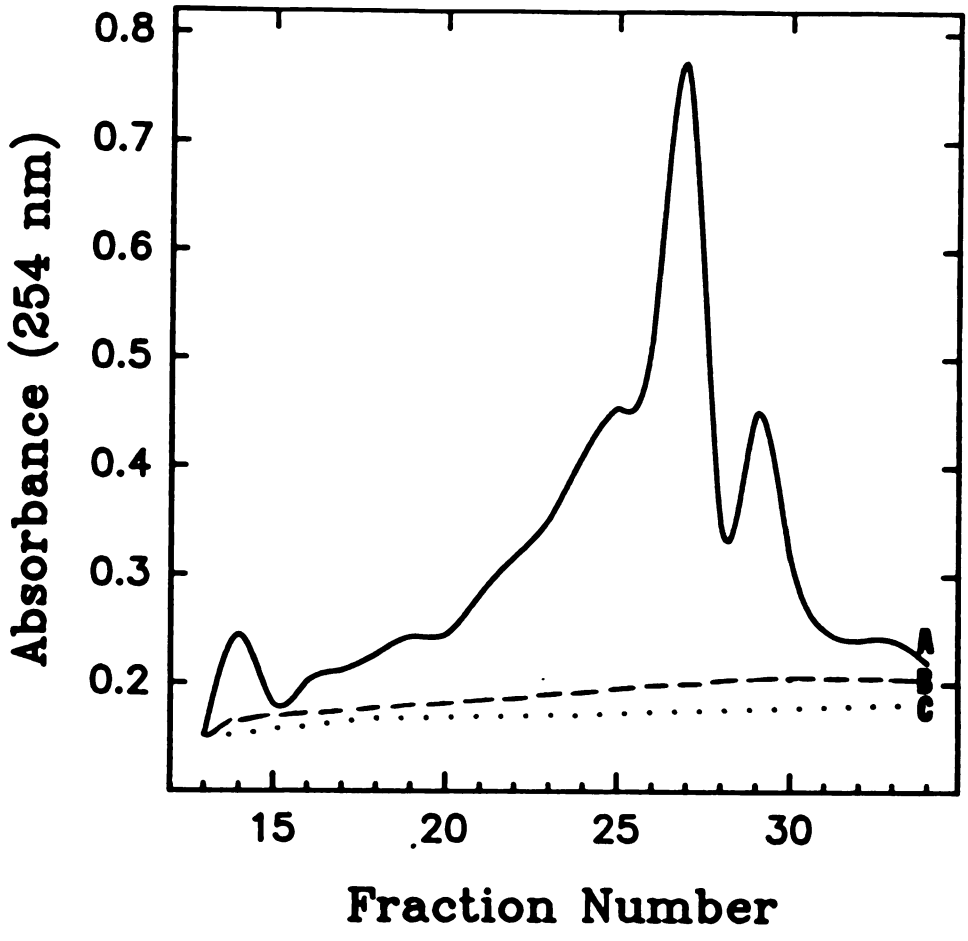
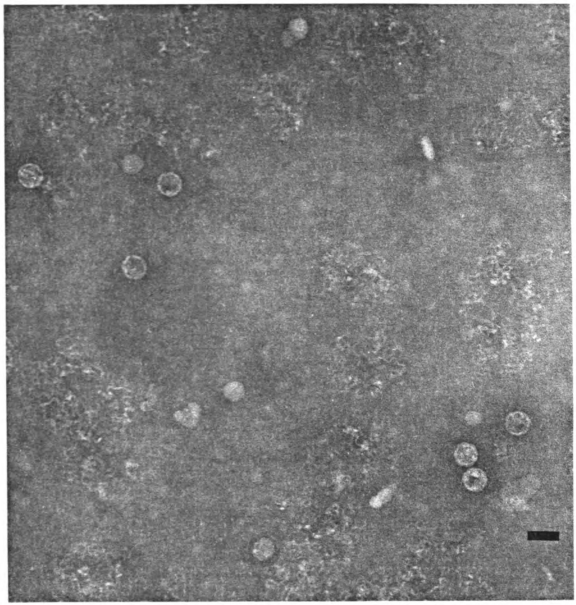


Figure 1. Absorbance profile (254 nm) of a 10 to 30% (w/v) linear-log sucrose density gradient following centrifugation of extracts from fungal hyphae for 1.5 hours at 245,000 G. Trace A: Extract from *L. persoonii* isolate NC 14.4A with two distinct peaks. Trace B: Extract from *L. persoonii* isolate HT, a partially cured isolate derived from NC 14.4A. Trace C: Extract from *L. persoonii* isolate MI 11.13, a dsRNA free isolate.

Figure 2. Electron micrograph of negatively stained isometric particles from the *L. persoonii* isolate NC 14.4A (bar = 50 nm).



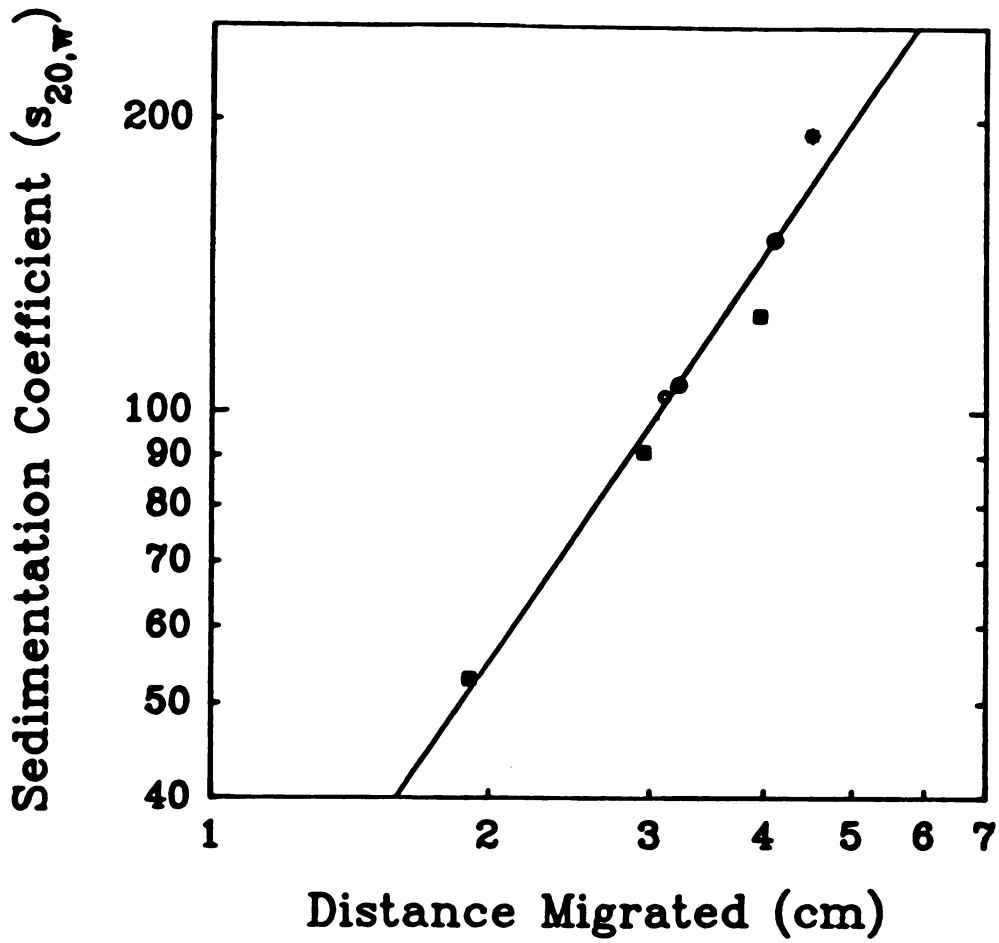


Figure 3. Determination of sedimentation coefficient of *Leucostoma persoonii* virus (Lp-V) by linear-log gradients. Size markers used were (o) = Sowbane Mosaic Virus (SMV) ($S_{w,20}$ = 104s), (▪) = Tobacco Ringspot Virus (TRSV) ($S_{w,20}$ = 53 s-Top component, 91 s-Middle component, and 126 s-Bottom component) and (*) = Tobacco Mosaic Virus (TMV) ($S_{w,20}$ = 196 s). Values for Lp-V are represented by (●). As determined by this plot, these values are 107 s for the top component and 151 s for the bottom component.

Figure 4. Coomassie blue stained proteins extracted from Leucostoma persoonii virus (Lp-V) particles after purification through differential and sucrose gradient centrifugation as observed in SDS-PAGE. Lane 1 and 6: size markers: Bovine albumin (MW = 66,000), Egg albumin (MW = 45,000), Glyceraldehyde-3-phosphate dehydrogenase (MW = 36,000), Carbonic anhydrase (MW = 29,000), Trypsinogen (MW = 24,000), Soybean trypsin inhibitor (MW = 20,100), and α-Lactalbumin (MW = 14,200) (Sigma Chemical Company, St. Louis, MO).; Lane 2: Protein extracted from top component of Lp-V; Lane 3: Protein extracted from bottom component of Lp-V; Lane 4: Protein extracted from isolate HT of gradient fractions corresponding to those which contain Lp-V particles in NC 14.4A extracts; Lane 5: Protein extracted from isolate 11.13 of gradient fractions corresponding to those which contain Lp-V particles in NC 14.4A.

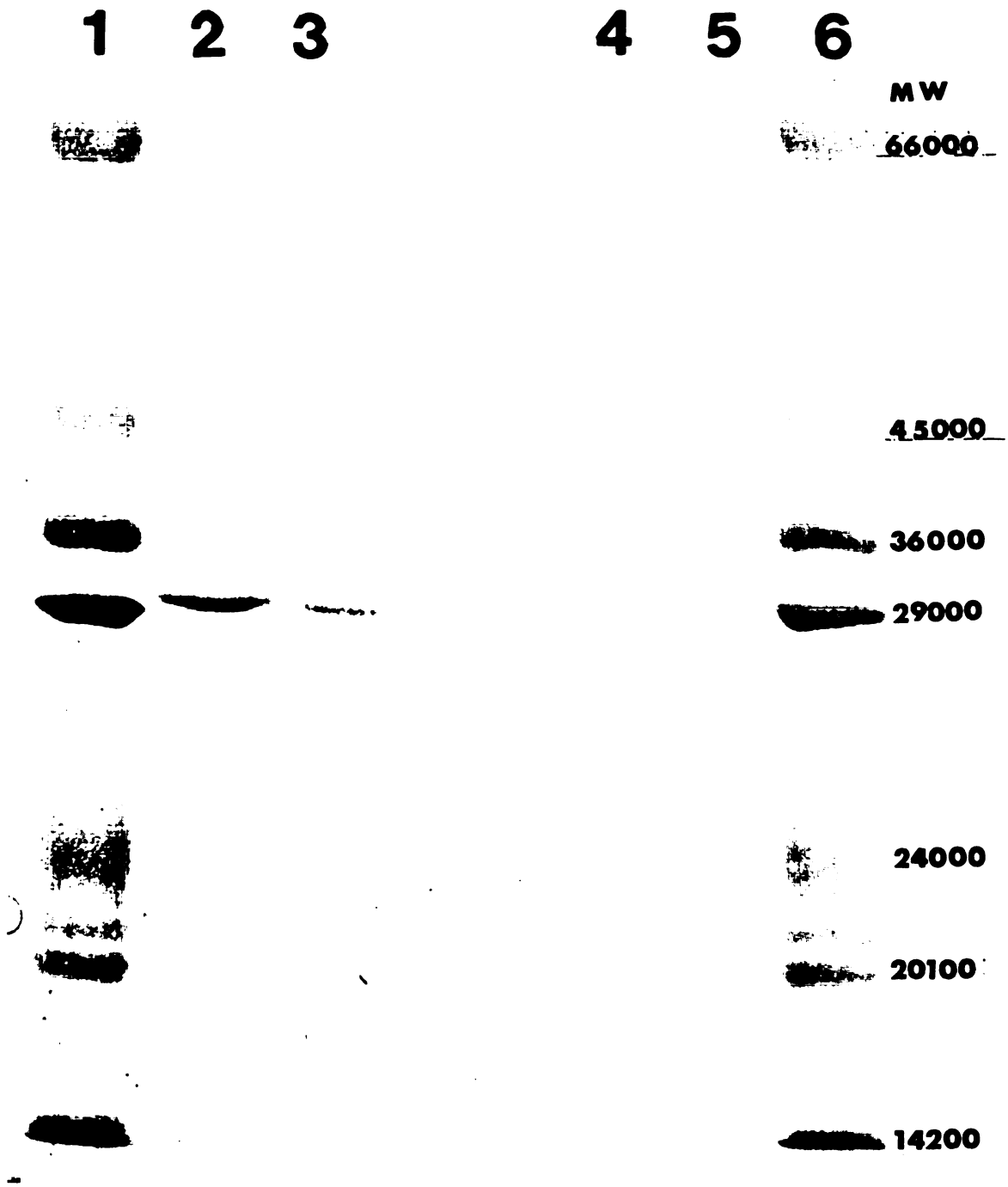
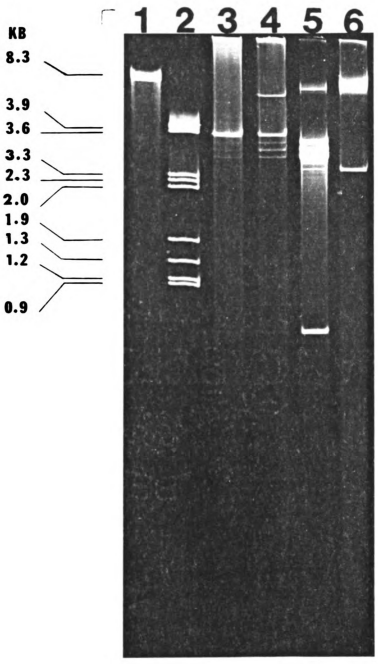
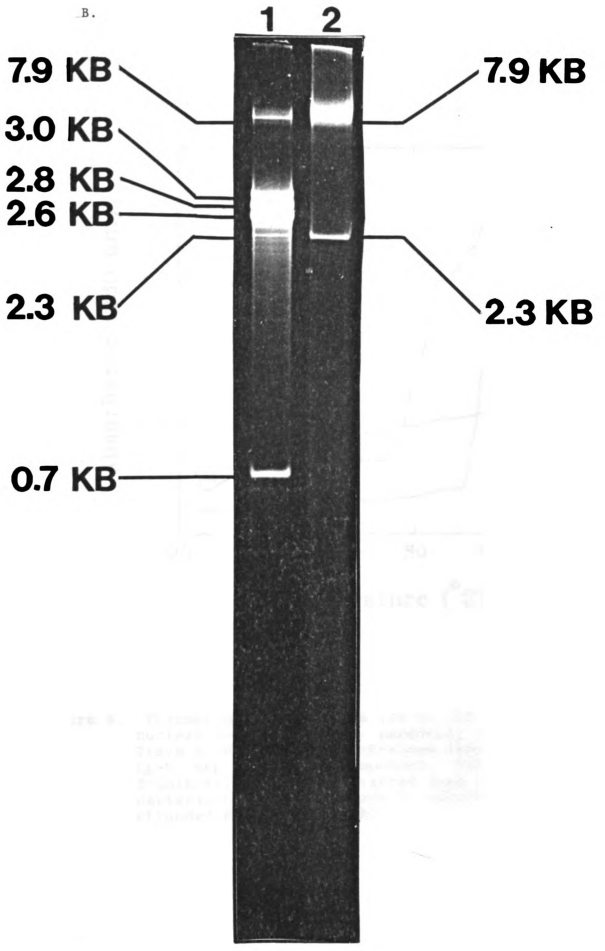

1 2 3
4 5 6
MW
66000
45000
36000
29000
24000
20100
14200

Figure 5. Ethidium bromide stained bands of dsRNA present in Lp-V and tissue extracted dsRNA as observed in PAGE. A.) Lane 1: dsRNA size standard from Hm-9, a virus of *Helminthosporium* maydis; Lane 2: dsRNA size standard from reovirus, serotype 3; Lane 3: dsRNA extracted from Lp-V particles, top component; Lane 4: dsRNA extracted from Lp-V particles, bottom component; Lane 5: dsRNA extracted from tissue of NC 14.4A; Lane 6: dsRNA extracted from tissue of HT. B.) Detail of dsRNA extracted from tissue of NC 14.4A (Lane 1) and HT (Lane 2) with sizes shown in kilobases (kb).

A.




B.
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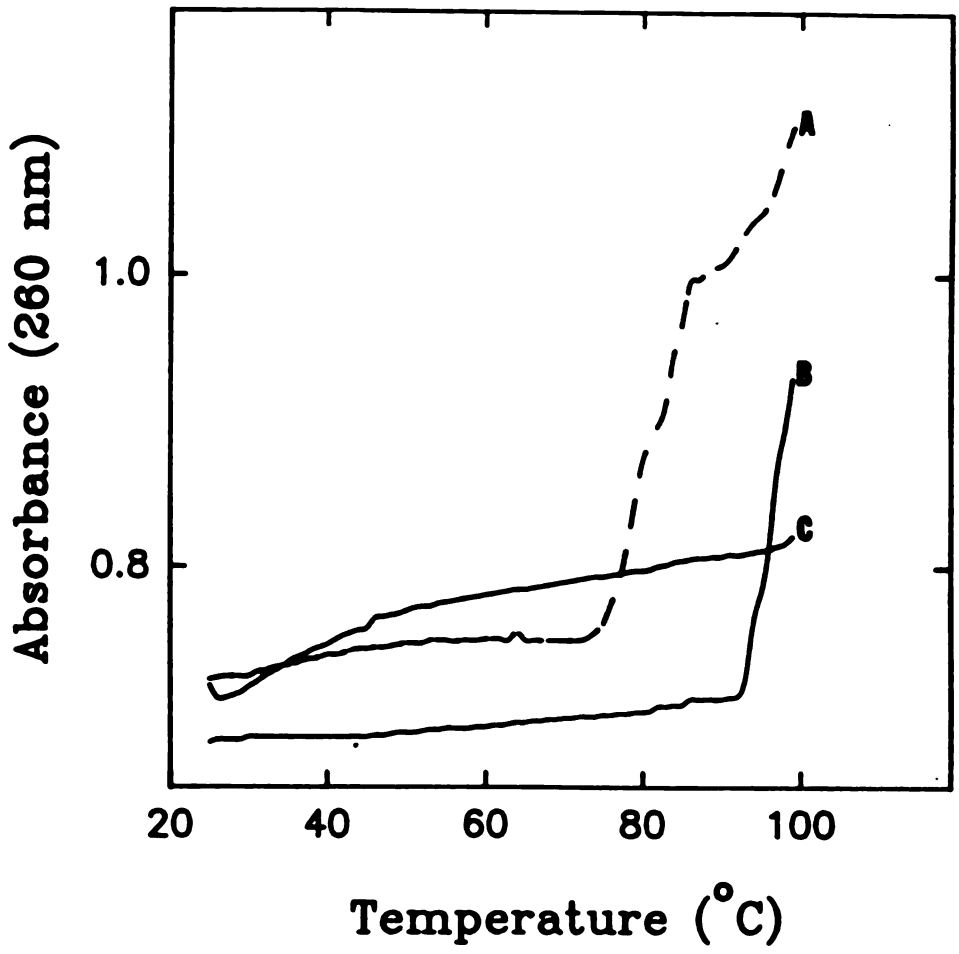

Figure 6. Thermal melting profile (20 to 100 °C) of the nucleic acid from the *L. persoonii* mycovirus Lp-V. Trace A: Nucleic acid extracted from particles of Lp-V, top and bottom component. Trace B: Control double-stranded RNA extracted from the bacteriophage, ϕ 6. Trace C: Control single-stranded RNA (yeast mRNA).

Figure 7. Ouchterlony double-diffusion test of antibodies produced by injection of purified particles of Lp-V top component. Center well: antiserum against Lp-V top component. Well A: purified particles of top component of Lp-V. Well B: purified particles of bottom component of Lp-V. Well C: extract from isolate HT of gradient fractions corresponding to those which contain Lp-V particles in NC 14.4A. Well D: extract from isolate MI 11.13 of gradient fractions corresponding to those which contain Lp-V particles in NC 14.4A.

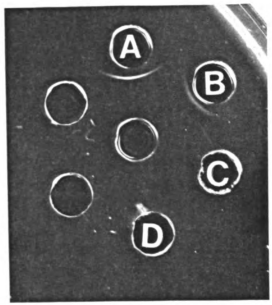
A
B
C
D

Figure 8. Northern blot analysis of dsRNA from *L. persoonii* isolates NC 14.4A and HT. Lanes 1 and 2 are total dsRNA from NC 14.4A and HT respectively in each section. A) total dsRNA from NC 14.4A and HT probed with 7.9 kb band of HT. B) total dsRNA from NC 14.4A and HT probed with 7.9 kb band of NC 14.4A. C) total dsRNA from NC 14.4A and HT probed with 2.3 kb band of HT. D) total dsRNA from NC 14.4A and HT probed with 3.0 kb band of NC 14.4A. E) total dsRNA from NC 14.4A and HT probed with 2.8 kb band of NC 14.4A. F) total dsRNA from NC 14.4A and HT probed with 2.6 kb band of NC 14.4A. G) total dsRNA from NC 14.4A and HT probed with 0.7 kb band of NC 14.4A. Exposure time for A, B and C was 72 hours with intensifying screen. Exposure time for D and G was 24 hours without intensifying screen. Exposure time for E and F was 12 hours with intensifying screen.

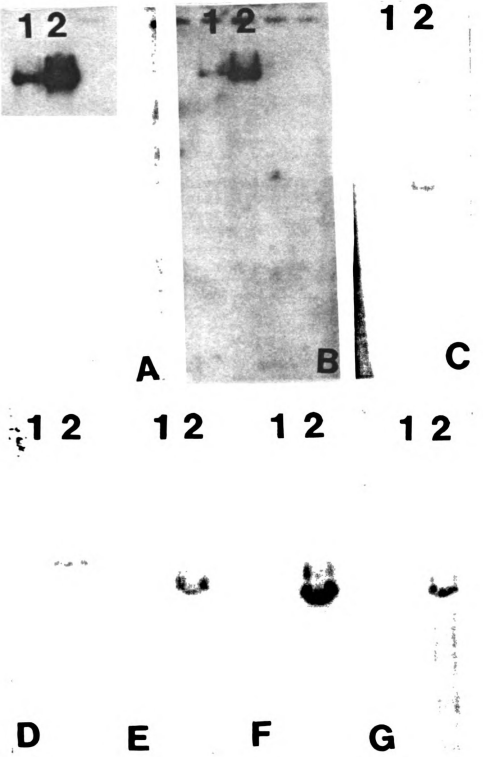
1 2
1 2
1 2
A
B
C
1 2
1 2
1 2
1 2
D
E
F
G

Table 1. Summary of biophysical properties of sucrose gradient-purified top and bottom components of the virus-like particle of *L. persoonii* isolate NC 14.4A, Lp-V .

	Top Peak	Bottom Peak
ρ	1.313 g/cm^3	1.339 g/cm^3
$S_{20,w}$	107 s	151 s
E.C.	1	1
Protein	32000	32000
Nucleic Acid	dsRNA	dsRNA
	5 bands	6 bands

Table 2. Summary of previous proposed dsRNA virus groups (1984 and Pereira, 1991).

Group	Host	Diameter, Density	$S_{20,w}$	ds RNA segments	No. of proteins
Reoviridae	Vertebrates, Invertabrates, Plants	50-80 nm, 1.36-1.44	370-730	10-12	6-10
Birnaviridae	Vertabrates, Invertabrates	60 nm, 1.32-1.35	435-460	2	5
Cryptoviridae	Plants	40-43 nm, 1.39-1.37	115-123	2	N.D.
Cystoviridae	Bacteria	75 nm, 1.27	406	3	12
Totioviridae	Fungi (protozoans)	40-43 nm, 1.40-1.42	161-172	1	2
Partiviridae	Fungi	30-35 nm, 1.35-1.36	101-145	2	
Unclassified					
mycoviruses	Fungi				
picobirnaviridae	Vertabrates, Bacteria	~40 nm		2 or 3	
protozoan	Protozoans	~40-43 nm			
naked ds RNA	Fungi, Plants	N.A.		1+ (sub genomic)	

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APPENDIX I: TRANSMISSION OF A VIRUS LIKE PARTICLE OF *LEUCOSTOMA PERSOONII* ASSOCIATED WITH HYPOVIRULENCE TO A NORMALLY VIRULENT ISOLATE.

INTRODUCTION:

In the process of screening isolates of *Leucostoma persoonii*, the causal agent of Cytospora canker of peach, for reduced virulence, an isolate containing dsRNA was found to exhibit greatly reduced virulence. A virus like particle (VLP) from this isolate of *L. persoonii* has been partially characterized and appears to be a unique VLP. Because of the highly debilitating nature of this VLP and the complexity of vegetative compatibility groups in this species, it would be highly desirable to achieve cell free transmission. Transmission of the dsRNA and its correlation with hypovirulence would complete Kochs postulates and add strong evidence for the causal nature of dsRNA as it relates to hypovirulence. It would also provide a system in which to study the specific role of individual bands of dsRNA and how they relate to the disease complex and virus infection.

The purpose of this study was twofold. First, field studies were initiated to determine if transmission in the field through hyphal anastomosis was possible. In addition, several system were tested for transmission to protoplasts either via protoplast fusion or through cell free transfer of dsRNA or VLPs to normal dsRNA free isolates.

Materials and Methods:

Transmission in the Field:

To determine if the dsRNA could be successfully transmitted in the field through hyphal anastomosis, a field test was established. Branches were initially inoculated with either NC 14.4A or HT. Branches of uniform size (2.5 cm) were inoculate in the fall using the freeze/wound technique outlined by Hammar *et al.* (1988) as adapted from Scorza and Pusey (1984) where branches were sterilized with alcohol, wounded with a staple gun, frozen and inoculated with a 5 mm plug of mycelium. The branches were then challenge inoculate with normally virulent isolates that were marked by benalate resistance. The branches were harvested in the spring, the resulting cankers measured and the fungi from the cankers isolated. The reisolated fungi were plated on double selective media to check for transmission. This media contained benalate for selection of the marked isolate and perchlorate, to select for the dsRNA containing isolates, taking advantage of the fact that the dsRNA containing isolate grows readily on media amended with 1.5% perchlorate, whereas the challenging fungi does not.

Protoplast formation:

Mycelium was grown in *Endothia* complete broth for 48 hours at room temperature. Mycelium was harvested by vacuum

filtration through sterile miracloth and used fresh for protoplast formation. Mycelial mats were placed into protoplast formation media (PFM) which contained 20 ml of 1 M sorbitol, buffered to pH 5.8 with a final concentration of 0.01 M sodium phosphate buffer, with 1 mg/ml Novazyme 234 and 0.1 mg/ml Chitanase. This mixture was incubated at 33°C for three to four hours. The protoplasts were harvested by filtering the mixture through four layers of cheese cloth, then through 45 μm mesh screen. The protoplasts were separated form the PFM by centrifugation (10 minutes at 1000 g) and washed twice in 1 M sorbitol. The protoplasts were further purified from cellular debris using the two phase system of Hasiba and Yamada (1982). Protoplasts resuspended in 1 M sorbitol were layer onto 1 M sucrose and centrifuged for 10 minutes at 500 g. Protoplasts were concentrated in the white interface and remove with a pasture pipette. Protoplasts were concentrated and counted for further use.

Transmission through protoplast fusion:

For protoplast fusion experiments, two approaches were used. First, protoplasts of a benalate resistant isolate were mixed 1:1 with virus containing protoplast in the presence of PEG (12.5% PEG 4000 and 0.05 M CaCl), pelleted together and incubated at 4 or 33°C for 3 hours. The protoplasts were then plated on benalate-perchlorate media, the same media used to detect fusion products from natural infection. To increase the pressure for fusion, rhodamine-

6-G (R6G) at a rate of 1, 10, 20 or 100 mg/ml of media was added to the liquid cultures of the benalate resistant strain. This compound is selectively toxic to mitochondria (Gear, 1974). This should have enhanced the chances of fusion in that the benalate resistant isolate had greatly reduced viability. In these experiments, the protoplasts were plated on benalate media, transferred and visually screened for altered colony morphology.

Transmission through Cell Free Systems:

Three approaches were used to achieve cell free transmission, electroporation, PEG-mediated transmission with heparin and mechanical transmission with silica fibers as vehicles of infection. In all systems, both intact virions and isolated dsRNA were used for transmission.

For electroporation, a kill curve was established to determine the point of 50% kill, often the optimum for transfection. Healthy protoplasts (200 μl of 10^6 cells in 1 M sorbitol) were mixed with virions (16 μl, ~40 μg) or dsRNA (10 μl, 5 μg) and electroporated in a Biorad electroporater set at 1000, 1250 or 1500 volts with resistance set at 100 ohms and capacitance set at 3, resulting in a time constant of 0.3. Treated protoplasts were dilution plated on *Endothia* complete agar amended with 1 M sorbitol.

Heparin enhanced PEG mediated transmission was attempted using the procedure outlined in for *Ustilago* (Cockburn and Silva, 1990). Purified dsRNA (5 μl, 4 μg) or

virions (5 μl, ~13 μg) were mixed with 1.5 μl of 10 mg/ml heparin and incubated for 20 minutes at 4°C. Protoplasts (4 x 10^6 cells) were suspended in 50 μl of STC. The cells were mixed with the heparin mixture and the solution incubated on ice for 10 minutes. At this time, the mixture was brought to 10% PEG by adding 5 μl of 40% PEG in STC. The mixture was incubated for 15 minutes. Treated protoplasts were dilution plated on *Endothia* complete agar amended with 1 M sorbitol.

A third method was used in an attempt to achieve transmission. Needle-like silica fibers (20 μl,) were vortexed for 3 minutes with dsRNA (20 μl, ~10 μg) or virions (20 μl, ~20 μg). The silica fiber mixture was added to 400 μl of protoplasts (3.5 x 10^6) and this solution was vortexed for 2 minutes. Treated protoplasts were dilution plated on *Endothia* complete agar amended with 1 M sorbitol.

Results:

Transmission in the field:

Two things were evident from this study. First, often NC 14.4A inoculations were unsuccessful. It appears that the isolate is too debilitated to consistently establish infection. Canker size in those infected with NC 14.4A or HT was greatly reduced and in fact, often it was zero. From sample with no visible infection, no fungi were reisolated. Second, transmission in the field was unsuccessful. None of the isolates tested were able to grow normally on the selective media.

Transmission through protoplast fusion:

Stable heterokaryons were never recovered in these experiments. Though protoplasts would regenerate on nonselective media, no regeneration on double selective media.

Treatment with pregrowth on R6G reduced viability of resulting protoplasts (83.6% of control at 1 ppm, 48.5% of control at 10 ppm and no regeneration at 100 ppm). Of 80 colonies derived from single protoplast isolations, none showed visible signs of infection.

Transmission through electroporation:

As with other fungi, the amount of voltage required to kill the protoplasts was quite high. A possible explanation for this could be that the protoplasts are in fact spheroplasts with most if not all of the cell wall intact.

Of the 200 single protoplast colonies from electroporation experiments, only one appeared morphologically altered and this isolate did not contain dsRNA.

Transmission through heparin assisted PEG mediated transmission:

For the experiments without silica fibers, of the 350 single protoplast colonies, none appeared visually different. When silica fibers were used to facilitate delivery, of the 437 single protoplast colonies isolated, three appeared morphologically different. None of these colonies contained dsRNA.

Discussion:

Though there has been extensive success in transmission of plant and animal virus in cell free systems, even the simplest most direct means to transmit fungal viruses have been unsuccessful. To date, only the VLPs of *Saccharomyces cervesia* have been successfully transmitted in a cell free system (Schmitt, *et al.*, 1990). Recent reports of transmission of cDNA of the dsRNA associated with hypovirulence in *Cryphonectria parasitica* have been successful as well (Nuss *et al.* in press). The strategy used in these two cases has been to use a cotransformation system. Transformation of fungal protoplasts in general can be difficult. Conditions for transformation of fungal protoplasts are often very rigorous. For example, electroporation conditions required for appreciable transformation rates rival those required for transformation of intact bacterial cells. Transformation efficiency are often very low especially when compared to plant systems. The use of a marker plasmid would greatly facilitate screening. Such a marker could select for competent cells (cells capable pf being transformed) thus reducing the number of protoplast needed for screening. In addition, plasmid transformation system can be used to optimize conditions for transformation and help bypass the process of visual screening for transformation.

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APPENDIX II: CONSTITUATIVE LIGNIN LEVELS IN INNER BARK OF LINES OF PEACH TOLERANT AND SUSCEPTIBLE TO *LEUCOSTOMA*

INTRODUCTION:

Cytospora canker on peach caused by *Leucostoma persoonii* and *L. cincta* is a devastating disease on peach. The pathogens establish infection by invasion of wounds or dead tissue (Willison, 1933). Infection can result in tip dieback and xylem necrosis. In older orchards, large perennial cankers often cause splitting of the scaffold branches thus ending the productive life of the tree. Chemical control of this disease has proven to be ineffective. Identification of potential tolerant varieties would be of great practical use, especially in the northern regions of the USA where this disease is often the limiting factor in peach production. In a program designed to screen peach lines for tolerance to this diseases, a line of peach was identified as having a high degree of tolerance. When trees of this line (Yennoh) were inoculated with the pathogens, the cankers that result were of much reduced size then those on susceptible trees. It was found that on the tolerant trees, the fungi were unable to invade the xylem. Progeny of a cross between this line and the highly susceptible variety of peach was shown to have similar levels of tolerance to the parental line. Preliminary evidence suggested that this resistance could be due to increased levels of lignin in branches not only in response to wounding but in normal nonwounded tissue. This study was initiated to determine if the two tolerant lines did indeed

have increased constitutive lignin levels when compared to the highly susceptible variety. In a concurrent study done by Y. Zeng, the activity of enzymes believed to be involved in lignin biosynthesis was also compared. Lignin levels where determined over the course of the growing season to see if the different levels were seasonally related.

MATERIALS AND METHODS:

Plant Material:

Vegetative clones of Yennoh and its progeny, 1-39, were generated by grafting branch scion of the original tree stock onto appropriate root stock. Highly SUSCEPTIBLE Loring trees were used for comparison. The trees used in this study were three year old field grown trees. The same branches were used for both lignin and enzyme determinations. Two year old branches with a diameter of 2.5 cm were excised from healthy trees and brought back on ice for processing.

Lignin Determination:

The lignin extraction and quantification was a modification of the method described by Doster and Bostock (1988). The dissection of samples was carried out at 4 ^{o}C to reduce oxidation. Strips of bark encompassing the entire girth of the branch were removed using a razor blade. The outer bark was removed and discarded. Inner bark composed of phloem and cortex was separated from the inner layer of first year xylem and both samples were quick frozen in liquid nitrogen then plunged into methanol with 5% citric acid to begin dehydration. This procedure greatly reduced oxidation of phenolic compounds in the samples that can lead to artificially high results. After 12 hours of extraction in methanol ascorbic acid, the solution was changed to pure methanol. The samples were extracted two more tines in pure

methanol to completely dehydrate the samples. The samples were further dried at room temperature overnight to evaporate the remaining methanol. The samples were weighed. To each sample, 5 mls of 2 N HCl and 0.5 ml thioglycolate (TGA) was added and the material was allowed to react at 97 ^{o}C for 4 hours. The liquid was removed and the samples were washed twice with distilled water. The samples were then extracted in 5 ml of 0.5 N NaOH for 18 hours. The extracting solution was removed and saved in 15 ml clinical centrifuge tubes. The samples were washed in 5 ml distilled water and this was saved as well. To precipitate the lignin-thioglycolate, 1 ml concentrated HCl was added to the tubes and the mixture was incubated at 4 ^{o}C for 4 hours. The tubes were centrifuged at 1000 g for ten minutes. The pellet lignin thioglycolate was resuspended in 5 ml of 0.5 N NaOH. The sample was centrifuged again to remove solid debris. The sample was diluted and the absorbance at 280 nm was measured in a Perkin Elmer spectrophotometer. The A_{280} was converted to a per gram or per cm^2 basis for comparison of lignin content between samples.

Experimental Design:

Due to limited tree material, the experiment was set up as a nested design. For each sample date, three branches from three trees was selected and four samples were taken from each branch. Relative lignin content for each sample was determined on a per unit weight basis for phloem,

cortex, and phloem and cortex and on a per unit surface area basis. The data was analyzed as a nested design using SAS.

RESULTS AND DISCUSSION:

Lignin levels:

When lignin levels were compared on a per weight basis, there was no significant difference between the three varieties for all sample dates except for one (Table 1). In general, lignin levels increased though the growing season. In some cases, it is of value to compare lignin levels on a per area basis. As with the per weight data, there was no significant difference between the three varieties, though in general, the parental line Yennoh had the highest level of lignin for all sample dates (Table 2). The enzyme levels paralleled the lignin levels, showing no significant difference between the three cultivars for all three enzymes tested. Thus, it appears as though the higher level of resistance in the two lines cannot be directly linked with constitutive levels of lignin.

Lignin deposition in response to wounding has been linked with disease resistance in other systems (Hammerschmidt *et al.*; Biggs, 1986;and Bostock and Middleton 1987; for review see Bostock and Stermer 1989). Initial studies on our cultivars suggested that the resistant clones contained constitutively high levels of lignin prior to wounding (Chang, 1989). In this study, prewounded lignin levels were tested at only one date. When lignin levels were tested over a range of dates, there appears to be no trend of increased constituative lignin levels in resistant

varieties throughout the course of the year, regardless of how these levels were compared.

Table 1: Relative lignification detected in peach cortex and phloem for clones sampled in 8 months[a]

Month	tissue sampled	Clone		
		Yennoh	Loring	I-39
April	cortex and phloem	53.0 ns	41.2 ns	54.2 ns
	phloem	41.7 ns	28.7 ns	41.6 ns
	cortex	67.6 ns	52.2 ns	65.1 ns
May	cortex and phloem	49.2 a[b]	38.0 b	34.9 b
	phloem	46.2 a	35.3 b	37.1 b
	cortex	52.3 a	39.9 b	31.8 b
June	cortex and phloem	47.7 ns	45.3 ns	38.6 ns
	phloem	50.3 ns	48.9 ns	42.7 ns
	cortex	40.9 ns	38.1 ns	32.5 ns
July	cortex and phloem	37.6 ab	28.1 b	41.9 a
	phloem	43.4 ab	30.9 b	51.3 a
	cortex	30.0 ns	24.5 ns	26.6 ns
August	cortex and phloem	40.5 a	37.8 ab	29.9 b
	phloem	45.5 a	42.7 ab	66.1 b
	cortex	31.4 a	30.3 ab	24.5 b
September	cortex and phloem	47.5 ns	43.6 ns	43.8 ns
	phloem	55.7 ns	52.2 ns	58.4 ns
	cortex	31.1 ns	30.3 ns	24.5 ns
October	cortex and phloem	38.0 ns	34.9 ns	25.0 ns
	phloem	43.0 a	32.1 b	26.5 b
	cortex	23.5 ab	34.0 a	21.9 b
November	cortex and phloem	42.7 ns	37.7 ns	32.4 ns
	phloem	47.3 ns	40.3 ns	38.1 ns
	cortex	33.5 ns	33.7 ns	27.7 ns

[a] LTGA yield expressed as A_{280}nm per gram tissues in 5 ml of 0.5 N NaOH. Each value is the mean of 4 samples per branch for 2 branches per tree in April and 3 Branches per tree in May through November. Only one tree was sampled per month.

[b] Different letters in rows are significantly different according to LSD (0.05).

Table 2: Relative lignification detected in peach cortex and phloem for clones sampled in 8 months[a]

	Clone		
Month	Yennoh	Loring	I-39
May	11.0 a[b]	7.5 b	8.2 ab
June	11.1 ns	11.0 ns	7.8 ns
July	9.3 ns	5.7 ns	9.0 ns
August	9.4 ab	10.0 a	6.0 b
September	13.1 ns	10.0 ns	9.9 ns
October	9.8 ns	7.7 ns	5.6 ns
November	10.1 ns	7.5 ns	6.3 ns

[a] LTGA yield expressed as A_{280}nm per square millimeter in 5 ml of 0.5 N NaOH. Each value is the mean of 4 samples per branch for 2 branches per tree in April and 3 Branches per tree in May through November. Only one tree was sampled per month.

[b] Different letters in rows are significantly different according to LSD (0.05).

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