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"DOWNY MILDEW: HOST SPECIALIZATION
AND EFFECTS ON
PHOTOSYNTHESIS AND CARBON
PARTITIONING IN 'NIAGARA' GRAPEVINES"

presented by

BRIAN LEE LEHMAN

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DOWNY MILDEW: HOST SPECIALIZATION AND EFFECTS ON PHOTOSYNTHESIS AND CARBON PARTITIONING IN 'NIAGARA' GRAPEVINES

By

Brian Lee Lehman

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ABSTRACT

DOWNY MILDEW: HOST SPECIALIZATION AND EFFECTS ON PHOTOSYNTHESIS AND CARBON PARTITIONING IN 'NIAGARA' GRAPEVINES

By

Brian Lee Lehman

Michigan is the fourth largest producer of juice grapes (*Vitis labrusca*) in the United States, averaging more than 400,000 tons annually. Downy mildew (*Plasmopara viticola*) is an important pathogen of juice grapes in Michigan and throughout the grape-growing regions of the United States. Downy mildew can damage leaves and fruit clusters, resulting in losses in fruit yield and quality. The objectives of this research were to: 1) study the effect of downy mildew on photosynthesis of 'Niagara' leaves, 2) study the effect of infection on dry weight and carbon partitioning in 'Niagara' vines and, 3) evaluate the ability of *P. viticola* to cross-infect different grape cultivars and species. In field studies, 'Niagara' leaves infected with downy mildew showed a reduction in total carbon assimilation with increasing disease severity. On potted 'Niagara' vines, inoculated leaves showed a significant reduction in photochemical efficiency, carboxylation efficiency, maximum rate of photosynthesis, and the stomatal limitation to photosynthesis before symptoms appeared. In field studies, downy mildew reduced total net dry weight in vines and affected the rate of carbon translocation, but had no effect on carbon partitioning. Cross infection studies carried out with isolates of *P. viticola* from different host species and isolates in Michigan showed that 'Niagara' leaves were resistant to infection by isolates of *P. viticola* obtained from several other host species.

DEDICATION

This thesis is dedicated to my wife Marcie for her continuous love and support.

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Chapter 1

Literature review

The grapevine

History

Written records of the European grape (*Vitis vinifera* L.) date back five or six thousand years making it one of the longest known and earliest reported cultivated fruits, but cultivation in North America did not happen extensively until the late 1700's (Anderson 1956, Hedrick 1945, Mullins *et al.* 1992). When several large companies failed at growing European varieties due to susceptibility to diseases in the New World, the colonists turned to the native grapevine species.

Most of the grapes in eastern North America were bred from two native species, *Vitis labrusca* L. and *V. rotundifolia* Michx. (Anderson 1956). *Vitis labrusca* was initially the grape the American viticulturist employed for wine and table grape production. Being native to North America, it is widely adapted to the climate and soils, and has furnished more varieties of cultivated grapes than all other American species combined (Hedrick 1945). *Vitis labrusca* is somewhat cold hardy and moderately resistant to downy and powdery mildews, but it is susceptible to black rot (Pearson and Goheen 1988). The first cultivated American grapevine variety was 'Alexander', a descendant of *V. labrusca* named after John Alexander, gardener to the governor of Pennsylvania (Bush *et al.* 1895).

Vitis labrusca 'Concord' originated from the seed of a wild grape planted in 1843 by Ephraim Wales Bull in Concord, Massachusetts. It was first displayed in 1853 at the

25th annual exhibition of the Massachusetts Horticultural Society (Bush *et al.* 1895). By 1860, it was the most popular grape used for juice production in America and still is today. Horace Greeley termed it "the grape for the millions" when awarding it the Greeley prize for the best American grape (Bush *et al.* 1895).

In 1868, the variety Niagara was produced from the seed of a 'Concord' grape fertilized by the variety Cassady by C.L. Hoag and B.W. Clark of Lockport, NY. 'Cassady' was a *V. labrusca* cross that originated in the yard of H. P. Cassady in Philadelphia. 'Niagara' was described as a "true Labrusca in habit and foliage but immensely productive" (Bush *et al.* 1895). Proprietors guarded the grape for many years, not allowing its distribution by others and only furnishing vines for vineyard planting on 'special terms' (Bush *et al.* 1895). This enabled the proprietors to market 'Niagara' grapes on a large scale and at premium prices. Today, 'Niagara' grapes are the leading American grape used for white grape juice production in the U.S. 'Niagara' and 'Concord' are similar in terms of vigor and productiveness, but 'Niagara' is slightly less cold hardy than 'Concord'(Bush *et al.* 1895).

Grape production

The United States is the third largest grape producer in the world, behind Italy and France, producing over six million tons annually (Amanor-Boadu 2003). California is the largest grape-producing state in the U.S., followed by Washington, New York, Michigan, and Pennsylvania. Of the grapes grown in the United States, approximately 87% are processed, while the remaining 13% are table grapes. Approximately 60% of all processed grapes is made into wine, 30% into raisins, 9% into juice, and less than 1% is canned (Amanor-Boadu 2003).

Michigan is the fourth largest producer of grapes in the U.S., currently producing over 90,000 tons annually (NASS 2004). Of the grapes grown in Michigan approximately 90% (by weight) are juice grapes ('Concord' and 'Niagara'), with the largest portion being 'Concord'. Figure 1.1 shows the total number of acres used for grape production since 1980 in Michigan. Michigan was the fourth largest producer of 'Concord' grapes by weight and the largest producer of 'Niagara' grapes in 2003. Figure 1.2 shows trends in the production of 'Concord' and 'Niagara' grapes in Michigan since 1992.

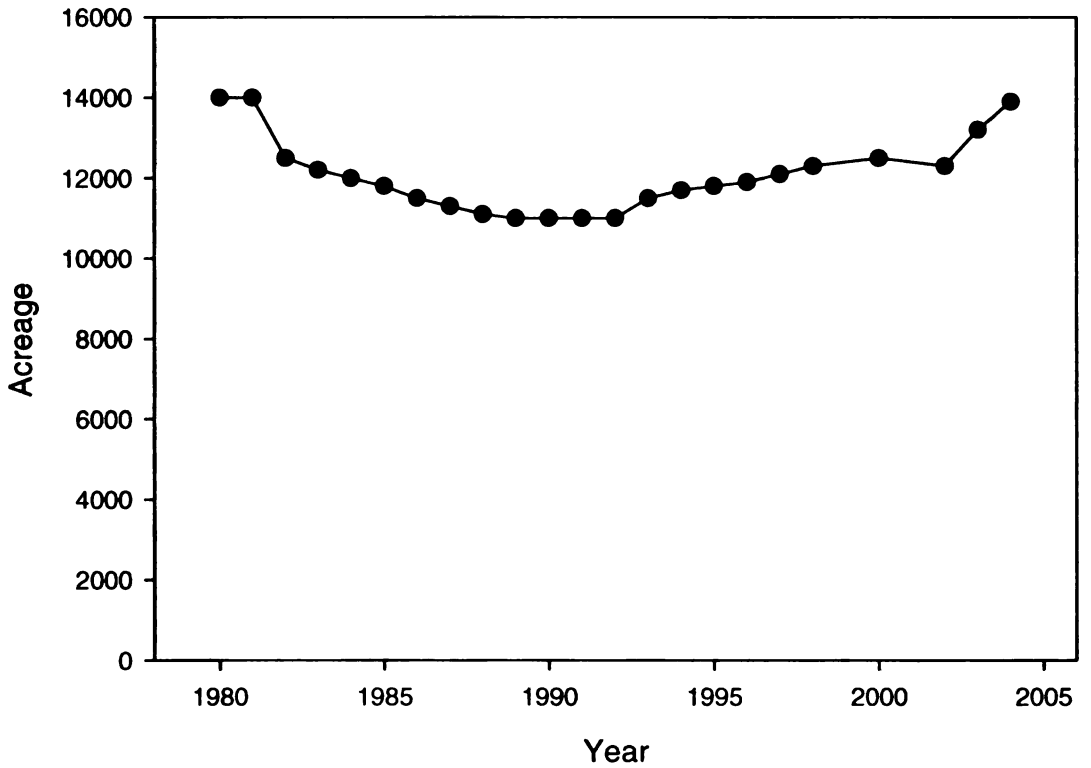

Figure 1.1. Total grapevine acreage in Michigan from 1980 to 2004 (National Agricultural Statistics Service 1980-2004).

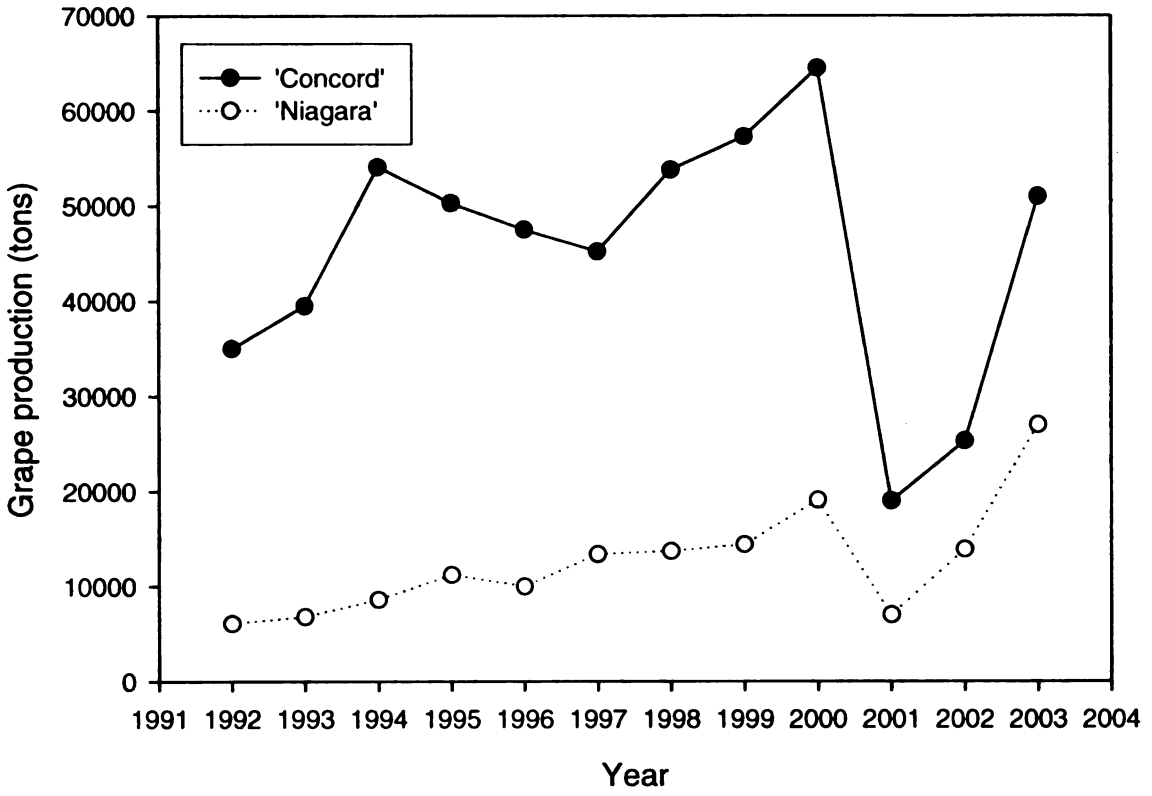

Figure 1.2. Total yield of 'Niagara' and 'Concord' juice grapes produced in Michigan from 1992 to 2003 (NASS, 1992-2003). Low yield was likely due to poor pollination and adverse weather conditions during flowering in 2001 and an early spring frost in 2002.

Grapevine physiology

The grapevine belongs to the family Vitaceae, a group of angiosperms containing 14 genera of both woody and herbaceous perennials characterized by the occurrence of tendrils arising opposite from a leaf (Mullins *et al.* 1992, Pearson and Goheen 1988). The grapevine is a woody perennial, and the woody parts of the vine contain the stores of carbohydrates and minerals needed for growth in the early parts of the next growing season. Grapevine shoots usually produce from one to three flower clusters. The number is dependent upon the variety and conditions of the previous season under which the dormant bud developed (Hellman 2003).

Carbon assimilation

Carbohydrates are produced in plants through the process of photosynthesis which utilizes solar energy for the reduction of carbon dioxide from the atmosphere, as follows:

$$CO_2 + 2H_2O \xrightarrow{\text{light}} (CH_2O) + O_2 + H_2O$$

Solar energy is converted to chemical energy by the light-harvesting antenna complexes (groups of pigment molecules that absorb solar energy) and photosystems I and II located in the chloroplasts. Electrons provided by the splitting of water molecules in Photosystem II provide energy in the form of ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) needed for the assimilation of CO_2 (Figure 1.3). The chloroplasts also contain high concentrations of ribulose 1, 5-bisphosphate carboxylase-oxygenase (Rubisco), the enzyme that catalyzes the reaction of ribulose 1, 5-bisphosphate and CO_2 to produce 3-phosphoglycerate, which is subsequently reduced to carbohydrates. These reactions are termed the photosynthetic carbon reduction (PCR) cycle or Calvin cycle and utilize the ATP and NADPH captured in the light reactions. The enzyme Rubisco has a dual function as a carboxylase as well as an oxygenase enzyme. Carbon dioxide and oxygen compete for binding sites at Rubisco, so the concentration of these molecules has a direct effect on carbon fixation. The oxygenase reaction (termed photorespiration) results in the uptake of O_2 and release of CO_2 resulting in a decrease in net photosynthesis (Lawlor 2001). The biological function of the oxygenase reaction is not known, but one possible explanation is that it helps to dissipate excess ATP under high light intensities and low intercellular CO_2 concentrations (Taiz and Zeiger 2002).

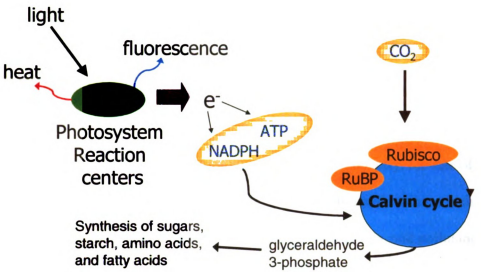

Figure 1.3. The transfer of energy during photosynthesis. Light is captured by pigments in the antenna complexes of the photosystems. Energy in the form of electrons is used for the production of NADPH and ATP. Energy from NADPH and ATP is needed for the regeneration of ribulose 1, 5-bisphosphate (RuBP) in the Calvin cycle. The enzyme Rubisco uses RuBP and CO_2 as substrates for synthesis of sugars, starch, amino acids, and fatty acids.

Photosynthetic intermediates produced in the PCR cycle are used to produce starch in the chloroplasts and sucrose in the cytoplasm of cells. Sucrose is the main carbohydrate translocated throughout the grapevine. If the rate of sucrose synthesis exceeds the removal or transport out of the cytoplasm, photosynthates are diverted into starch synthesis (Mullins *et al.* 1992). These reserves are utilized in the absence of newly formed photosynthates, mainly at night and early in the growing season. The amount of carbon assimilated and partitioned to woody parts of the vine varies with vine age, genotype, and time of year (Mullins *et al.* 1992).

Effect of stomata and leaf age on photosynthesis

Grape leaves are hypostomatal, meaning that the majority of the stomata are located on the lower leaf surface (Mullins *et al.* 1992). Diffusion of carbon dioxide through the cuticle is minimal. As a consequence, the size of the stomatal opening can directly affect the diffusion of carbon dioxide and consequently, the rate of photosynthesis in the leaf. Several factors can affect stomatal function including light, CO_2 concentration, vapor pressure differential, water deficits, and pollutants (Mullins *et al.* 1992). The photosynthetic apparatus of young grape leaves is not fully developed and, therefore, photosynthates from other parts of the vine must be imported to support their growth (Kriedman 1968, Mullins *et al.* 1992). Photosynthesis in mature grapes leaves generally reaches a maximum when the leaves are fully expanded and may maintain this rate for extended periods before decreasing towards senescence (Kriedman 1968). Developing berries are photosynthetically active, but their contribution to the carbon budget of the berry is probably insignificant (Mullins *et al.* 1992).

Effect of light on photosynthesis

Solar radiation provides the energy needed for photosynthesis to occur. Approximately 90% of solar radiation striking a leaf is absorbed. Light saturation (the point where an increase in solar radiation will not increase the rate of photosynthesis) generally occurs at 600-700 $\mu mol\ m^{-2}\ s^{-1}$ PAR (photosynthetically active radiation) which is roughly a third of full sunlight. The light compensation point (amount of light needed for net photosynthesis to occur) of grapes is about 50 μmol PAR (Mullins *et al.* 1992). Some enzymes important in berry ripening are light regulated. Low light intensities can

decrease berry size, Brix (a measure of soluble solids), and pH, and increase the titratable acidity (measure of organic acids) in grape berries (Mullins *et al.* 1992).

The emission of chlorophyll fluorescence, a process occurring from the deactivation of excited pigments, is a minor process competing with photosynthesis (Krause and Weis 1991). Excess light energy absorbed exceeds the electron carrying capacity and the capacity of Rubisco to fix CO_2 and is dissipated by the release of heat or as fluorescence. Most chlorophyll *a* fluorescence is emitted by Photosystem II at normal temperatures. Photosystem I, which is relatively stable, traps excitation energy and dissipates excess energy as heat (Lawlor 2001).

Source-sink relationships and carbon partitioning

'Source' refers to plant tissue that provides and 'sink' to plant tissue that consumes photosynthetic carbon compounds (Herold 1980). Sinks are comprised of all non-photosynthetic parts of a plant such as roots, trunks, ripening fruits, and immature leaves. Large sinks tend to dominate the overall supply of photosynthates, while small sinks must obtain them from more localized supplies and storage (Wardlaw 1990). Fruits and seeds act as large sinks and therefore, generally dominate as sinks over vegetative growth (Wardlaw 1990).

Carbon partitioning refers to the differential distribution of photosynthates to multiple sinks within the plant (Taiz and Zeiger 2002). Carbon partitioning in plants can be controlled by many factors including the supply of photosynthate, the size and number of sinks, vascular connections between compartments, and the potential for temporary storage of carbohydrates in leaves (Wardlaw 1990). Individual plant cell tissues may be

spatially separated and have different functions, but they all function as a tightly networked system which comprises the whole plant. Therefore, small changes in the demand for assimilates produced from photosynthesis in the grapevine can have significant effects on the partitioning of carbon or a change in the rate of photosynthesis or both (Wardlaw 1990).

Defoliation (a source reduction) can lead to reduced growth and yield, while the removal or thinning of grapes (a sink reduction) can lead to reduced rates of photosynthesis (Candolfi-Vasconcelos and Koblet 1990, Petrie *et al.* 2000, Quereix *et al.* 2001). Grape berries act as a large carbon sink, and the removal or lack of fruit can affect source-sink relationships in vines (Coombe 1989). Since fruiting vines tend to allocate more carbon to the fruit clusters rather than to roots and canes for storage, heavy fruiting can result in reduced shoot growth and poor fruit production the following year (Edson 1993, Kaps and Cahoon 1989, Wardlaw 1990). Excess crop loads and reduced leaf area on vines can also delay fruit ripening, reduce yield, and reduce total vine size and wood maturity (Howell 2001). Reducing crop loads in 'Seyval blanc' grapevines increased berry weight, Brix, and vegetative growth (Kaps and Cahoon 1989). Heavy crop loads in 'Concord' grapes caused greater carbohydrate partitioning to the fruit, but at the expense of vegetative tissues (Miller and Howell 1998). High crop loads in 'Seyval' vines reduced dry weight to the roots but did not affect total vine dry weight (Edson 1995b). Younger vines typically bear little or no fruit, and if fruit develops, it is usually removed to encourage vine growth and carbohydrate storage for future growth.

Defoliation of vines during ripening of fruit can cause major changes in dry matter partitioning and root growth, but this effect is strongly dependent on the timing of

the defoliation (Candolfi-Vasconcelos *et al.* 1994). Partially defoliated vines compensated for lost leaf area by increasing leaf chlorophyll content and increasing stomatal and mesophyll conductance in the remaining leaves to allow an increase in photosynthetic activity (Candolfi-Vasconcelos 1991). A low source:sink ratio also causes vines to delay senescence (Howell 2001). Two years of defoliation of *V. vinifera* vines caused a significant reduction in starch reserves in the wood. Complete recovery occurred the second season after the removal of the stress (Candolfi-Vasconcelos and Koblet 1990). Retranslocation of carbon from the trunk and roots was shown to be much higher in the defoliated vines three weeks after veraison (the onset ripening in berries) (Candolfi-Vasconcelos *et al.* 1994). In order to maintain the balance in the source-sink relationships, maximum root growth usually occurs in the spring and fall of the year when growth of other plant organs is less rapid (Mullins *et al.* 1992).

The grapevine does show some capacity for compensation. The extent to which a vine can compensate is highly dependent on the timing and amount of stress applied to the vine such as very large crop loads or excessive defoliation. 'Chardonnay' vines with young fruit removed showed no difference in the partitioning of newly fixed carbon as well as the rate of sucrose and starch synthesis compared to vines with fruit (Chaumont *et al.* 1994). The effects are more noticeable during the cluster ripening periods when berries become the dominant sinks. Large vegetative and fruiting sinks have been shown to influence photosynthetic rates of vines (Edson 1995a). The photosynthetic rates of vines (*V. vinifera*) were shown to increase from veraison until harvest (Petrie *et al.* 2000). The photosynthetic capacity of grapevines before veraison typically does not exceed 50% of the total photosynthetic capacity and therefore, is not source limited (Howell 2001).

Understanding the balance between crop load and the photosynthetic capacity of the vine enables growers to maximize yields without sacrificing juice quality or vine health.

Downy mildew of the grapevine

Classification

The causal agent of downy mildew of grapes is *Plasmopara viticola* (Berk. & Curt.) Berlese & de Toni, and belongs to a group of biotrophic pathogens (organisms which can obtain food in nature only from living host cells) called downy mildews (Burruano 2000). Downy mildews are fungal-like organisms belonging to the kingdom Chromista (Stramenopila), phylum Oomycota, family Peronosporaceae (Agrios 1997, Alexopoulos *et al.* 1996). The kingdom Chromista also contains brown algae and diatoms (Agrios 1997). The phylum Oomycota is characterized by organisms containing a diploid thallus, biflagellate zoospores produced in sporangia, and cell walls containing glucans, with small amounts of hydroxyproline and cellulose. Members of the family Peronosporaceae have sporangia borne on sporangiophores of determinate growth (Agrios 1997). In the genus *Plasmopara*, sporangia germinate indirectly (via zoospores) as opposed to *Peronospora* and *Bremia* spp., where germination is usually direct (formation of a germ tube) (Shaw 1981).

The first collection and identification of the causal organism of grape downy mildew was made in North America in 1837 by L.D. Schweinitz, who identified it as *Botrytis cana* Lk. (Anderson 1956, Gregory 1915, Viennot-Bourgin 1981). In 1848, it was renamed *Botrytis viticola* Berk. & Curt. by Berkeley and Curtis (Anderson 1956, Berkeley and Curtis 1848). Anton de Bary published an extensive study on *Botrytis* and

other fungal parasites in 1863, and changed the name to *Peronospora viticola* de Bary (Anderson 1956, Bary 1863). After Schroeter established the genus *Plasmopara* in 1886, Berlese and de Toni (1888) renamed *Peronospora viticola* to *Plasmopara viticola* (Berk. & Curt.) Berlese & de Toni (Gregory 1915, Lafon 1981).

History

Plasmopara viticola is believed to be native to North America. Prior to 1878, the disease was reportedly well established throughout southern Canada and most of the eastern and central United States (Agrios 1997, Alexopoulos *et al.* 1996, Anderson 1956, Singh 2000,Viennot-Bourgin 1981). Cultivation of grapevines in America in the 1800's was risky due to the possibility of downy mildew outbreaks. One limitation to the introduction of wine grapes (*Vitis vinifera*) from Europe was the fear of losses due to their susceptibility to *P. viticola* (Viennot-Bourgin 1981).

By the late 1800's, Europe had already suffered losses from several introduced pathogens and pests before the introduction of downy mildew. In 1845, a few years following the potato late blight epidemic (caused by *Phytophthora infestans* (Mont.) de Bary), grape powdery mildew (*Uncinula necator* (Schw.) Burr.) was inadvertently introduced into Europe from the United States (Viennot-Bourgin 1981). It spread throughout the grape-growing regions in Europe, causing extensive damage until the introduction of sulfur in the 1850's to control the spread of the disease (Viennot-Bourgin 1981). Grapevines were imported from America in the hopes of finding and breeding plant material resistant to powdery mildew. These imports were responsible for the introduction of grape phylloxera (*Daktulosphaira vitifoliae* Fitch), a root-attacking aphid, which destroyed vineyards across Europe (Anderson 1956,Viennot-Bourgin 1981). Soon

after in 1881, it was discovered that a European vine could be grafted to an American vine rootstock making the vine resistant to phylloxera (Viennot-Bourgin 1981). There was concern in Europe that the introduction of plant material from America could introduce downy mildew and have devastating effects, but the need to replant the vineyards after the phylloxera destruction was more urgent. In his bulletin, Farlow (1876) mentioned that if the vine mildew was introduced into Europe, it would prove a repetition of what had already happened with phylloxera due to the susceptibility of *V. vinifera* to the disease. In addition, the warmer temperatures and greater moisture in areas of Europe compared to the northeastern U.S., would allow infections to begin before the vine had attained substantial growth. In August 1878, downy mildew was identified and reported in France (Viennot-Bourgin 1981). By the early 1880's, it had spread across Europe and the Mediterranean, causing severe crop losses until the Bordeaux mixture (copper sulfate and lime) was discovered and found to be effective (Lafon 1981,Viennot-Bourgin 1981).

Host range

Downy mildews are generally limited in their host range, possibly due to the close association with the host plant with which they evolved as biotrophic pathogens (Renfro 1981). Most *Plasmopara* species infect broadleaf hosts, although a few have been reported on Graminaceae such as *P. oplismeni* Viennot-Bourgin on basketgrass and *P. penniseti* Kenneth & Kranz on pearl millet (Alexopoulos *et al.* 1996, Kenneth 1981, Shaw 1981). Woody hosts of the genus *Plasmopara* include *Vitis* spp. (*P. viticola*), *Ribes* spp. (*P. ribicola* J. Schröt. Ex J.J. Davis), and *Viburnum* spp. (*P. viburni* Peck) (Shaw 1981).

Numerous endogenous grape species have been identified in North America, including *Vitis aestivalis* Michx. (summer grape), *Vitis vulpina* L. (frost grape), *Vitis riparia* Michx. (riverside grape), *Vitis labrusca* L. (fox grape), and *Vitis rotundifolia* Michx. (muscadine grape). *Plasmopara viticola* is known to infect all of these grape species but virulence of the pathogen is generally low (Agrios 1997, Emmit 1992, Viennot-Bourgin 1981). However, Farlow (1876) reported that downy mildew was very abundant on *V. aestivalis*, *V. labrusca*, *V. cordifolia* Michx. (syn. *V. vulpina*), and all American cultivated varieties, and by the middle of September most leaves had been infected and "hang dead on the branches" (Farlow 1876). The European grape, *Vitis vinifera,* is also highly susceptible. Little is known about the role of infected wild hosts in the spread of the disease to cultivated grapes, but it appears to be minimal (Emmett 1992, Renfro 1981). In addition to *Vitis* species, *P. viticola* also infects *Parthenocissus quinquefolia* (L.) Planch., (Virginia creeper) and *Parthenocissus tricuspidata* (Sieb. & Zucc.) Planch. (Boston ivy), both woody perennials in the family Vitaceae (Anderson 1956).

Symptoms

Downy mildew can infect all green parts of the vine including leaves, flowers, petioles, tendrils, and young shoots and berries (Figure 1.4). Young tissue is the most susceptible to infection, becoming more resistant to infection at it matures (Pearson and Goheen 1988). Mature berries and immature developing leaves are more resistant to infection than other tissues, probably due to the presence of non-functioning stomata, although infection of the rachis can spread into mature berries (Lafon 1981). Infected young berries become discolored and develop white, felt-like sporulation. Infected berries

or entire clusters may drop prematurely. Young infected shoots are typically shorter and thicker than normal due to hypertrophy of the cells (Anderson 1956). Infected shoots may also curl, forming a 'shepherd's crook', become white with sporulation and eventually die (Pearson and Goheen 1988). Leaves develop circular yellow lesions on the upper leaf surface commonly called 'oil spots' due to their greasy or translucent appearance. Sporulation occurs on the underside of the leaf and appears as a dense white, downy growth giving rise to the common name of the pathogen (Pearson and Goheen 1988). The infection of older leaves results in small angular lesions due to the inability of the organism to spread across veins. Infected leaf surfaces eventually become necrotic and leaves may drop prematurely. Symptoms typically take 1-2 days longer to appear from the time of infection on American than on European species (*V. vinifera*) (Farlow 1876). Leaf infection is the most important source of inoculum for berry infection and overwintering oospores (Pearson and Goheen 1988).

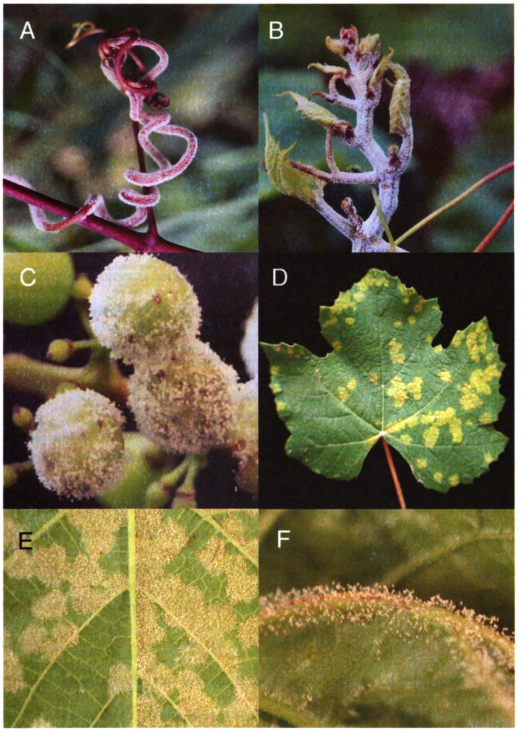

Figure 1.4. Signs and symptoms caused by *Plasmopara viticola*. A) Sporulation on a tendril of *Vitis riparia*, B) Sporulation and hypertrophy on a young shoot of *V. riparia*, C) Sporulation on berries of 'Mars' grapes, D) 'Oil spots' on the upper surface of a 'Niagara' leaf, E) Sporulation on the lower leaf surface of *V. aestivalis*, and F) Sporulation along the midvein of a *V. riparia* leaf.

Morphology

Plasmopara viticola produces a diploid, thin-walled coenocytic (multinucleate), nonseptate mycelium that occupies the intercellular spaces of the host (Anderson 1956, Gregory 1915, Singh 2000). Hyphal size can vary from 1-2 to 60 μm wide, which is partially determined by the size of the intercellular spaces in which they grow. Upon contact with the host cells, the hyphae invaginate the cells and form pear-shaped structures called haustoria. These haustoria are the host-parasite interface through which the exchange of nutrients between the host and pathogen occurs (Langcake and Lovell 1980). They are produced abundantly in tissues where mycelium is found, but are generally smaller and less abundant on resistant varieties (Gregory 1915). Haustoria are generally 4-10 μm in diameter (Singh 2000).

A swollen cushion of hyphae in the substomatal space gives rise to sporangiophores, which eventually emerge through the stomata or lenticels (Figure 1.5A, B) (Anderson 1956). Sporangiophores range from 140-250 μm long and are dichotomously branched at the tips (Emmett 1992).

Sporangia are produced on branches formed at right angles to the main stem of the sporangiophore (Figure 1.5C) (Anderson 1956, Singh 2000). Sporangia are hyaline and range from 11-18 μm in width to 15-31 μm in length, but size can vary with environmental conditions and among hosts (Anderson 1956, Singh 2000). A sporangium typically releases from 5-8 zoospores (Figure 1.5D, E) (Gregory 1912). Zoospores are pear-shaped and range in size from 4-5 μm in width and 7-9 μm in length (Singh 2000). Motility is by means of two apical flagella, one longer tinsel flagellum with hairs and a shorter whiplash flagellum that trails behind (Alexopoulos *et al.* 1996).

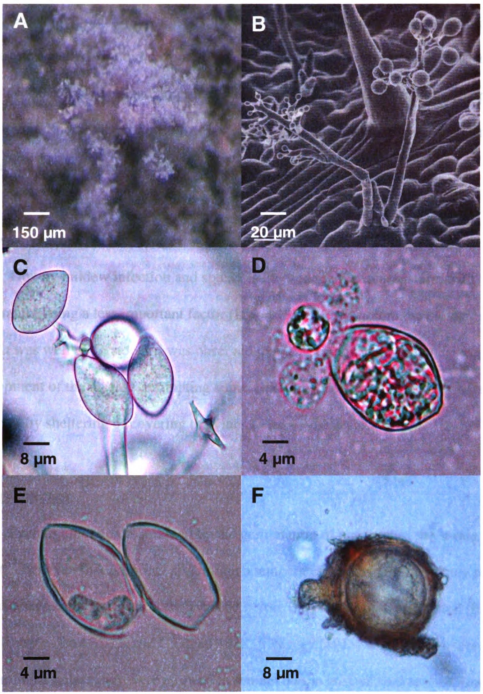

Figure 1.5. Morphological structures of *Plasmopara viticola*. A) Sporangiophores with sporangia on 'Niagara' grape leaves, B) Electron micrograph of sporangiophore and sporangia growing out of a stoma (Alexopoulos *et al.*1996), C) Sporangiophore and sporangia from 'Niagara' grape leaves, D) Sporangium releasing zoospores, E) Empty sporangium and sporangium with a few zoospores inside, and F) Oospore taken from a 'Niagara' grape leaf.

Sexual reproduction begins by the fusion of an antheridium and an oogonium to form a thick-walled, diploid oospore (Figure 1.5F). Oospores range from 28-40 μm in diameter. Oospores germinate to produce a sporangiophore with a single, large apical sporangium. Sporangia produced from oospores are 25 μm in width and 35-40 μm in length, and typically produce 8-20 zoospores (Singh 2000).

Disease cycle and epidemiology

Downy mildew infection and spread is favored mainly by moisture, with temperature being a less important factor (Lafon 1981). Even before the biology of *P. viticola* was well understood, it was observed that moisture from morning dew favored development of the disease, prompting some viticulturists to try to prevent condensation on leaves by sheltering or covering the vines (Viennot-Bourgin 1981).

Primary infection

Primary infection is initiated by the germination of oospores in the spring (Figure 1.6). It is believed that oospores go through a temperature-dependent dormancy period, but it is uncertain if maturation can proceed before the dormancy is completed (Emmit 1992, Ronzon-Tran Manh and Clerjeau 1988). Gregory (1915) found that oospores would not germinate unless they were allowed to remain on the ground until at least January or February in the northeastern United States. Maturation is influenced primarily by winter precipitation and soil temperature (Emmit 1992). Rainy conditions with temperatures above 10°C favor the germination of oospores (Emmett 1992, Serra and Borgo 1995).

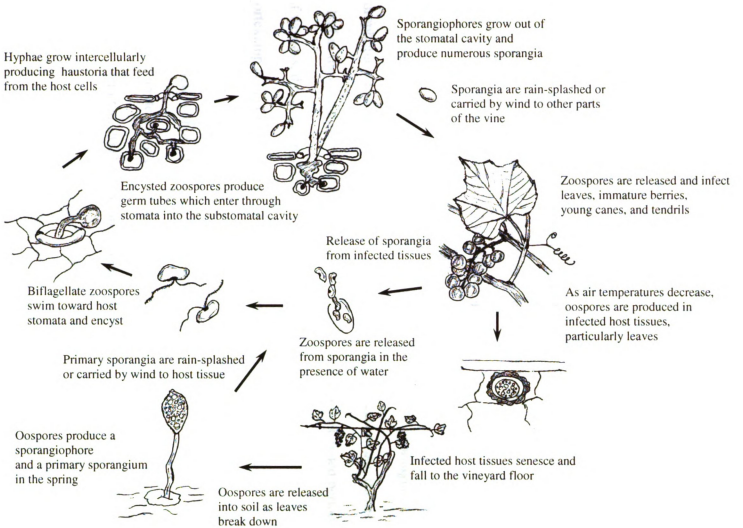

Figure 1.6. Disease cycle of *Plasmopara viticola*.

The optimum temperature for germination is 20-22°C (Populer 1982, Ronzon-Tran Manh Sung and Clerjeau 1988). At optimum temperatures, the formation of the sporangium and release of zoospores is usually completed in 24 hours (Gregory 1915). Oospores germinate to form a single sporangiophore and primary sporangium. Moisture is required to dissolve the cross-wall of callus connecting the sporangia to the sporangiophores, permitting their dispersal, and for the liberation of zoospores from sporangia (Emmett 1992, Lafon 1981). The detachment of sporangia is aided by twisting in response to a vapor pressure differential or a decrease in relative humidity (Pinckard 1941). Primary inoculum consists of windborne sporangia or rain-splashed sporangia or zoospores (Emmett 1992, Gregory 1912, Singh 2000).

Release and movement of zoospores requires free water. The sporangia are hydrophobic which allows them to resist water and adhere to the cuticle and trichomes of the leaf as they are dispersed. During periods of high humidity and extended periods of leaf wetness, the sporangia become hydrophilic and begin to differentiate into zoospores (Kortekamp *et al.* 1999). The optimum temperature for differentiation is 15-25°C (Gregory 1915, Lafon 1981, Emmit 1992). The differentiation and release of zoospores is usually complete within 30 minutes (Langcake and Lovell 1980). Zoospores are attracted to stomata by unidentified chemical signals, which vary with the size of the stomatal opening (Kiefer *et al.* 2002, Royle and Thomas 1973). Upon contact with stomata, zoospores encyst, shed their flagella, and produce a germ tube that enters the substomatal cavity (Emmit 1992). The rate of zoospore release, development of the germ tube, and stomatal targeting can be affected by the absence or presence of host tissue as well as stomatal closure (Kiefer *et al.* 2002). Several zoospores can encyst and produce germ

tubes that penetrate a single stoma (Royle and Thomas 1973). In the substomatal cavity a vesicle is formed which produces a short hypha. The production of a haustorium is initiated upon contact of the infection hypha with a host cell (Langcake and Lovell 1980). Further hyphal development into the mesophyll and palisade cells occurs 12 to 15 hours after the production of the initial haustorium (Emmett 1992). Additional haustoria are produced with additional hyphal growth. Hyphal growth ceases at 30°C, but hyphae can remain viable for more than 10 days at temperatures of 42-43°C (Singh 2000).

Sporulation

Under favorable conditions, sporulation occurs on the infected tissue and the secondary infection cycle begins. The major factors affecting sporulation are relative humidity, temperature, light/darkness, and the condition of the host tissue (Gregory 1915, Emmit 1992). A continuous relative humidity of 95-100% is required for sporulation (Blaeser 1979, Pearson and Goheen 1988, Emmit 1992). A dark period of at least 4 hours followed by light is required for the production of sporangia (Rumbolz *et al.* 2002, Singh 2000). Continuous darkness or light produces little or no sporulation (Anderson 1956, Yarwood 1937). White light irradiation prevents formation of sporangia and produces abnormally shaped sporangiophores (Brook 1979, Rumbolz *et al.* 2002, Yarwood 1937).

Sporulation can occur at temperatures as low as 10°C and as high as 29°C but the optimal temperature is 18-22°C (Blaeser and Weltzien 1978, Gregory 1915, Pearson and Goheen 1988). Production of sporangia and sporangiophores can be completed in as little as 7 hours (Rumbolz *et al.* 2002). As many as 20 sporangiophores can arise through a

single stomatal opening (Anderson 1956, Singh 2000). Sporulation can continue from the same infected tissue for several days under favorable conditions.

Survival of sporangia

Survival of sporangia is much shorter than oospore survival and decreases with decreasing humidity and increasing temperature (Blaeser and Weltzien 1978, Blaeser 1979, Singh 2000). Sporangia in a vineyard showed a viability of 4 to 8 days when shaded by the canopy, provided temperatures did not exceed 22°C (Kast and Stark-Urnau 1999, Zachos 1959). Gregory (1915) found that sporangia cannot survive sunlight exposure at temperatures above 30°C. Sporangia on detached leaves survived less than 48 hours at 20°C with 70% relative humidity, and less than 24 hours at 20°C with 30% relative humidity. Viability was maintained longer when sporangia remained attached to sporangiophores (Blaeser and Weltzien 1978). Older sporangia required more time to release zoospores and showed declining viability with age (Kast and Stark-Urnau 1999). Sporangia produced at night between 0200 and 0600 h had the highest germination rates compared to sporangia produced at any other time during the diurnal cycle (Srinivasan 1976). Production of sporangia can continue for several successive nights from the same lesion under ideal conditions (Singh 2000). Secondary sporangia are wind dispersed or water-splashed to other plant tissues where they release zoospores in free water to continue the secondary infection cycle.

Overwintering

Late in the season, temperatures favor sexual reproduction, and overwintering oospores are produced in infected host tissue. *Plasmopara viticola* is reported to be heterothallic, requiring two different mating types for sexual reproduction to occur (Wong *et al.* 2001). Oospores are formed in lesions on infected leaves in late summer and fall (Figure 5.1). Oospore numbers in lesions can be as high as 250 per square mm (Populer 1982) and are most concentrated adjacent to the leaf midrib or a major vein (Singh 2000). Overwintering occurs mainly in the form of oospores in leaf debris on the soil surface, however, in milder climates, *P. viticola* may survive as mycelium in buds and infected shoots (Emmit 1992, Pearson 1988, Singh 2000).

Survival of oospores is favored by moisture and their close proximity to the soil surface with temperature being a less important factor (Lafon 1981). Oospores have been known to survive temperatures of -26 °C for five days (Gäumann 1950). Before germination, oospores must go through a temperature-dependent dormancy period (Singh 2000). Under controlled conditions oospores mature at alternating weekly temperatures of -5 and 10°C. Oospores germinate within 6-10 days after shifting to 20°C, but a 4-month minimum maturation period is required (Ronzon-Tran Manh Sung and Clerjeau 1988).

Distribution and economic importance

Downy mildew is the most destructive fungal disease of grapevines and has been reported in 91 countries (Emmett 1992, Singh 2000). Downy mildew is particularly destructive in parts of the world that receive frequent summer rains that maintain high

humidity at night and temperatures over 13°C, such as France, Germany, and South Africa (Emmett 1992). In areas with drier climates such as Afghanistan, California, Chile, Egypt, and Western Australia, downy mildew is of little importance (Emmett 1992, Weltzien 1981).

The pathogen infects all green tissues of the vine causing loss of photosynthetic area and cluster infections that can lead to 100% yield loss (Emmett 1992, Singh 2000). Early infections can lead to defoliation of vines that prevents cluster maturation resulting in low sugar content as well as exposing fruits to sun scald (Singh 2000). Vine vigor, grape production, and grape quality may be reduced in years following epidemics due to depletion of carbohydrate reserves (Emmett 1992). Heavy infections may have an effect on winter hardiness making vines more susceptible to colder temperatures. In addition, bud burst may be slowed and crop potential may be reduced due to a lower number of viable buds (Emmett 1992).

Disease Management

Since the discovery of the Bordeaux mixture by Millardet in 1885, fungicides have been used extensively to control downy mildew, although recently canopy management practices are gaining attention (Emmett 1992, Schwinn 1981, Singh 2000). Preventative methods such as cleaning up infected leaves can be used to help reduce downy mildew inoculum in the vineyard, but these methods are impractical and often only provide limited control. Vines should be spaced and pruned to increase air circulation and decrease humidity when practical (Mullins *et al.* 1992).

Fungicides

Application of fungicides provides the best method of control (Emmett 1992, Schwinn 1981, Singh 2000). Several different fungicides can provide effective control of downy mildew if applied at recommended intervals. Protectant fungicides include captan, mancozeb, ziram, and copper fungicides. Copper-containing fungicides such as the Bordeaux mixture provide preventative control when used with proper timing. Copper fungicides are still used frequently for downy mildew control but are often mixed with other fungicides to improve effectiveness and increase the spectrum of control (Schwinn 1981). Some varieties such as 'Catawba', 'Merlot', and 'Chancellor', are sensitive to copper, so alternative fungicides must be employed (Wise *et al.* 2004). Mancozeb is a protectant EBDC (ethylene bisdithiocarbamate) fungicide that is effective against downy mildew as well as black rot and Phomopsis cane and leaf spot. Even though it is primarily a protectant, it does have some anti-sporulant activity when applied post-infection (Wong and Wilcox 2001); however, it has a 66-day preharvest interval (Wise *et al.* 2004). In addition, some juice grape processors do not allow sprays of mancozeb after bloom. Captan is also effective for control of downy mildew and Phomopsis cane and leaf spot but not for black rot. While Captan currently has a 0-day preharvest interval for grapes, its use is not allowed by some juice grape processors (Wise *et al.* 2004). These fungicides are all preventative, providing control before infection occurs, and normally have little or no effect on disease that is already established.

Ridomil Gold (mefenoxam) is a systemic fungicide that prevents hyphal growth and the production of haustoria and provides excellent control of downy mildew (Singh 2000). Ridomil can provide complete control if applied before infection and can

significantly reduce disease severity if applied up to five days after infection (Wong and Wilcox 2001). Post-symptom application is effective at reducing further sporulation. Other fungicides such as copper and mancozeb are added to Ridomil as a resistance management tool to reduce the likelihood of pathogens developing resistance, however these additions increase the pre-harvest interval (42 days for Ridomil Gold Copper and 66 days for Ridomil Gold MZ) (Wise *et al.* 2004).

ProPhyt (potassium phosphite) and Aliette (fosetyl-Al) are formulations of phosphorous acid that are considered true systemics and an alternative to mefenoxam (Singh 2000). Application up to 12 days after infections occur can reduce the incidence and severity of infection by reducing sporulation (Wicks *et al.* 1991). These fungicides are translocated through the plant via the xylem and phloem and provide protection to actively growing foliage and clusters for 14 to 21 days (Singh 2000).

Strobilurins are a class of protectant and curative fungicides that prevent infection as well as provide some post-infection activity. Strobilurin fungicides include Abound (azoxystrobin), Sovran (kresoxim-methyl), Flint (trifloxystrobin), and Pristine (pyraclostrobin and boscalid). Pristine can be phytotoxic to 'Concord', 'Fredonia', 'Worden' and other related varieties (Wise *et al.* 2004). On young plants, the preventative efficacy of azoxystrobin was shown to be similar to that of mancozeb (Singh 2000). When applied up to five days after the start of infection, azoxystrobin can significantly reduce sporulation and lesion size, but has little effect on disease incidence (Wong and Wilcox 2001). Strobilurins have a broad spectrum of activity, yet they have a single mode of action, so management practices must be used to limit the development of fungicide resistance (Wise 2004).

Biological control

Fusarium proliferatum (T. Matsushima) Nirenberg is a fungus that has been shown to act as a biocontrol agent by parasitizing the sporangiophores of *Plasmopara viticola*. Post-infection application on leaf disks reduced sporulation by more than 90%, and weekly applications of conidial suspensions in vineyards reduced disease development on leaves and clusters (Falk *et al.* 1996). Although it is not currently registered as a fungicide, it could potentially prove effective in areas where downy mildew is not severe or when used in conjunction with other fungicides in resistance management programs (Falk *et al.* 1996).

Forecasting

Forecasting methods can be used to determine if disease severity warrants fungicide application. These systems measure the duration of leaf wetness, relative humidity, and temperature to identify infection periods and determine fungicide application intervals (Agrios 1997). Since downy mildew climatic conditions and disease outbreaks can vary from region to region, disease forecasting can be helpful in predicting when outbreaks might occur. Several disease-forecasting systems are available for downy mildew and many used in Europe are based on the findings of Blaeser and Weltzien (1979) which include infection conditions, sporulation conditions, and viability of sporangia. Models designed to predict outbreaks in the U.S. may be more suited to predict outbreaks on American vines and French hybrids (Madden and Ellis 2000, Park *et al.* 1994). DMCast is a forecasting system that also considers ontogenic resistance (increasing resistance as tissues mature) of berries and cluster stems, the period of

oosporic inoculum release, the development and components of lesion productivity, and sporangium survival (Park *et al.* 1994).

Rationale and objectives of the research

Plasmopara viticola has been infecting grapevines for many years in the United States and around the world. Most research over the last century has focused on understanding the biology of *P. viticola* and developing management strategies which incorporate the use of fungicides. In order to establish damage thresholds caused by foliar infections of *P. viticola* in grapevines, it is important to have an understanding of how the pathogen affects carbon assimilation and allocation in the vine. An investigation of host specialization in *P. viticola* can give a better understanding of how the pathogen spreads between vine species and cultivars, and also improve inoculation procedures for 'Niagara' grapevines. This research will ultimately assist in the development of minimal fungicide strategies and better alternate methods of control that allow maximum grapevine productivity.

The objectives of this research were to: 1) Determine the effects of *P. viticola* on photosynthesis of infected 'Niagara' leaves, 2) Determine the effects of infection on biomass accumulation and carbon partitioning in 'Niagara' vines, and 3) Evaluate the ability of *P. viticola* sporangia to cross-infect different grape cultivars and species.

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Chapter 2

The effects of downy mildew (*Plasmopara viticola*) infection on photosynthesis of 'Niagara' grapevine leaves

Introduction

Many studies have been done to determine the effect of pathogens on physiological and biochemical changes in plants. However, little is known about the effects on photosynthesis at the biochemical level (Scholes 1992). Studies using leaves infected with viruses, bacteria, and fungi have shown that the rates of photosynthesis in infected plants may decrease, remained unchanged, or in some cases may even increase (Buchanan 1981). Blueberry leaves infected with Septoria leaf spot showed exponential reductions in photosynthesis as disease severity increased (Roloff *et al.* 2004). Potato leaves infected by *Phytophthora infestans* (Mont.) de Bary showed an increase in the photosynthetic rate in infected areas. This increase was presumably caused by the opening of stomata allowing more efficient gas exchange (Farrell 1971). Many of these effects depend on the pathogen, host and the specific stage of disease development (Bassanezi *et al.* 2002, Shtienberg 1992).

Estimating photosynthesis and gas exchange parameters

The measurement of CO_2 uptake by plants provides a direct measure of productivity and is instantaneous and nondestructive. It can also provide information on individual leaves or parts of leaves (Long 1985). Photosynthesis measurements on a leaf or leaf section can accurately reflect changes in photosynthesis within the leaf but do not

necessarily reflect a whole-vine response, due to the variation in age and position of leaves on the vine (Edson 1993, Kriedman 1968, Miller 1996, Mullins *et al.* 1992, Nobel 1985). The rate of photosynthesis can also be affected by the environmental conditions in which it is measured, such as temperature and humidity (Scholes *et al.* 1994). The effects of pathogens on photosynthesis can be evaluated by comparing the response of diseased leaves and healthy leaves to different levels of light, CO_2, temperature, or vapor pressure changes. The analysis of chlorophyll fluorescence can be used as an indicator of the photochemical efficiency of photosystem II (Scholes and Rolfe 1996). The analysis of response curves of the photosynthetic rate and absorbed light allows the changes in quantum efficiency, maximum light intensity, the light compensation point, and dark respiration to be identified (Figure 2.1) (Baker 1996, Rossing 1992). Photosynthetic efficiency or yield is the number of moles of CO_2 absorbed by the leaf per mole quanta of light absorbed, giving a direct measure of the efficiency of light utilization by the leaf. There is evidence that the CO_2 assimilation rate of mature crop canopies is determined more by the photosynthetic efficiency than by the light-saturating rate of photosynthesis (Long 1985). Plots of the photosynthetic rate vs. intercellular CO_2 concentration (A/C_i curves) allow identification of changes in Rubisco activity and regeneration rate of ribulose 1, 5-bisphosphate (RuBP) (Figure 2.2) (Baker 1996, Farquhar and Sharkey 1982). A/C_i curves can also be useful in separating stomatal and mesophyll limitations to photosynthesis (Farquhar and Sharkey 1982, Long 1985).

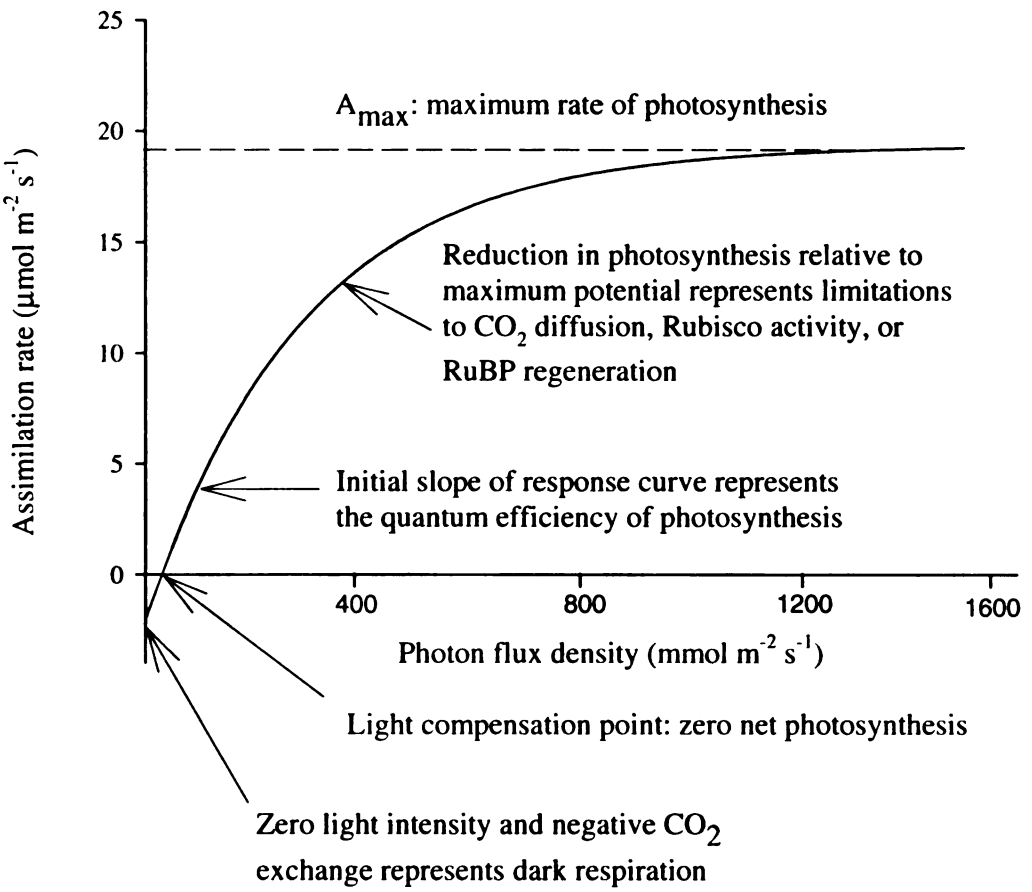


Figure 2.1. A representation of a light response curve showing the relationship between the carbon assimilation rate and light intensity. Variables can then be calculated from the curve (modified from Baker 1996).

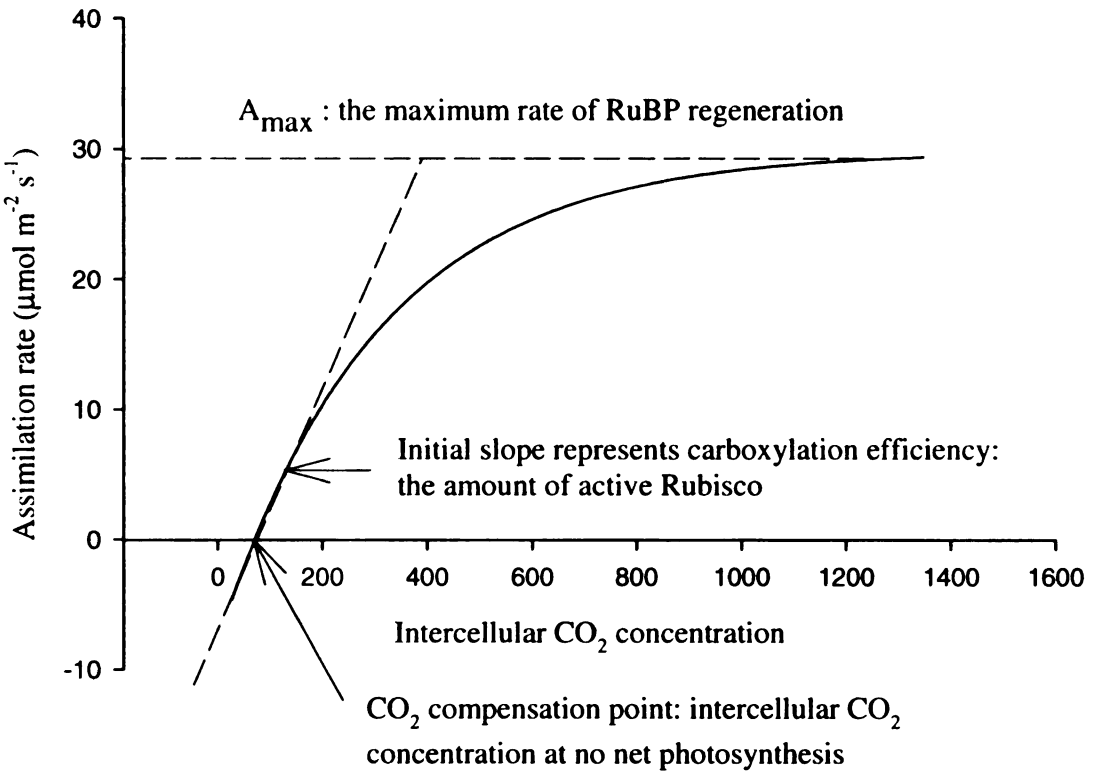


Figure 2.2. A representation of a CO_2 response curve showing the relationship between the carbon assimilation rate and the intercellular CO_2 concentration. Variables can then be calculated from the curve. The maximum rate of RuBP (ribulose 1, 5-bisphosphate) regeneration is assumed equal to the rate of coupled photosynthetic electron transport (modified from Long 1985).

The intercellular CO_2 concentration (C_i) can also provide a method for assessing limitations to photosynthesis caused by stomatal (resistance to CO_2 movement across stomata) and mesophyll limitations (resistance to CO_2 movement across cell walls, cell membranes, cytosol, or chloroplast membranes). If a reduction in the assimilation rate is a result of stomatal limitation, then C_i should decrease; if the reduction is caused by mesophyll limitations, then C_i should increase (Long 1985). Under normal CO_2 concentrations and saturating light conditions, the rate of photosynthesis is largely controlled by Rubisco activity. Under saturating light and saturating CO_2 conditions, the rate of photosynthesis is determined by the availability of inorganic phosphate (P_i) (Scholes *et al.* 1994). The data obtained from these measurements can provide a mechanistic understanding of how pathogens alter photosynthesis in infected plants.

Effects of biotrophic pathogens on photosynthesis

Many studies have examined the effects of infection by biotrophic pathogens, particularly rusts and powdery mildews, on photosynthesis and respiration (Farrar 1987, Magyarosy *et al.* 1976, Owera *et al.* 1981, Scholes and Farrar 1986, Whipps and Lewis 1981). Biotrophic pathogens typically cause a general decline in the photosynthetic rates of plants by reducing the efficiency of, or decreasing functional leaf area (Bassanezi *et al.* 2002, Bastiaans 1991, Black 1968, Daly 1976, Livne 1964, Raggi 1978, Shtienberg 1992). The reduction in net photosynthetic rates of infected leaves can be a consequence of increased stomatal or mesophyll resistance (Ahmad *et al.* 1983, Owera *et al.* 1981, Rabbinge *et al.* 1985, Scholes 1992). In addition, reduced rates of photosynthesis can be a result of reduced Rubisco activity (Bassanezi *et al.* 2002, Bertamini *et al.* 2002),

destruction of chloroplasts (Ahmad *et al.* 1982, Mignucci and Boyer 1979, Sziraki *et al.* 1984), alterations of the cytochrome content of membranes (Magyarosy and Malkin 1978, Walters 1985), or increased respiration (Scholes 1992). Radish leaves infected with white rust (*Albugo candida* (Pers.) Kuntze) showed a reduction in photosynthesis several days before an increase in respiration rates and the appearance of symptoms, but the cause of the decrease was unknown (Black *et al.* 1968). Rust-infected beans (Bassanezi *et al.* 2002), wheat (Rabbinge *et al.* 1985), and barley (Bassanezi *et al.* 2002, Owera *et al.* 1981) showed a decrease in the net photosynthetic rate and an increase in the internal CO_2 concentration, although significant decreases in these parameters in beans were not seen until after symptom development. A reduction in Rubisco activity was the major cause of the decreased photosynthetic activity of phytoplasma-infected apple leaves (Bertamini *et al.* 2002) and rust-infected bean leaves (Bassanezi *et al.* 2002) after the appearance of symptoms. Stomata did not significantly limit photosynthesis in diseased bean leaves (Bassanezi *et al.* 2002). There was also less damage to photosynthetic regulation mechanisms in the remaining healthy parts of rust-infected bean leaves than in bean leaves infected by a hemibiotrophic pathogen (a pathogen initially requiring living host cells but eventually killing host cells and living on the remains) such as *Phaeoisariopsis griseola* (Sacc.) Ferraris (angular leaf spot) (Bassanezi *et al.* 2002). Powdery mildew infecting the upper surface of barley leaves had little effect on gas exchange, because gas exchange was for the most part through stomata of the lower leaf surface (Aust *et al.* 1977). However, powdery mildew was shown to reduce photosynthetic rates by lowering mesophyll conductance in infected leaves of sugar beet (Gordon and Duniway 1982). Scholes (1992) reported that the reduction in

photosynthesis in barley leaves infected with powdery mildew was primarily due to loss of activity or quantity of photosynthetic enzymes in the Calvin cycle (reductive pentose phosphate [RPP] cycle). Down-regulation of the Calvin cycle was thought to be caused by the effect of carbohydrates on gene expression encoding photosynthetic enzymes. Electron transfer rates also decreased due to decreased demand for ATP and NADPH, while loss of chlorophyll occurred later. Montalbini and Buchanan (1974) found that rust-infected bean leaves had significantly fewer electron carriers than healthy leaves, causing a disruption of electron transport. In addition to decreased photosynthetic rates in rust-infected beans (Bassanezi *et al.* 2002, Raggi 1978), barley (Aust *et al.* 1977) and wheat (Bethenod *et al.* 2001), diseased leaves also showed an increase in respiration. Rust-infected beans showed an increase in the CO_2 compensation point (CO_2 concentration where there is zero net photosynthesis) that was highly correlated with a decrease in respiration (Raggi 1978). Rust-infected wheat leaves showed an increase in the intercellular CO_2 concentration (C_i) with increased respiration (Bethenod *et al.* 2001). Increased rates of respiration of the pathogen, host or both could increase C_i, causing an apparent decrease in net photosynthesis, although the pathogen contribution to increased respiration rates is thought to be small due to the greater biomass of plant tissue (Ayres 1981, Daly 1976, Walters 1985). Reduction in photosynthesis caused by the loss of chlorophyll or destruction of chloroplasts has typically been evident after the appearance of disease symptoms in soybean (Mignucci and Boyer 1979), barley (Ahmad *et al.* 1983), and dry beans (Lopes and Berger 2001, Sziraki *et al.* 1984, Wagner and Boyle 1995).

Transient increases in photosynthetic rates have been known to occur in uninfected areas of diseased leaves, but these increases are usually short lived and vary

with disease severity and leaf age (Allen 1942, Aust *et al.* 1977, Daly 1976, Livne 1964). Depending on the pathogen-host combination, the visual size of the lesion may not always provide an accurate estimate of physiological damage to a leaf, because the area affected can be larger than the area invaded by the pathogen (Bastiaans 1991, Giuntoli and Orlandini 2000, Shtienberg 1992). This effect can lead to a stronger or weaker pathogen effect on photosynthesis than what may be expected based on lesion size. Increased rates of photosynthesis in healthy areas of diseased leaves could contribute to this effect.

A decrease in the export of photosynthates from source leaves caused by a strong pathogen sink at the site of infection can cause an accumulation of carbohydrates in the leaf. This effect has been shown in rust-infected beans (Zaki 1965) and radishes (Black 1968). Powdery mildew infections on cereals tend to show this effect as well (Scholes 1992). Carbohydrate accumulation at infection sites can cause a down regulation of the Calvin cycle, thereby causing an inhibition of photosynthesis (Livne 1966, Scholes 1992, Wagner and Boyle 1995).

Effects of biotrophic pathogens on respiration

Leaves infected with biotrophic fungi generally have greater rates of dark respiration, and this is likely due to both the pathogen and the host (Farrar 1992, Scholes 1992). Daly (1976) attributed most of the respiration observed in a powdery mildew infection to the host. Since rates of respiration are typically higher in infected plant tissues, the flux of CO_2 into intercellular spaces from respiration raises the intercellular CO_2 concentration above that of healthy plants and thus reduces the measured net

photosynthesis (Farrar 1987). Increases in the intercellular CO_2 concentration were reported for some rust diseases (Owera *et al.* 1981), but not powdery mildew of beet (Gordon and Duniway 1982).

Effects of biotrophic pathogens on chlorophyll fluorescence

Chlorophyll fluorescence can be used as a non-destructive measure of photosynthetic activity in photosynthetic parts of plants (Chaerle and Van der Straeten 2000, Daley *et al.* 1989). The measurement of F_v/F_m (ratio of variable to maximum fluorescence) provides information about the potential photosynthetic efficiency of photosystem II (Bassanezi *et al.* 2002, Genty *et al.* 1989). Rust-infected beans showed no significant reduction in F_v/F_m after symptoms appeared. Neither photosystem II efficiency nor chlorophyll abundance changed in powdery-mildew-infected sugar beet leaves even in 60-day-old infections (Bassanezi *et al.* 2002, Magyarosy *et al.* 1976). Reductions in F_v/F_m may be a consequence of reduced capacity of electron transport or regeneration of ATP and NADPH, causing excess absorbed energy in photosystem II to be dissipated as fluorescence (Bassanezi *et al.* 2002).

Pathogen effects on grapevine photosynthesis

Few studies have focused on the impact of pathogen infection on photosynthesis of the grapevine and knowledge is limited (Giuntoli 2000). Mature grapevine leaves infected with powdery mildew show a decreasing photosynthetic rate with increased infection caused by damage to the palisade cells (Lakso *et al.* 1982). Powdery mildew on grape leaves reduced the net assimilation rate, stomatal conductance, carboxylation

efficiency, and quantum efficiency on potted 'Chardonnay' grapevines (Nail and Howell 2004). Similar results were found with infection on 'Riesling' vines as well as an increase in respiration in the healthy areas of infected leaves (Clearwater 2002). A decrease in Rubisco activity was the primary mechanism causing a substantial decrease in photosynthesis in grape leaves infected with grapevine fan leaf virus (GFLV), while inhibition of the primary light reactions (conversion of light energy to NADPH and ATP) was only a minor effect (Sampol *et al.* 2003).

Effects of *Plasmopara viticola* on photosynthesis of grapevines

The majority of studies on the effect of biotrophic pathogens on photosynthesis have focused on rusts and powdery mildews, and little is known about plants infected with downy mildews (Walters 1985). Downy mildews are known to induce many alterations in the host physiology, but the exact mechanisms for these alterations have only been identified in a small number of pathosystems (Giuntoli 2000, Scholes 1992). Grapevine leaves infected with downy mildew showed decreased rates of photosynthesis, and when only symptomatic areas were measured the net photosynthetic rate fell below zero (Giuntoli 2000). Healthy areas of diseased leaves did not show a significant difference in their photosynthetic rates compared to healthy leaves (Giuntoli 2000). There is no evidence to show the mechanism of the decrease in net photosynthesis in downy-mildew-infected grapes.

Rationale and objectives

Plant pathogens can cause a reduction in photosynthetic rates of plant tissues, which in turn can potentially reduce total dry weight accumulation or adversely affect carbon allocation in plants. Understanding the effects that pathogens have on carbon assimilation in plants is the first step to predicting when pathogens will negatively affect crop productivity.

The objectives of this study were to: 1) Determine the relationship between foliar disease severity and the rate of photosynthesis and 2) Use gas exchange and chlorophyll fluorescence measurements to determine the sequence of changes in carbon assimilation following inoculation of grape leaves with *Plasmopara viticola*.

Material and methods

Assimilation rates of leaves of downy mildew-infected 'Niagara' vines in the field

Carbon assimilation rates were measured on *Vitis labrusca* 'Niagara' leaves in vineyards located at the Trevor Nichols Research Complex in Fennville, MI in 2002, in a commercial vineyard in Lawton, MI in 2003, and at the Clarksville Horticultural Experiment Station in Clarksville, MI in 2004. All vines measured were mature and had fruit at the time of measurement. Measurements were taken on October 1st in 2002, September 11th and 16th in 2003, and September 16th in 2004 when infections were well established throughout the vineyard. Fully expanded leaves of approximately the same age (first or second fully expanded leaf) were chosen for the experiment. Measurements of assimilation rate were taken with a CIRAS I infrared gas analyzer (PP Systems, Amesbury, MA) equipped with a Parkinson leaf cuvette.

Well-developed, sporulating downy mildew lesions on infected leaves were enclosed in the leaf chamber of the cuvette. The location of the cuvette on the leaf was adjusted so that areas with estimated disease severities ranging from 0 to 90% were enclosed in the cuvette. Noninfected leaves of a similar age in close proximity to infected leaves were used as a control. Sunlight was used for all measurements at saturating values of 1400 μmol m^{-2} s^{-1} PAR or greater. Measurements were taken between 1000 and 1500 h. Temperatures were 22 ± 5°C. A total of 9 healthy and 9 diseased leaves were measured in 2002, 16 healthy and 16 diseased leaves in 2003, and 12 healthy and 12 diseased leaves in 2004. Values for the assimilation rate, stomatal conductance, and intercellular CO_2 concentration were obtained for analysis.

Data analysis

The assimilation rate (A) was plotted against disease severity, stomatal conductance (g_s), and intercellular CO_2 concentrations (C_i). The data were plotted and analyzed with nonlinear regression analysis using Sigmaplot statistical software (Systat Software Inc., Richmond, California).

Leaf photosynthesis in potted vines as infection progresses

Plant material

Two-year-old *Vitis labrusca* 'Niagara' grapevines were potted in 5 gallon pots and maintained outdoors at the Michigan State University Plant Pathology farm, East Lansing, MI in 2003 and the Michigan State University greenhouse courtyard in 2004. Plants were watered regularly and fertilized every two weeks with 20-20-20 N-P-K

fertilizer. Any flowers that developed were removed. The potting medium was a mixture of steam-sterilized 60% loam and 40% sand. Vines were sprayed regularly with Sevin (carbaryl, Bayer Cropscience, Research Triangle Park, NC) to control Japanese beetle and Nova (myclobutanil, Dow Agrosciences, Indianapolis, IN, USA) to control powdery mildew. The experiment began on October 4 in 2003 and October 28 in 2004.

Inoculation

The first or second fully expanded leaf was selected from each of five vines for the experiment. For inoculum, naturally infected leaves collected from 'Niagara' vines in the field were placed in plastic bags with moistened paper towels. Bags were placed in the dark for 12 h at 22°C to induce sporulation. Sporangia were washed into a beaker from the leaves using a pipette and deionized water. The concentration was determined using a hemacytometer and adjusted to $5x10^4$ sporangia per ml. A suspension of sporangia was applied to one half of the leaf using an atomizer while the other half of the leaf was kept dry to avoid infection. The entire leaf was covered for 6 hours with a moistened plastic bag to retain moisture. Inoculations were done before sunrise to avoid any negative effect of light on sporangial germination. Measurements began the day after inoculation in 2003 and the day before inoculation in 2004.

Measurements

Gas exchange measurements were taken every 48 hours after the initial measurement for 14 days after inoculation in 2003 and 10 days after inoculation in 2004. Each measurement was taken on the inoculated half of the leaf, and the noninoculated

half was used as a control. A small spot was marked on both sides of the leaf with a permanent marker so measurements could be taken in the same location. Gas exchange measurements were taken with a CIRAS I infrared gas analyzer and a Parkinson leaf cuvette (18-mm diameter chamber) (PP Systems, Amesbury, MA). Measurements were taken between 1000 and 1600 h. Temperatures ranged from 24 ± 3°C. Light values of 0, 50, 100, 200, 300, 400, 500, 700, 900, 1100, 1300, and 1500 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation) and ambient CO_2 concentrations were applied to the leaves to develop the light response curves. Concentrations of 0, 100, 200, 300, 400, 500, 700, 900, 1100, 1300, and 1500 μmol mol^{-1} of CO_2 and s1400 μmol m^{-2} s^{-1} PAR were applied to leaves to develop A/C_i curves. The experiment was similar in 2004 with the exception that younger leaves were used, and only four vines were used.

Fluorescence measurements were taken with a Hansatech plant efficiency analyzer (Hansatech Instruments, Norfolk, England). Infected and control areas were measured twice (close to the same time) every 48 h and the average was used in the analysis. Leaves were dark acclimated for 30 minutes before measurements were taken.

Data analysis

Data obtained from the light experiments in both years include the maximum assimilation rate (A_{max}), light compensation point, and photosynthetic efficiency (Φ). In 2004, the dark respiration rate was also obtained. Data obtained from the CO_2 response curves included the maximum assimilation rate (A_{max}), assimilation rate at ambient CO_2 (A_{amb}), carboxylation efficiency (k), CO_2 compensation point (Γ), and intercellular CO_2 concentration (C_i). Stomatal conductance (g_s) was obtained directly from the

measurements. The stomatal limitation ($l_{g(ii)}$) was calculated according to Layne (1989). Fluorescence was expressed as the optimal yield of PSII, the ratio between variable fluorescence to maximum fluorescence (F_v/F_m). Data were plotted and subjected to nonlinear regression analysis using Sigmaplot software (Systat Software Inc, Richmond, CA) using the following equation from Layne (1989):

$$y = a \times (1.0 - b) \times e^{(-c \times X)}$$

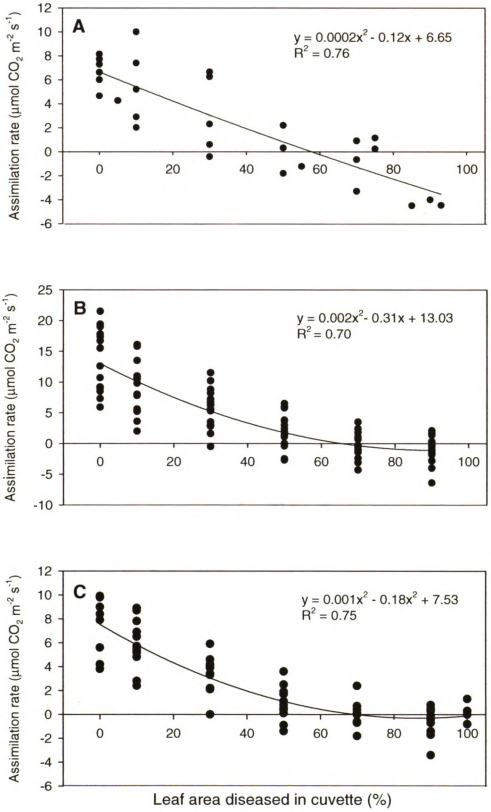
Where "y" is the net CO_2 assimilation, "x" is the intercellular CO_2 concentration, "a" is the asymptotic value of the curve, "b" is the minimum value of the curve, and "c" is the rate constant. The value of "e" is 2.7182818. Statistical analysis was done with Sigmastat statistical software using a repeated measures analysis of variance.

Results

Assimilation rates of leaves of downy mildew-infected 'Niagara' vines in the field

Downy mildew infection caused a significant reduction in photosynthesis after the appearance of symptoms. The assimilation rate of diseased leaves decreased with increasing severity of infection. There was a linear reduction with increasing disease severity in 2002 and a nonlinear reduction in 2003 and 2004. A 60% disease severity in 2002 and 70% severity in 2003 and 2004 produced a net assimilation rate of zero in symptomatic infections (Figure 2.3). There was a strong negative correlation between the assimilation rate and C_i in the infected leaf (Figure 2.4A).

Figure 2.3. Relationship between the carbon assimilation rate and increasing disease severity in field-grown *Vitis labrusca* 'Niagara' vines in 2002 (A), 2003 (B), and 2004 (C) measured in an 18-mm diameter cuvette of a CIRAS I infrared gas analyzer. The zero values for disease severity represent measurements on healthy leaves.


A
y = 0.0002x² - 0.12x + 6.65
R² = 0.76
Assimilation rate (µmol CO2 m-2 s-1)
B
y = 0.002x² - 0.31x + 13.03
R² = 0.70
Assimilation rate (µmol CO2 m-2 s-1)
C
y = 0.001x² - 0.18x² + 7.53
R² = 0.75
Assimilation rate (µmol CO2 m-2 s-1)
Leaf area diseased in cuvette (%)

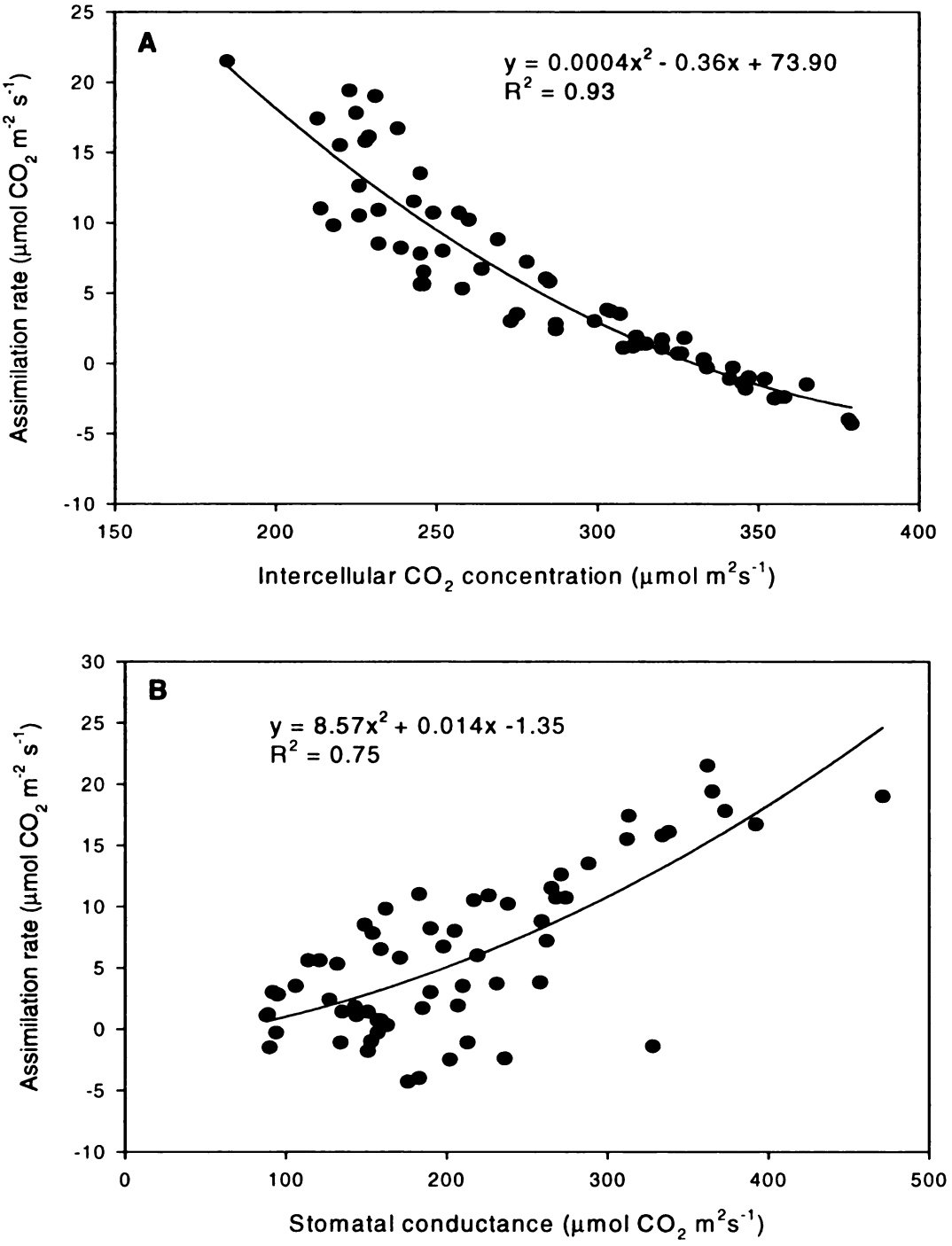


Figure 2.4. Relationship between carbon assimilation rate and intercellular CO_2 concentration (A) and stomatal conductance (B) in healthy and downy mildew-infected leaves of field-grown *Vitis labrusca* 'Niagara' vines measured with a CIRAS I infrared gas analyzer in Lawton, MI in 2003.

There was a positive correlation between the assimilation rate and stomatal conductance (Figure 2.4B). Stomatal conductance values increased with increasing assimilation rates.

Leaf photosynthesis in potted vines as infection progresses

In 2003, symptoms first appeared on leaves 7 days after inoculation as slight, pale yellow lesions which became darker and more distinct as the disease progressed. Symptoms appeared 8 days after inoculation in 2004. Infected leaves showed differences from the healthy leaves in photosynthetic efficiency, A_{amb}, carboxylation efficiency, intercellular CO_2, and A_{max} in 2003 and A_{amb} in 2003 and 2004 before symptoms appeared. However, chlorophyll fluorescence, the light compensation point, CO_2 compensation point, stomatal conductance, and stomatal limitation were not significantly affected until after symptoms appeared in 2003. Chlorophyll fluorescence did not decrease significantly until eight days after inoculation in 2003 and ten days after inoculation in 2004 (Figure 2.5A, B). In 2003, the light compensation point was variable, becoming significantly different from control values 8 days after inoculation (Figure 2.6A). In 2004, the light compensation point increased slowly and became significantly different from the control 10 days after inoculation (Figure 2.7B). The photosynthetic efficiency (θ) of infected leaves decreased significantly 6 days after inoculation in 2003 and 2 days after inoculation in 2004, although there was an increase in the control values 2 days after inoculation in 2004 (Figure 2.7A, B). The CO_2 compensation point (Γ) increased steadily in 2003 and became significantly higher than control values 6 days after inoculation (Figure 2.8A). In 2004 the CO_2 compensation point became significantly higher 8 days after inoculation and the infected half of the leaf showed more

variability (Figure 2.8B). Carboxylation efficiency (k) became significant 4 days after inoculation in 2003 and 2004 (Figure 2.9A, B) and decreased sharply 4 days after inoculation in 2003. Photosynthesis at ambient CO_2 decreased sharply at 2 days after inoculation and became significantly lower than control values 4 days after inoculation (Figure 2.10A). In 2004, the control values also decreased and photosynthesis in infected areas was lower on 4, 8, and 10 days after inoculation (Figure 2.10B). Maximum photosynthesis at increased CO_2 levels (A_{max}) decreased significantly 4 days after inoculation in 2003 and 6 days after inoculation in 2004 (Figure 2.11A, B). Control values in 2004 also dropped sharply 6 days after inoculation. Stomatal conductance decreased significantly in infected leaf areas 8 days after inoculation in 2003 and 10 days after inoculation in 2004 (Figure 2.12A, B). Stomatal conductance also decreased in healthy leaf areas in 2004. The stomatal limitation to photosynthesis was significantly lower in infected leaf areas 6, 8, and 10 days after inoculation in 2003, but was not significantly different from healthy leaves in 2004 (Figure 2.13A, B). The intercellular CO_2 concentration (C_i) increased sharply in infected leaf areas 6 days after inoculation in 2003 compared to healthy leaf areas (figure 2.14A). In 2004, C_i was only significantly higher on the 8th day after inoculation (Figure 2.14B). Dark respiration was not significantly affected by downy mildew infection of *P. viticola* (Figure 2.15). Table 2.1 shows the number of days after inoculation of leaves with *P. viticola* each parameter was affected in 2003.

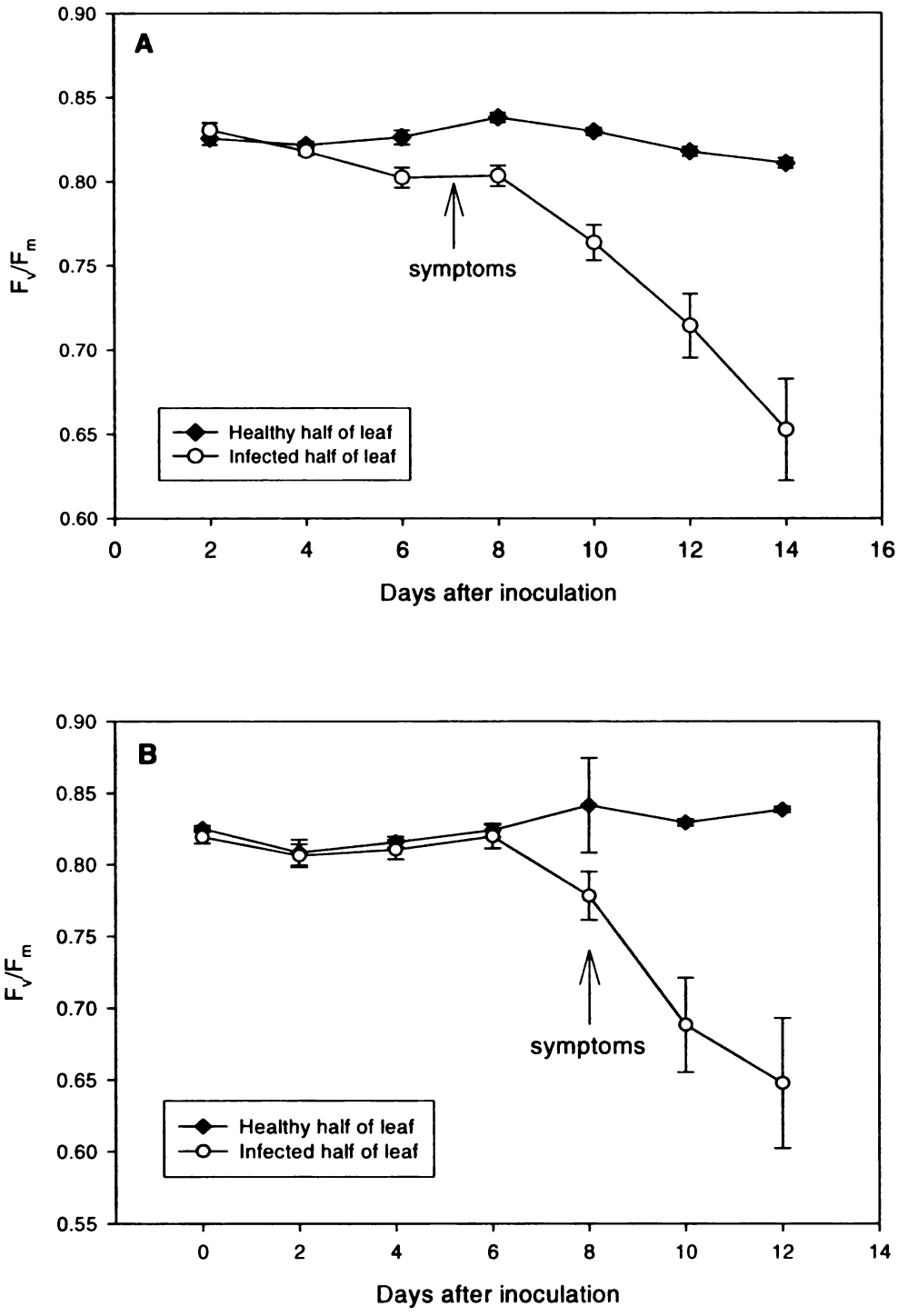


Figure 2.5. The effect of downy mildew disease development on chlorophyll fluorescence measured as the ratio of F_v/F_m in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

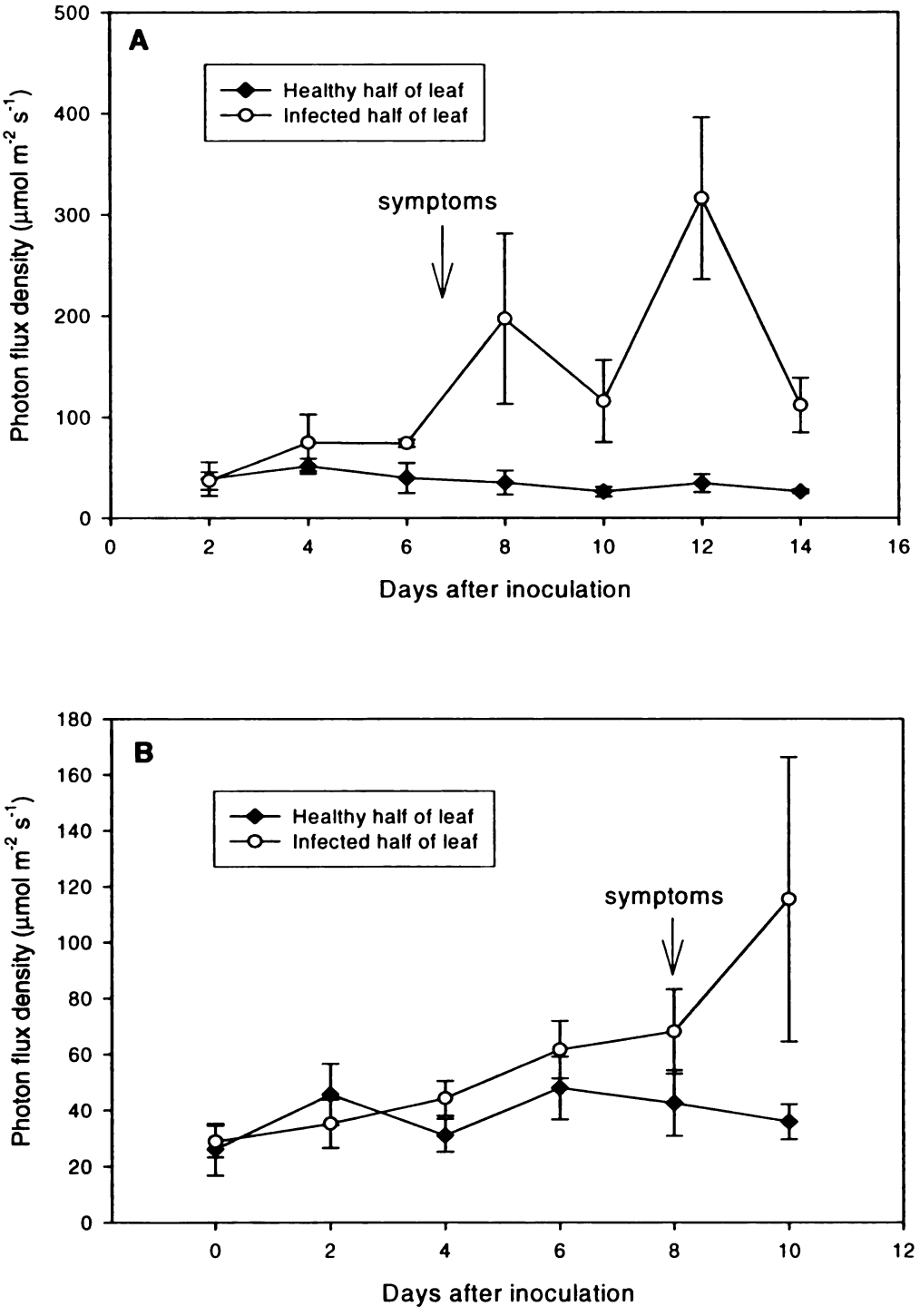


Figure 2.6. Effect of downy mildew disease development on the light compensation point in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

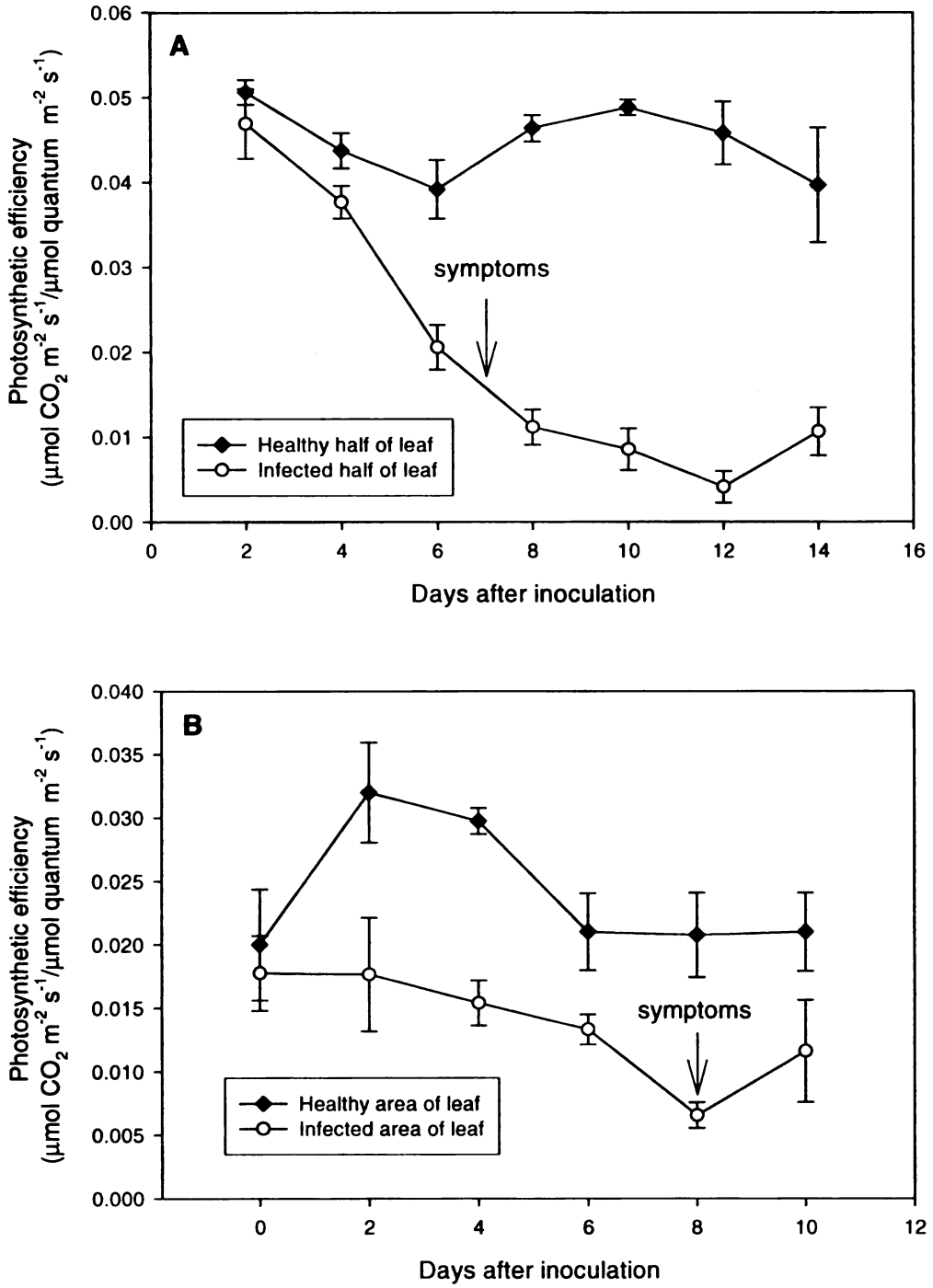


Figure 2.7. The effect of downy mildew disease development on the photosynthetic efficiency in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

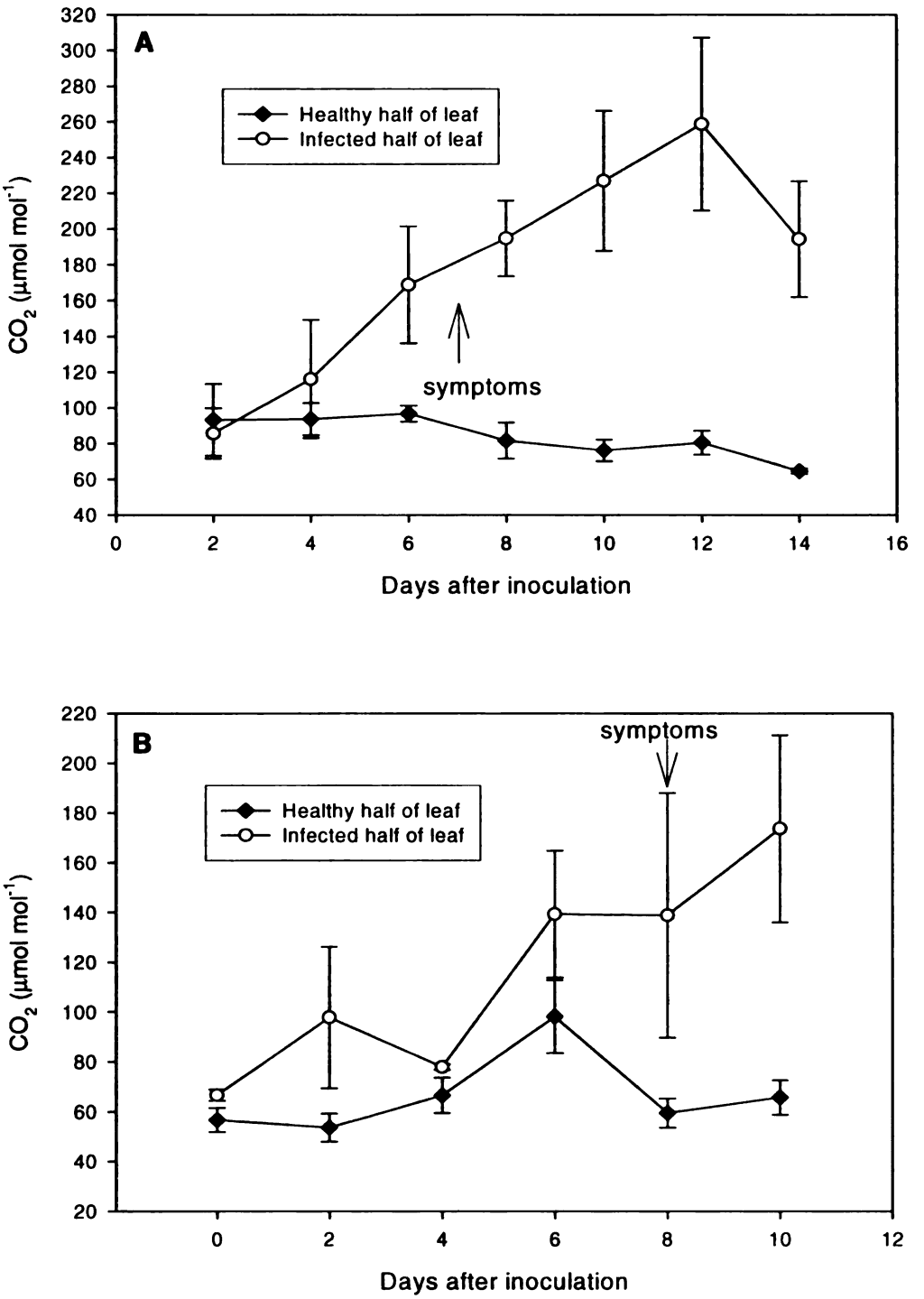


Figure 2.8. The effect of downy mildew disease development on the CO_2 compensation point in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

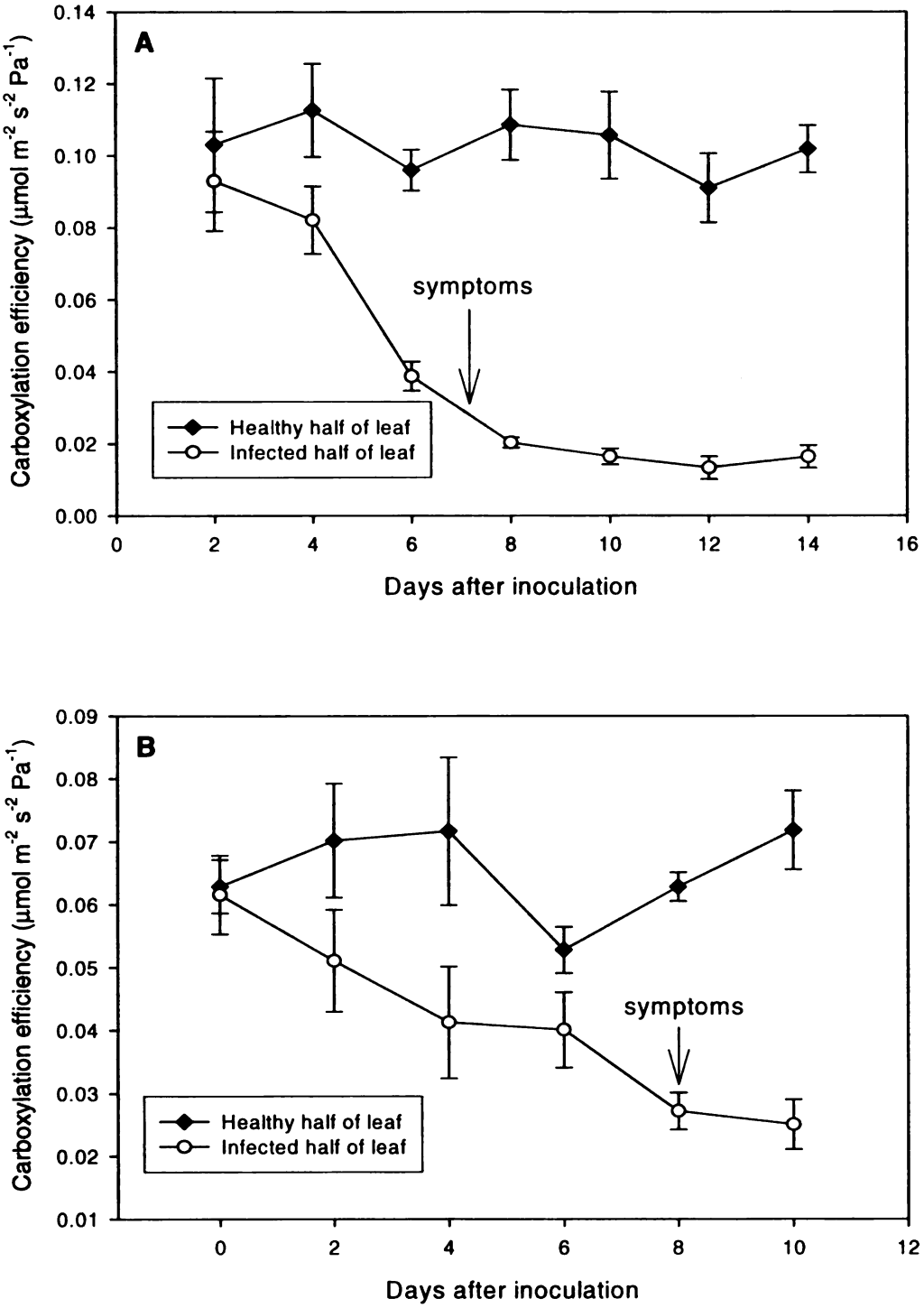


Figure 2.9. Effect of downy mildew disease development on the carboxylation efficiency in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

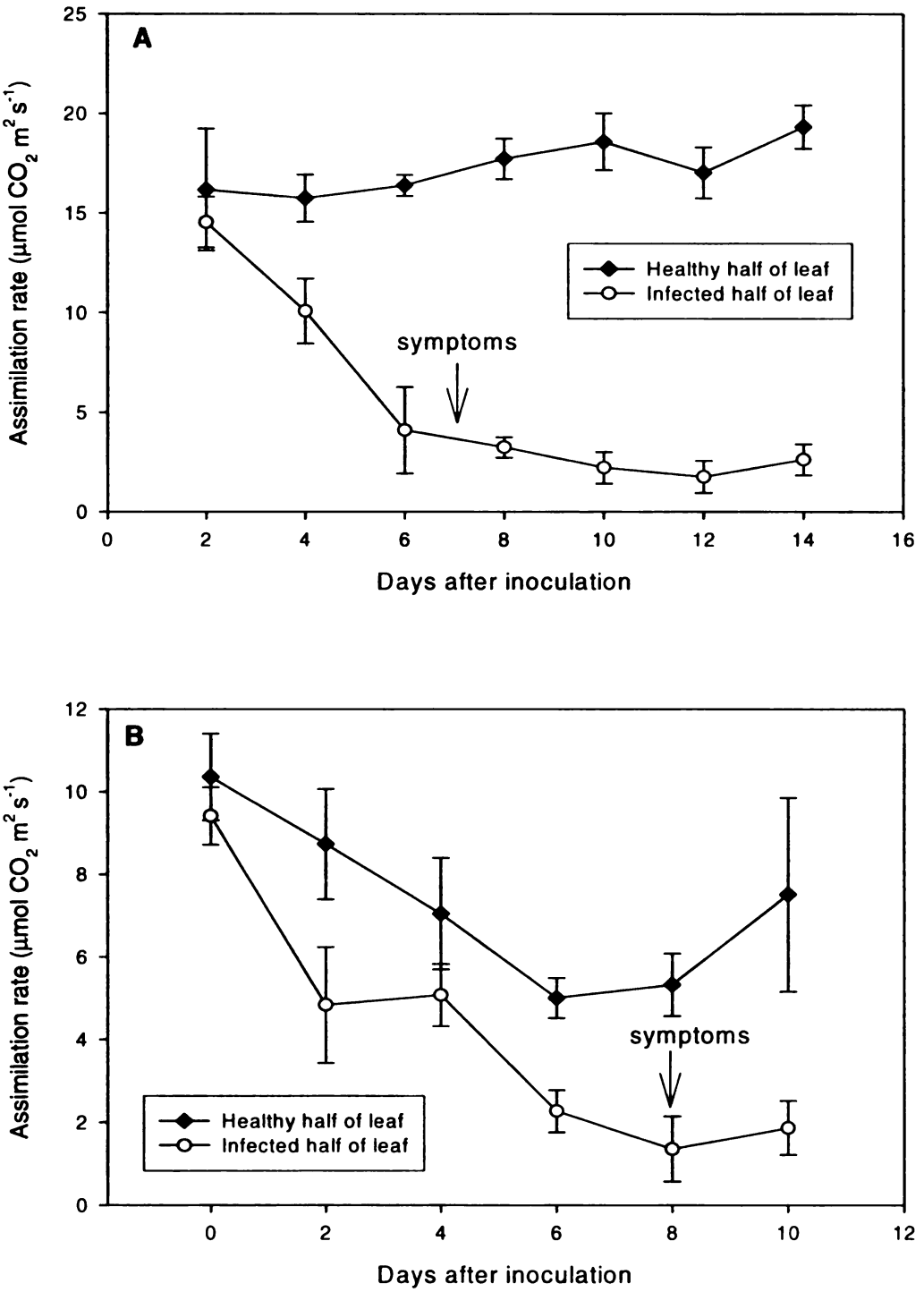


Figure 2.10. Effect of downy mildew disease development on the rate of photosynthesis at ambient CO_2 levels in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

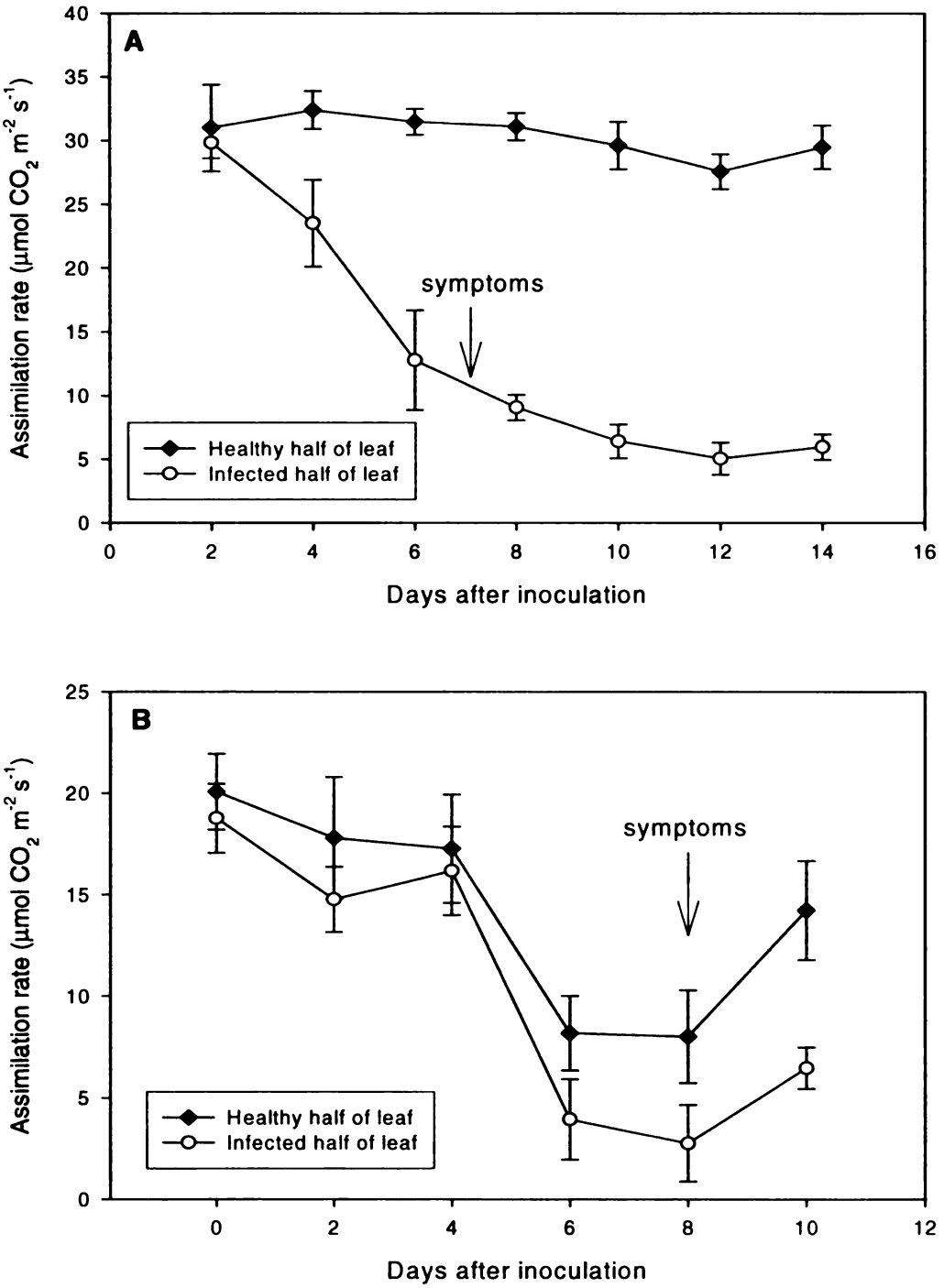


Figure 2.11. Effect of downy mildew disease development on the assimilation rate at increased CO_2 concentrations (1400 µmol mol^{-1}) in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

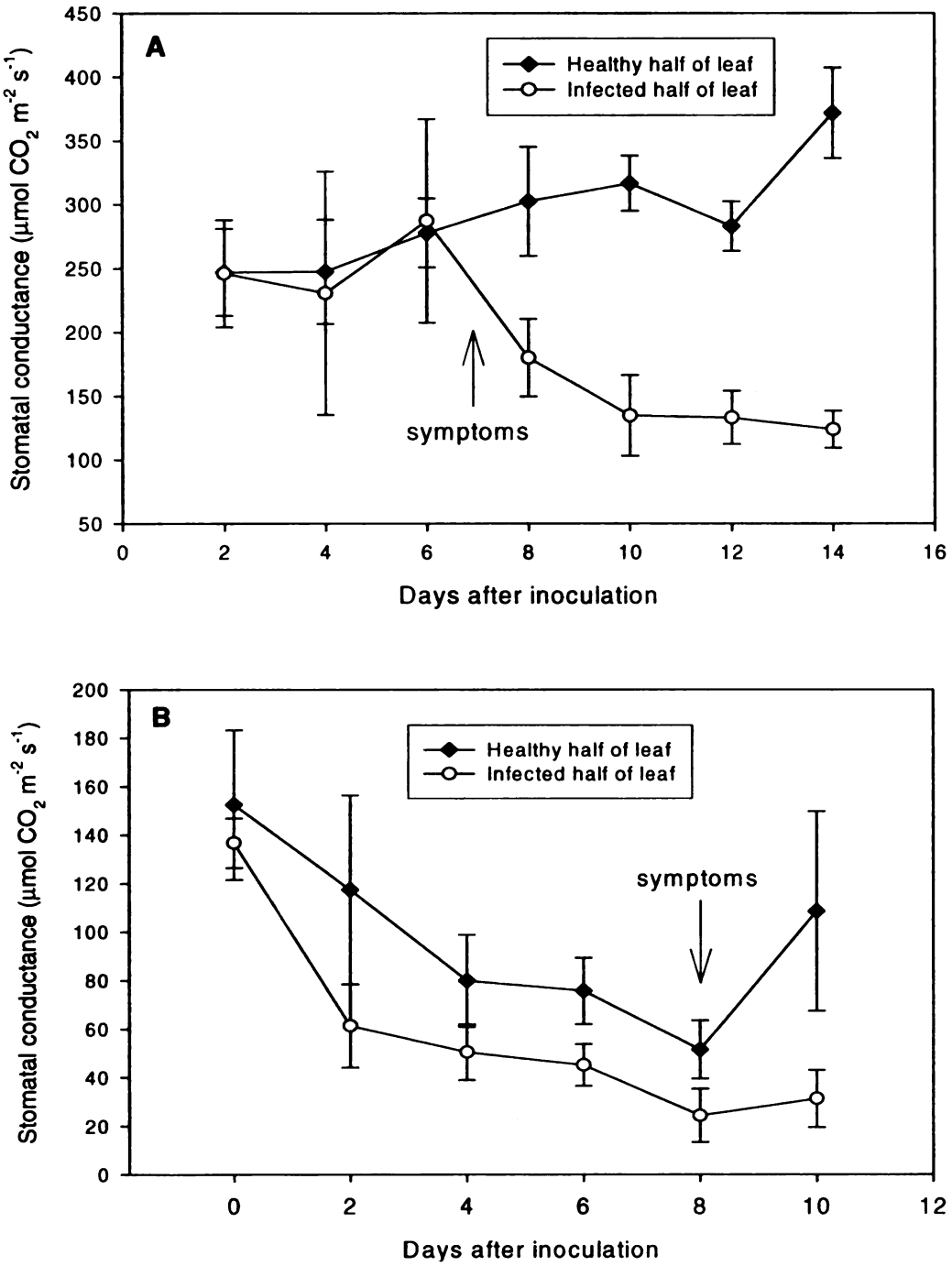


Figure 2.12. Effect of downy mildew disease development on the stomatal conductance in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

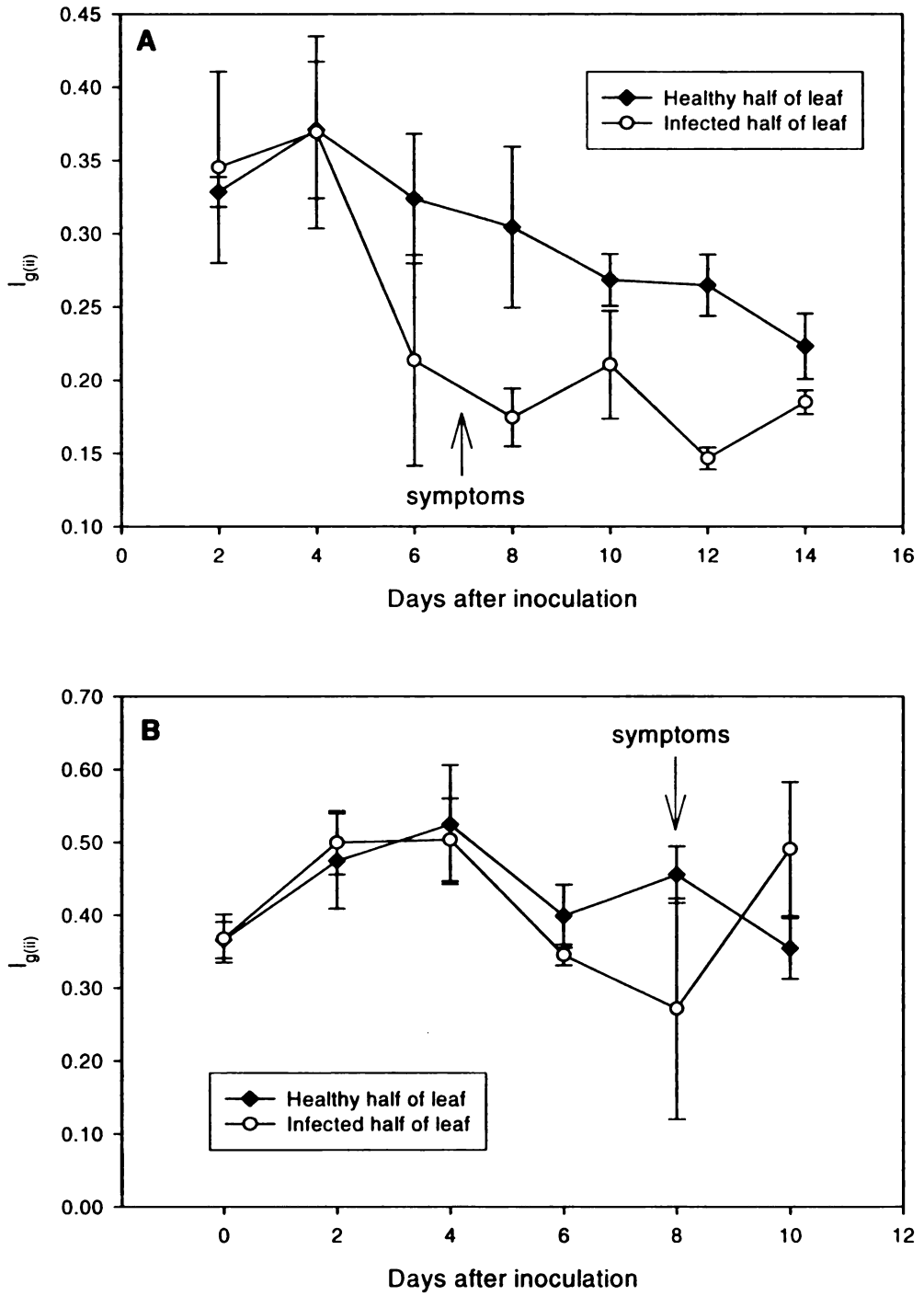


Figure 2.13. Effect of downy mildew disease development on the stomatal limitation to photosynthesis ($l_{g(ii)}$) in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

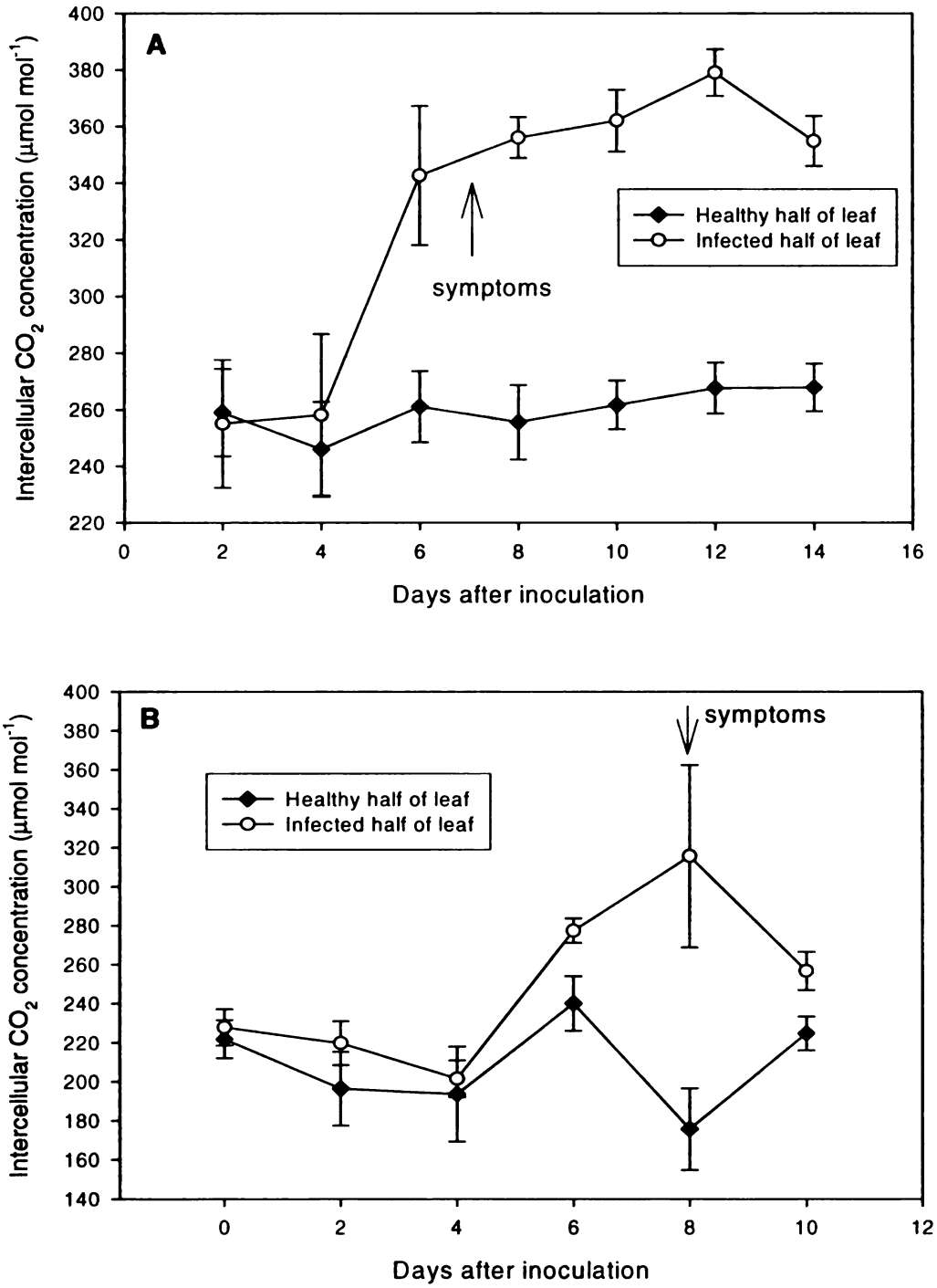


Figure 2.14. Effect of downy mildew disease development on the intercellular CO_2 concentration in leaves of two-year-old potted 'Niagara' grapevines in 2003 (A) and 2004 (B). Means are averages of five replications in 2003 and four replications in 2004. Bars indicate the standard error of the mean.

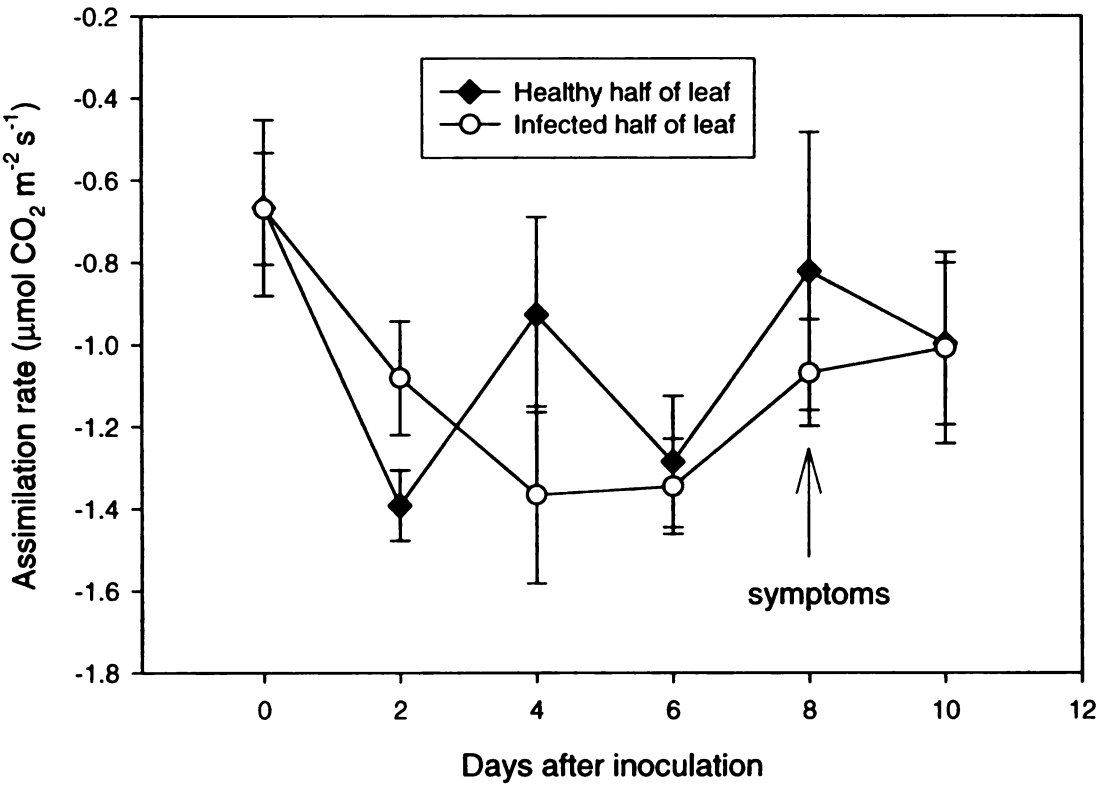


Figure 2.15. Effect of downy mildew disease development on dark respiration in leaves of two-year-old potted 'Niagara' grapevines in 2004. Means are averages of four replications. Bars indicate the standard error of the mean.

	Days after inoculation						
Parameter	**2**	**4**	**6**	**8**	**10**	**12**	**14**
Symptoms				X	X	X	X
Fluorescence				X	X	X	X
Light compensation point				X	X	X	X
Photosynthetic efficiency			X	X	X	X	X
Dark respiration							
Carboxylation efficiency		X	X	X	X	X	X
Intercellular CO_2			X	X	X	X	X
CO_2 compensation point			X	X	X	X	X
A_{amb}		X	X	X	X	X	X
A_{max}		X	X	X	X	X	X
Stomatal conductance				X	X	X	X
$l_{g(ii)}$			X	X		X	

Table 2.1. Photosynthetic parameters affected in 'Niagara' grapevine leaves inoculated with *Plasmopara viticola* in 2003. An "X" indicates a significant difference from healthy leaves at $P \leq 0.05$.

Discussion

Downy mildew infection of grape reduced net photosynthesis in the leaves of field-grown vines. The decrease in photosynthesis as severity increased in 2002 was more linear than in 2003 and 2004. A similar linear decrease in photosynthesis in *Vitis vinifera* infected with downy mildew was also reported by Giuntoli (2000). The differences between the seasons could be due to the age of infection in the leaves when measurements were taken. The nonlinear decreases in photosynthesis in 2003 and 2004 as disease severity increased could be a result of compromised healthy tissues surrounding the symptomatic areas or "oil spots". A similar effect was found for leaf blast on rice (Bastiaans 1991), Septoria leaf spot on blueberry (Roloff *et al.* 2004), and downy mildew on *Vitis vinifera* (Orlandini 1998). The effect could be the result of toxin production, a reduced relative water potential, or accumulation of carbohydrates at the host-pathogen interface (Bastiaans 1991, Scholes 1992). This would result in a larger relative reduction in photosynthesis under small disease severities due to the presence of more non-symptomatic tissue, some of which could be photosynthetically compromised.

The intercellular CO_2 concentration (C_i) of leaves had a strong negative relationship to the rate of photosynthesis, suggesting that C_i decreased as disease severity increased. Stomatal conductance increased with an increasing assimilation rate, but the relationship was weaker. The reduction in photosynthesis could be caused in part by reduced CO_2 flow across stomata. Sporulation through the stomata could reduce the flux of CO_2 across the stomata. Sporulation was present on the leaves of all vines measured in the field. Closure of the stomata from the physiological effects of the pathogen in symptomatic areas or non-symptomatic areas could also have this effect. Slight decreases in stomatal conductance have been measured in rust-infected barley (Owera *et al.* 1981).

Powdery mildew of grape also showed a decrease in stomatal conductance, but carboxylation efficiency and intercellular CO_2 concentration decreased as well (Nail and Howell 2004), suggesting the reduced assimilation rate may have been caused by biochemical as well as physical mechanisms.

Carboxylation efficiency, A_{max}, and A_{amb} were the first significant reductions in leaves infected with *P. viticola* relative to healthy leaf areas in 2003. Carboxylation efficiency and A_{amb} are indicators of Rubisco efficiency suggesting the ability of Rubisco to fix CO_2 is compromised 4 days after the infection process begins. Rubisco activity in powdery mildew and rust-infected plants have been attributed to a reduction of ribosomes and rRNA in chloroplasts and RNA metabolism (Ayres 1979, Chakravorty 1982, Walters 1985). A_{max}, a measurement of the ability to regenerate RuBP, also decreased significantly 4 days after inoculation. However, if Rubisco is compromised, RuBP levels will remain high, and the rate of photosynthesis will subsequently decrease.

Six days after inoculation, photochemical efficiency decreased significantly in infected leaves, while the CO_2 compensation point and intercellular CO_2 concentration increased significantly in 2003. Photosynthetic efficiency is the amount of CO_2 fixed relative to the amount of light absorbed. The amount of RuBP produced in infected leaves cannot be used by Rubisco due to decreasing Rubisco efficiency and therefore, the ability to fix CO_2 decreases while the same amount of light is being absorbed. As a result, photosynthetic efficiency decreases. However, reductions in photosynthesis in the chloroplasts of rust-infected beans were shown to be a result of reduced electron transport (Sziraki *et al.* 1984). The sharp increase in the intercellular CO_2 concentration in infected leaves 6 days after inoculation suggest there is a mesophyll limitation to CO_2 diffusion,

which can also be attributed to Rubisco damage. As damage to Rubisco occurs, its ability to use CO_2 as a substrate becomes limited and intercellular CO_2 levels increase relative to levels in healthy leaves. The CO_2 compensation point can also be affected by Rubisco. Transgenic tobacco plants with less Rubisco than control plants showed substantially higher CO_2 compensation points (Lawlor 2001). The CO_2 compensation point can also be affected by temperature and light, but no significant change in the compensation point was seen in the healthy grape leaves, suggesting that the increase in infected leaves was caused by the pathogen. The stomatal limitation to photosynthesis ($l_{g(ii)}$) was significantly lower in infected leaves at 6, 8, and 12 days after inoculation. As the mesophyll limitation to photosynthesis (Rubisco limitation) increases in infected plants, the percent of stomatal limitation should decrease which is further evidence that the decrease in photosynthesis in infected leaves is a result of a mesophyll limitation.

The light compensation point and chlorophyll fluorescence increased significantly 8 days after inoculation, while the stomatal conductance decreased significantly 8 days after inoculation. Similar levels of the light compensation point in infected leaves before symptoms compared to healthy leaves suggest that infected leaves have the ability to fix CO_2 at low light levels even with damage to Rubisco. At low light levels, the production of ATP and NADPH and subsequently, the regeneration of RuBP are limiting. The damage to Rubisco in infected leaves by day 8 may be enough to become the limiting factor to photosynthesis, or possibly damage to the electron transport system has occurred, either of which could increase the compensation point. The increase in chlorophyll fluorescence (measured as a decrease in F_v/F_m) after symptoms appeared could also be a result of damage to the electron transport system. Light energy absorbed

by PSII can no longer be used to produce ATP and NADPH, and a relatively larger proportion of the excess absorbed energy is emitted as fluorescence. The increase in fluorescence could also be a result of damage to PSII. Phytoplasma infection of apple leaves was shown to inhibit photosystem II efficiency and electron transport chain activity (Bertamini *et al.* 2002), but the increase in fluorescence after the appearance of symptoms in downy mildew-infected leaves suggests that damage to photosystem II is not the initial cause of the decreased rates of photosynthesis in infected leaves.

The decrease in stomatal conductance 8 days after inoculation suggests that there was a stomatal closure or blockage, but this was not the primary cause of the reduction in photosynthesis rates in infected leaves since there was still a greater stomatal limitation ($l_{g(ii)}$) in healthy leaves compared to infected leaves. Decreased photosynthetic rates on powdery mildew-infected wheat, barley, and oak leaves have also been shown not to be a result of stomatal closure or blockage (Ayres 1979).

A stomatal limitation could be imposed initially during the infection process of *Plasmopara viticola* when the germ tube is formed in the substomatal cavity, but the restriction is short lived because the primary hypha then extends into the mesophyll. Therefore, it is not likely that this would cause a substantial reduction in the assimilation rate. Sporulation through stomata could also cause a stomatal limitation, but this usually occurs when lesions are well developed, and by this time in the infection process numerous other physiological processes have been photosynthetically compromised in the leaf.

The results in 2004 were more variable and photosynthesis was generally lower in healthy leaves than in 2003. The lower rates of photosynthesis were due in part to

younger leaf age in 2004. Also, lesions that developed did not fill the entire cuvette area in two of the leaves that were measured. The decreases in photosynthetic efficiency, carboxylation efficiency, maximum assimilation rate at ambient CO_2 (A_{amb}), maximum assimilation rate at 1400 μmol mol^{-1} CO_2 (A_{max}), and stomatal conductance in healthy leaf tissue in 2004 suggest there may be some other factor affecting these parameters. The effects could be due to temperature fluctuations in the greenhouse, the amount of natural light the plants were receiving during the experiment, or from moving the plants while performing the experiment

The reduction in the photosynthetic rate in infected leaf tissues may be caused by physical or biochemical limitations to CO_2 assimilation. The data from the response curves suggest that the reduction in photosynthesis in infected leaves is due to a mesophyll limitation rather than reduced photosystem II efficiency or a stomatal limitation. Similar effects were found with Fusarium wilt on tomato (Nogues 2002), phytoplasma infection on apple (Bertamini *et al.* 2002), powdery mildew on sugar beet leaves (Gordon and Duniway 1982), and grapevine fan leaf virus-infected grapes (Sampol *et al.* 2003). Understanding the infection process is important for finding ways to manage diseases and preventing economic losses. Since carbon allocation in the vine is ultimately affected by the carbon assimilation mechanisms, the reductions in photosynthesis in vine leaves caused by downy mildew could potentially reduce the amount of energy available for dry matter accumulation and thus affect crop yield, quality, and vine hardiness.

In conclusion, reduced rates of photosynthesis in leaves of 'Niagara' vines infected by *P. viticola* are likely biochemical in nature and could be a result of damage to

photosynthetic enzymes (such as Rubisco) and/or decreased rates of RuBP regeneration possibly due to damage to electron transport components.

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Chapter 3

The effect of downy mildew infection on carbon partitioning and biomass accumulation in 'Niagara' vines

Introduction

Pathogen effects on carbon allocation

Growth of crops is usually measured by the amount of dry weight added over a period of time and determined by destructive sampling (Long 1985). Infection by plant pathogens often results in a change in the movement of carbon assimilates among plant organs (de Nooij 1992). Pathogens invading leaves have access to large pools of carbon and can cause a movement of photoassimilates into infected areas, as well as a reduction of export to other organs. Foliar pathogens frequently increase the shoot:root ratio so higher proportions of assimilates in infected plants are exported to the shoots (Farrar 1992, Walters 1985). In potato leaves infected with *Phytophthora infestans*, there was a retention of assimilates at the infection sites (Farrell 1971). Carbohydrate concentrations were found to be as much as ten times higher in the immediate area of rust infection on bluegrass (Hodges and Robinson 1977). Assimilates are known to accumulate in radish leaves infected with downy mildew (Coffey 1975, Williams 1964), but it is not known whether they are retained by the pathogen or imported from other sites (Walters 1985).

The flux of carbon into the apoplast of plants has been shown to be sufficient to support fungal growth (Ahmad *et al.* 1982, Farrar 1992, Kneale and Farrar 1985). The photosynthetic capacity of one mesophyll cell of barley was shown to be enough to

support a colony of powdery mildew, suggesting that the carbohydrate supply was not likely to be limiting to growth of powdery mildew (Farrar 1987). Accumulation of carbohydrates in the leaf at the sites of infection generally appears to be at the expense of the roots or non-infected leaves (Walters 1985).

Effects of biotrophic pathogens on carbon allocation

Biotrophic pathogens, unlike necrotrophic fungi, depend upon the host metabolism for nutrients (Wright *et al.* 1995). Because of the close nutritional association of biotrophic pathogens with their hosts, studies on the effects of carbon assimilation and metabolism caused by the infection of these types of pathogens have been more numerous than for other types of pathogens (Daly 1976). Biotrophic pathogens can cause internal recycling of CO_2 through repeated respiration and photosynthesis and, as a result, there is a smaller amount of net photosynthates available for translocation (Farrar 1987).

Leaves infected by biotrophic pathogens often show a large increase in invertase activity. Sucrose hydrolization (into glucose and fructose) requires the enzyme invertase, but it is difficult to tell whether invertase is produced by the plant or pathogen. Downy mildew (*Plasmopara viticola* (Berk. & Curt.) Berlese & de Toni) infection on leaves of grape (*Vitis vinifera* L.) caused a relatively small increase in invertase activity compared to powdery mildew infection (*Uncinula necator* (Schwein.)) (Brem *et al.* 1986).

The increased accumulation of carbohydrates resulting from an increase in invertase activity can eventually cause a down-regulation of the Calvin cycle leading to inhibition of photosynthesis (Scholes *et al.* 1994). Reduced photosynthesis can reduce the potential sucrose supply for translocation from infected leaves to other parts of the plant.

Brown rust (*Puccinia hordei* Otth.) infection has been shown to reduce carbon translocation in barley leaves (Owera *et al.* 1983). Infection of grapevine leaves by biotrophic pathogens can also have significant effects on carbon partitioning in grapevines. Downy mildew (*P. viticola*) has been shown to cause a depletion of sucrose in grape leaves (Brem *et al.* 1986). 'Concord' grapevines infected with powdery mildew showed reduced berry quality and wood maturity (Gadoury *et al.* 2001).

Carbon isotope composition in plant tissues

Two carbon isotopes occur in nature, one being the more abundant ^{12}C, and the other, the less abundant ^{13}C. The isotope ^{13}C comprises approximately 1.11% of the total carbon (Boutton 1991, Farquhar *et al.* 1989). The ratio of $^{13}C/^{12}C$ varies slightly in natural materials due to fractionation during chemical, physical, and biological processes (Farquhar *et al.* 1989). Very small differences in the carbon fractionation of materials can be measured by mass spectrometry and can be of use to biologists (Boutton 1991).

Most of the natural isotopic variation of interest to biologists results from the fractionation of carbon during photosynthesis (Boutton 1991, Farquhar *et al.* 1989). Carbon isotopes in plant tissues are measured as the relative difference between the isotope ratios of the sample and standard gases, and are known as the delta (δ) notation. The $\delta^{13}C$ values are expressed relative to a calcium carbonate standard, Pee Dee Belemnite (PDB), a limestone fossil formation of *Belemnitella americana* of the Cretaceous period (Boutton 1991). The $\delta^{13}C$ value is calculated as the $^{13}C/^{12}C$ ratio of the sample relative to the PDB standard as follows:

$$\delta\,^{13}C = [\,(R_{sample} - R_{standard})/\,R_{standard}] \times 1000$$

where $\delta^{13}C$ is the parts per thousand difference in ^{13}C between that of the sample and the standard, R_{sample} is the $^{13}C/^{12}C$ mass ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ mass ratio of the Pee Dee Bee Belemite (PDB) limestone standard which has a value of 0.0112372 (Boutton 1991).

The abundance of ^{13}C in plant tissues is normally lower than that of atmospheric carbon dioxide largely due to isotopic fractionation (change in the ^{13}C fraction) during the photosynthetic process (Boutton 1991, Oleary 1988). In C_3 plants, the enzyme Rubisco discriminates against the reaction of $^{13}CO_2$ due to small differences in chemical and physical properties imparted by the difference in the mass of the isotopes (Farquhar *et al.* 1989, Oleary 1988). Plants, including grapevines, using the conventional C_3 pathway to fix carbon have relatively low ^{13}C values because CO_2 is reduced to phosphoglycerate by the enzyme Rubisco which discriminates against $^{13}CO_2$ (Boutton 1991). Conversely, C_4 plants reduce CO_2 to aspartic or malic acid via the enzyme PEP carboxylase which discriminates less against ^{13}C than Rubisco. Therefore, C_3 plants generally have lower $\delta^{13}C$ values ranging from -32 to -20, whereas C_4 plants have $\delta^{13}C$ values ranging from -17 to -9 (Bender 1971, Boutton 1991, Farquhar 1982). Tissue samples of *Vitis vinifera* grapes were shown to have ^{13}C values of -24.6 to -26.3 (Dimarco *et al.* 1977). Fractionation can also occur through limitations to diffusion of CO_2 into the leaf, allowing some aspects of stomatal control of photosynthesis to be studied (Ehleringer 1991, Oleary 1988). Natural variation in carbon isotope abundance can be useful in studying biochemical and physiological characteristics of photosynthesis

in plants under various environmental stresses such as drought stress (Ehleringer 1991, Long 1985, Oleary 1988). Isotopic enrichment can also be used to study storage and mobilization of carbon compounds, carbon transport between root grafts or mycorrhizal connections, and the effects of herbicides or disease on carbon allocation (Svejcar *et al.* 1990).

Isotopes and carbon partitioning in grapevines

Carbon isotope labeling has been a valuable tool in studying the carbon partitioning among sinks in plants. Plants can be enriched with ^{13}C or ^{14}C through the uptake of $^{13}CO_2$ or $^{14}CO_2$. Translocation of carbon from leaves has been measured by the loss of the radioactive isotope ^{14}C from the leaf after being pulsed with labeled $^{14}CO_2$ (Wardlaw 1990). Downy mildew has been shown to cause a change in assimilate translocation in infected grape leaves using isotopic labeling (Brem *et al.* 1986, Owera *et al.* 1983). Mature grape leaves labeled with ^{14}C have shown that assimilates can be translocated acropetally to young leaves and shoots or basipetally to clusters and permanent vine structures depending on their proximity to the sink (Hale 1962). Leaves of 'Delaware' fed with ^{14}C exported an increasing percentage of assimilates as the number of fruit clusters increased (Motomura 1990). A high percentage of these assimilates came from the side of the plant in which the clusters were located. Top leaves generally supply carbon to the apical buds and young leaves, middle leaves supply to the stem, and basal leaves supply carbon to the stem and roots (Motomura 1990, Palit 1985).

Rationale and objectives

Little is known about the effect of downy mildew infection on the movement of carbon in plants. However, grapevines infected with *P. viticola* do show the ability to compensate for leaf damage by mobilization of reserves from the roots (Jermini 2003). Stable carbon isotope (^{13}C) techniques can be used to follow the movement of carbon in plants and determine the effect pathogens have on carbon allocation. The use of stable carbon isotopes in agricultural research has recently become more frequent due to the regulatory constraints and radioactive properties associated with ^{14}C labeling (Svejcar *et al.* 1990). In addition, photosynthetic and metabolic processes discriminate much less against the ^{13}C isotope than ^{14}C (Van Norman 1952) making ^{13}C attractive for studying pathogen effects on carbon allocation in plants. These studies may ultimately aid in understanding the effects of pathogens on carbon assimilation and allocation. Understanding the effects pathogens have on biomass accumulation and carbon allocation in plants will aid in developing management practices that incorporate minimal fungicide use and optimize plant growth and yield.

The objectives of this experiment were to: 1) Determine the effect of *P. viticola* infection on dry weight accumulation and partitioning in 'Niagara vines and, 2) Determine the effect of *P. viticola* infection on short-term carbon movement in vines by using stable isotope labeling.

Materials and Methods

Effect of downy mildew on plant dry weight and dry weight partitioning

Two-year-old bare-rooted 'Niagara' vines were planted in 5-gallon pots in a mixture of 80% loam and 20% sand. Plants were grown outdoors from the beginning of July until the end of September at the Plant Pathology farm, Michigan State University, East Lansing, MI. Vines were fertilized every two weeks with a soluble 20-20-20 N-P-K fertilizer at a rate of 0.2 g N per vine. All vines were sprayed every two weeks at the recommended rates with Sevin (carbaryl) (Bayer Cropscience, Research Triangle Park, NC) to control Japanese beetle and Nova (myclobutanil) (Dow Agrosciences, Indianapolis, IN) to control powdery mildew. Control vines were sprayed once a month at the recommended rate with Ridomil Gold MZ (mefenoxam and mancozeb) (Syngenta Crop Protection, Greensboro, NC) to prevent downy mildew infection.

Inoculation

Vines were arranged in a completely randomized design and inoculated with a suspension of distilled water and *P. viticola* sporangia collected from infected field-grown 'Niagara' vines in Lawton MI. The suspension was adjusted to 5 x 10^4 sporangia per ml using a hemacytometer. Inoculations were done between 0500 and 0700 h in the first week of August and the beginning of September. The undersides of the leaves were sprayed with the suspension using an atomizer to provide uniform coverage for infection. Vines were covered with plastic for five hours after the inoculation to retain moisture. Two treatments and a control were used. Each treatment was replicated five times (one vine per replicate) in a completely randomized design. One treatment was inoculated at

the 5-mm berry stage and the other at bunch closure. Control vines were kept free of infection. Three separate control vines of the same age with fruit were exposed to the same conditions and used to determine the growth stages, but were not used in the analysis. The disease severity was estimated at harvest as a percent of the total leaf area on the vine covered by downy mildew lesions. Abscised leaves were collected and added to the remaining leaf samples at harvest. Vines were destructively harvested at the end of September, seven weeks after the first inoculation, and separated into leaves, canes, and roots. Samples were then placed in a drying oven at 65°C for 7 days and dry weights were obtained for each tissue type.

Statistical analysis

Statistical analysis was done using an analysis of variance with Sigmastat statistical software (Systat Software Inc, Richmond, CA). Regression analysis was performed using Sigmaplot statistical software (Systat Software Inc, Richmond, CA).

^{13}C translocation and dry weight accumulation in infected vines

Eight two-year-old, bare-rooted 'Niagara' vines were potted in 10-gallon pots in a mixture of 80% loam and 20% sand in 2003. Vines were arranged in a completely randomized design. Four vines were inoculated and four vines were used as non-inoculated controls. Plants were allowed to grow outdoors for two summers at the Plant Pathology Research Farm, Michigan State University, East Lansing, Michigan. Vines were fertilized every two weeks with a soluble 20-20-20 N-P-K fertilizer at a rate of 0.2 g N per vine. All vines were sprayed every two weeks with Sevin to control Japanese beetle

and Nova to control powdery mildew. Control vines were sprayed with Ridomil Gold MZ to prevent downy mildew infection.

Inoculation

Vines were initially inoculated with *P. viticola* beginning in August (around bunch closure) and inoculations continued once every week for five weeks to simulate a natural infection process. Flowers that developed on vines were removed. Inoculations were done by washing sporangia from infected leaves taken from field-grown vines in Lawton, MI. Infected leaves were incubated in the dark for 12 h at 22°C to obtain fresh sporangia for inoculations. Sporangia were suspended in sterile, distilled water, and the concentration was adjusted to 5 x 10^4 sporangia per ml using a hemacytometer. The abaxial sides of the leaves were sprayed with the suspension using an atomizer. Inoculations were done in the morning between 0400 and 0600 h while leaves were wet from dew to aid in the infection process and to eliminate the need to cover leaves with plastic bags to retain moisture.

Administration of $^{13}CO_2$ to plants (pulsing)

Six weeks after the initial inoculation, all plants were exposed to CO_2 labeled with ^{13}C. Vines were covered with plastic bags and the opening of the bag was sealed to the pots to create an airtight chamber around each plant. Two 6-mm holes were drilled into each of the pots 5 cm above the soil line, and plastic tubing was installed in each hole to allow $^{13}CO_2$ to enter the chamber. $^{13}CO_2$ was produced by mixing 20 ml of 85% lactic acid (Baker, Phillipsburg, NJ, USA) with 600 mg $Ba^{13}CO_3$ (Icon Isotopes, Summit, NJ,

USA) per plant. The reagents were mixed using 20-ml syringes. Syringes were attached to the application tube by a two-way valve. Upon mixing, $^{13}CO_2$ was released into the chamber. Administration of $^{13}CO_2$ was done between 0900 and 1200 h. Plastic chambers were removed from plants 30 minutes after administration of $^{13}CO_2$.

Sampling for ^{13}C

Sampling of plant tissues was done at 1 h, 24 h, 48 h and 7 days after the administration of $^{13}CO_2$. Tissue was sampled randomly at every interval from healthy leaves (HL), non-symptomatic tissue on diseased leaves (HDL), symptomatic (chlorotic) leaf tissue from well-developed lesions (DL), and roots (R). New shoots (current year's growth) and woody tissues (stem and older canes) were sampled at 7 days only. Samples from all tissues of plants that were not exposed to $^{13}CO_2$ were taken to determine a baseline for natural abundance of ^{13}C relative to the experimental vines. Harvested samples were placed in aluminum foil packs and immediately placed in liquid nitrogen to stop respiration. They were subsequently transferred to a freezer at -80°C for storage prior to processing.

Dry weight analysis

The vines were destructively harvested at 7 days, and total leaf area and disease severity were determined. Leaf area was measured with a leaf area meter to obtain total leaf area for each vine. Disease severity was estimated on each leaf, and disease severity on the entire vine was calculated as a ratio of total diseased area relative to total leaf area.

Harvested plant material was dried in a drying oven at 65°C for 7 days to obtain dry weights from leaves, roots, shoots, and woody parts (stem and older canes) of the vines.

Stable isotope analysis

Frozen samples were removed from the freezer and placed in a drying oven at 65°C for seven days. Once dry, leaf and root samples were ground to a powder in liquid nitrogen using a mortar and pestle. Cane samples were ground using a Wiley Mill with a 40-mesh screen. A 1 to 2-mg sample of each of the tissues was weighed with a microgram scale and placed in pressed tin capsules (Costech Analytical Technologies, Valencia, CA) for analysis. One sample of each tissue type from each plant was taken. Samples were sent to the UC Davis Stable Isotope Facility (University of California, Davis, CA) and analyzed for ^{13}C content using a mass spectrometer (Integra automated carbon and nitrogen gas analyzer, Sercon Ltd., Cheshire, U.K.).

Data analysis

The carbon isotope composition ($\delta\ ^{13}C$) was calculated by the following formula:

$$\delta^{13}C = [(R_{sample} - R_{standard})/ R_{standard}] \times 1000$$

where $\delta^{13}C$ is the parts per thousand difference in ^{13}C between that of the sample and standard, R_{sample} is the $^{13}C/^{12}C$ mass ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ mass ratio of the Pee Dee Bee Belemite (PDB) limestone standard which has a value of 0.0112372.

From this value several other values can be calculated in order to determine the amount of ^{13}C in plant tissues. The absolute ratio (R) of a sample is calculated by the formula:

$$R = {}^{13}C/{}^{12}C = [(\delta^{13}C/1000) + 1] \times R_{PDB}$$

Where R_{PDB} equals 0.0112372. From the absolute ratio (R), the fractional abundance (F) of ^{13}C in plant tissue can be calculated as follows:

$$F = R/(R+1)$$

Atom % is defined as the percent of ^{13}C in plant tissues and is calculated as:

$$\text{Atom \%} = F \times 100$$

The amount of ^{13}C in the plant was calculated by multiplying the amount of carbon in the sample (mg) by its corresponding fractional abundance. The fractional abundance of ^{13}C in each sample was adjusted for plant size by multiplying it by the fraction of each plant's dry weight relative to the largest plant. The amount of ^{13}C attributed to labeling was determined by subtracting the ^{13}C present in the natural abundance of the tissue from the total amount of ^{13}C in each tissue sample. The amount of ^{13}C in each plant organ was expressed as the ratio of excess ^{13}C (amount of ^{13}C

attributed to labeling) in the plant to the excess in each plant organ at the time of sampling.

Statistical analysis

Statistical analysis using a repeated measures analysis of variance and multiple comparisons using the Fisher's LSD was used to separate differences in isotope composition at sampling time. Separation of means of plant samples collected at seven days was performed with a t-test. All statistical testing was done using Sigmastat statistical software.

Results

Effect of downy mildew on plant dry weight and dry weight partitioning

Total estimated disease severity of the infected vines at the time of plant harvest in 2003 averaged 22.5% for vines inoculated at the 5-mm berry stage and 16% for vines inoculated at bunch closure. Total disease severity of the infected vines in 2004 averaged 11.4% of the total leaf area at the time of plant harvest. Whole vine estimates of disease severity in 2003 gave an appearance of a higher overall disease severity than estimates done on a leaf by leaf basis in 2004 due to the precision of the leaf area meter.

'Niagara' vines inoculated with *P. viticola* in 2003 accumulated less dry weight than non-inoculated vines (Figure 3.1). Vines in 2004 did not show a significant difference in total dry weight (Figure 3.2). Vines inoculated at the 5-mm berry stage and bunch closure did not show any significant differences in the percent of dry weight partitioning between the leaves, canes, or roots compared to non-inoculated vines in 2003

(Figure 3.3). Vines inoculated in 2004 showed a significantly higher percent of dry weight allocation to the new shoots of infected vines and a lower percent of dry weight allocation to the woody parts of the vine compared to the control vines (Figure 3.4). There was no significant difference in percent of dry weight allocation in the leaves or roots. The 2004 vines showed a higher leaf dry weight and lower root dry weight than vines in 2003. The total dry weight of the canes was similar in 2003 and 2004. There was a significant negative relationship ($P<0.05$) between severity of the infection and leaf dry weight (Figure 3.5), and between severity and total dry weight, but the latter relationship was not as strong (Figure 3.6).

^{13}C translocation in infected vines

The total enriched ^{13}C content was slightly higher in the leaves and lower in the roots of non-infected plants compared to the infected plants 7 d after exposure to $^{13}CO_2$, but the differences were not significant (Figure 3.7). Total enriched ^{13}C in the leaves of infected vines was similar to the control vines after seven days, but the rate at which the carbon was translocated was different. Infected leaves retained the labeled carbon longer than the non-infected leaves. Labeled carbon accumulation in the roots showed similar, but opposite trends (Figure 3.8). Roots of non-infected plants accumulated more labeled ^{13}C in 24 hours than infected plants, but after 168 hours the amount of ^{13}C in the roots was similar (Figure 3.9). Symptomatic tissues accumulated nearly twice the amount of ^{13}C in 24 hours as they had at the time of exposure to $^{13}CO_2$, decreasing slightly after 24 hours (Figure 3.10).

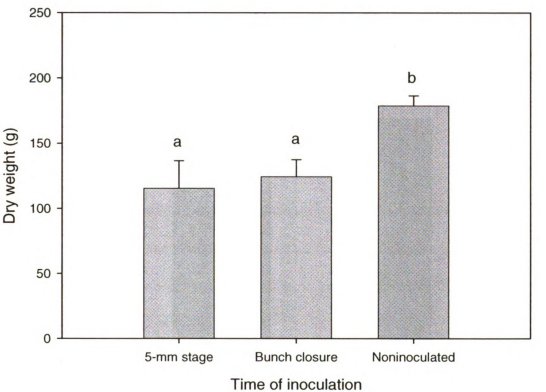

Figure 3.1. Total dry weight of two-year-old potted 'Niagara' grapevines inoculated with *Plasmopara viticola* at the 5-mm berry stage and bunch closure compared to non-inoculated vines in 2003. Each mean represents the average of five replications. Error bars represent the standard error of the mean. Means sharing the same letter are not significantly different at $P \leq 0.05$.

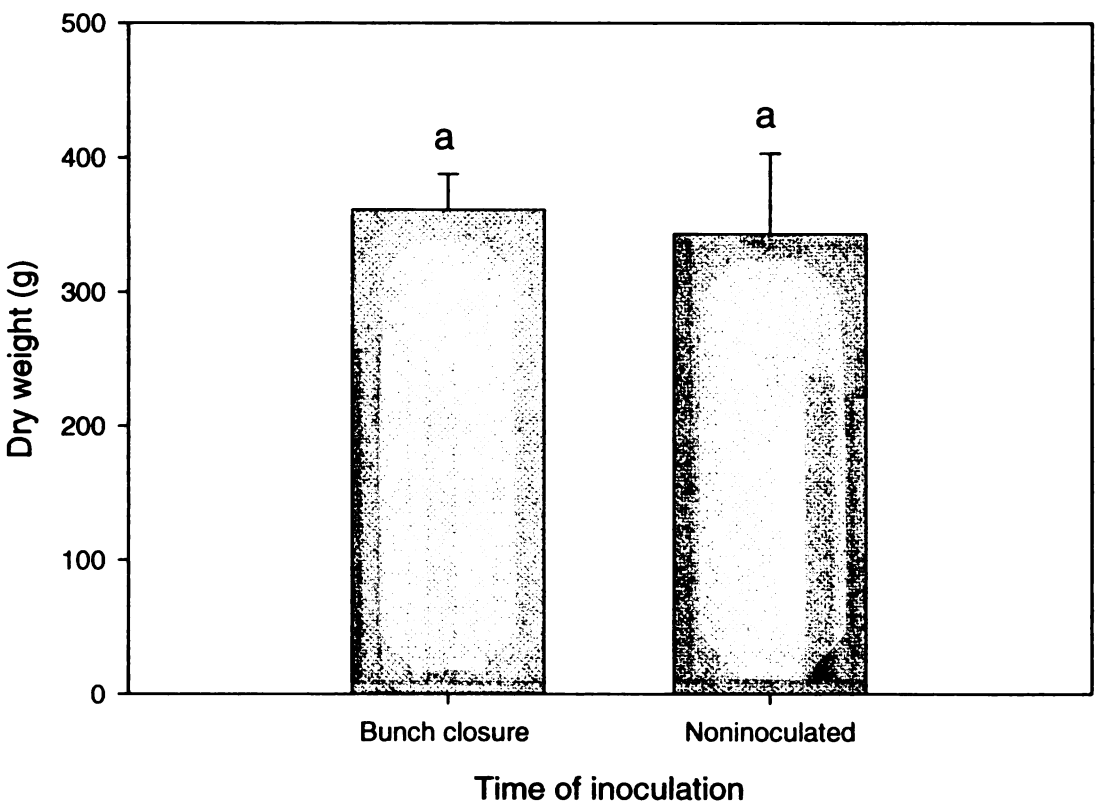

Figure 3.2. Total dry weight of three-year-old potted 'Niagara' grapevines inoculated with *Plasmopara viticola* at bunch closure compared to non-inoculated vines in 2004. Each mean represents the average of four replications. Error bars represent the standard error of the mean. Means sharing the same letter are not significantly different at $P \leq 0.05$.

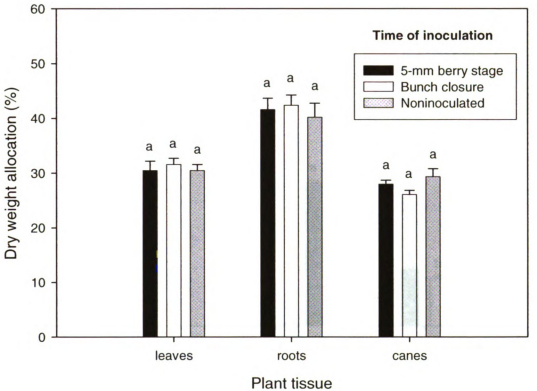

Figure 3.3. Percent of total dry weight in leaves, roots, and canes of two-year-old potted 'Niagara' grapevines inoculated with *Plasmopara viticola* at the 5-mm berry stage and bunch closure compared to non-inoculated vines in 2003. Each mean represents the average of five replications. Error bars represent the standard error of the mean. Means sharing the same letter are not significantly different at $P \leq 0.05$.

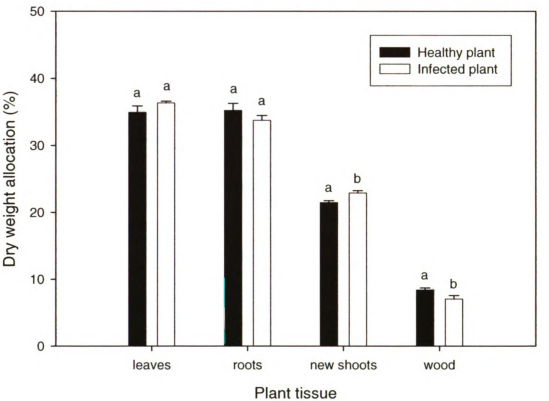

Figure 3.4. Percent of the total dry weight in leaves, roots, new canes, and woody parts (including canes and stem) of three-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* compared to non-inoculated vines in 2004. Treatment vines were inoculated once a week for six weeks before plant harvest. Each mean represents the average of four replications. Error bars represent the standard error of the mean. Means sharing the same letter are not significantly different at $P \leq 0.05$.

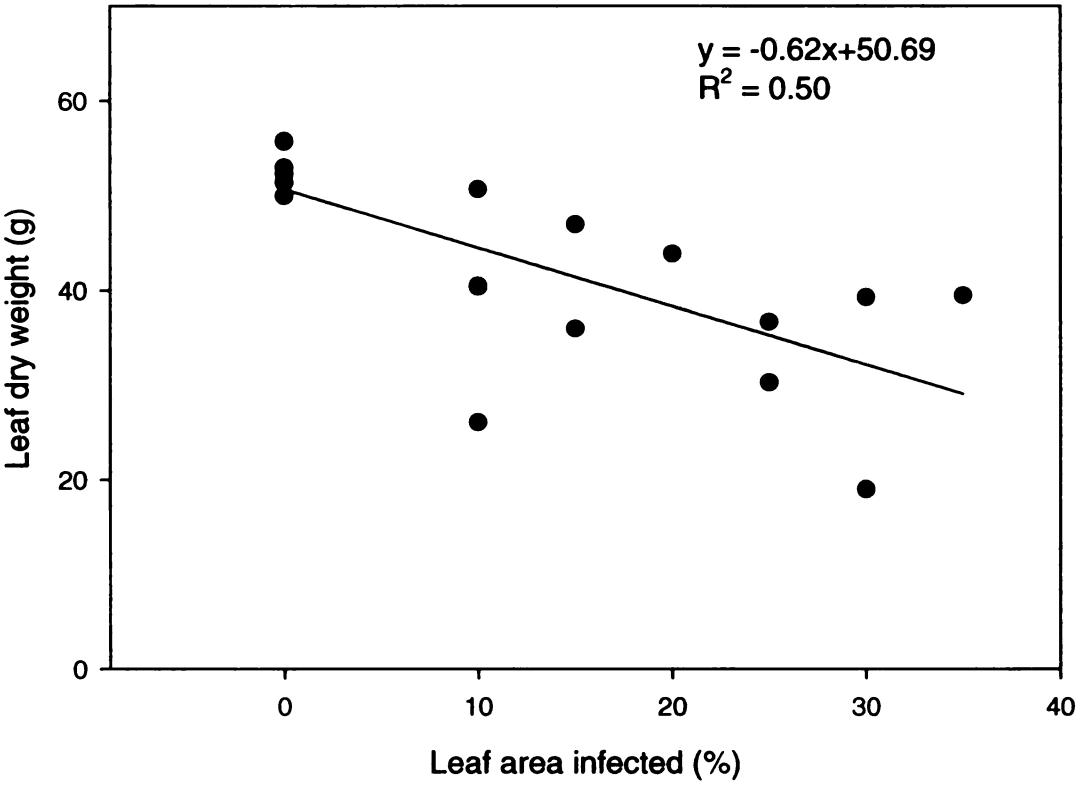

Figure 3.5. The relationship between leaf dry weight and disease severity of two-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* in 2003. Each point represents one plant.

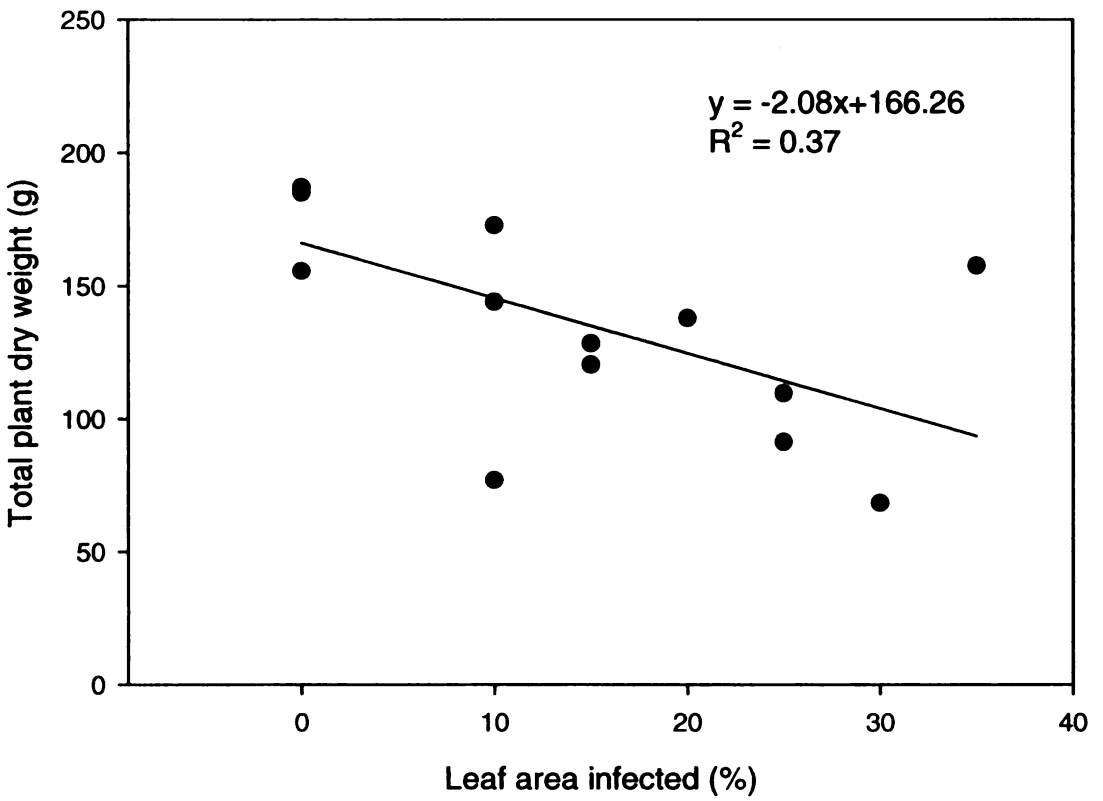

Figure 3.6. The relationship between total plant dry weight and disease severity of two-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* in 2003. Each point represents one plant.

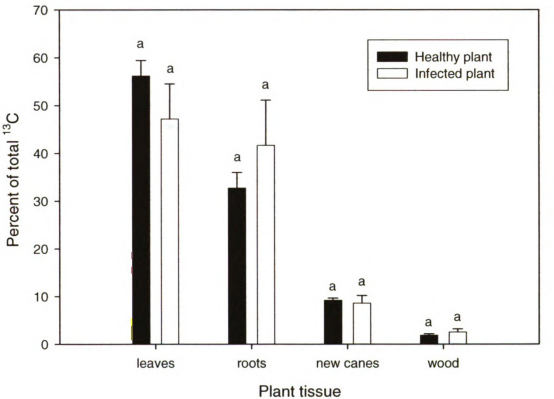

Figure 3.7. Percent of the total ^{13}C sampled relative to the natural abundance in leaves, roots, new shoots, and woody (including canes and stem) parts of three-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* compared to non-inoculated vines in 2004. Treatment vines were inoculated once a week for six weeks before plant harvest. Samples were taken 7 d after labeling. Each mean represents the average of four replications. Error bars represent the standard error of the mean. Means sharing the same letter are not significantly different at $P \leq 0.100$.

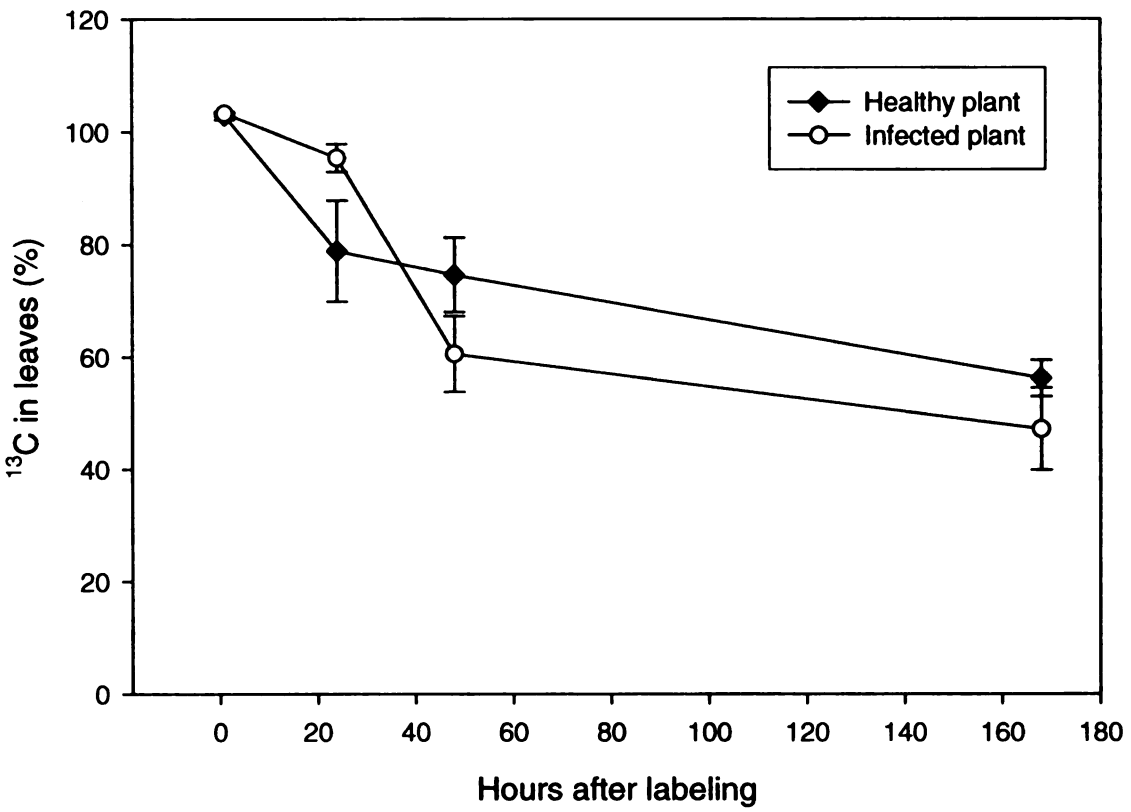

Figure 3.8. Percent of total labeled ^{13}C sampled relative to the natural abundance in non-symptomatic downy mildew-infected leaves compared to healthy leaves of 3-year-old potted 'Niagara' grapevines in 2004. Samples were taken at 1, 24, 48, and 168 h after exposure to $^{13}CO_2$. Each value is an average of four replications. Bars represent the standard error of the mean.

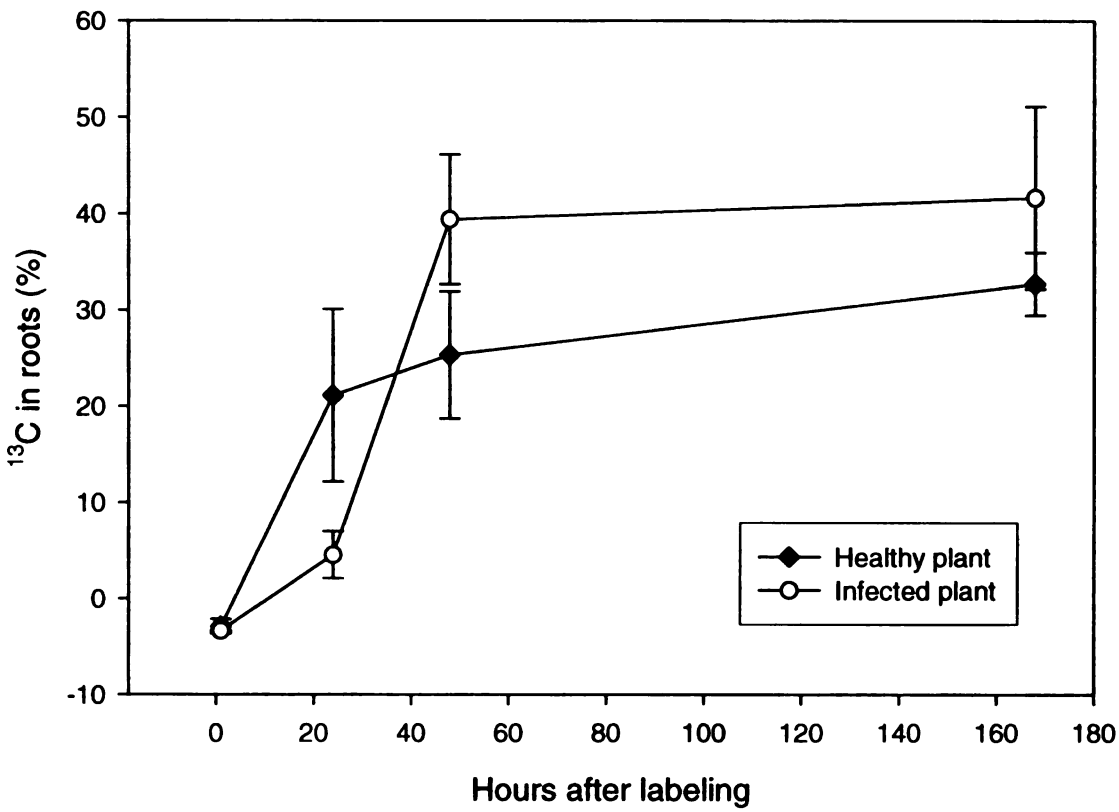

Figure 3.9. Percent of total ^{13}C sampled relative to the natural abundance in the roots of downy mildew-infected vines compared to the roots of healthy three-year-old potted 'Niagara' grapevines in 2004. Samples were taken at 1, 24, 48, and 168 hours after exposure to $^{13}CO_2$. Each value is an average of four replications. Bars represent the standard error of the mean.

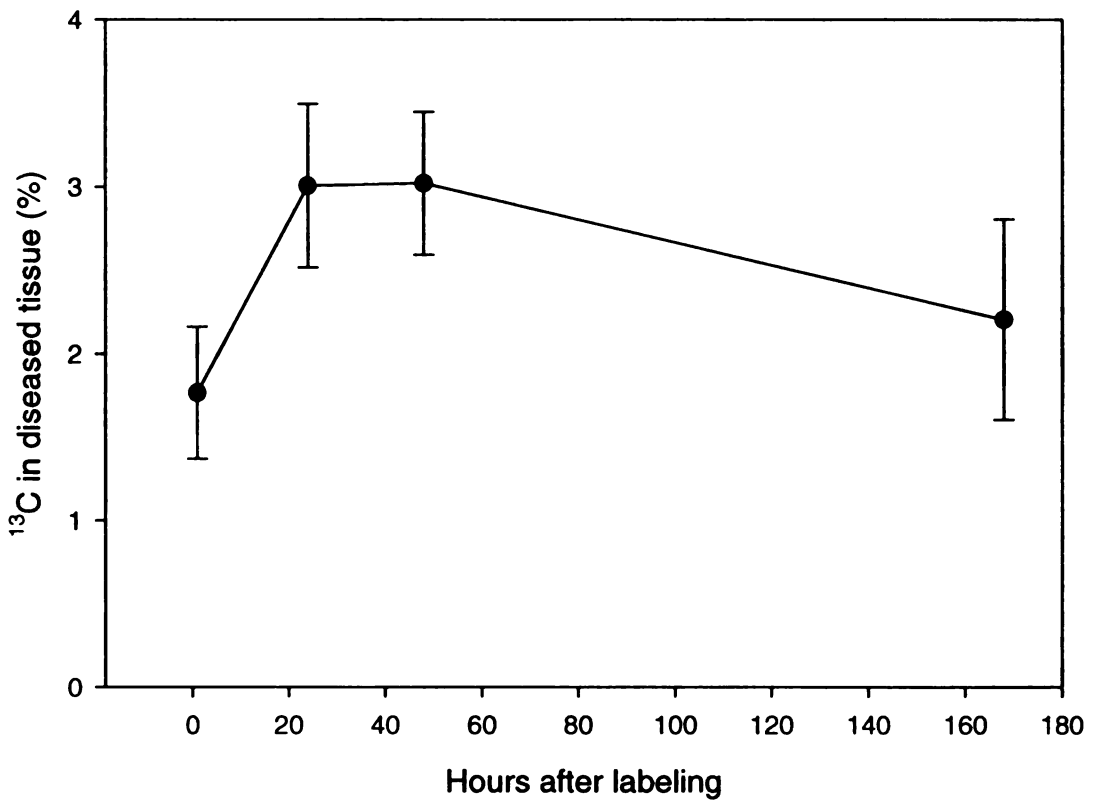

Figure 3.10. Percent of total ^{13}C sampled relative to the natural abundance of ^{13}C in the lesions of leaves of three-year-old 'Niagara' grapevines infected with *Plasmopara viticola* in 2004. Samples were taken at 1, 24, 48, and 168 hours after exposure to $^{13}CO_2$. Each value is an average of four replications. Bars represent the standard error of the mean.

Discussion

Dry weight accumulation and allocation

In order to develop damage thresholds that can be used in integrated disease management, it is essential to understand the effects of disease on carbon partitioning and biomass accumulation in plants. Since biotrophic pathogens typically cause a general decline in the photosynthetic rates of plants, there is a smaller amount of net photosynthates available for translocation, and thus total biomass could ultimately be reduced (Farrar 1987). Infection by *P. viticola* generally caused a reduction in the dry weight of grapevines for infections lasting at least three weeks in 2003, but did not affect dry weight in 2004. In 2004, overall dry weights were much higher and there was more variability. This could be attributed to fewer plants. Also, vines used in 2003 were potted in 5-gallon pots and allowed to grow for one growing season. Vines used in 2004 were potted in 10-gallon pots and allowed to grow for two seasons which could better simulate field conditions.

Downy mildew infection did not affect the percent of dry weight partitioning to different plant tissues considerably in 2003, at least not for infections lasting up to a 7-week period. Infected vines allocated more biomas to new shoots and less to the cane and stem (wood) in 2004. Although canes and shoots were not sampled separately in 2003, there was more leaf biomass as well as total biomass in 2004, which could have resulted in a larger percent of dry weight allocated to new shoots of infected vines.

^{13}C distribution

Seven days after labeling, infected vines had generally allocated more enriched carbon to the roots and less to the leaves than the non-infected vines, but this effect may be due to the timing of the sampling, since carbon reaching the roots in healthy plants may have already been respired. At 24 hours, the healthy vines had allocated a larger portion of the labeled carbon from the leaves to the roots than infected vines, suggesting that the rate of translocation of assimilates is slowed by downy mildew infection. Diseased bean leaves infected by rust (*Uromyces phaseoli* (Pers.) Wint.) also showed reduced rates of translocation at five hours after labeling with ^{14}C (Livne 1966). Powdery mildew infection of barley also showed transient decreases in the percentage of assimilates translocated to the roots and subsequently, had little effect on assimilate distribution (Walters and Ayres 1982).

White rust on radish showed an accumulation of assimilates at infection sites (Williams 1964), a trend similar to what was seen for downy mildew on grapes although the extent to which carbon accumulated was less for downy mildew. It is important to remember that the disease was fully developed and some lesions had sporulated at the time of labeling and sample collection. Downy mildew has an incubation period of approximately seven days, and the accumulation of assimilates during that period could be significantly different from leaves with fully developed and/or sporulating lesions. Changes in photosynthesis and respiration can ultimately alter the movement of carbon within and between diseased tissues and to non-infected plant tissues (Daly 1976). Since there is a significant change in the photosynthetic processes before the appearance of downy mildew symptoms in grape leaves, it is possibly that there could be changes in

carbon translocation during the incubation period as well. Fungal metabolites in some biotrophic infections increase linearly after the third day of inoculation with little or no increase after sporulation occurs (Farrar 1987). If this is the case with *P. viticola*, the accumulation of ^{13}C in lesions of infected grapevine leaves may have been greater before symptoms appeared, or possibly after symptoms appeared if sporulation was prevented. Another consideration is that it was not possible to know if a natural infection was established in apparently healthy leaves of infected plants and symptoms had not yet appeared during sampling, but it is unlikely that all apparently healthy leaves were infected.

Respiration can also affect the amount of ^{13}C in the leaves and roots of infected and non-infected grapevines. Of the total carbon fixed each day by barley plants, 25% was translocated to the roots, and half of what was translocated was respired (Farrar 1980). Starch reserves in barley roots are relatively small compared to perennial plants, and pathogens causing small changes in the assimilate supply could greatly affect the availability of energy for respiration. In contrast, grapevines have large carbohydrate reserves in the roots and trunk (Winkler 1945), and moderate downy mildew infections may not affect the allocation of carbon substantially.

Other implications

Infected leaves eventually become necrotic and fall, allowing light to reach other leaves in the canopy but exposing the fruit to sun scald. Defoliation was a common occurrence on American vines in the late 1800's before the introduction of chemical control methods, but it did not normally have an adverse affect on fruit ripening (Farlow

1876). However, defoliated *V. vinifera* vines with ripening fruit changed their translocation patterns by directing carbon stored in roots of the vine to the fruit (Candolfi-vasconcelos *et al.* 1994). If carbon allocation to the roots is affected by prolonged infection of downy mildew and too few reserves are stored in the roots, cold hardiness and fruit set (transition of flowers into young fruit) the following year could be compromised.

Conclusion

Downy mildew infection affects the rate of carbon translocation and the total dry weight accumulation in 'Niagara' grapevines. Carbon allocation and dry weight accumulation in 'Niagara' vines infected with *P. viticola* still needs further investigation to determine the damage thresholds of 'Niagara' vines infected with *P. viticola.*

The effects of longer periods of infection and vine defoliation need to be studied under field conditions to determine when control measures need to be taken in order to prevent losses. Data also need to be obtained on the effects of the disease on carbon allocation of fruiting vines. Finally, the effects of *P. viticola* infection on cold hardiness and growth and fruit production during the next growing season should be examined. Carbon isotope techniques can be further implemented to study the movement of assimilates during the incubation period of the infection. The effects of disease on carbon translocation should be extended to field studies using fruiting vines as well. Stable isotope techniques are not frequently employed but can serve as a powerful tool to study many aspects of the carbon partitioning in diseased plants, and ultimately aid in the refinement of disease management practices.

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Chapter 4

Variation among *Plasmopara viticola* isolates from different host species

Introduction

Downy mildew pathogens are generally specific to a single host order or family, a characteristic that may stem from co-evolution with their hosts (Crute 1981). As a result, the movement or spread of pathogen populations may be limited by the host species. *Plasmopara viticola* (Berk. & Curt.) Berlese & de Toni is a highly specialized pathogen that has persisted on a few genera within one host family (Vitaceae). Wild plant hosts have been an important factor in the development of downy mildew pathogens (Renfro 1981). *Plasmopara viticola* is commonly found on wild hosts in the U.S., but it does not appear that wild hosts have a significant role in disease epidemiology on cultivated grapevines (Renfro 1981). Severely infected wild grapes in close proximity to cultivated grapes do not appear to be the source of infection for the cultivated grapes (Barrett 1939, Renfro 1981), suggesting the possibility of the existence of physiological races. Physiological races or pathotypes have been identified in downy mildew species parasitizing crops including sunflower (Vear *et al.* 1997), cucurbits (Lebeda and Widrlechner 2003), lettuce (Zinkernagel 1983), pearl millet (Thakur *et al.* 2004), and spinach (Irish *et al.* 2003), but races may also occur in downy mildews adapted to wild or non-crop plants. Although a few studies have mentioned the variation in pathogenicity between isolates of *P. viticola* on different grape cultivars (Kast 2001, Santilli 1957), physiological races or pathotypes have not been identified.

Host range of *P. viticola*

Plasmopara viticola has been reported to cause losses in *Vitis vinifera* L., the most economically important grapevine species in Europe, for more than 100 years (Agrios 1997, Anderson 1956, Emmett 1992, Viennot-Bourgin 1981). North America has various species of cultivated grapes including *Vitis labrusca* L. (fox grape), *V. vinifera* (wine grape), *V. rotundifolia* Michx. (muscadine grape), and wild hosts including *V. riparia* Michx. (riverside grape), *V. aestivalis* Michx. (summer grape), and *Parthenocissus quinquefolia* (L.) Planch. (Virginia creeper), that can harbor the pathogen (Pearson and Goheen 1988, Renfro 1981). Although Europe has a large pool of *V. vinifera* available to *P. viticola*, most of the acreage of *V. vinifera* in the U.S. is located in California where conditions are not conducive to infection by the pathogen (Gubler 1994). *Plasmopara viticola* is native to North America, and natural selection had produced a balance so it could parasitize wild American grape hosts without seriously affecting the grape's survival while the European grape had little or no natural resistance (Agrios 1997, Alexopoulos *et al.* 1996). Since fungal pathogens on crop species can become specialized to host genotypes when the host provides a uniform environment (Crute 1981), there is the possibility that physiological forms exist in *P. viticola*.

Host resistance to *P. viticola*

Many grape species and hybrids show at least partial resistance to *P. viticola* (Barlass 1986, Dai 1995, Demaree 1937, Denzer *et al.* 1995, Di Gaspero and Cipriani 2002, Eibach 2000, Kast 2001, Matthews 1981, Staudt and Kassemeyer 1995). The most likely sources of resistance are found in the American grape species, particularly *V.*

labrusca and *V. aestivalis* (Matthews 1981). V*itis labrusca* (depending on cultivar) and *V. aestivalis* are less susceptible to downy mildew than *V. vinifera*, and the American species *V. riparia* is classified as highly resistant (Demaree 1937, Lafon 1981, Pearson and Goheen 1988). However, infection studies using *P. viticola* inoculum taken from *V. vinifera* cv. Muller-Thurgau showed that *V. riparia* was moderately resistant while *V. aestivalis* showed susceptibility to infection (Staudt and Kassemeyer 1995). Studies using *P. viticola* isolates collected from different hybrid varieties in Europe showed that disease severity on leaves, especially on resistant hybrid varieties, was dependent upon the fungal isolate being tested, suggesting that different physiological races may exist (Kast 2001). Isolates taken from *Vitis californica* Benth., a wild grape species in California, were not able to complete their life cycle on *Vitis vinifera* or any other species that served as hosts outside of California (Santilli 1957). A few attempts have been made to classify *P. viticola* into distinct physiological forms or pathotypes based on differences in sporangiophore and sporangia morphology, but differences in morphology have been shown to be highly dependent on environmental conditions, particularly humidity and temperature (Rafaila 1968).

Growth of *P. viticola* on resistant cultivars

On resistant grape species and cultivars, the infection process by *P. viticola* is initiated, but is terminated several days after the initiation (Kortekamp *et al.* 1998, Dai 1995, Langcake and Lovell 1980). The lesions on infected leaves of resistant grape species and cultivars became necrotic soon after the infection process was terminated, whereas on susceptible species and cultivars, the tissues did not become necrotic until

after sporulation occurred (Dai 1995, Kortekamp *et al.* 1998, Langcake and Lovell 1980). Infection on leaves of *V. riparia* by isolates taken from *V. vinifera* caused brown necroses around the stomata and produced sporulation only under highly favorable conditions, and even then sporangia were few in number (Langcake and Lovell 1980). The necrosis, which was seen 2-3 days after the infection began, was apparently triggered by a hypersensitive response. Increased peroxidase activity and the accumulation of phenolic compounds in the leaves of other resistant cultivars have also been reported (Dai 1995, Kortekamp *et al.* 1998). Oospore formation, however, was shown to occur more readily on species and cultivars that do not support sporangial production (Grunzel 1961, Populer 1981). Sexual reproduction by means of oospores has the potential to increase genetic diversity through recombination, potentially yielding more virulent isolates. There is also evidence that *P. viticola* is heterothallic which can increase the potential for pathogenic specialization among isolates relative to grapevines species and cultivars (Wong *et al.* 2001). New isolates of *P. viticola* could pose a problem with control of downy mildew over time on resistant grapevines, even though resistance of most hybrid varieties is assumed to be polygenic (controlled by more than one gene) and generally not complete (Eibach 2000, Kast 2001, Kortekamp *et al.* 1998).

Molecular studies on *P. viticola*

Various molecular markers have been used on plant pathogens to determine the phylogeny of related organisms, study the diversity of populations, and follow the movement of specific genes and genotypes (McDermott and McDonald 1993). Techniques such as restriction fragment length polymorphisms (RFLPs), randomly

amplified polymorphic DNA (RAPDs), microsatellite DNA markers, and direct sequencing of nuclear ribosomal internal transcribed spacer (ITS) regions have been used to create a better understanding of the evolution and population dynamics of *P. viticola*. Studies using large subunit ribosomal DNA sequences have shown genetic relationships between downy mildews such as *Peronospora*, *Bremia*, and *Plasmopara* spp. (Goker *et al.* 2003, Riethmuller *et al.* 2002, Voglmayr 2003, Voglmayr *et al.* 2004), but no studies have focused on variation within *P. viticola* isolates themselves. Although there have been an increasing number of studies on the genetic diversity of downy mildews, the knowledge is generally limited.

Molecular studies can also be used to follow the movement and spread of pathogens. Using microsatellite DNA markers, it was determined that at least some of the disease establishment in an Italian vineyard was imported on either young plants or by some type of human transport rather than wind-mediated introduction (Gobbin *et al.* 2003). Studies using RAPD techniques from isolates of *P. viticola* from the leaves of several grapevine varieties in a single vineyard in Germany showed a high genetic diversity among primary infections and decreasing diversity with each subsequent cycle of infections (Stark-Urnau *et al.* 2000). The decreasing diversity in subsequent cycles was attributed to selection of the fittest isolates to carry on the cycles. However, in a Greek vineyard in consecutive years, it was shown that there was very low diversity among primary infections, and that oospore germination and infection occurred throughout the season (Rumbou and Gessler 2004). The ability of secondary sporangia to cause infections was low, and infections occurred over short distances. The differences in diversity of primary infections in these studies could be attributed to the environmental

conditions during the previous season and the amount of primary inoculum present. Since it has been traditionally believed that secondary sporangia are largely responsible for the temporal and spatial spread of the disease (Agrios 1997, Blaise 1996, Gobbin *et al.* 2003, Pearson and Goheen 1988), findings in these studies could change theories on the disease cycle and epidemiology of *P. viticola*, and could have implications for disease management strategies.

Rationale and objectives

Most of the studies on *P. viticola* in the past century have focused on understanding the biology of the pathogen and developing management strategies to control the disease. Not much attention has been given to the possibility that physiological races exist in *P. viticola* or if the taxa contains more than one species. If races or other species of *P. viticola* exist, it could affect the way the disease is managed on different grape species and cultivars as well as in different geographical areas.

The objectives of this study were to: 1) Determine if downy mildew isolates collected from different grape cultivars were able to infect other grape cultivars, and 2) Determine if *P. viticola* isolates collected from wild host species were able to infect leaves of cultivated grapevines.

Materials and Methods

Plant material and isolate collection

Healthy, fully expanded leaves of field-grown *Vitis labrusca* 'Niagara' and 'Delaware', interspecific hybrids 'Seyval' and 'Vignoles', and wild vines of *Vitis riparia*, *V. aestivalis*, *V. vinifera*, and *Parthenocissus quinquefolia* (used as a positive control when *P. quinquefolia* inoculum was used) were collected in July in 2003 and July through September in 2004. Healthy leaves used for inoculation were collected at the Clarksville Horticultural Station in Clarksville, MI and a greenhouse courtyard of Michigan State University in East Lansing, MI. Healthy leaves of field-grown vines were chosen from untreated vineyards or untreated vines in experimental plots to avoid leaves containing fungicide residues. Isolates of *P. viticola* were collected from leaves and fruit of wild (*V. riparia*, *V. aestivalis*, and *P. quinquefolia*), potted (*V. labrusca* 'Niagara' and 'Delaware') and field-grown (*V.* interspecific hybrid 'Vignoles' and 'Seyval') vines in Lawton, Clarksville, Onondaga, East Lansing, and Jackson, MI. The isolates collected and the date and location of collection are shown in Table 4.1.

Inoculum preparation

Infected leaves, shoots, and clusters were collected from the field and any sporulation was removed by rinsing with deionized water. Infected tissues were then placed in plastic bags with moistened paper towels, sealed, and incubated overnight in the dark at 22°C to induce sporulation. The following morning the sporangia were washed from sporulating tissues with sterile, deionized water into a 20-ml vial to create a

suspension of inoculum. The suspension was adjusted to a concentration of 5 x 10^4 sporangia per ml using a hemacytometer.

Leaf disk preparation

Healthy leaf disks measuring 2.5 cm in diameter were taken from a leaf of each of the six vine species/cultivars and placed abaxial side up in a 150 mm Petri dish. Another Petri dish was prepared with six more leaf disks that were left uninoculated to serve as a control. Leaf disks that served as the inoculated and the control were taken from the same area of the same leaf to confirm that an infection did not already exist in the leaf. The abaxial surfaces of the leaves were wetted with sterile, deionized water using an atomizer before inoculation. Leaf disks in one Petri dish were inoculated with a sporangial suspension, while sterile, deionized water was added to leaf disks of the control. Each Petri dish (inoculated and control) was replicated five times (a total of ten Petri dishes).

Inoculation

A 200-µl volume of each prepared sporangial suspension was applied to each leaf disk using a pipette. The same amount of sterile water was applied to the control leaf disks. Sterile filter paper was wetted and applied to the inside of the lid of each Petri dish to maintain humidity. The plates were sealed with parafilm and incubated under fluorescent light at 21°C for 24 hours with a 12-hour light and 12-hour dark period. In the 2004 experiments, the leaf disks were removed after the incubation period and washed with sterile water to remove any remaining sporangia from the inoculation to avoid

counting them during the analysis. Leaf disks were patted dry with sterile towels and the plates were resealed with parafilm and incubated for 7 additional days at 21°C.

After a total of 8 days of incubation, the leaf disks were examined under a dissecting microscope to see if any sporulation had occurred. The leaf disks were then removed from the Petri dish, and the sporangia were removed by drawing them into a pipette using 60 µl of sterile, deionized water. The total number of sporangia produced per leaf disk was determined using a hemacytometer. The average of two counts (one on each side of hemacytometer) per leaf disk was used in the analysis. If sporulation was seen under the dissecting microscope and the hemacytometer counts were zero, it was given a count of one to indicate that there were sporangia on the leaf disks. Experiments with isolates not collected in 2003 were repeated twice in 2004. Isolates taken from wild hosts were also included in 2004.

Data analysis

The analysis of the isolate-host combinations was conducted using a two-factor analysis of variance (ANOVA) in both years. In addition, results from isolates used in both seasons were analyzed together using a three-factor analysis of variance using year as a factor. Statistical analysis was done using Sigmastat statistical software (Systat Software Inc., Richmond, California, USA).

Table 4.1. *Plasmopara viticola* isolates used in cross-infection studies on different host species and cultivars in 2003 and 2004.

Isolate	Host isolated from	Tissue isolated from	Type of grape	Location	Date of collection
Ni-03	*Vitis labrusca* 'Niagara'	leaves	juice	Lawton, MI	9/5/03
Se-03	*Vitis* hybrid 'Seyval'	leaves	wine	Clarksville, MI	9/7/03
Ta-03	Unidentified grape (*Vitis* sp.)	clusters	table	Clarksville, MI	9/1/03
Ma-03	'Mars' (*Vitis* sp.)	leaves	table	Onondaga, MI	9/7/03
Ni-04	*Vitis labrusca* 'Niagara'	leaves	juice	Lawton, MI	7/9/04
Se-04	*Vitis* hybrid 'Seyval'	leaves	wine	Clarksville, MI	7/26/04
Ta-04	Unknown grape (*Vitis* sp.)	clusters	table	Clarksville, MI	7/18/04
Ma-04	'Mars' (*Vitis* sp.)	leaves	table	Onondaga, MI	7/9/04
Ri-04	*Vitis riparia*	leaves and shoots	wild	East Lansing, MI	7/5/04
Ae-04	*Vitis aestivalis*	leaves	wild	Lawton, MI	7/14/04
Ge-04	*Vitis vinifera* 'Gewurztraminer'	leaves	wine	Jackson, MI	8/27/04
Pa-04	*Parthenocissus quinquefolia*	leaves	---	Lawton, MI	7/14/04

Results

In 2003, four different isolates of downy mildew were used to inoculate vines. There was substantial variation in the number of sporangia produced on inoculated leaf disks for each host-isolate combination (Figure 4.1). The effect of the host was statistically significant ($P<0.001$), while the effect of the isolate was not ($P<0.526$). However, there was a significant host-isolate interaction ($P<0.001$) (Table 4.2). Generally, each host-pathogen combination resulted in an infection that was capable of producing sporulation. The highest production of sporangia on 'Niagara' leaves was produced by the Ni-03 isolate, and it was the only isolate that caused significant sporulation on 'Niagara' leaves in 2003. In addition, the Ni-03 isolate was not effective at producing sporulation on *Vitis riparia*, but isolates Ta-03, Se-03, and Ma-03 all did. Sporangium production on 'Delaware' was generally low in 2003 with all isolates. All isolates produced some sporulation on 'Seyval' and 'Vignoles'.

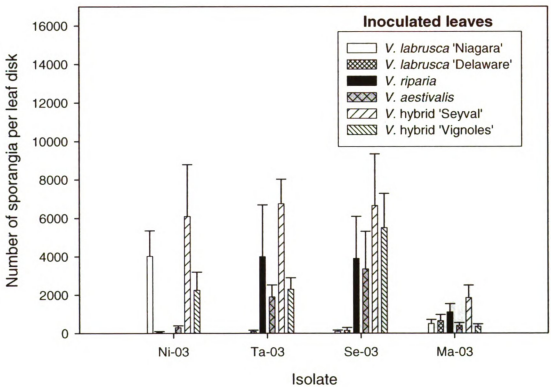

Figure 4.1. Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape cultivars and species in 2003. Isolate origin: Ni-03 ('Niagara' leaves in Lawton, MI), Ta-03 (unidentified table grape clusters, Clarksville, MI), Se-03 ('Seyval' leaves, Clarksville, MI), and Ma-03 ('Mars' table grape leaves, Onondaga, MI). Values are mean sporangium counts from five leaf disks. Error bars represent the standard error of the mean.

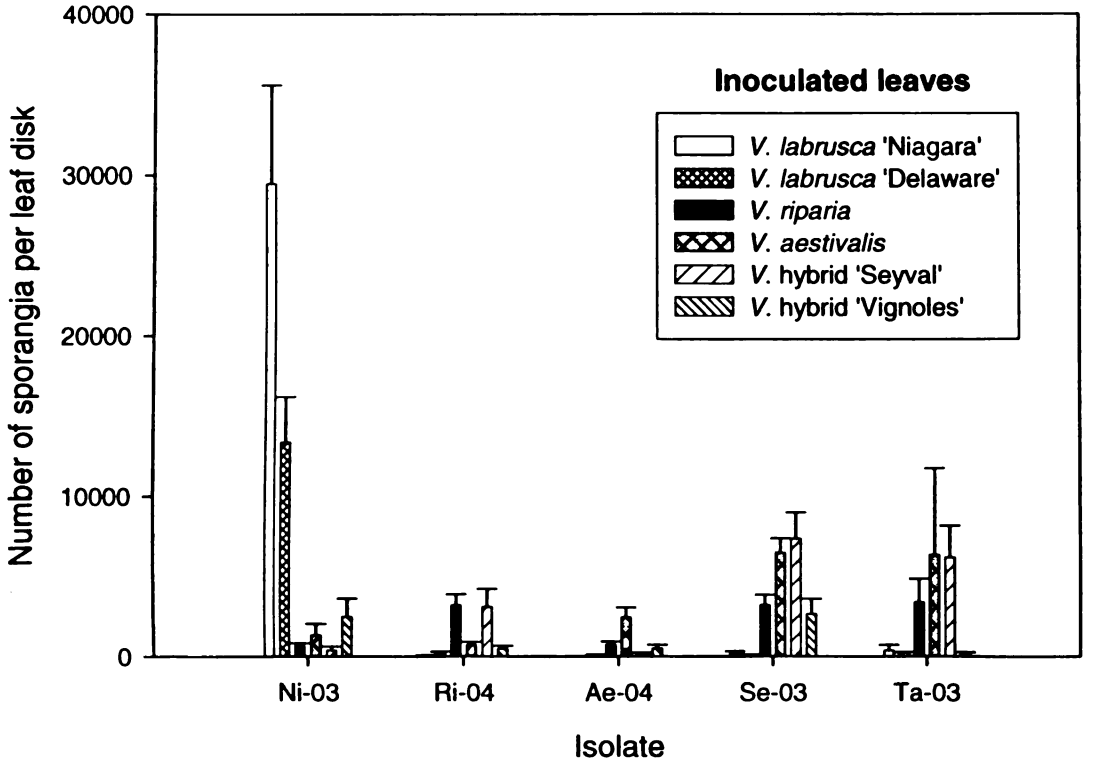

Figure 4.2 Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape cultivars and species in 2004. Isolate origin: Ni-04 ('Niagara' leaves in Lawton, MI), Ri-04 (*Vitis riparia* leaves, East Lansing, MI), Ae-04 (*V. aestivalis* leaves, Lawton, MI), Se-04 ('Seyval' leaves, Clarksville, MI), and Ta-04 (unidentified table grape clusters, Clarksville, MI). Values are mean sporangium counts from five leaf disks. Error bars represent the standard error of the mean.

Table 4.2. Analysis of variance of isolate-host combinations used in 2003.

Source of Variation	DF	SS	MS	F	*P*
Isolate	3	14472675	4824225	0.75	0.526
Host	5	382195438	76439088	11.82	<0.001
Isolate x host	15	368584438	24572296	3.80	<0.001
Residual	136	879413000	6466272		
Total	159	1631517437	10261116		

Table 4.3. Analysis of variance of isolate-host combinations used in 2004.

Source of Variation	DF	SS	MS	F	P
Isolate	4	73724	18431	25.21	<0.001
Host	5	3431	686	0.94	0.456
Isolate x host	20	280455	14023	19.19	<0.001
Residual	240	175388	731		
Total	269	533440	1983		

Table 4.4. Analysis of variance of isolate-host combinations used in 2003 and 2004.

Source of Variation	DF	SS	MS	F	P
Year	1	4191	4191	4.88	0.029
Isolate	3	10869	3623	4.22	0.007
Host	4	16999	4250	4.95	<0.001
Isolate x year	3	9948	3316	3.86	0.011
Host x year	4	22943	5736	6.68	<0.001
Isolate x host	12	134673	11223	13.07	<0.001
Isolate x host x year	12	45020	3752	4.37	<0.001
Residual	160	137391	859		
Total	199	382034	1920		

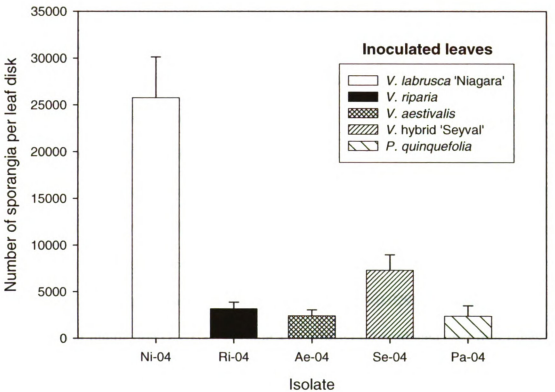

Figure 4.3. Sporulation of *Plasmopara viticola* isolates inoculated onto the same host from which they were collected in 2004. Isolate origin: Ni-04 ('Niagara' leaves in Lawton, MI), Ri-04 (*Vitis riparia* leaves, East Lansing, MI), Ae-04 (*V. aestivalis* leaves, Lawton, MI), Se-04 ('Seyval' leaves, Clarksville, MI), and Pa-04 (*Parthenocissus quinquefolia* leaves, Lawton, MI), Values are mean sporangia counts from five leaf disks. Error bars represent the standard error of the mean.

In 2004, the Ni-04 isolates produced more sporangia on *Vitis labrusca* 'Niagara' vines than any other host-isolate combination (Figure 4.2), while producing little sporulation on *V. riparia*, *V. aestivalis*, *V.* hybrid 'Seyval' and *V.* hybrid 'Vignoles'. Sporulation on *V. labrusca* 'Delaware' with the Ni-04 isolate was substantially higher than with Ni-03 in 2003. Isolates Ri-04, Se-04, and Ta-04 produced little sporulation on 'Niagara' leaves and Ae-04 produced none at all. The other isolates generally produced more sporulation on leaf disks other than 'Niagara'. Isolates Ni-04, Ae-04, and Se-04 all produced more sporangia on the leaves of the species or cultivar from which they were collected than on other species and cultivars. Isolates Ta-03, Se-03, Ta-04, and Se-04 produced similar results on all leaf disks. There was a significant difference among the isolates ($P<0.001$) in 2004, but not among the hosts (Table 4.3). There was also a significant host-isolate interaction ($P<0.001$) in 2004. Analysis of the data from both seasons showed a significant difference between years ($P=0.029$) and a significant host x isolate x year interaction ($P<0.001$) (Table 4.4). The Ni-04 produced over four times the number of sporangia in 2004 than Ni-03 did in 2003. Sporangia collected from *Parthenocissus quinquefolia* did not cause sporulation or visible infection on any host except for on leaves of *P. quinquefolia*, on which it sporulated readily. Figure 4.3 shows the number of sporangia produced on each leaf disk by isolates collected from the same host species or cultivar. Inoculum from Ge-04 did not cause any sporulation or sign of infection on any host in either replication, but a leaf of *V. vinifera* could not be obtained for a positive control.

Discussion

Data obtained in 2003 and 2004 suggest that *P. viticola* isolates collected from *V. labrusca* 'Niagara' vines (Ni-03, Ni-04) are more efficient at infecting and causing sporulation on 'Niagara' leaves than on any other host tested, with the exception of 'Seyval' in 2003. In addition, all other isolates were not very successful at causing sporulation on 'Niagara' vines, suggesting that the Ni-03 and Ni-04 isolates may be adapted specifically to infect 'Niagara' vines or possibly other cultivars of the species *V. labrusca*. These results are similar to those of Kast (2001), who found variation in the ability of *P. viticola* isolates collected in Germany and Switzerland to infect the hybrids 'Johanniter', 'Regent' and 'Trollinger'. The significant host-isolate interaction in both seasons may be due in large part to the ability of the Ni-03 and Ni-04 to produce large quantities of sporangia on 'Niagara' leaves consistently but not on other grape species. Isolates Ta-03, Se-03, and Ma-03 all showed the ability to produce infection and sporulation on the wild and interspecific hybrid leaves somewhat consistently, suggesting they may be more similar to each other than the Ni-03 and Ni-04 isolates. Interestingly, isolates obtained from other wild host species (Ri-04, Ae-04) produced little or no sporulation on any 'Niagara' leaves, but were able to infect the host from which they were collected (*V. riparia* and *V. aestivalis*). The isolates Se-04 and Ta-04 were able to infect the wild hosts as well, suggesting they may be able to overcome resistance factors in these grapevines but not in the Labrusca vines. Isolates taken from the unidentified grape and 'Seyval' (Ta-03, Ta-04, Se-03, Se-04) were collected from the same location and produced similar results, so it is possible that they were the same isolate or closely related. Inoculum taken from *P. quinquefolia* seems to be very specific to the leaves of *P.*

quinquefolia, suggesting physiological races or species of *P. viticola* may exist, but little has been reported about downy mildew infection of *P. quinquefolia*, and infection is generally considered to be caused by *P. viticola* (Renfro 1981).

The significant interaction between host and isolate in both seasons indicates that the quantity of sporangia produced on a specific host greatly depends on the isolate being used for the inoculation. The significant interaction between isolate, host, and year suggests that the variability in the number of sporangia produced could also be affected by conditions that isolates and hosts are exposed to during a particular season, particularly temperature and moisture. Isolates from Romania showed high morphological variability, particularly in sporangia, which was shown to be influenced by temperature and humidity (Rafaila 1968). Favorable conditions in 2004 could have allowed isolate Ni-04 to produce more sporulation on leaf disks of *V. labrusca* than isolate Ni-03 in 2003. The same conditions could be true for the host, *V. labrusca*, being less resistant to certain isolates during a particular season. Leaf age can also strongly influence susceptibility (Emmett 1992, Pearson and Goheen 1988, Rafaila 1968). Even though leaves of approximately the same age were chosen for the experiment, there was inevitably some variability in susceptibility of individual leaves. Although healthy leaves were chosen from untreated vineyards, they could have also contained fungicide residues that drifted from adjacent vineyards or plots. Washing the leaf disks after inoculations in 2004 may have provided a more realistic count of sporangia produced by the infection, although the sporangia counts were generally higher in 2004 when the leaf disks were washed, and it is doubtful that this had much of an effect on total counts of sporangia between 2003 and 2004.

There is other evidence that could possibly support the idea of physiological races of 'Niagara' isolates. Downy mildew infection is not typically seen on cultivated *V. labrusca* vines until July or August in Michigan. Farlow (1876) reported that the fungus did not usually appear in New England on American species and cultivars until August and was very common from the middle of August until frost. Other reports in the late 1800's stated that downy mildew did not appear in the eastern United States until late summer and fall (Bush *et al.* 1895), similar to what is typically seen on *V. labrusca* in Michigan today (personal observation). In contrast, substantial downy mildew infections were seen in late June on table grape clusters and *V. riparia* in 2004, two weeks before the first infection was seen on leaves of 'Niagara' vines. The difference could be due to susceptibility of the vine at different times throughout the growing season, or that host-specific sporangia were released earlier or later in the season from germinating oospores.

Immature berries are reported to be highly susceptible to downy mildew infection (Pearson and Goheen 1988). Inoculations with *P. viticola* on 'Niagara' in New York showed that flowers remained susceptible until several days after bloom (Kennelly 2002). Inoculations done on 'Niagara' and 'Delaware' in the early 1900's showed that *P. viticola* attacked the leaves, stems, tendrils, and fruit on American varieties (Gregory 1912). However, infections of flowers and clusters of 'Niagara' were not commonly observed under field conditions in the northeastern U.S. (Farlow 1876). In his study on the biology of the pathogen, Farlow (1876) stated that in New England, "*Peronospora viticola* infection was limited to the leaves and stems of American species and cultivars, and does not attack the fruit", suggesting that berry infection was rare or non-existent on field vines in the late 1800's. This conflicting evidence may be a result of the young

berries of some American species or cultivars being resistant during the period of oospore germination, or that there are isolates infecting berries of certain cultivars that do not have the ability to infect the berries of others. The lack of resistance factors in *V. vinifera* in Europe may have limited the need for the pathogen to carry specific virulence factors that enable it to overcome resistance found in its native range.

Knowledge of host-pathogen interactions can aid in analyzing and predicting disease pressure and epidemics. If different physiological races do exist, there is the need to identify them and determine how frequently they can be found, where they are found, and how they change with different selection pressures (Crute 1981). An increased understanding of host specificity of *P. viticola* isolates and a better understanding of individual host resistance and tolerance could ultimately lead to alternative and more efficient methods of disease control. Future studies should focus on the relationship between the genetic diversity of *P. viticola* isolates and the pathogenicity on different host species over a wide range of environmental conditions and geographic areas.

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APPENDIX A

Table A.1. The effect of downy mildew disease development in 'Niagara' grapevine leaves on chlorophyll fluorescence, photosynthetic efficiency (θ), A_{amb}, and the light compensation point determined by fluorescence measurements and light response curves in 2003. Values in columns with *, **, and *** are significantly different at $P<0.05$, $P<0.0.1$ and $P<0.001$, respectively, compared to healthy control leaves on the day measured.

Days after inoculation	Symptoms	Fluorescence	θ	A_{amb}	Light compensation point
2	no	0.830	0.047	16.915	36.96
4	no	0.817	0.021	10.309**	74.67
6	no	0.802	0.011***	3.530***	74.02
8	yes	0.803*	0.007***	1.559***	196.90**
10	yes	0.763***	0.004***	1.269***	115.62*
12	yes	0.714***	0.004***	0.790***	315.98***
14	yes	0.653***	0.011***	2.523***	111.49*

Table A.2. The effect of downy mildew disease development in 'Niagara' grapevine leaves on carboxylation efficiency (k), CO_2 compensation point (Γ), internal CO_2 concentration, A_{max}, stomatal conductance (g_s), and stomatal limitation ($l_{g(ii)}$) determined by CO_2 response curves in 2003. Values in columns with *, **, and *** are significantly different at $P<0.05$, $P<0.0.1$, and $P<0.001$, respectively compared to healthy control leaves on the day measured.

Days after inoculation	Symptoms	k	Γ	C_i	A_{max}	g_s	$l_{g(ii)}$
2	no	0.093	85.66	255.00	29.83	246.05	0.345
4	no	0.082*	116.07	258.10	23.51**	136.13	0.369
6	no	0.039***	168.77*	342.63***	12.77***	157.44	0.214*
8	yes	0.020***	194.70**	356.06***	9.05***	179.98**	0.174*
10	yes	0.016***	226.93***	362.06***	6.41***	134.77***	0.211
12	yes	0.013***	258.70***	378.99***	5.05***	133.13***	0.147*
14	yes	0.016***	194.28***	354.82***	5.96***	123.88***	0.185

Table A.3. The effect of downy mildew disease development in 'Niagara' grapevine leaves on chlorophyll fluorescence, photosynthetic efficiency (θ), A_{amb}, and the light compensation point determined by fluorescence measurements and light response curves in 2004. Values in columns with *, **, and *** are significantly at $P<0.05$, $P<0.0.1$, and $P<0.001$, respectively, compared to healthy control leaves on the day measured.

Days after inoculation	Symptoms	Fluorescence	θ	A_{amb}	Light compensation point	Dark respiration
0	no	0.819	0.0179	9.17	28.88	-0.669
2	no	0.806	0.0177***	5.17*	35.27	-1.081
4	no	0.811	0.0154***	2.74	44.28	-1.336
6	no	0.820	0.0133***	1.95	61.67	-1.345
8	yes	0.778*	0.0066***	1.01*	68.10	-1.069
10	yes	0.688**	0.0116*	1.99***	115.41**	-1.008

Table A.4. The effect of downy mildew disease development in 'Niagara' grapevine leaves on carboxylation efficiency (k), CO_2 compensation point (Γ), internal CO_2 concentration, A_{max}, stomatal conductance (g_s), and stomatal limitation ($l_{g(ii)}$) determined by CO_2 response curves in 2004. Values in columns with *, **, and *** are significantly different at $P<0.05$, $P<0.0.1$, and $P<0.001$, respectively compared to healthy control leaves on the day measured.

Days after inoculation	Symptoms	k	Γ	C_i	A_{max}	g_s	$l_{g(ii)}$
0	no	0.062	66.60	227.87	18.77	136.63	0.368
2	no	0.051	97.75	219.74	14.76	61.25	0.499
4	no	0.041**	53.73	201.55	16.17	50.38	0.503
6	no	0.040	139.29	277.42	3.92*	45.11	0.345
8	yes	0.027**	138.81*	315.61***	2.74**	24.34	0.155
10	yes	0.025***	173.60**	256.73	6.45***	31.27*	0.598

Table A.5. Repeated measures analysis of variance of fluorescence in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	0.0756	0.0126	26.25	<0.001
Treatment	1	0.0529	0.0529	31.32	0.005
Day x treatment	6	0.0518	0.0086	21.10	<0.001
Residual	24	0.0098	0.0004		
Total	69	0.2110	0.0031		

Table A.6. Repeated measures analysis of variance of the assimilation rate at ambient CO_2 in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	684.55	114.09	7.87	<0.001
Treatment	1	2206.81	2206.81	247.89	<0.001
Day x treatment	6	574.37	95.73	6.94	<0.001
Residual	24	331.20	13.80		
Total	69	4204.40	60.93		

Table A.7. Repeated measures analysis of variance of the light compensation point in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	107690.14	17948.36	6.31	<0.001
Treatment	1	112135.58	112135.58	14.673	0.02
Day x treatment	6	78134.65	13022.44	6.42	<0.001
Residual	18	36542.19	2030.12		
Total	63	533103.61	8461.96		

Table A.8. Repeated measures analysis of variance of the photosynthetic efficiency in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	0.0048	0.0008	14.64	<0.001
Treatment	1	0.0110	0.0110	347.10	<0.001
Day x treatment	6	0.0037	0.0006	14.37	<0.001
Residual	24	0.0010	0.0001		
Total	69	0.0200	0.0003		

Table A.9. Repeated measures analysis of variance of the assimilation rate at maximum CO_2 in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	1721.88	286.98	14.70	<0.001
Treatment	1	4577.35	4577.35	124.00	<0.001
Day x treatment	6	1131.78	188.63	20.86	<0.001
Residual	22	198.96	9.04		
Total	67	8909.31	132.98		

Table A.10. Repeated measures analysis of variance of the stomatal conductance in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	31230.68	5205.11	0.44	0.847
Treatment	1	180369.48	180369.48	15.98	0.016
Day x treatment	6	150749.72	25124.96	5.54	0.001
Residual	24	108839.29	4534.97		
Total	69	907623.96	13153.97		

Table A.11. Repeated measures analysis of variance of the CO_2 compensation point in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	44271.07	7378.51	2.56	0.046
Treatment	1	155115.63	155115.63	25.77	0.007
Day x treatment	6	68580.45	11430.08	6.06	<0.001
Residual	23	43383.53	1886.24		
Total	68	427452.50	6286.07		

Table A.12. Repeated measures analysis of variance of the carboxylation efficiency in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	0.0207	0.0035	6.34	<0.001
Treatment	1	0.0686	0.0686	72.05	0.001
Day x treatment	6	0.0148	0.002	8.93	<0.001
Residual	24	0.0067	0.0003		
Total	69	0.1290	0.0019		

Table A.13. Repeated measures analysis of variance of the intercellular CO_2 concentration in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	48061.23	8010.21	6.30	<0.001
Treatment	1	85424.50	85424.50	26.07	0.007
Day x treatment	6	32025.71	5337.62	9.94	<0.001
Residual	24	12883.99	536.83		
Total	69	231815.59	3359.65		

Table A.14. Repeated measures analysis of variance of the stomatal limitation to photosynthesis in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2003.

Source of Variation	DF	SS	MS	F	*P*
Day	6	0.252	0.0420	4.31	0.004
Treatment	1	0.074	0.0738	6.63	0.061
Day x treatment	6	0.056	0.0094	1.52	0.216
Residual	23	0.142	0.0062		
Total	68	0.835	0.0123		

Table A.15. Repeated measures analysis of variance of fluorescence in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	6	0.0491	0.0082	5.44	0.002
Treatment	1	0.0487	0.0487	20.86	0.020
Day x treatment	6	0.0722	0.0120	13.48	<0.001
Residual	18	0.0161	0.0009		
Total	55	0.2280	0.0042		

Table A.16. Repeated measures analysis of variance of the assimilation rate at ambient CO_2 in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	149.77	29.95	4.54	0.010
Treatment	1	85.97	85.97	10.53	0.048
Day x treatment	5	52.06	10.41	6.89	0.002
Residual	15	22.66	1.51		
Total	47	476.22	10.13		

Table A.17. Repeated measures analysis of variance of the light compensation point in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	11585.15	2317.03	2.14	0.116
Treatment	1	5152.83	5152.83	5.24	0.106
Day x treatment	5	9750.74	1950.15	1.20	0.137
Residual	15	14640.55	976.04		
Total	47	69727.48	1483.56		

Table A.18. Repeated measures analysis of variance of the photosynthetic efficiency in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	0.0007	0.0001	2.30	0.097
Treatment	1	0.0013	0.0013	125.69	0.002
Day x treatment	5	0.0002	0.0001	1.69	0.197
Residual	15	0.0004	0.0001		
Total	47	0.0036	0.0001		

Table A.19. Repeated measures analysis of variance of the dark respiration in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	2.191	0.438	3.66	0.023
Treatment	1	0.067	0.067	0.21	0.679
Day x treatment	5	0.641	0.128	1.61	0.219
Residual	15	1.197	0.080		
Total	47	8.380	0.178		

Table A.20. Repeated measures analysis of variance of the assimilation rate at maximum CO_2 in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	1416.65	283.33	9.25	<0.001
Treatment	1	171.62	171.62	15.14	0.030
Day x treatment	5	64.65	12.93	3.80	0.020
Residual	15	51.08	3.41		
Total	47	2288.40	48.689		

Table A.21. Repeated measures analysis of variance of the stomatal conductance in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	53613.26	10722.65	4.93	0.007
Treatment	1	18586.67	18586.67	6.58	0.083
Day x treatment	5	5201.28	1040.26	0.85	0.539
Residual	15	18455.85	1230.39		
Total	47	146071.32	3107.90		

Table A.22. Repeated measures analysis of variance of the CO_2 compensation point in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	29494.60	5898.92	3.15	0.039
Treatment	1	24245.76	24245.76	6.68	0.081
Day x treatment	5	19442.76	3888.55	2.38	0.088
Residual	15	24484.29	1632.29		
Total	47	144303.37	3070.28		

Table A.23. Repeated measures analysis of variance of the carboxylation efficiency in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	0.0022	0.00045	3.11	0.040
Treatment	1	0.0071	0.00714	68.82	0.004
Day x treatment	5	0.0027	0.00054	2.67	0.064
Residual	15	0.0030	0.00020		
Total	47	0.0185	0.00039		

Table A.24. Repeated measures analysis of variance of the intercellular CO_2 concentration in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	21901.48	4380.30	3.74	0.021
Treatment	1	20255.28	20255.28	29.90	0.012
Day x treatment	5	25036.73	5007.35	2.68	0.064
Residual	15	28078.74	1871.92		
Total	47	119831.05	2549.60		

Table A.25. Repeated measures analysis of variance of the stomatal limitation to photosynthesis in leaves of 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves in 2004.

Source of Variation	DF	SS	MS	F	*P*
Day	5	0.181	0.0362	2.56	0.067
Treatment	1	0.001	0.0003	0.04	0.852
Day x treatment	5	0.026	0.0053	0.39	0.844
Residual	15	0.161	0.0134		
Total	47	0.692	0.0157		

APPENDIX B

Figure B.1. Equation used to calculate percent uptake of $^{13}CO_2$ from $Ba^{13}CO_2$ in 'Niagara grapevines in 2004.

$$Ba^{13}CO_3 + 2H^+ \longrightarrow Ba^{+2} + {}^{13}CO_2 + HOH$$

Table B.1. Percent of pulsed $^{13}CO_2$ taken up by 'Niagara' grapevines in 2004.

Vine replication	Treatment	% uptake
1	Healthy plant	14.67
2	Healthy plant	15.78
3	Healthy plant	14.03
4	Healthy plant	11.41
1	Infected plant	17.56
2	Infected plant	19.16
3	Infected plant	14.42
4	Infected plant	14.49

Table B.2. Analysis of variance of total dry weight of 'Niagara' grapevines infected with *Plasmopara viticola* at the 5-mm berry stage and bunch closure compared to healthy vines in 2003.

Source of Variation	DF	SS	MS	F	*P*
Treatment	2	11192.94	5596.47	5.45	0.017
Residual	15	15399.97	1026.66		
Total	17	26592.90			

Table B.3. Analysis of variance of dry weight of individual organs of 'Niagara' grapevines infected with *Plasmopara viticola* at the 5-mm berry stage and bunch closure compared to healthy vines in 2003.

Source of Variation	DF	SS	MS	F	*P*
Treatment	2	9.1E-02	4.6E-02	3.6E-02	1.000
Tissue	2	1376.15	688.08	53.83	<0.001
Treatment x tissue	4	34.78	8.70	0.68	0.611
Residual	33	421.83	12.78		
Total	41	1890.36	46.11		

Table B.4. Repeated measures analysis of variance of ^{13}C content in leaves of three-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves at 1, 24, 48, and 168 h in 2004.

Source of Variation	DF	SS	MS	F	*P*
Treatment	1	18.41	18.41	0.11	0.763
Hour	3	12099.05	4033.02	110.98	<0.001
Treatment x hour	3	1090.58	363.53	2.20	0.158
Residual	9	1489.36	165.49		
Total	31	16094.41	519.17		

Table B.5. Repeated measures analysis of variance of ^{13}C content in roots of three-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* compared to roots of healthy vines at 1, 24, 48, and 168 h in 2004.

Source of Variation	DF	SS	MS	F	*P*
Treatment	1	17.93	17.93	0.09	0.787
Hour	3	8280.56	2760.19	63.71	<0.001
Treatment x hour	3	1088.19	362.73	1.97	0.189
Residual	9	1655.99	184.00		
Total	31	12708.11	409.94		

Table B.6. Repeated measures analysis of variance of ^{13}C content in lesions of leaves of three-year-old potted 'Niagara' grapevines infected with *Plasmopara viticola* compared to healthy leaves at 1, 24, 48, and 168 h in 2004.

Source of Variation	DF	SS	MS	F	*P*
Rep	3	8.38	2.79	8.69	0.005
Hour	3	4.61	1.54	4.78	0.029
Residual	9	2.89	0.32		
Total	15	15.89	1.06		