An Abstract of the Thesis of

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Patrick J Breen

Death of flower parts near bloom due to inflorescence necrosis (IN) is associated with high ammonium (NH_4^+) concentration in flower clusters, shade, cool wet weather preceding bloom, and excessive vigor. Faults in NH_4^+ assimilation are suspected to cause a rise in NH_4^+ concentration to toxic levels in flower cluster but not other tissues.

In this study, shading whole vines of Pinot noir increased flower NH_4^+ concentration if applied at budbreak (early) or 1wk before bloom (late), but only late application of shade to individual shoots raised it. Late, complete shoot defoliation raised flower cluster NH_4^+ in all three years of the experiment. Early or late removal of lower leaves on a shoot also increased flower cluster NH_4^+. Lamina, petiole, and other shoot tissue NH_4^+ levels responded differently to treatments than flower clusters. IN severity was not affected by treatments; however, on shoots whose primary clusters had been removed at bloom secondary clusters did show significant differences, with IN being more severe in the early defoliation and late, lower defoliation treatments.

Ethephon, sprayed on whole vines, slowed shoot growth to zero and increased IN severity greatly, however, flower cluster NH_4^+ concentration was increased only 20% over the controls. Methionine sulfoximine applied as a cluster dip, increased flower cluster NH_4^+ by 100%, yet resulted in little necrosis.

Rootstock and clone affected Pinot noir flower cluster NH_4^+ and IN severity. Rootstocks 420A caused lower and 101-14 and 3309 higher NH_4^+ concentrations than the

average. IN severity and flower cluster NH_4^+ varied between vineyard sites, possibly due to environment and management differences. Pinot noir clones UCD23 and 32 had lower and UCD4 higher than average flower cluster NH_4^+. IN in clones UCD4 and DJN115 was the least and UCD23 the most severe.

A single 73kDa protein from grape shoot tissues reacted with anti-rice Fd-GOGAT IgG. An extraction method and assay for Fd-GOGAT activity from grape tissues was developed and gel filtration was used to show that the native enzyme is a dimer or trimer of the 73kDa protein. Activity was found in lamina, petiole, flower, rachis, and tendril, but not pedicel tissue.

Inflorescence Necrosis, Ammonium, and Evidence for Ferredoxin-Glutamate Synthase Activity in Grape (*Vitis vinifera* L.)

by

Glen L. Creasy

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APPROVED:

Major Professor, representing Horticulture

Head of Department of Horticulture

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Glen L. Creasy, Author

Acknowledgments

Well. Where to begin? I guess first I'd like to thank the members of the Academy... No, wait, that's the wrong one! ☺

Seriously, though, I find this part of the thesis the most difficult to write, as I have been so long a part of the Horticulture Dept. here. My committee members, Drs. Breen, Arp, Righetti, Chen and Yoder deserve thanks for agreeing to serve (and I hope it wasn't like serving a prison sentence!), and making such a great group. I must also thank the Oregon Grape industry, who helped fund both my MS and PhD research, as did the Weatherspoon Foundation. I hope that I have contributed, and will contribute in the future, something back to the Oregon industry after they have been so generous to me.

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Table of Contents

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

Page

List of Figures

List of Figures (Continued)

List of Tables

Dedication

This thesis is dedicated to my parents, whose
love and support I cherish.

Inflorescence Necrosis, Ammonium, and Evidence for Ferredoxin-Glutamate Synthase Activity in Grape (*Vitis vinifera* L.)

Chapter 1: Introduction

Not too long ago in the wine industry, the situation for the future looked bleak. Per capita consumption of still wines has been on the decline worldwide, falling prey to a younger generation brought up on cola and beer and the perception that wine is an alcoholic beverage rather than a food. But there is a glimmer of hope now, due to lifestyle changes, increased public awareness of health issues, and medical studies finding that wine may actually benefit the health of the moderate drinker (Frankel *et al.*, 1993; Renaud and De Lorgeril, 1992). The point is still argued, but as the results of more studies that focus on wine consumption and health come to bear fruit, the more it seems that yes, wine, a beverage historically considered not only a perfect complement to a meal, but also as a 'tonic' that could strengthen the imbiber's resistance to maladies, can contribute to one's well being.

So there is a mood of optimism now, which accompanies marketing savvy and niche filling that results in an increase in the number of (smaller) wineries and the vineyards that support them.

There are the giants of the industry, some with long histories (Italy, France, Spain, *etc.*) and some belonging to the "new world" of winemaking (*e.g.* the United States, Chile, Australia, *etc.*). The wine industry of these countries are made up of many small, individual wineries, though in some cases not so small: one Californian winery has almost 40 million gallons of wine storage capacity (Cooke and Vilas, 1989)! The smaller wineries are what give each region it's character, however.

In the US, California dominates the wine industry, but there are bonded wineries in virtually every state. Oregon, as a grape growing area, is garnering more interest within the industry, partly due to excellent wines coming from areas well suited to the traditional

winegrape varieties (*Vitis vinifera* L.), and partly due to shrewd marketing of one of the world's most coveted varieties, Pinot noir.

In most cool climate grape growing regions, the benefit of higher quality fruit comes at the cost of more problems to overcome, or at least, different ones (the motto of the Alexis Bailly Vineyard in Hastings, Minnesota, a region where temperatures often reach the lower limits of grapevine winter hardiness, is prominently displayed on each of their labels: "Where the vines can suffer"). Vineyards in Oregon are considered to be growing under cool climate conditions, and while managers do not normally have to face such problems as winter injury, they do have to deal with powdery mildew, insufficient warm weather to ripen crops, or rainy weather when it is time to harvest.

Another problem, which has caused significant crop loss in many cool climate areas, is poor fruit set. This reduction in fertilized ovaries results in smaller clusters with fewer berries, and concomitantly low yields. Rain during bloom can cause pollen to be washed from the flower, and encourage fungi such as *Botrytis cinerea* to infect the inflorescences. Cool weather is also associated with the formation of shot berries, which fail to develop into full-sized grapes (Kliewer, 1983; Winkler *et al.*, 1974).

However, a new physiological fruit set disorder in Australia and New Zealand was reported in 1988 by Jackson and Coombe. They described a necrosis of flower tissue including the florets, the pedicels, and the rachis subtending them, and proposed the name early bunchstem necrosis for it, due to the similarity in affected tissues to a late season berry ripening problem known as bunchstem necrosis. Upon its description, researchers in Oregon noted its appearance (Jordan, 1989; Lombard *et al.*, 1989) and proposed another name for the disorder, inflorescence necrosis (IN).

As word of the IN symptoms spread in the scientific community, additional sightings of IN were noted (Creasy, 1995), many in other cool climate viticulture regions. Since IN had seriously affected Oregon winegrape harvests in multiple years (Lombard *et al.*, 1991), it has been a focus of study at Oregon State University. Jordan (1989) linked IN symptoms with high levels of NH_4^+ in the flower clusters, results that were confirmed by the work of Ibacache (1990) and Gu (1992). Research also suggested that high NH_4^+

concentrations in the flower clusters may be due to inadequacies in the enzyme system utilized by plants to detoxify, or assimilate NH_4^+ (Gu *et al.*, 1994). So far, research, as it so often does, has raised many more questions than it has answered.

The objectives of this research: 1) investigate the dynamics of NH_4^+ levels in grape shoot tissues and IN severity in response to various defoliation, tipping, shading, and salt solutions/growth regulators presumed to affect IN, 2) explore the effect of rootstock or clone on flower cluster NH_4^+ and IN severity, and 3) follow up on previous research that suggested that the NH_4^+ assimilation pathway may not be functioning in parts of the flower cluster near bloom, leading to NH_4^+ accumulation and appearance of IN symptoms. The latter goal was limited to investigating Fd-glutamate synthase, since its activity had not been demonstrated in grape tissues before.

Chapter 2: Literature Review

Introduction

The Oregon grape industry has been steadily expanding for more than a decade and a half. In 1981, there were only about 1000 acres of winegrapes planted in the state. By 1994, that figured had increased to about 6500, with additional acreage being planted every year (Watson and Price, 1995). Most of the vineyards are concentrated in the Willamette Valley, with significant acreage also found in southern Oregon (the Rogue and Umpqua river valleys). Pinot noir is the most widely produced variety, making up about 40% of the acreage (Watson and Price, 1995). Consistent fruit set has been a continuing problem in Oregon vineyards, with poor weather during bloom often being blamed for the small clusters and low yields that result.

There are numerous reasons for low yields in grape vineyards. Flower bud initiation occurs in the season prior to its emergence, coincidentally, during flowering of the current year's growth (Pratt, 1971). If buds and the leaves subtending them are shaded, the framework of cluster primordia developing in these buds may be smaller than if well-exposed to light (May, 1965). In the following year, fewer and smaller clusters on shoots are a result of the conditions in the previous year. In some years winter freezes can cause a reduction in yield, due to death of grape bud tissue. In February of 1989, temperatures in the Willamette Valley reached as low as -18°C, causing damage in many vineyards and resulting in sparse budbreak and low yields (Lombard *et al.*, 1990). Crop can also be lost to near freezing temperatures after budbreak, which damages the tender shoot tissue, including flower clusters. At the other end of the growing season, fall frosts can damage buds on green shoots, resulting in poor budbreak the following spring. In 1990, 81% of the total crop loss in Oregon was attributed to frosts (Lombard *et al.*, 1991). However, fruit set, the percentage of florets on a flower cluster that are pollinated, fertilized, and develop into mature berries, varies significantly from year to year in

Oregon, resulting in reduced yields when fruit set is poor, or the necessity of thinning clusters from vines when excessive (Price *et al.*, 1995).

Fruit set

Factors affecting fruit set

Fruit set is affected by many variables, the most important being unfavorable weather during bloom. Grapevines are self-fertile, but if the weather is cool, pollen may not grow quickly enough to fertilize the ovule and in rainy periods pollen may be washed from the clusters (Kliewer, 1983; Winkler *et al.*, 1974). Ovule fertilization in several winegrape varieties was reduced at 15°C day/10°C night temperatures versus 25°C/20°C regimes (Ewart and Kliewer, 1977). Mean temperature in the Willamette Valley during the month of flowering (June) is 16°C, with the average low being 10°C and the high 22°C (Oregon Climate Service figures, averages from 1961 to 1990). Thus, weather that may impede the fertilization of grape flowers commonly occurs in Oregon, where poor fruit set is a continuing problem. Excessively warm temperatures, on the other hand, may compress the flowering period such that a short period of rain that coincides with it may reduce fruit set, and hot temperatures (>35°C) actually inhibit pollination of flowers (Winkler *et al.*, 1974).

In some cases, fungal or insect attack can damage flowers, resulting in loss of potential crop. Thrips can feed on flower tissues as they are developing, distorting them as they grow, or causing the flower to abort (Winkler *et al.*, 1974). Flower clusters can be attacked by fungal agents, such as *Botrytis cinerea*, *Xanthomonas ampelina* (bacterial blight), or *Psuedopezicula tracheiphila* (rotbrenner), causing parts of the cluster to die and become necrotic (Schüepp, 1988; Siegfried, 1996; Winkler *et al.*, 1974).

Nutritional status of the vine results in more common causes of reduced fruit set. A lack of boron in the soil and vine causes abnormal shoot growth and the abortion of many flowers. Zinc deficiencies can also cause poor fruit set, but both of these problems can be remedied with foliar and/or soil applications of micronutrients (Kliewer, 1983; Winkler *et al.*, 1974). An excess of nitrogen in vines can also cause abnormally low fruit set, though perhaps in an indirect way by increasing vigor (Kliewer, 1983). Necrotic flower clusters found in vineyards of South Africa were said to be caused in part by excess vigor, brought on by high availability of nitrogen in the soil (Saayman, 1983b).

Improving fruit set

Since fruit set has been a problem for grape growers for centuries, several methods to increase the percentage of flowers that develop into fruits have been developed. Even at very early stages of formal grape research, in the 1880s, it was thought that competition for photoassimilates/ carbohydrates (though expressed in terms of shoot tip growth, importance of the presence of leaves, and cloudy weather) within the vine contributed to the fruit set problem (Müller-Thurgau, quoted in Sartorius, 1926). The actively growing, and numerous, shoot and root tips are better able to draw nutrients needed for cell division and expansion (Hale and Weaver, 1962), leaving flower clusters at a disadvantage. Based on this reasoning, researchers found that if shoot tips (about 5cm) were removed at or slightly before bloom, thus eliminating strong competitors for photoassimilates and stored carbohydrates, fruit set was improved (Coombe, 1962; Oinoue, 1940; Skene, 1969). Another way of improving the competitive ability of the flower cluster was to isolate the subtending shoot from the rest of the vine. Since the shoot is a net exporter of assimilates near flowering, if those assimilates are prevented from leaving the shoot, more should be available for the cluster. The process used to achieve this is called girdling - the removal of a small (about 5mm) ring of shoot or cane tissue that includes everything down to the cambium (Winkler *et al.*, 1974). This

temporarily interrupts flow through the phloem from shoots to roots increasing the carbohydrate status of the shoots and resulting in greater fruit set (Weaver and McCune, 1959). It must be noted, that since phloem flow is cut off, all materials normally transported through it (*e.g.* growth regulators, amino acids, mineral nutrients, *etc.*) are stopped, which also may have an impact on fruit set.

Exogenously applied growth regulators have also been found to affect fruit set. Auxins and gibberellin (GA) have been applied to vines or clusters to increase fruit set, but usually in seedless varieties. Applied at bloom on seeded varieties, with almost all winegrapes being in this group, fruit set is not improved and may actually worsen (Christodoulou *et al.*, 1968; Theiler and Coombe, 1985; Weaver and Pool, 1971). Growth retardants, such as ethephon and cycocel, result in a positive effect on fruit set in seeded varieties when used at moderate concentrations (Coombe, 1965; Naito and Kawashima, 1980). Application of high levels of ethephon actually worsens fruit set (Creasy and Breen, 1995; Jackson, 1991).

Decreasing fruit set

Indeed, there are multiple ways to decrease fruit set, though there is usually less need for this in the grape industry. As mentioned above, ethephon, when applied at high levels (>500ppm), as well as GA on seeded varieties reduces the number of berries per cluster. Shading vines reduces fruit set (Gu *et al.*, 1996; Ibacache *et al.*, 1991; Ollat, 1992), as does defoliation of whole vines, parts of shoots, or severe pruning of shoots before flowering (Aravindakshan and Krishnamurthi, 1969: Jordan, 1993). Another possible early season stress, water deficiency, can also lead to reduced fruit set (Hardie and Considine, 1976; Jackson, 1991).

Fruit set in general

A French term to describe poor fruit set in general is "coulure." This broad term includes filage, where parts of flower clusters develop into tendril-like structures, with small and sometimes unviable sets of flowers on the end, and millerandage, which results from unfertilized flowers developing into shot berries (Rives, 1961; Winkler, *et al.*, 1974). But historically, the term has been used to describe any fruit set condition where flowers fail to develop into normal berries.

In 1988, Jackson and Coombe reported a previously undescribed fruit set disorder, which they termed early bunchstem necrosis. Research into this disorder has been a focus of the Oregon State University viticulture program, starting with Jordan (1989), who suggested a different term to better describe it: inflorescence necrosis.

Inflorescence Necrosis

Background

Inflorescence necrosis (IN) has no doubt occurred for many years, but been attributed to coulure, rotbrenner, or botrytis infection of the flowers and rachis, whose symptoms are similar. Researchers (Jackson and Coombe, 1988; Keller and Koblet, 1994) have not been able to isolate any pathogens from affected tissue, suggesting that IN has a physiological origin. Symptoms manifest themselves as a necrosis of flower and pedicel tissue in mild cases, but in severe cases, the necrosis can progress to encompass rachis or peduncle tissue (Fig 2.1). The disorder appears on flower clusters from just before bloom through fruit set (Ibacache, 1990). In Oregon, IN has contributed to low yields across the state in 1981, 1983, 1988, and 1990 (Jordan, 1989; Lombard *et al.*,

1991). In other years it has not been a statewide problem, but probably contributed to crop loss in individual vineyards.

Figure 2.1. Example of a Pinot noir cluster severely affected by inflorescence necrosis. Note that parts of the rachis distal to the main axis have died back. Photo taken near fruit set. Note that most of the necrotic florets on this cluster retained their calypteras, though their bases had separated from the ovary's base.

There are other, distinct, fruit set problems that have symptoms similar to that of IN. As mentioned earlier, several fungal agents that infect florets and pedicels cause death and browning of the tissue that looks similar to IN. In South Africa, Growth Arrestment Phenomenon, which appeared in Sultanina (syn. Thompson Seedless)

expressed symptoms very similar to severe IN. Further research suggested it was caused by mineral imbalances and "faulty carbohydrate metabolism" (Saayman, 1983a,b).

Factors that affect IN

Differences in susceptibility to IN have been found between grapevine varieties in Oregon. Jordan (1989) reported that Pinot noir, Gewurztraminer, Early Muscat, and Muscat Ottonel were more likely to show IN symptoms than other varieties, but added that even Riesling, traditionally regarded as having better set than other varieties, can be affected. Lombard *et al.* (1989) noted that Pinot noir, Chardonnay, Riesling, Gewurztraminer, Cabernet Sauvignon, and Early Muscat in the Willamette Valley were affected by poor set in 1988, though this list includes both IN and shot berry affected clusters. In 1990 the most affected varieties were Pinot noir, Riesling, Gewurztraminer, Pinot gris, and Sauvignon blanc. Chardonnay was singled out as not showing symptoms of IN in that year, though it suffered from shot berries (Lombard *et al.*, 1991).

In New Zealand, field observations showed moderate levels of IN in Malbec, Pinot noir, Merlot and Meunier and very little IN on Riesling, Chardonnay, Cabernet Sauvignon, Pinotage, and Chasselas (Jackson and Coombe, 1988). In greenhouse trials varieties were ranked as to the appearance of IN on clusters, with the high group containing the varieties Alicante (58% necrosis), Brown Muscat, Muscat Hamburg, Muscat Ottonel, Queen of the Vineyard, Black Hamburg, and Italia (16% necrosis). Varieties showing little IN in the greenhouse were Schuyler, Cabernet Sauvignon, Pearl of Csaba, Fiesta, Chasselas, Flora, and Cardinal (Jackson and Coombe, 1988).

So far, there seems to be little convergence as to varieties that are particularly susceptible to IN, with the exception of Riesling, Pinot noir, and Gewurztraminer in Oregon. There have been seasons since 1991 where there was significant IN occurring in Oregon vineyards (personal observation), but there are no reports of surveys that specifically looked for differences between varieties. Pinot noir was also affected in New

Zealand, suggesting that it is chronically susceptible to IN. However, it must be noted that in Oregon and New Zealand significant acreage is planted to Pinot noir, so it receives more attention than other varieties.

Varietal clones may differ in susceptibility to IN. Jordan (1989) reported that Pinot noir clone UCD4, or Pommard clone, had greater IN severity than the UCD2A (Wädenswil) clone. In a Willamette Valley Pinot noir clonal trial the Colmar 538 clone had the highest IN severity (72% necrotic flowers) and UCD2A the lowest (25%) (Jordan, 1989).

The disorder also seems to be associated with regions that experience cool and/or wet weather preceding bloom (Jackson and Coombe, 1988; Jordan, 1989; Lombard *et al.*, 1993), although symptoms can also be brought on by drought stress (Jackson, 1991; Jackson and Coombe, 1988; Lombard *et al.*, 1993). Thus site, which affects these factors, would appear to have an effect on IN. In general, it would seem stresses to vine growth tend to worsen IN severity.

Similarity to a late-season cluster disorder

Because similar tissues are affected and early season cool weather may exacerbate its symptoms (Theiler and Müller, 1986), IN has been likened to bunchstem necrosis (BSN). BSN causes berries on parts of the grape cluster to become flaccid and accumulate less color and sugars (Ureta *et al.*, 1981). Symptoms, which include necrotic regions on the cluster rachis, are not noticed until after veraison, the beginning of the ripening phase of berry development. In addition to the association of BSN with cool spring temperatures, mineral imbalances have also been implicated, and symptoms could be induced with the application of diammonium phosphate to the clusters (Christensen and Boggero, 1985; Theiler and Coombe, 1985).

Beginnings of research on IN

Due to the similarity, first research on IN started from what was known about BSN (Jackson and Coombe, 1988; Jordan, 1989). Both disorders were associated with vineyards showing excessive vigor, shade, and other stresses. Ethylene was found to induce IN symptoms (Jackson, 1991), as could chemicals containing ammonium (NH_4^+) (Gu *et al.*, 1994; Holzapfel and Coombe, 1991; Jackson and Coombe, 1988; Jordan, 1989; Keller and Koblet, 1995). The cations Ca^{2+}, Mg^{2+}, and K^+ have been implicated, but their effects in reducing or inducing IN have been conflicting (Jackson and Coombe, 1988; Keller and Koblet, 1995; Gu, 1992).

Though shoot tipping increases fruit set, if it is severe enough (*e.g.* removal of the shoot at the second node above the distal cluster), it can actually increase IN severity (Jordan, 1993). Since vigor is associated with IN, Jordan (1993) thinned the number of shoots per Cabernet Sauvignon vine several weeks after budbreak to either 10 or 50 shoots. Vines with fewer shoots had greater growth and number of active shoot apices, and also increased incidence of IN. Ibacache (1990) thinned shoots of Pinot noir to 10 or 24 shoots per meter, but found no difference in IN between the treatments. This was attributed to the high background levels of IN in the experimental vineyard (60% necrotic florets).

Leaf removal can also affect IN. Jordan (1993) stripped leaves off all the shoots of vines two weeks before bloom, which resulted in more severe IN. In a study with similar treatments, Aravindakshan and Krishnamurthi (1969) found that complete defoliation as well as removal of the leaves basal to the flower clusters reduced fruit set. Unfortunately, as in many earlier studies of coulure and fruit set, a sufficiently detailed description of the characteristics of the increased shatter were not given, so in the 1969 study it is unclear if the reduction in fruit set was due to IN. Leaves are still indicated as being important to fruit set, however. In a model system using one node green shoot cuttings, Keller and Koblet (1995) found that the presence of a leaf on the cutting prevented necrosis of the cluster tissue.

Shade and IN

One of the surest ways to induce IN is to shade vines for at least several weeks before bloom. Gu *et al.* (1996) shaded (to 50% of ambient light) potted own-rooted Pinot noir vines from budbreak, which resulted in 27% necrotic tissue on flower clusters, versus less than 1% on control vines. Keller and Koblet (1994) grew potted vines of Müller-Thurgau on SO4 rootstock in growth chambers at different light levels and found that necrosis in the clusters increased when light was limiting. In a research vineyard, Ibacache (1990) showed that shading whole vines to 50% ambient light increased the percentage of necrotic florets by 240%. Shade was also found to increase NH_4^+ levels in flower cluster tissue (Ibacache *et al.*, 1991; Gu *et al.*, 1996).

Ammonium and IN

Much of the ensuing research on IN has centered on the involvement of the NH_4^+ cation. Taking clusters affected by various levels of IN, rating the severity visually, and then measuring the NH_4^+ in the cluster, Jordan (1989) found that flower clusters with more severe IN tended to have higher NH_4^+ levels. That relationship was formalized in both greenhouse and field-grown vines (Gu *et al.*, 1994; Ibacache *et al.*, 1991), showing that necrosis increased with increasing rachis and pedicel NH_4^+ concentration.

Although Jordan's data (1989) suggested that there was an absolute level of NH_4^+ that corresponded to the appearance of severe IN, further data collected do not support this hypothesis, as the relationship between IN and pedicel and rachis NH_4^+ is progressive and linear (Gu *et al.*, 1994; Ibacache *et al.*, 1991). Also, necrosis greater than 25% of rachis tissue in green shoot cuttings that were incubating in NH_4^+ solutions occurred at tissue NH_4^+ levels in the range of 6 to 8mg NH_4^+-N/g dry rachis weight (Gu *et al.*, 1994). Yet in the greenhouse, these same researchers found that 25% IN occurred at NH_4^+ levels of less than 3mg/g dry weight (Gu *et al.*, 1996). In shaded field vines (Ibacache *et al.*,

1991) 25% necrosis was found on clusters with 7 to 9mg NH_4^+/g rachis dry weight. So under different conditions, IN and NH_4^+ seem to vary somewhat independently, though high NH_4^+ is always found in flower clusters exhibiting symptoms. The reverse, however, is not true: high NH_4^+ can be found in tissues without IN symptoms (*e.g.* flower cluster NH_4^+ concentration is high from before bloom, but necrosis appears only near bloom (Gu, 1992), or a chemical treatment that increases NH_4^+ two-fold over controls in the flower cluster does not result in severe IN (Creasy and Breen, 1995)). Nevertheless, under a given set of conditions, there is a moderate relationship between IN and NH_4^+ (Gu *et al.*, 1994; Ibacache *et al.*, 1991).

Model systems

Jordan (1989) found that NH_4^+ would induce IN symptoms in flower clusters, and developed a model system that took advantage of the readily available tendrils (which derive from the same primordial tissue in the grape shoot apex) over hard to obtain flowers. Using tendrils from Cabernet Sauvignon with their cut ends incubating in various solutions, he determined that NH_4^+ was toxic to grape tissue. Working on the hypothesis that elevated NH_4^+ may be caused by inadequate NH_4^+ assimilation, Jordan introduced exogenous α-keto glutarate (α-KG), which is a substrate of the NH_4^+ assimilation pathway (described in more detail later in this chapter), to the system. Addition of this substrate to solutions with high NH_4^+ concentration that the tendrils were incubating in, reduced their fresh weight loss and tissue NH_4^+ levels. Additional evidence showing that amino acid synthesis in the model system was also stimulated upon the addition of α-KG led him to the conclusion that NH_4^+ accumulated in the flower tissues due to inadequate supply of substrates to the NH_4^+ assimilation pathway (Jordan, 1989).

This model system has been criticized as not always being indicative of flower cluster responses (Holzapfel and Coombe, 1991), but Gu (1992) developed a similar

incubation method that used green shoot cuttings that included a leaf and a cluster from the field. With it, he was able to show that the NH_4^+ cation could duplicate IN cluster symptoms while not affecting leaf tissues, and that the addition of α-KG could reduce tissue NH_4^+ and severity of necrosis. Keller and Koblet (1995) used a similar system and found that the addition of sucrose would delay the onset of necrosis in the cluster. This was more evidence that carbohydrate nutrition was involved in the appearance of IN. Sugars are broken down and used in the tricarboxylic acid cycle to supply organic acids used in NH_4^+ assimilation (Matsumoto and Wakiuchi, 1974). NH_4^+ has also been reported to stimulate the tricarboxylic acid cycle (Paul *et al.*, 1978).

NH_4^+ assimilation

The principal NH_4^+ assimilation enzymes in higher plants are glutamine synthetase [GS, EC 6.3.1.2] and glutamate synthase [syn. glutamine: oxoglutarate aminotransferase, or GOGAT] (Givan, 1979). Glutamate formed in the cycle generally is used in the synthesis of asparagine or other amino acids (Joy, 1988). Much of the work in elucidating the pathway (Fig. 2.2) has been through the use of rice, maize, and bean plants. Work on either GS or GOGAT in woody perennial species has been limited, and only a small amount of work has been reported in grapevine. GS activity has been found in roots and leaves of Chenin blanc (Roubelakis-Angelakis and Kliewer, 1983), and in leaves and fruits of Merlot (Ghisi *et al.*, 1984). Roubelakis-Angelakis and Kliewer (1983) were unsuccessful in their attempts to obtain GOGAT activity from grape tissue, but this was probably due to difficulties in extracting the active enzyme. Indeed, almost a decade later, Jordan *et al.* (1992) reported evidence of NADH-GOGAT activity in grape leaf extracts.

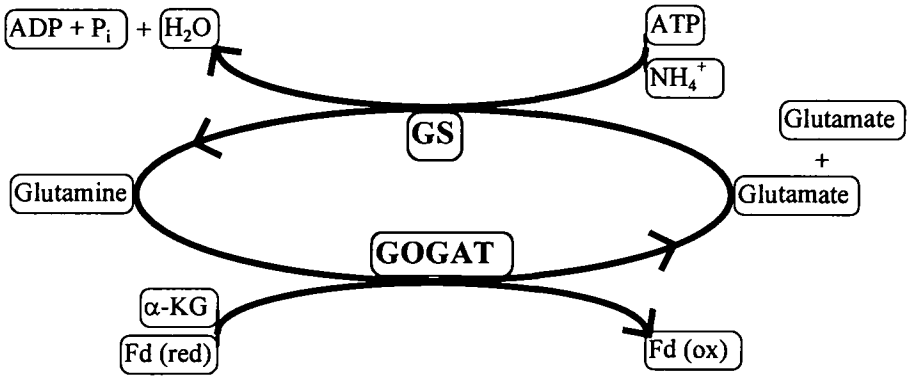

Figure 2.2. The glutamine synthetase [EC 6.3.1.2] / ferredoxin-glutamate synthase [EC 1.4.7.1] (GS/GOGAT) cycle.

Before the early 1970s, glutamate dehydrogenase [GDH, EC 1.3.1.2], located in the mitochondria, was thought to be the principal agent for ammonium assimilation, using a reducing agent to aminate α-KG, thus forming glutamate:

$$NH_3 + \alpha\text{-KG} + NAD(P)H + H^+ \leftrightarrow \text{glutamate} + H_2O + NAD(P)^+$$

But GS was found to have a much higher affinity for NH_4^+ than did GDH (Lea and Miflin, 1974; Stewart *et al.*, 1980), and was located in the cytoplasm and chloroplasts, where it could act on NH_4^+ diffusing out of the mitochondria (Keys *et al.*, 1978). However, there still is controversy as to the exact role of GDH in plant tissues, be it anabolic or catabolic (Oaks, 1994; Oaks and Yamaya, 1990; Yamaya and Oaks, 1987). GDH may function in an NH_4^+ assimilatory role in mitochondria if NH_4^+ released by photorespiration reaches sufficiently high levels (Yamaya and Oaks, 1987).

Glutamate Synthase

The counterpart of GS, GOGAT, is found in chloroplasts and root plastids. Two forms of GOGAT are generally recognized and distinguished by the source of reducing power they use, either NAD(P)H or ferredoxin (Fd). NAD(P)H-GOGAT [EC 1.4.1.13

and 1.4.1.14] is found in higher amounts in non-photosynthetic tissues, whereas Fd-GOGAT [EC 1.4.7.1] predominates in leaf and other photosynthetically active tissues (Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992). These forms are immunologically distinct, and probably coded for by separate genes (Anderson *et al.*, 1989; Hawakawa *et al.*, 1992; Sakakibara *et al.*, 1991; Suzuki *et al.*, 1982; Suzuki *et al.*, 1983; Suzuki *et al.*, 1987; Suzuki and Gadal, 1984).

The importance of NAD(P)H-GOGAT in legume nodule nitrogen fixation, along with the relative ease in assaying for its activity (usually by following the decrease in absorbance at 340nm as the reductant is used by the enzyme), has resulted in a large body of literature on this enzyme. Fd-GOGAT activity is harder to quantify, and is usually assayed by measuring the accumulation of glutamate product. This method often involves the use of ion-exchange to separate glutamate from other reaction constituents, followed by reaction with ninhydrin reagent to develop color that is proportional to the amount of glutamate formed (Matoh *et al.*, 1980). Or, for more precise determination of glutamate formed during test reactions, derivitization of glutamate (and other amino acids) with o-phthaldialdehyde (Lindroth and Mopper, 1979) is done prior to separation and detection using HPLC (Martin *et al.*, 1982).

Fd-GOGAT has been purified in several different higher plant species. Molecular weights range from 145kDa in pea (Wallsgrove *et al.*, 1977) to a 224kDa dimer in rice (Suzuki and Gadal, 1982). There seems to be little consensus in the size and make-up of the enzyme (see Suzuki and Gadal, 1984 for review), and even the statement that prosthetic groups are not associated with it have since been challenged (Hirasawa and Tamura, 1984). Similarly, calculated K_m values for glutamine and α-KG vary widely between species (Suzuki and Gadal, 1984), with one report showing that the enzyme from rice leaf has a different K_m for glutamine dependent on the glutamine concentration (Suzuki and Gadal, 1982).

Polyclonal antibodies have been raised against Fd-GOGAT purified from a range of plant species (Botella *et al.*, 1988; García-Gutiérrez *et al.*, 1995; Marquez *et al.*, 1988; Suzuki *et al.*, 1982; Zehnacker *et al.*, 1992) and antibody from one species has successfully been used to screen other species, including monocot to dicot probes

(Becker *et al.*, 1993; Commere, *et al.*, 1986; García-Gutiérrez *et al.*, 1995). Recently, three monoclonal hybridoma cell lines for tobacco Fd-GOGAT were isolated and used to show that methyl viologen and Fd share the same electron donor sites (Suzuki *et al.*, 1994).

DNA of Fd-GOGAT from a variety of plant species has been sequenced at least in part, and the protein appears to be highly conserved among higher plants, as well as having similarities to bacterial GOGAT (Avila *et al.*, 1993; García-Gutiérrez *et al.*, 1995; Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992).

The function of the GS/GOGAT cycle in photosynthetic tissues

As mentioned earlier, the primary role of the GS/GOGAT cycle is to assimilate NH_4^+ generated in tissues. Two large sources of NH_4^+ in plants are primary nitrogen assimilation (*i.e.* the reduction of NO_3^- to NH_4^+) and photorespiration (Joy, 1988). In relatively recent times, the development and use of mutant plants deficient in Fd-GOGAT activity have done much to determine the role of this enzyme (see review by Blackwell *et al.*, 1988a). Such mutants develop and grow normally under non-photorespiratory conditions (*i.e.* O_2 concentration is lowered or CO_2 raised), but when taken out of those conditions, they rapidly accumulate NH_4^+ to the point where CO_2 fixation stops (Blackwell *et al.*, 1988b). Production of NH_4^+ from NO_3^- reduction was not found to be significant in these test plants, or at least, the action of GDH was sufficient to keep NH_4^+ from reaching toxic levels.

GS/GOGAT in woody perennials

GS activity has been assayed from a few woody plant species, including avocado fruit (Loulakakis *et al.*, 1994), the leaves of several deciduous tree species, elderberry, and horsechestnut (Pearson and Ji, 1994), jack pine seedling roots (Vézina, *et al.*, 1989), Scots pine seedling cotyledons (Elmlinger *et al.*, 1994), and rose cell cultures (Mohanty and Fletcher, 1980). GS activity has been demonstrated in grape leaf, root, and berry tissues. Roubelakis-Angelakis and Kliewer (1983) partially purified GS using L-cysteine and polyethylene glycol in the grinding and homogenizing buffers followed by centrifugation to separate plastidic and mitochondrial forms. Ghisi *et al.* (1984) obtained GS activity in crude extracts of grape leaves and berries through the use of EDTA, L-cysteine, and PVPP. These researchers also determined that GS activity was greater than that of GDH and used that as evidence that NH_4^+ is mainly assimilated by the GS/GOGAT pathway. The interest in GS activity is usually connected to trying to elucidate nitrogen metabolism within plants, be it from primary N-assimilation, photorespiration, or amino acid formation for protein synthesis.

Fd-GOGAT has not been isolated, nor its activity determined previously in grape tissue. Roubelakis-Angelakis and Kliewer (1983) specifically reported that "numerous attempts to obtain active GOGAT preparations from grapevine roots have not yet been successful." This is not to say that it isn't present and active in grape tissues, as extraction of active enzymes from woody perennial species is often difficult (Loomis, 1974). Phenolic compounds, which are released and subsequently mix with the other cellular components, complex with proteins, inactivating and precipitating them out of solution. Successful extraction of GOGAT requires the inclusion of PMSF, β-mercapto-ethanol (β-ME), and EDTA in the homogenizing buffer for herbaceous species. Enzyme stability is also improved with the addition of PMSF, dithiothreitol, and/or β-ME to the buffer solution (Hirasawa and Tamura, 1984; Suzuki and Gadal, 1982; Wallsgrove *et al.*, 1977). In one report, the bacterial (*Chlamydomonas reinhardii*) enzyme is able to withstand significant heating and still retain some activity (Galván *et al.*, 1984), but this has not been reported in plant species. On the contrary, Tamura *et al.* (1980) reported that the

purified Fd-GOGAT from spinach was unstable in solution, even at temperatures of 0 to 4°C.

Fd-GOGAT activity has been assayed or isolated in several pine species (Elmlinger and Mohr, 1991; García-Gutiérrez *et al.*, 1995; Vézina, *et al.*, 1989) and rose (Mohanty and Fletcher, 1980). Note, however, that material in these studies were either from seedlings or dark-grown suspension cultures, to avoid tissues high in phenolic compounds. Extraction buffers contained detergents (Triton X-100), metal chelators (*e.g.* EDTA), and phenolic compound scavengers (*e.g.* PVPP). To this author's knowledge, Fd-GOGAT activity in other woody species has not been demonstrated as of yet.

Inhibitors of GS and GOGAT

A much more accessible way of studying the activity of GS and GOGAT, especially in woody perennials, is through the use of inhibitors. Much work has been done on the effect of such inhibitors as the glutamate analogs methionine sulfoximine (MSO) and phosphinothricin (PPT) on GS activity (see Lea and Ridley, 1989), which are generally regarded to be fairly specific in nature. Included in this specificity is that MSO does not affect GOGAT activity. Specific inhibitors of GOGAT are not available, but there has been widespread use of azaserine, an analog of glutamine, to study both NAD(P)H- and Fd-GOGAT. Azaserine is an inhibitor of all amido-transfer reactions, but when used under controlled conditions, can be a good tool to investigate GOGAT in plant tissues.

The use of GS inhibitors in grapes

Since GS has been only lightly studied in *Vitis*, use of inhibitors has actually been limited to work on IN. Jordan (1989) used MSO in his tendril model system and concluded that because the addition of MSO failed to increase NH_4^+ levels, GS was not actively assimilating it. Gu *et al.* (1994) showed that MSO resulted in an increase in tissue NH_4^+ when added to their green cutting model system. Pinot noir lamina, petiole (Gu *et al.*, 1991), flower, and pedicel tissue NH_4^+ rose significantly with the introduction of MSO, however, rachis tissue NH_4^+ was not affected. The authors concluded that GS was not active in rachis tissue, since there was no response to MSO in only that part of the cluster. In a similar study (Keller and Koblet, 1995) PPT was added to the incubation solution of green Müller-Thurgau cuttings, which resulted in more severe IN symptoms. These authors also concluded that GS must be active in cluster tissues, as disruption of GS should lead to NH_4^+ accumulation in and necrosis of the cluster. Note that they did not test specific parts of the flower cluster with the inhibitor.

These GS inhibitors have also been used in vineyards. Creasy and Breen (1995) used MSO in a cluster dipping solution, which increased Pinot noir flower cluster tissue NH_4^+ two-fold over that of controls. However, the incidence of IN could not be accurately assessed because clusters were harvested before bloom. Keller and Koblet (1995) used PPT to successfully induce necrosis in Müller-Thurgau flower clusters, and noted that symptoms were first seen in flower and pedicel tissue, followed by rachis and peduncle tissue. Naturally occurring IN follows the same pattern of necrosis (Ibacache, 1990).

From these experiments, the activity of GS can be inferred in grape cluster tissues, as well as in laminas and petioles. Keller and Koblet (1995), however, suggested that rachis tissue does have GS activity, which runs contrary to statements by Gu *et al.* (1994). It must be noted that in the latter group's experiments flower cluster tissues were separated, necrosis rated as "percentage of necrotic tissue area on the rachis and pedicel," and then followed by NH_4^+ determination. In the experiments using PPT (Keller and Koblet, 1995), NH_4^+ was not measured and necrosis was presented as percent necrotic

flowers per cluster. These slight differences in measuring IN severity may obfuscate direct comparisons, but there still remains the discrepancy of GS being active in rachis tissue. Gu *et al.* (1994) reported necrosis of rachis tissue without detecting an increase of NH_4^+ in that specific tissue due to MSO, while Keller and Koblet (1995) reported only visual cues as to the action of the inhibitor on GS. Keller and Koblet (1995) as well as Ibacache (1990) noted that IN symptoms start with the flowers and pedicels, and only then progress to the more basal rachis tissues. The results of Gu *et al.* (1994) indirectly demonstrated activity of GS in pedicel tissue, but not rachis tissue, so symptoms would seem to appear in a tissue that has, at least, some capacity for NH_4^+ utilization before a tissue that does not have that capacity. Gu *et al.* (1996) suggest that rachis acts as a pool of high NH_4^+, but why it is not affected by what should be a toxic NH_4^+ concentration is unknown. However, there are more variables here, as Gu *et al.* also demonstrated temporal differences in inhibitor response. There was differential response to inhibitors as the flower clusters approached and passed anthesis, with the greatest response occurring one week post-bloom in one year, yet in the following year, responses were seen only at the one week pre-bloom. So sampling time could have a great effect on results reported. Additionally, it is known that there are varietal differences in susceptibility to IN, which might mean variations in enzyme activities. These two studies used Pinot noir (Gu *et al.*, 1994) and Müller-Thurgau (Keller and Koblet, 1995). In Oregon, Pinot noir is regarded as a susceptible variety (Jordan, 1989; Lombard *et al.*, 1989; Lombard *et al.*, 1991), while there is insufficient acreage of Müller-Thurgau in Oregon to know its susceptibility to IN.

The use of GOGAT inhibitors in grapes

As previously reviewed, NAD(P)H- or Fd-GOGAT enzymes have not been studied extensively in grape. Azaserine has been used in two studies to provide evidence of GOGAT activity. Gu *et al.* (1994) used azaserine in the same set of experiments that

MSO was used in, and obtained similar results. That is, the response in tissue NH_4^+ was the same as that seen for MSO, *viz.* it elevated NH_4^+ when the inhibitor was applied to flower or pedicel tissue, but not rachis tissue. Jordan *et al.* (1992) used azaserine to verify their assay of NADH-GOGAT from crude extracts of grape leaf tissue. In this case, the inhibitor slowed the oxidation of NADH in the enzyme assay compared to the assay without azaserine.

Chapter 3: Vine Manipulation Effects on Shoot Tissue Ammonium and Inflorescence Necrosis

Abstract

The fruit set disorder of grape, inflorescence necrosis (IN), has been associated with elevated ammonium (NH_4^+) in flower cluster tissues, but little is known about how NH_4^+ varies in shoot tissues under conditions that may affect IN severity. Beginning in 1993, shoots and vines of Pinot noir in a commercial vineyard were subjected to defoliation, tipping, or shade treatments and tissue NH_4^+ measured at bloom in three seasons. IN severity in the defoliation and tipping treatments was visually rated after fruit set in 1994 and 1995. Treatments were applied from budbreak (early) or from one week before bloom (late). Defoliation treatments were 1] removal of all leaves (complete), 2] removal of leaves from the 9th node and below (lower), and 3] removal of leaves from the 9th node and above (upper) of a single shoot. Treatments were applied to ten shoots each. Shoot growth was adversely affected by removal of leaves as they appeared on the shoot tip from budbreak (early-complete and -lower treatments). Complete defoliation of a shoot was the only treatment to increase flower cluster NH_4^+ levels in all three years, but there was significant variation in concentration and responses to treatments from year to year. In the year with the lowest overall NH_4^+ levels (1994, 1.79mg NH_4^+-N/gdw), removal of leaves from part or all of a shoot either early or late increased flower cluster NH_4^+ significantly. Shoot tip NH_4^+ was most affected by early treatments, but was increased over control levels for all but the late-lower treatment. Severe shoot tipping in 1994 (removal of shoot above the 9th node) increased flower cluster NH_4^+, but removal of just the shoot tip itself (top 5cm) in 1995 had no effect on flower cluster NH_4^+.

Shade was applied early and late to individual shoots or to whole vines. Whole vine shade increased flower cluster NH_4^+ regardless of when it was applied, but late

application to individual shoots increased flower cluster NH_4^+ more than early application. Early shoot shade increased mature lamina tissue NH_4^+ six-fold over those from the controls while other treatments had no effect. Petioles from leaves opposite the cluster had much higher levels of NH_4^+ than lamina or shoot tip tissues, but less than that found in clusters.

IN severity in primary and secondary clusters was not affected by any treatments, but significant differences did appear on secondary clusters of shoots whose primary cluster had been removed at bloom. In this case, IN on secondary clusters was worsened by any early defoliation or late-lower defoliation treatment the most, though there were year to year differences.

These results show that NH_4^+ concentration and possibly IN in shoots can be manipulated up or down through the use of defoliation, tipping, or shading treatments, though there is a significant year to year variation.

Introduction

Inflorescence necrosis (IN, syn. early bunchstem necrosis), is a fruit set disorder of grape that has significantly reduced economic yields in some viticultural areas (Jackson and Coombe, 1988; Jordan, 1989; Lombard *et al.*, 1991). However, IN doesn't appear regularly, and its severity varies considerably in a given growing region. The full extent of symptoms is often not evident until after fruit set, but the necrotic flowers and, if severe, rachis tissue usually remain on undisturbed clusters (Creasy, 1995; Ibacache, 1990).

IN has been linked to ethylene/general stress (Jackson, 1991), low carbohydrate status (Gu *et al.*, 1996; Jordan *et al.*, 1991; Keller and Koblet, 1994), and to high ammonium (NH_4^+) concentration in affected tissues (Gu *et al.*, 1994; Ibacache *et al.*, 1991; Jordan *et al.*, 1991). External stresses to plant growth and development may induce production of NH_4^+ in tissues (Feng and Barker, 1992; Lovatt, 1990). Low

carbohydrate status due to low ambient light or shading of vines may lead to inadequate resources for NH_4^+ assimilation, causing NH_4^+ to accumulate and necrosis to occur, especially in flower clusters (Gu *et al.*, 1996; Keller and Koblet, 1994). The NH_4^+ cation, in salt solutions, has been reported to induce IN symptoms (Gu *et al.*, 1994; Jackson and Coombe, 1988; Keller and Koblet, 1995). The presence of α-ketoglutarate (Gu *et al.*, 1994), a substrate of the ammonium assimilation pathway, or sucrose (Keller and Koblet, 1995) in those solutions can reduce or prevent IN symptoms.

Though evidence suggests that NH_4^+ is a causal agent of IN symptoms, there is still little known about why NH_4^+ accumulates to toxic levels in the flower cluster and not in other tissues. Shade applied to whole vines has been used to induce IN (Gu *et al.*, 1996; Ibacache *et al.*, 1991; Jackson, 1991; Jackson and Coombe, 1988), and has been found to raise vine NH_4^+ status (Gu *et al.*, 1996; Ibacache *et al.*, 1991; Jordan, 1989; Smart *et al.*, 1988). Techniques of manipulating shoots to increase IN have been demonstrated (Jordan, 1993), and those to reduce IN suggested (Jackson, 1991; Lombard *et al.*, 1993). Prevention primarily consists of efforts to reduce shade in the early season canopy. The connection between IN symptoms induced by some treatments and NH_4^+ in the flower cluster is still ambiguous.

There is little known about the implied association between IN and NH_4^+ levels within the grape shoot. In this report, the effects of shoot defoliation treatments, shoot tipping, and applied shade on shoot tissue NH_4^+ levels and IN severity are presented. All treatments should affect carbohydrate status of the shoot/vine, which should also have an impact on NH_4^+ levels and/or IN severity. According to present knowledge of IN, more available carbohydrates should decrease IN severity and NH_4^+ concentration.

Materials & Methods

Vineyard: A high-vigor commercial Pinot noir vineyard with a history of IN near Corvallis, Oregon was the site of these experiments. Vines were cane pruned and trained

to a Scott Henry system. All treatments were applied to shoots on the upper cane of the system, from which shoots are vertically positioned with catchwires.

Treatments. Defoliation and tipping: Three early defoliation treatments were imposed in 1994. From budbreak, leaves emerging from the shoot tip were removed from 20 randomly distributed shoots. Ten of these continued to have their leaves removed until fruit set (early defoliation, complete treatment), and the remaining 10 had their leaves removed up to and including the 9th node, after which leaves were allowed to develop normally (early defoliation, lower). When an additional 10 randomly distributed shoots grew to 10 nodes, leaves were removed as they emerged from the shoot tip starting from the 10th node (early defoliation, upper) and on through fruit set.

The same three defoliation treatments were applied to 30 additional shoots one week before bloom in 1993, 1994, and 1995 and given a "late" designation (Fig. 3.1). "Control" shoots were also randomly distributed throughout the trial and allowed to develop normally. Laterals were not allowed to develop in any of the treatments.

Also at one week before bloom in 1994 and 1995, ten shoots were tipped (Tip treatment): in 1994 the part of the shoot above the 9th node was cut off, which amounts to approximately 4 nodes above the secondary cluster (equal to about 70cm from the shoot base). In 1995 the top 5cm of the shoot tip was pinched off, leaving the rest of the shoot intact. Laterals on all shoots were removed as they appeared. Jordan (1993) reported that severe shoot tipping worsened IN severity, while removal of just the growing tip near bloom is considered a method to improve fruit set (Coombe, 1962).

Shoot and vine shade: At budbreak in 1994 and 1995, a 1.3m high cylinder of 50% transmission shadecloth (Ibacache, 1990) was erected around each of 10 buds on different vines (early shoot shade, or early-SS, treatment). In 1994 shadecloth was used to cover four whole vines, also at budbreak (early whole vine shade, or early-WS, treatment). Shoots developed inside the shaded environment until after fruit set, when IN was rated in 1995.

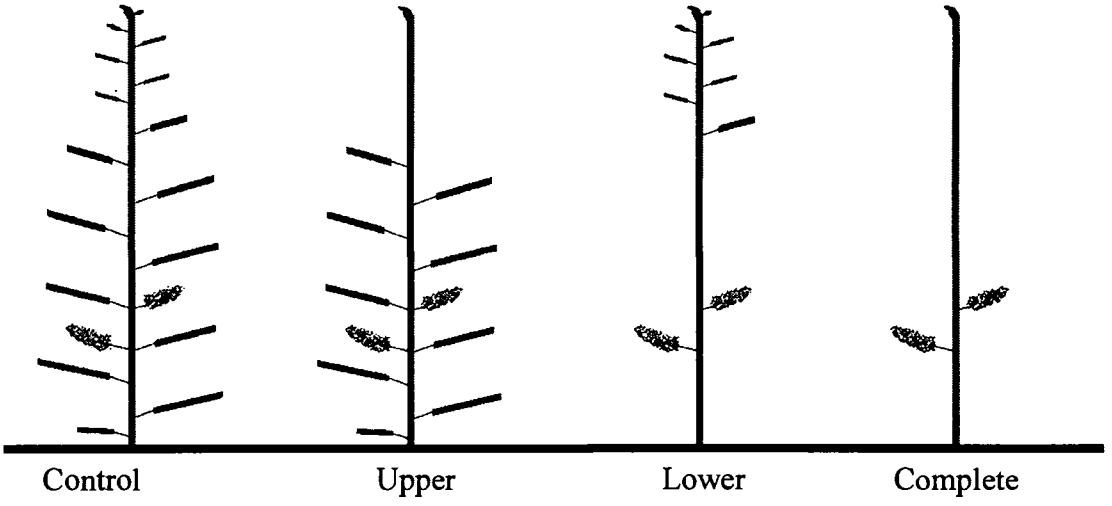

Figure 3.1. Diagrammatic representation of shoot treatments applied in 1993, 1994, and 1995. Upper defoliation was removal of all leaves above the 9th node. Lower defoliation was removal of all leaves at the 9th node and lower. Shown here is how shoots appeared at one week before first bloom, along with the approximate locations of the two clusters on the shoot.

One week before bloom in 1994 and 1995, shadecloth was applied to ten additional shoots each (late shoot shade, or late-SS, treatment). Similarly, shade cloth was erected around three whole vines (late whole vine shade, or late-WS, treatment) at this time in 1994 only. In 1993, ten individual primary clusters were covered loosely with aluminum foil at one week before bloom to look at the effect of very localized shade on tissue NH_4^+.

In 1995, ten additional shoots each received the late lower, complete, and upper defoliation treatments as well as Tip, early-SS, and late-SS, to be used solely for rating IN severity after fruit set.

Sample collection: Primary flower clusters were collected from all treatments at bloom. Additionally, in 1994 and 1995 the leaf blade and petiole opposite the cluster were collected from the shade treatments and control shoots. Shoot length and number of nodes were recorded from all shoots in defoliation treatments in 1994 only. Shoot tips (10cm) from shade (including ten shoots each in the early-WS and late-WS) and defoliation treatments in 1994 were collected and separated into young leaf, young

petiole, young tendril, the shoot tip itself, and analyzed separately, where possible. The amount of young petiole and tendril tissue collected from each shoot tip was inadequate for individual NH_4^+ analysis, so they were pooled before NH_4^+ determination.

After berry set in 1994 and 1995, IN on secondary clusters (when present) was visually rated on treatment shoots whose primary clusters had been harvested at bloom for NH_4^+ determination. The following scale was used: 1= <5% (little or no necrosis); 2= 5 to 20% (moderate); 3=20 to 40% (significant); 4= 40 to 60% (severe); 5= >60% (very severe). In 1995 ratings were taken from primary and secondary clusters on shoots with duplicate treatments.

Sample analysis: Plant tissue was dried in a forced-air oven at 55°C and ground in a Wiley mill with a #20 mesh screen. Ten milliliters of 2% (v/v) acetic acid were added to approximately 0.1g of each sample in a 16x100mm tube, which was capped, vortexed briefly, and then placed horizontally on a rotating (35rpm) shaker for 24 hours at room temperature.

An in-tube Plasma/Serum Separator (Karlan Research Products Corp., Santa Rosa, CA 95403 USA) was used to filter the solution in each tube. Ammonium concentration in the filtrate was determined with a Wescan Model 360 Ammonia Analyzer (Alltech Associates, Deerfield IL 60015 USA) and expressed on a mg NH_4^+-N per gram dry weight (NH_4^+ mg/gdw) basis.

Data were expressed as mg NH_4^+ - N per gram of tissue dry weight. Data were analyzed using Statgraphics v5.0 by ANOVA using a Tukey HSD separation for means, when necessary.

Results

Defoliation and tipping treatment effects on shoot growth: In 1994, the two defoliation treatments that removed leaves from budbreak up until flower cluster harvest had a negative effect on shoot growth (Fig. 3.2). Shoots from early, complete and lower

defoliation treatments had fewer nodes (except for the tipped shoots, which were cut off at the 9th node) and shorter internode lengths than other treatments. Early, complete defoliation shoots averaged 27cm in length and 11.2 nodes and early, lower defoliation shoots 48cm and 13.3 nodes. Control shoot values were 116cm and 15.4, respectively. Late, lower defoliation shoots showed a small effect due to removal of leaves, with slight reductions in both node number and shoot length. Late defoliation had little effect on shoot length, but node number varied slightly. Shoot length and node number on the early, upper defoliation treatment was virtually identical to that of the late, upper defoliation treatment. Removal of leaves on that early treatment started only three weeks before all of the late treatments were applied at one week before bloom.

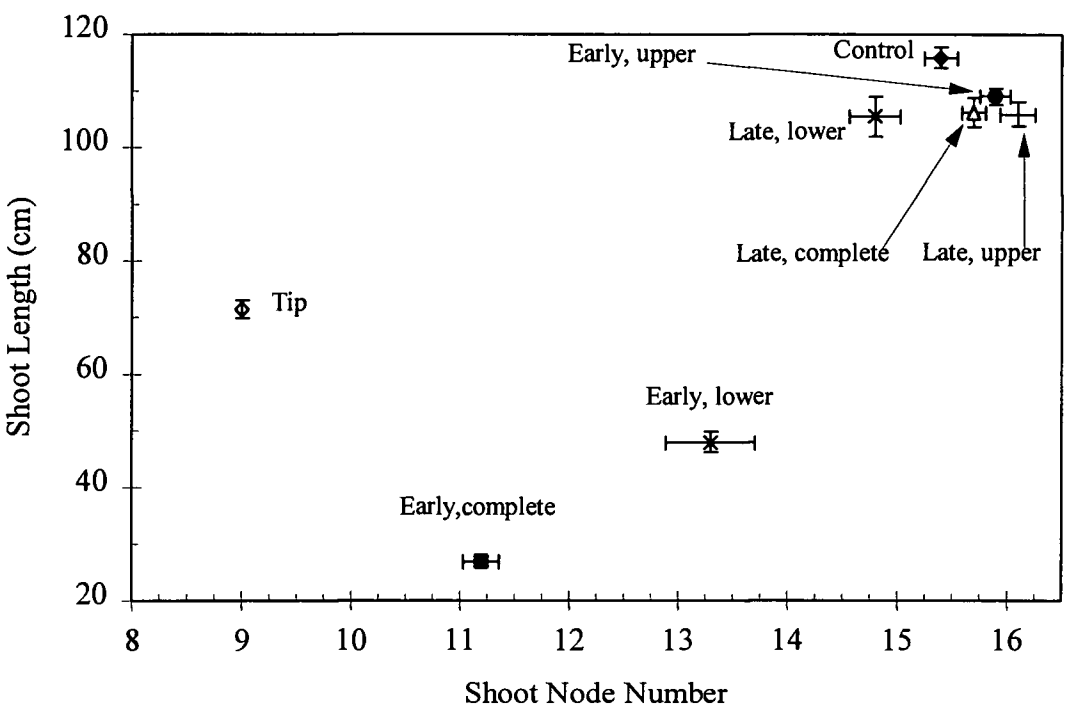

Figure 3.2. Averages of 1994 shoot node number versus shoot length as affected by defoliation and tipping treatments in a commercial Pinot noir vineyard. For complete description of treatments, see text. Measurements taken at bloom; bars indicate standard error.

Defoliation and tipping: The responses of flower cluster NH_4^+ levels to early and late applied defoliation and tipping treatments are shown in Table 3.1. Significant differences between controls and treatments, as determined by Tukey HSD separation of means at a 0.05 level were apparent only in 1993 and 1994. Tipping or late defoliation of part or all of a shoot increased flower cluster NH_4^+ in 1994, but only the complete defoliation-late treatment increased cluster NH_4^+ to any extent in all three years. The defoliation treatments in 1993 resulted in a different response relative to the control, with flower cluster NH_4^+ being significantly lower in the lower defoliation treatment, but slightly higher in the upper and complete treatments. Shoot tipping at the 9th node increased flower cluster NH_4^+ in 1994, but less severe tipping in 1995 had no effect. On a yearly basis, NH_4^+ concentrations were much lower, especially for the controls, in 1994 compared to 1993 and 1995, with values of 1.79, 2.88 and 3.15mg/gdw, respectively.

In 1994 when defoliation treatments were applied early, but flower clusters not harvested until first bloom, the response in cluster NH_4^+ was similar to late treatments (Table 3.1). Both early and late complete defoliation treatments increased flower cluster NH_4^+, but the late treatment more so. Removal of the upper leaves near budbreak increased NH_4^+ more than the other two early defoliation treatments.

NH_4^+ levels in shoot tips were lower than those found in flower clusters, even though both tissues were collected at first bloom. In 1994 the NH_4^+ concentration in control flower clusters was 1.8mg NH_4^+-N/gdw versus 0.6 in the tips. Shoot tip NH_4^+ was affected by treatments (Fig. 3.3), but responses differed from those of flower clusters. Compared to controls, all treatments except the late, lower treatment caused a significant increase in shoot tip NH_4^+. Defoliation of the entire shoot from near budbreak markedly increased shoot tip NH_4^+ above that of the control, the other early treatments resulted in smaller increases. The late treatments had a lesser effect on raising shoot tip NH_4^+, with the exception of upper defoliation-late, which had significantly higher NH_4^+ than its early season equivalent.

Table 3.1. Pinot noir flower cluster NH_4^+ levels as affected by defoliation and tipping treatments in 1993, 1994, and 1995. Vines were part of a commercial vineyard near Corvallis, Oregon, with treatments applied as noted. Tip = shoot cut off above 9th node (1994) or top 5cm removed (1995) one week before bloom (late). For complete description of the treatments, see text.

	Flower Cluster NH_4^+ (mg/gdw)		
Treatment	1993	1994	1995
Early Defoliation			
Complete		2.26*	
Lower		2.27*	
Upper		2.65*	
Late Defoliation			
Complete	3.45	2.65*	3.72
Lower	2.65*	2.33*	3.15
Upper	3.43	2.51*	3.02
Tip		2.34*	3.06
Control	2.88	1.79	3.15

* Indicates difference from control at p = 0.05 (Tukey HSD)

In 1994, early treatments resulted in no significant change in IN scores, although all of the treatments tended to have higher ratings than controls (Table 3.2). In the late treatments, IN severity was greater on lower defoliation shoots but unaffected by the other defoliation treatments. Shoot tipping reduced IN severity in both years, but the reduction was not statistically significant. Separate shoots with these treatments, where the primary clusters were not removed at bloom, were available for IN severity ratings in 1995, but there were no significant differences between treatments on either primary or secondary clusters (data not shown). There was, however, a strong positive relationship between the IN rating of the primary clusters and the rating of the secondary clusters on the same shoot ($p<0.001$).

In 1995, no treatment's IN rating of secondary clusters on shoots that had had their primary clusters removed at bloom differed from that of the control (Table 3.2). The Tip treatment, however, had a rating significantly lower than that of the late, complete

treatment. There was a trend for late, upper treatments to reduce, and lower defoliations to increase, IN severity in these secondary clusters.

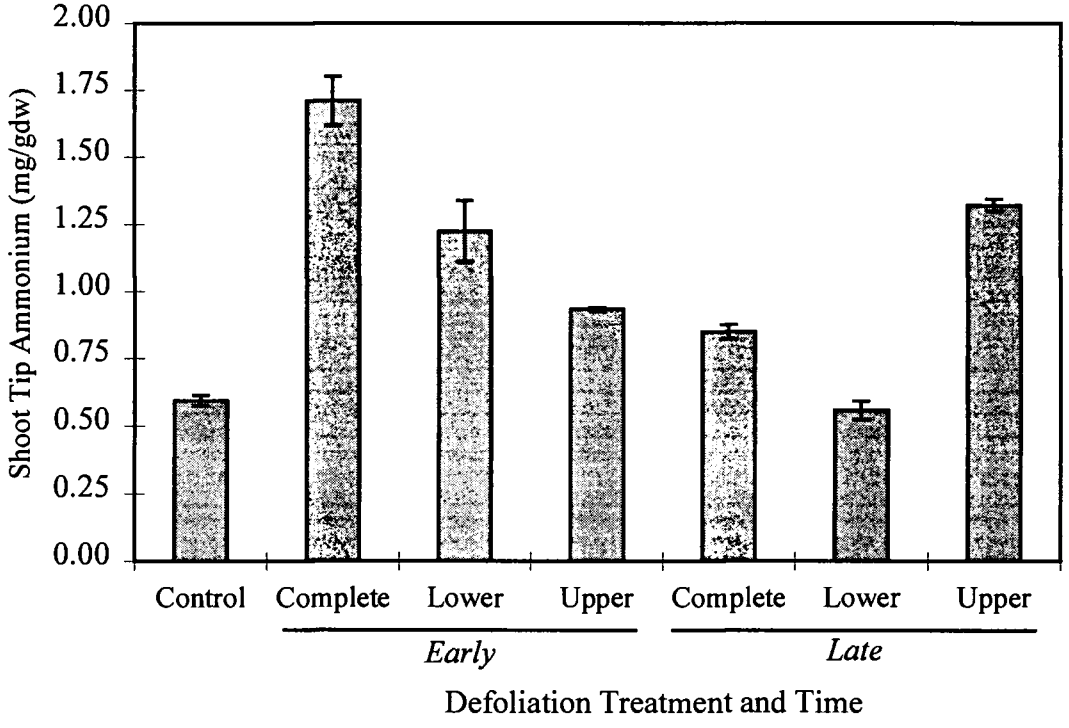

Figure 3.3. Pinot noir shoot tip NH_4^+ as affected by defoliation treatments in 1994. Vines were part of a commercial vineyard near Corvallis, Oregon and tissue collected at bloom. For complete description of the treatments, see text. Bars indicate standard error.

Shading: In 1994, shading shoots and whole vines increased flower cluster NH_4^+ (Fig. 3.4), while covering individual clusters with aluminum foil had no effect (data not shown). Also in 1994, flower cluster NH_4^+ from the early shoot treatment was significantly lower than that of the remaining three treatments. Early shading of shoots raised flower cluster NH_4^+ slightly in 1994 and reduced it slightly in 1995 relative to controls, but the late-SS treatment increased flower cluster NH_4^+ in both years. Shading whole vines increased flower cluster NH_4^+ by about 55% over that of the controls.

Table 3.2. Visual rating of IN severity in secondary Pinot noir clusters as affected by defoliation treatments applied at budbreak or one week before bloom. The primary clusters of these shoots had been removed at bloom. Vines were part of a commercial vineyard near Corvallis, Oregon and rated after fruit set. Tip = shoot cut off above 9th node (1994) or top 5cm removed (1995) one week before bloom (late). For complete description of the treatments, see text. IN ratings were taken after fruit set according to the following scale: 1= <5% (little or no); 2= 5 to 20% (moderate); 3=20 to 40% (significant); 4= 40 to 60% (severe); 5= >60% (very severe).

	Inflorescence Necrosis Rating	
Treatment	1994	1995
Control	2.40a[z]	2.89ab
Early defoliation		
Complete	3.33a	
Lower	3.33a	
Upper	3.60a	
Late defoliation		
Complete	2.20a	3.60b
Lower	3.78b	3.44ab
Upper	2.20a	2.55ab
Tip	1.90a	2.10a

[z]Separation of means by Tukey HSD 95%

Shoot shade applied from budbreak (early-SS) or one week before bloom (late-SS) had no effect on IN severity in 1995, the only year shoots were available for rating (data not shown).

Shade applied from budbreak (early-SS, early-WS) had a large effect on mature petiole NH_4^+ concentration, almost doubling the value compared to controls (Table 3.3). Mature petiole NH_4^+ was much higher than that found in the lamina tissue. The early-SS treatment had a large effect on lamina NH_4^+ concentration, increasing the value to six-fold over that of the controls. Shade applied one week before bloom (late-SS, late-WS) effected a moderate increase in mature petiole NH_4^+. Only shading of shoots increased the NH_4^+ of young lamina tissue, with shading from budbreak raising NH_4^+ levels to three-fold over that of the controls. Young tendril tissue had responses to shade similar

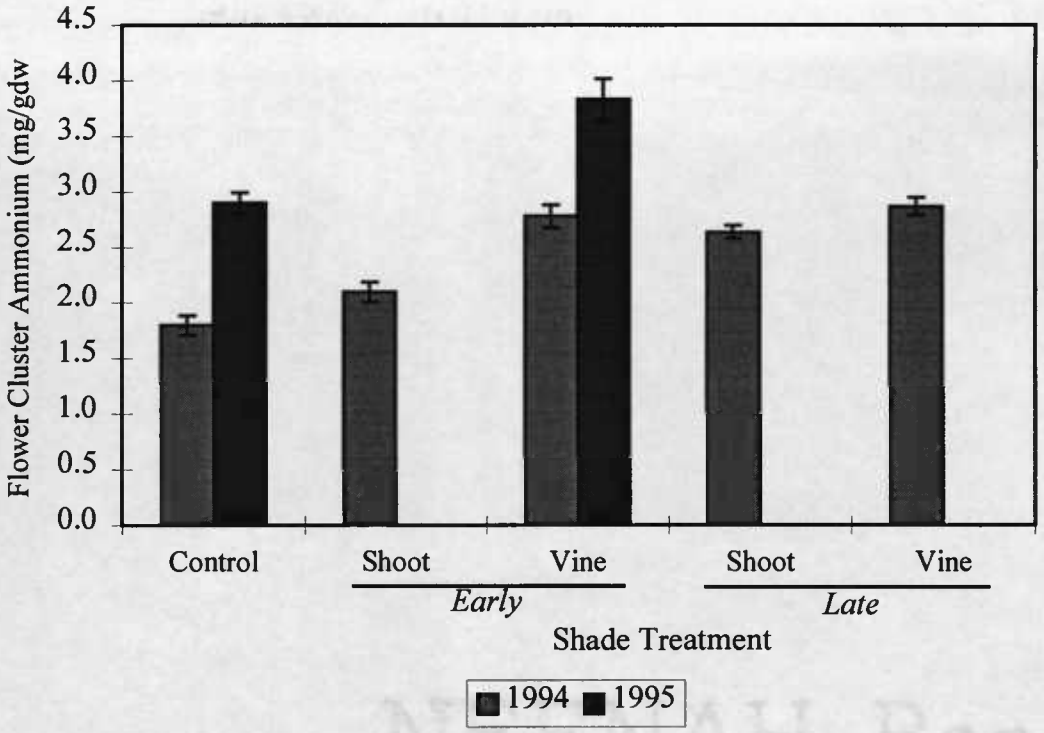

Figure 3.4. Effect of shade treatments on flower cluster NH_4^+ concentration in 1994 and 1995. Vines were part of a commercial Pinot noir vineyard near Corvallis, Oregon. Shoot = shade applied to individual shoots, Vine = shade applied to whole vines. For complete description of the treatments, see text. Flower clusters were collected at bloom; bars indicate standard error.

to that of young petiole with the exception of the late shade treatments, where tendrils had higher NH_4^+ than in young petioles. NH_4^+ in shoot tips was most affected by shoot shade treatment applied from budbreak. In 1995, NH_4^+ concentration from mature lamina and petioles from early-SS and late-SS treatments followed a pattern similar to that in 1994, though overall levels were approximately twice as high. As in 1994, the early-SS treatment had a large effect on lamina and petiole NH_4^+ concentration, with a three-fold increase in the former and a doubling of the latter (data not shown).

Table 3.3. Effect of shade treatments on shoot tissue NH_4^+ concentration in 1994. Vines were part of a commercial Pinot noir vineyard near Corvallis, Oregon. Flower clusters were collected at bloom. For complete description of the treatments, see text.

	Tissue NH_4^+ (mg/gdw)					
Shade Treatment	Mature Lamina	Mature Petiole	Young Lamina	Young Petiole[z]	Young Tendril[z]	Shoot Tip
Control	0.17a[y]	1.10a	0.68a	0.51	0.64	0.59a
Early application						
Shoot	1.09b	2.23b	2.27c	0.93	0.91	1.28c
Whole vine	0.41a	2.14b	0.77a	1.21	0.90	0.99abc
Late application						
Shoot	0.43a	1.68ab	1.09b	1.03	1.64	1.07bc
Whole vine	0.29a	1.58ab	0.83a	0.69	1.36	0.86ab

[z] Insufficient tissue for individual analysis. Value for pooled samples shown

[y] Separation of means by Tukey HSD test, 95%

Discussion

Defoliation - effects on shoot growth: Shoot growth was severely retarded by early and sustained removal of leaves from the entire shoot or only the lower portion (Fig 3.2). Shoot length in these treatments was 23% and 41%, respectively, that of control shoots, and node number was reduced by four and two respectively. Without growth of leaves, the shoot has no local supply and/or may be a poor competitor for nutrients from the parent vine. In this case, insufficient amounts of water and associated compounds could be drawn to the shoot tip to support cell growth and expansion (evidenced by the shortness of the internodes). It should be noted that there were only 3 weeks between the first leaf removal in the early and late upper defoliation treatments, leaving little time for a treatment effect to develop.

Defoliation and tipping: The loss of leaves on a grape shoot has been found to have an negative impact on fruit ripening (Kliewer, 1970; Smart *et al.*, 1988). Early in shoot growth, defoliation also has an effect on flower and fruit development resulting in

reduced fruit set, which may include losses by IN (Aravindakshan and Krishnamurthi, 1969; Coombe, 1962; Mullins, 1967; Nii, 1973). The experiments reported here have shown that shoot tissue NH_4^+ concentration can be increased by early season defoliation.

That leaves play a role in fruit set of grapes has been known for some time. Coombe (1962) showed that on shoots that had been girdled to isolate them from the rest of the vine, fruit set was proportional to the number of leaves on the shoot. He also demonstrated that the presence of young leaves and also shoot tips detracted from fruit set, something that was pointed out as early as 1883 by Müller-Thurgau (quoted by Sartorius, 1926). Mullins (1967), working with grape cuttings, found that leaves are not required for fruit set as long as the shoot is able to draw upon the reserves in the cutting itself. When growing grapes from cuttings in the greenhouse, Mullins (1966) found that removal of leaves as they emerged from the shoot was actually advantageous to flower cluster development and fruit set in potted grape cuttings. He hypothesized that young developing leaves were in competition with the flower cluster for nutrients coming from the cutting, and since they were a stronger sink than the flowers, the clusters would atrophy if the leaves were allowed to develop. This hypothesis was supported by data showing that photoassimilates were preferentially drawn to growing leaves and shoot tips rather than the flower cluster (Hale and Weaver, 1962). The situation in older vines and those in the field is slightly different, where shoot development is further advanced than flower development. By the time flower clusters are ready to bloom there are a number of fully expanded leaves able to supply photoassimilates to the shoot for growth. Older vines, with their massive root and trunk systems, have a much larger supply of stored nutrients to draw upon during early season growth. Though the availability of photosynthates and stored nutrients should be greater in field grown vines, so is the rate of vine growth (largely dependent on the temperature, light, and water availability) and number of strong sinks (developing leaves and shoot tips). Under these conditions, flower clusters are still poor sinks by comparison (Coombe, 1962; Hale and Weaver, 1962), and may draw insufficient photoassimilates for their development.

A defoliation study using five vinifera grape varieties (Aravindakshan and Krishnamurthi, 1969) found that removal of all leaves or just those basal to the cluster

before bloom reduced fruit set, while removal of leaves above the cluster had no effect. Unfortunately, it cannot be discerned from the text whether these treatments were applied to potted or field-grown vines, but similarities to these results were found in the present study (Table 3.2) related to IN reducing fruit set.

Since defoliation one week before sample collection leaves a short time between treatment and measurement of effect, it would be logical that it result in a higher flower cluster NH_4^+ concentration than in the early defoliation treatment, where leaves are removed as they appear, rather than after they are fully developed. Contrary to what happened in flower clusters, defoliation from early in the season raised shoot tip NH_4^+ much more than late defoliation (Fig. 3.3). Removal of the upper leaves had the opposite effect, with the later removal increasing shoot tip NH_4^+ more than early season leaf removal. The large increase in shoot tip NH_4^+ level in response to late defoliation of the shoot may be due to the proximity of leaf removal to the shoot tip, though this tendency for localized NH_4^+ increase was not seen in other shoot organs. For example, removal of leaves near the flower cluster did not consistently increase flower cluster NH_4^+.

Visual scoring of IN severity of secondary clusters after fruit set (Table 3.2) showed that late, lower defoliation treatments in 1994 and 1995 and the late, complete defoliation treatment in 1995 increased IN. Jordan (1993) found similar results when all shoots of Cabernet franc vines in the field were completely defoliated two weeks before bloom. The IN rating in 1994 and 1995 was not significantly affected by the late upper defoliation treatment or any of the early defoliation treatments (1994 only), though in the latter case IN was more severe in the treated than the control shoots (about twice as many necrotic flowers than controls). These data imply that the lower leaves of the shoot are important in preventing loss of fruit to IN, possibly because they are a close source of photoassimilates.

Unexpectedly, there were no significant differences in IN ratings of primary or secondary clusters on shoots treated expressly to gather that information in 1995. Responses to treatments only seemed to manifest themselves in secondary clusters on shoots whose primary clusters were removed at bloom for NH_4^+ analysis. The removal of a reproductive organ may result in a change in plant hormonal balance: Theiler and

Coombe (1985) reported that grape florets produce auxins that are important to the development of the cluster, so removal of a whole cluster may have an adverse, or stressing, effect on the shoot, aggravating IN in the remaining cluster. Since no secondary clusters were harvested and their NH_4^+ determined, the effect of primary cluster removal at bloom on secondary clusters is unknown. Keller and Koblet (1994) remarked that IN was usually more severe on primary than secondary clusters. The present study supports this finding, as shoots where IN severity ratings could be taken on both clusters (1995) showed a positive relationship between IN on the primary and the secondary ones, with the latter having less severe IN (data not shown). This trend could be due to the slight difference in time of bloom between primary and secondary clusters.

Removing the upper leaves of a shoot raised flower cluster NH_4^+ in 1993 and 1994, but had no effect on the IN rating of secondary clusters in 1994 or 1995. Since most of the leaves removed in this treatment are still developing and expanding, their elimination means significant sinks are no longer competing for carbohydrates from storage or photosynthesis. Under current thinking (Jordan *et al.*, 1991; Keller and Koblet, 1994; Lombard *et al.*, 1993), this change would increase other tissues' ability to assimilate NH_4^+ by making carbohydrate more available, thus lowering NH_4^+ concentration and reducing the severity of IN symptoms. Since flower cluster NH_4^+ levels were increased in all but one year by the removal of upper leaves, but IN was not affected, this study did not provide evidence to support the idea that removal of net sinks on a shoot may decrease flower cluster NH_4^+ or IN.

Shoot tipping shortly before or at bloom, known to increase fruit set in grapes (Brown *et al.*, 1988; Coombe, 1962; Oinoue, 1940) and reported to decrease flower cluster NH_4^+ and IN (Lombard *et al.*, 1993), resulted in a non-significant decrease in IN severity in secondary clusters in these experiments. Despite this slight reduction in IN, severe tipping (shoot above 9th node in 1994) resulted in an increase in flower cluster NH_4^+, while moderate tipping (top 8cm in 1995) had no effect on flower cluster NH_4^+. In a study using Cabernet franc, shoot tipping equivalent to removal of everything above the 7th node at two weeks before bloom did increase IN severity (Jordan, 1993), however, NH_4^+ was not measured.

Shading: Shade increased grapevine tissue NH_4^+. In flower clusters, shading individual shoots starting at one week before bloom had an effect of similar magnitude to applying shade to the whole vine at the same time (Fig. 3.4), demonstrating that even localized shade can increase flower cluster NH_4^+. That persistent or more localized shade (*e.g.* shoot shade from budbreak or individual cluster shade from one week before bloom) elicits a lesser or no response in flower cluster NH_4^+ suggests that the vine is able to compensate somewhat for increased stress in one area if that area is small and/or the organ develops under the stress conditions from budbreak. Since a flower cluster is not very photosynthetically active (Blanke and Leyhe, 1989), shading it (with aluminum foil in this case) should not place the organ under much stress, limiting NH_4^+ production. When the whole vine was shaded, flower cluster NH_4^+ rose to similar levels whether shading began at budbreak or one week before bloom, supporting the idea that shade induced NH_4^+ can be compensated for only when part of the vine remains unstressed.

Lamina tissue, the primary site of NH_4^+ generation (due to photorespiration, Lea and Miflin, 1974), from control shoots contained the lowest NH_4^+ levels of any shoot organs measured. Compared to other shade treatments, shoot shade applied from budbreak resulted in a six-fold higher lamina NH_4^+ concentration, a response unlike that found in the flower clusters, where there was a comparatively moderate increase in NH_4^+. Possibly, leaves developing under low light conditions have a reduced capacity for photosynthesis with a concomitant reduction in the ability to assimilate NH_4^+. In studies with corn leaves transferred from dark to light conditions (Suzuki *et al.*, 1987) the production of key NH_4^+ assimilation enzymes was induced, so shading may have resulted in lower production of these enzymes in grape leaves.

Assuming that factors other than photorespiration are the source of NH_4^+ that is associated with IN symptoms (supported by work of Gu, 1992), leaves on shoots developing under shade would still be subject to high NH_4^+ levels, but have less ability to assimilate it. Compared to leaves, flower clusters have little capacity for photosynthesis (Blanke and Leyhe, 1989). Therefore, under shade or light conditions flower clusters may have a limited ability to assimilate NH_4^+, which may be why NH_4^+ levels are high in comparison to the leaf blades. Since the source of NH_4^+ found in the cluster is still

unknown, the possibility that it is internally generated, or enters the flower cluster via the peduncle must be considered.

When shoots or entire vines were shaded one week before bloom (late-SS and late-WS), leaves that had developed under ambient light are subjected to a sudden, close to cluster sampling, stress (*i.e.* change from ambient to half as much light, Ibacache, 1990), which may result in NH_4^+ production (Rabe, 1990). However, since these leaves developed an active NH_4^+ assimilation pathway needed for the rapid turnover of photorespiratory NH_4^+, under low light conditions they contained the enzymes and substrates necessary to assimilate NH_4^+ associated with the stress. In whole vine shade applied from budbreak, lamina NH_4^+ concentration was similar to the late applied shoot or whole vine shade. This result may be because the entire vine developed under low light conditions so that no single shoot was developing in a light environment different from the rest (*i.e.* the vine is equally shaded, and thus balanced). Development under these conditions means that the vine was not subjected to a severe stress, that is, there was no change in its environment. In contrast, leaves on shoots that had shade applied from budbreak have nearby shoots that are exposed to full sunlight and photosynthesizing rapidly, which could result in an accumulation of NH_4^+ in those leaves developing in shady conditions.

Other shoot tissues, mature petiole, young lamina, young petiole, and young tendril, responded in a different manner to the treatments than flower cluster or shoot tip tissues. Mature petiole seemed most affected by shade, especially from budbreak, though it was increased in all treatments. This pattern was also seen in flower cluster tissue, but was not the same as the pattern found in the leaf blade itself, however. If the petiole is thought of as a conduit, delivering materials to and from the leaf blade, then for all but the early shoot shade treatment (where lamina NH_4^+ was high) the leaf is either exporting NH_4^+ into the petiole or rapidly assimilating the NH_4^+ that is being brought in through the petiole. In the case of early applied shoot shade, perhaps the imbalance of having a shaded shoot surrounded by unshaded shoots contributes to the high NH_4^+ levels in both the petiole and the leaf blade. Alternatively, the argument that petioles, like flower clusters, are not actively photosynthesizing and thus do not have the enzymes and

substrates to adequately assimilate NH_4^+ could be defended. However, recent research shows that at least one of the NH_4^+ assimilation enzymes is present in the petiole (Chapter 6).

Young lamina tissue NH_4^+ was affected by both early and late applied shoot shade, which was also true for the shoot tip tissue. Though there was insufficient material available for statistical analysis of the young petiole and tendril tissues, pre-bloom treatments tended to raise NH_4^+, with the exception of young petiole tissue, which was most affected by the early applied whole vine shade treatment. Why the young petiole and tendril tissue would be differently affected by shade than the young leaf tissue or the shoot tip itself is unknown.

There was not a direct relationship between IN ratings and NH_4^+ found in flower clusters in this study. Jordan *et al.* (1991) reported that visual ratings of IN severity in Riesling flower clusters were associated with NH_4^+ concentration in those clusters. A positive linear relationship between IN and NH_4^+ concentration in rachis tissue has been reported in a model system (Gu *et al.*, 1994) and in the field (Ibacache *et al.*, 1991). However, in these reports, clusters already showing symptoms were sampled for NH_4^+ determination. Since IN may not manifest itself until after bloom (Ibacache, 1990; Jackson and Coombe, 1988), it is impossible to accurately gauge IN severity of a particular cluster if it must also be sampled for NH_4^+ determination at bloom. The between-cluster variability may account for the discrepancy between this study and others.

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Chapter 4: Do Chemical Treatments that Influence Inflorescence Necrosis Severity Affect Grape Flower Cluster NH_4^+?

Abstract

A fruit-set disorder of grape, called inflorescence necrosis (IN), causes death of flower cluster tissue near bloom. Various chemical treatments have been reported to increase IN severity. Separate studies show that high flower cluster NH_4^+ is associated with naturally occurring IN. A field trial was designed using Pinot noir to determine if treatments thought to influence IN severity also affect flower cluster NH_4^+ One week before first bloom, flower clusters were dipped in solutions of either methionine sulfoximine (MSO, an inhibitor of NH_4^+ assimilation), α-keto glutarate, $(NH_4)_2HPO_4$, KNO_3, NAA, GA, or an emulsion solution reported to increase transpiration. Also, ethephon was sprayed on whole vines 1 week before first bloom. Flower clusters were collected 5d after treatment. IN-like symptoms were seen in MSO, KNO_3 and ethephon treated flower clusters at the time of collection, but necrosis caused by KNO_3 was atypical of that associated with IN. MSO increased tissue NH_4^+ levels by two-fold over that of controls. NAA, GA, and emulsion treatments reduced NH_4^+ concentration a small amount, and none of the other dip treatments affected had any appreciable effect. Ethephon increased cluster NH_4^+ levels slightly over those of controls but caused the most severe IN. These data show that increased flower cluster NH_4^+ concentration is not always coupled with IN symptoms.

Introduction

Inflorescence necrosis (IN, syn. early bunchstem necrosis), a fruit set disorder of grapes, was first described in Australia and New Zealand (Jackson and Coombe, 1988). Since then it has been discovered in other viticultural areas, and has been found to be the cause of significant crop losses in Oregon (Jordan, 1989; Lombard, *et al.*, 1991). The necrosis of flower and pedicel tissue occurs from shortly before bloom up until fruit set, and may affect the rachis tissue in more severe cases (Ibacache, 1990)

Little is known about the disorder's physiology. Researchers have reported that salt solutions including Ca^{2+}, Mg^{2+}, K^{+}, NH_4^{+}, Cl^{-}, and/or NO_3^{-}, as well as ethephon applied to clusters or whole vines increase IN severity (Gu *et al.*, 1994; Jackson, 1991; Jackson and Coombe, 1988; Keller and Koblet, 1995). The ammonium cation (NH_4^{+}) was found at high concentrations in affected tissues, and there appeared to be a positive relationship between the severity of IN symptoms and NH_4^{+} found in the flower cluster (Gu *et al.*, 1994; Ibacache *et al.*, 1991; Jordan *et al.*, 1991).

Symptoms similar to IN were duplicated using NH_4^{+} solutions applied to green-shoot cuttings, and results of studies using NH_4^{+} assimilation enzyme inhibitors and substrates suggest that NH_4^{+} accumulation was the causal agent of the necrosis (Gu *et al.*, 1994; Keller and Koblet, 1995).

Studies of substances applied to vines in the field and those following NH_4^{+} levels in the clusters were conducted separately, leaving unanswered the question whether treatments reported to increase IN severity also affect NH_4^{+} concentration in flower cluster tissue.

In the present study we examined the effects of treatments that have already been reported to increase the severity of IN on flower cluster NH_4^{+} concentration. Additionally, using existing knowledge about the physiology of IN, we applied treatments that affect the nitrogen status, growth, or transpiration of the cluster.

Materials & Methods

The experimental vines were in a commercial Pinot noir vineyard in Oregon's Willamette Valley. Treatments were applied approximately one week prior to first bloom and flower clusters collected 5 days later. There were two broad categories of treatments. Those affecting I) nitrogen status of tissue, and II) flower cluster development.

Category I: The following solutions were applied as 10sec dips to 10 primary clusters each: 10m*M* methionine sulfoximine (MSO), an inhibitor of glutamine synthetase (Stewart and Rhodes, 1976); 80m*M* α-keto glutarate (α-KG), a substrate of the NH_4^+ assimilation pathway; 160m*M* KNO_3, a source of reducible N; 15m*M* $(NH_4)_2HPO_4$ (DAP), a source of NH_4^+ and previously reported to increase IN severity (Jackson and Coombe, 1988).

Category II: Ethephon was applied as a 7mM spray to four whole vines and 10 clusters collected from those vines. Jackson (1991) reported that ethephon exacerbated IN and it has also been used to arrest shoot growth (Szyjewicz and Kliewer, 1982). The remaining treatments were applied as previously described: 0.4m*M* gibberellic acid (GA), 0.5m*M* α-naphthalene acetic acid (NAA), and an emulsion solution (19m*M* Na_2CO_3 with 2% olive oil) reported to strip the waxy cuticle from berries and promote transpiration (Düring and Oggionni, 1986).

All solutions used, except that for the emulsion, contained 500ppm Tween as a surfactant. Concentrations of materials were drawn from existing literature (Gu *et al.*, 1994; Jackson, 1991; Jackson and Coombe, 1988; Theiler and Coombe, 1985; Szyjewicz and Kliewer, 1982).

The length of five shoots in each of the ethephon treated and control vines was measured at 2 to 3 day intervals to gauge the effect of the treatment on vine growth. Any necrosis on flowers was also noted at the time of collection.

Flower clusters were dried in a forced-air oven at 55°C and ground in a Wiley mill with a 20-mesh screen. NH_4^+ in 0.1g tissue was extracted with 10ml 2% acetic acid for 24h on a rotary shaker at 30rpm. Solutions were filtered with in-tube plasma/serum

separators (Karlan Research Products Corp., Santa Rosa, CA 95403 USA) and NH_4^+ concentration read with a Wescan Model 360 Ammonia Analyzer (Alltech Assoc., Deerfield, IL 60015 USA). Data are expressed as mg NH_4^+-N per gram dry weight (NH_4^+ mg/gdw).

Results

Observations, Category I: Necrosis of florets and groups of florets was noted on MSO and KNO_3 treated clusters. Necrosis seen in the MSO treatment most closely resembled naturally occurring IN, while the necrosis in KNO_3 treated clusters was more pale in color, with florets appearing more desiccated. Some calypteras on α-KG treated clusters browned by the collection date, but the ovaries and rest of the flower cluster remained green. In any of the treatments that resulted in necrosis, there was much variability between individual clusters, with some showing no necrosis. DAP did not result in the appearance of IN symptoms, unlike reported earlier (Jackson and Coombe, 1988).

Observations, Category 2: The rate of shoot growth was severely curtailed after application of the ethephon spray (Fig. 4.1). Ethephon also caused IN-like symptoms within 2d of application, the most severe of any of the treatments. Ethephon dramatically lowered flower cluster dry weights, with clusters from treated vines having an average weight of 0.56g compared to 1.15g for control vines. Visually, GA seemed to increase the length of the rachis and pedicels, and both GA and NAA had slightly higher fresh and dry weights than controls (data not shown).

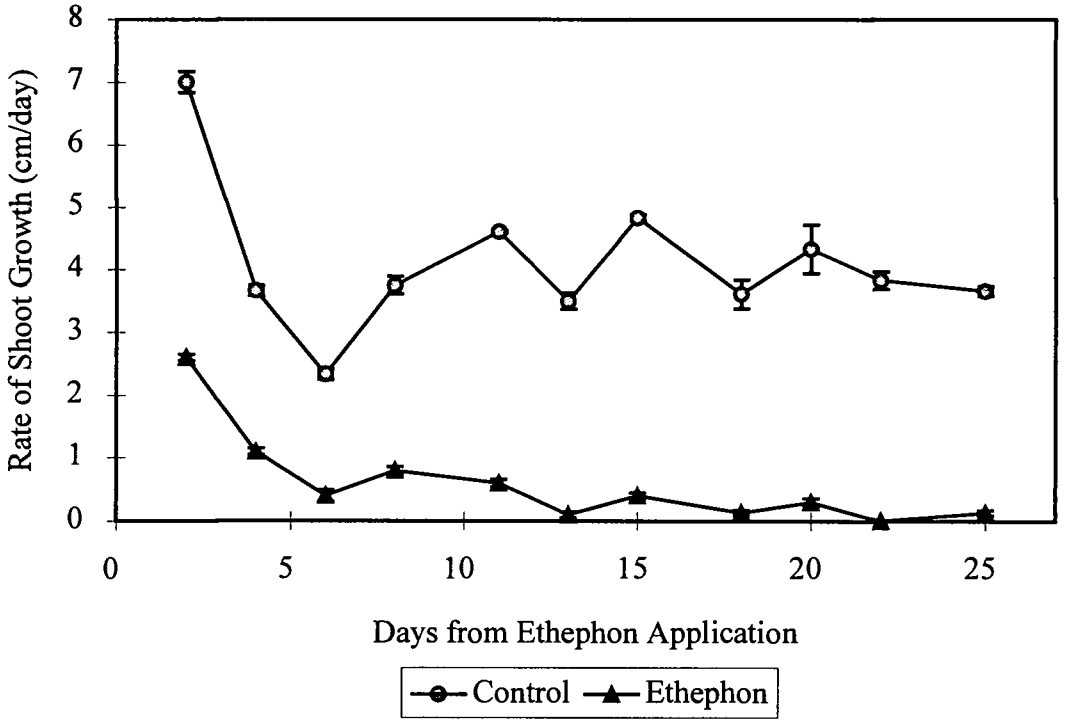

Figure 4.1. Shoot growth rate of Pinot noir control and ethephon treated vines. Vines were part of a commercial vineyard near Corvallis, Oregon. Bars indicate standard error.

Treatments, NH_4^+ concentration: There was a significant treatment effect on flower cluster ammonia (Fig. 4.2). MSO raised NH_4^+ concentrations by 100% over controls, with the only other increase found in the ethephon treated vines (a non-significant increase of 20% over its controls). GA, NAA and emulsion treated clusters had slightly lower flower cluster [NH_4^+], but the differences were not significant at p=0.05.

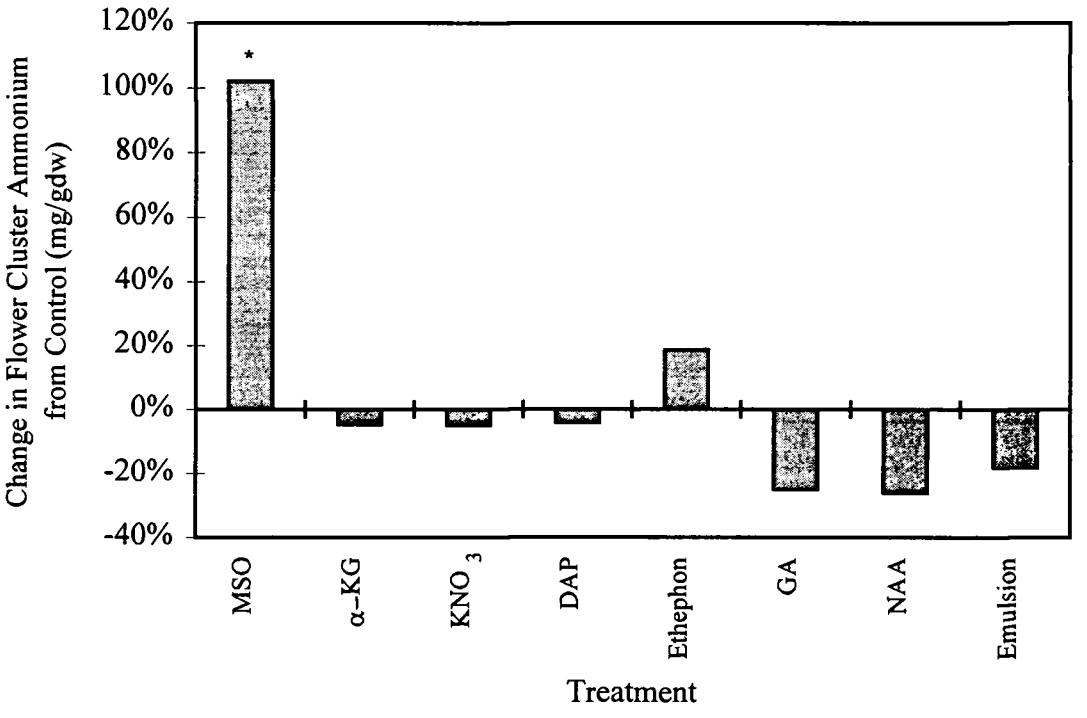

Figure 4.2. Effect of cluster dipping in various solutions and whole-vine ethephon spray on flower cluster NH_4^+. Flower clusters were immersed in solutions for 10sec 1wk before bloom and collected 5d later. Ethephon was applied to vines 1wk before bloom and clusters collected 5d later. Ten clusters per treatment were collected. Data are expressed in terms of percent change from control clusters. See text for precise nature of solutions. Only the MSO treatment was significantly different from controls ($p<0.001$), as indicated by an "*."

Discussion

Brief exposure of flower clusters to several of the solutions used in this study affected NH_4^+ concentration within 5d of application. MSO, the inhibitor of glutamine synthetase, resulted in a large increase in NH_4^+ levels within the flower cluster. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are considered to be the primary means of ammonium assimilation within aerial parts of the plant (Keys *et al.*,

1978). Inhibiting one of the enzymes should result in the accumulation of the corresponding substrates. In the case of glutamine synthetase, NH_4^+ is one of its substrates. There are multiple sources of NH_4^+ within plant tissues, among which are primary nitrogen assimilation ($NO_3^- \rightarrow NH_4^+$), photorespiration, and protein degradation (Joy, 1988). In grape, most primary nitrogen assimilation occurs in the roots, as little NH_4^+ is found in the xylem exudates: glutamine is the most abundant N-containing compound there (Roubelakis-Angelakis and Kliewer, 1979). Nitrate reductase activity is present in grape leaf tissue (Pérez and Kliewer, 1978), though little free NH_4^+ is found in grape cane exudates (Roubelakis-Angelakis and Kliewer, 1979). Photorespiration is responsible for most of the NH_4^+ turnover in photosynthetic tissues (Keys *et al.*, 1978), but research of Gu (1992) suggests that photorespiration is not a significant source of NH_4^+ in grape flower clusters. Protein degradation, such as occurs in senescing tissues, can be a significant source of NH_4^+, as it is released from the amino acids that make up the proteins (Joy, 1988). At bloom, the calyptera senesces and is released from the floret. Thus, the flower cluster experiences the formation of many abscission zones in a localized area and short time period. Data collected at this same vineyard site shows a peak in flower cluster NH_4^+ at the start of bloom, declining by full bloom (Ibacache *et al.*, 1991), which may suggest a connection between the senescence of many calypteras, the presumed protein degradation associated with it, and the release of NH_4^+ due to catabolism of amino acids.

α-KG is also necessary for the complete assimilation of NH_4^+ in plant tissues. Both glutamate dehydrogenase (GDH) and GS transfer N from NH_4^+ to α-KG, forming glutamate. The exact role of GDH in plant tissues is still under debate (Oaks, 1994; Oaks and Yamaya, 1990), but its K_m for NH_4^+ is much higher than that for glutamine synthetase (Lea and Miflin, 1974), and it is not considered to be active in grape inflorescences (Gu *et al.*, 1994). Studies by Gu *et al.* (1994) and Jordan *et al.* (1991) on IN suggest that NH_4^+ accumulates in flower cluster tissue due to a shortage of α-KG. Indeed, they showed that addition of α-KG to high NH_4^+ systems can reduce tissue NH_4^+ and prevent necrosis. In the present study, there was no reduction in flower cluster NH_4^+

in response to treatment with α-KG, but there was necrosis of a few calypteras on some clusters. It is possible that α-KG in the solution was not able to penetrate and enter but a few of the calypteras, or that the surfactant and α-KG combination had a desiccating effect on them. In the previous study on flower clusters (Gu *et al.*, 1994), α-KG was fed to tissues through the cut end of the organ, while here results were dependent on the material being absorbed by the intact organ.

Treatment of clusters with KNO_3 was an attempt to supply cluster tissue with a source of N that would be reduced to NH_4^+. Nitrate reductase is present in leaf tissues (Pérez and Kliewer, 1978), so the enzyme system to reduce NO_3^- may also be present in clusters. In this and previous studies (Gu *et al.*, 1994; Jackson and Coombe, 1988) IN could not be induced with the NO_3^- ion. The occasional browning of tissues seen in this treatment was atypical of that for IN. Necrosis may have been due to salt accumulation and concentration on the flowers, similar to the situation in the α-KG treatment. NH_4^+ in the flower cluster was not affected by the KNO_3 treatment, which is in agreement with a previous study that measured NH_4^+ in flower cluster tissues after incubating green cuttings with KNO_3 (Gu, 1992).

DAP was shown to be effective in inducing IN in a previous study (Jackson and Coombe, 1988), but did not in these experiments. Neither did this treatment raise tissue ammonium. Concentrations that promoted symptoms in that study, however, were five and ten times that used here. In this case, the concentration of the salt may not have been high enough to elicit a response.

The GA, NAA, and emulsion treatments caused non-significant decreases in flower cluster NH_4^+. GA is known to loosen developing flower clusters, and this effect is commonly exploited to improve the quality of grapes destined for the fresh market (Winkler *et al.*, 1974). When applied before bloom, GA reduces fruit set (Theiler and Coombe, 1985). Visually, this treatment affected the cluster even though there was only a 5d period for action. In this pre-bloom application, clusters were more elongate, and pedicels noticeably longer than in other treatments, resulting in a more open cluster. No necrosis was noted in the GA treatment. Rachis elongation was seen in a previous study

that treated only clusters with GA (Gil *et al.*, 1994). The NAA treated clusters did not have any noticeable necrosis at sampling time, and clusters looked similar to, though more twisted than, controls. A previous study with NAA showed no effect on the structure of the cluster, though fruit set was reduced (Theiler and Coombe, 1985). The emulsion had no effect on NH_4^+ levels, but since this treatment was originally developed for use on berries, not flowers and transpiration was not measured in the present study, any effect of the treatment on the flower cluster cannot be verified.

Ethephon has been widely studied in grapevines as a growth retardant and for other actions (for review, see Szyjewicz *et al.*, 1984). The shoot growth of treated vines in this study was slowed to near zero by 6d after application (Fig. 4.1). Just as dramatic was the effect on the flower clusters, which exhibited severe IN symptoms soon after application of the spray. Jackson (1991) reported that ethephon sprayed on either whole vines or just leaves increased IN severity. In the present study, flower cluster NH_4^+ concentration was increased by ethephon, but the magnitude was much smaller than that seen in the MSO treatment. Although MSO resulted in the greatest increase in flower cluster NH_4^+ level, IN symptoms were much less severe than treatments such as ethephon, which caused a non-significant increase in NH_4^+. This result indicates that the appearance of IN symptoms is not always linked to elevated NH_4^+ levels in the tissue as been suggested by past research (Gu *et al.*, 1994; Jordan *et al.*, 1991). It is notable that ethephon, a growth regulator that is often associated with senescence, induced IN symptoms without increasing NH_4^+ concentration. As mentioned earlier, the flower cluster forms many abscission layers at bloom, as the calypteras separate from the floret.

The latest proposals for mechanisms behind the appearance of IN suggest that an insufficient supply of carbohydrates may be the root of the problem (Gu *et al.*, 1994; Jordan, 1991; Keller and Koblet, 1994). This possibility was postulated by previous researchers, who demonstrated that increasing the availability of α-KG reduced NH_4^+ levels and IN symptoms (Gu *et al.*, 1994; Jordan, 1989; Jordan *et al.*, 1991). It is likely that NH_4^+ is not alone the causal agent of IN, but one of several factors that lead to the appearance of it's symptoms. The source of NH_4^+ remains to be discovered, and further

research into the nitrogen and carbon cycling in flower cluster tissues would be helpful in elucidating how IN symptoms develop.

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Chapter 5: Flower Cluster and Petiole Ammonium Content and Inflorescence Necrosis: A Survey of Rootstock and Pinot noir Clonal Effect

Abstract

Because the fruit-set disorder inflorescence necrosis (IN) has been associated with NH_4^+ in grape flower clusters at bloom, the possibility that rootstock or clonal selection may affect petiole or cluster tissue NH_4^+ was investigated. Three replicated rootstock trials in Oregon, two located in the Willamette Valley (Willamette-1 and Willamette-2) and one in the Rogue River Valley (Rogue), and a clonal trial in Alpine, Oregon, were sampled in this study. At bloom in 1994 and 1995, flower clusters and petioles from the leaf opposite were collected from Pinot noir on own-roots or grafted on rootstocks 5C, 44-53, 101-14, 3309, 420A, or Harmony. Samples were also collected from a block of twenty own-rooted Pinot noir clones. IN severity was visually rated in all vineyards 3 weeks after fruit set in 1995. In the rootstock trials, site had a greater effect on flower cluster NH_4^+ levels than rootstock in one year but not the other. Only at the site with the highest NH_4^+ levels (Rogue) were there significant differences in cluster NH_4^+, with 420A being the lowest in both years and 101-14 and 3309 the highest in 1994 and 1995, respectively. Petiole NH_4^+ concentration was about one-third that in the clusters and differed significantly at two sites in 1994 (Willamette-1 and Rogue) and one in 1995 (Rogue). Petioles from Pinot noir on 420A rootstock tended to have the lowest NH_4^+, while those on 3309 and own-rooted had higher NH_4^+. Site had a significant effect on IN in the rootstock trials, with it being most severe at Willamette-2. Severity as affected by rootstock varied widely between the vineyards surveyed. Clones exhibited significant differences in cluster, but not petiole NH_4^+ levels. UCD23 and UCD32 were lower than average in NH_4^+-N, and UCD4 concentrations the highest. Clones differed in IN, with UCD4 and DJN115 having the least and UCD23 the most severe symptoms. There was a

significant correlation between NH_4^+ in flower clusters and the petioles opposite, though there was no significant relationship between NH_4^+ concentration in tissues and IN severity. Rootstock and clone do seem to have a significant effect on tissue NH_4^+ and IN, and these factors may be used in planting decisions. However, site, year, and management also have a large effect on NH_4^+ levels and IN severity.

Introduction

Inflorescence necrosis (IN) is a fruit set disorder that results in the death of grapevine (*Vitis vinifera* L.) flower cluster parts prior to the fruit set stage. IN may manifest itself as the death of a single floret on an otherwise healthy-looking cluster, to necrosis of all tissue back to the central axis of the rachis (Ibacache, 1990). First reported in 1988 (Jackson and Coombe, 1988), the disorder has not been associated with any pathogenic agent; it seems to have a physiological cause (Jackson and Coombe, 1988; Keller and Koblet, 1994).

IN has been linked to vine stresses, such as shade, excessive vigor, and water stress (Ibacache, 1990; Ibacache *et al.*, 1991; Jackson, 1991; Jackson and Coombe, 1988; Jordan, 1989; Jordan *et al.*, 1991; Keller and Koblet, 1994;), and also low carbohydrate status of vines (Keller and Koblet, 1994). The disorder has also been associated with ammonium (NH_4^+): clusters with severe IN have a higher concentration of NH_4^+ (Ibacache *et al.*, 1991; Jordan, 1989; Jordan *et al.*, 1991), and the ion can induce IN symptoms in green shoot cuttings (Gu *et al.*, 1994; Keller and Koblet, 1995). Since vine growth factors are dependent on rootstock and some clonal selections seem more susceptible to IN than others (Lombard, *et al.*, 1993; S.F. Price, unpublished data), the effect of rootstock and clone on IN and NH_4^+ concentration in flower clusters and petioles was investigated.

Materials & Methods

Experimental Material - Rootstock: Material was from three grower cooperative rootstock trials established in 1992 in Oregon. Two of the replicated sites were in the Willamette Valley, Willamette-1 and Willamette-2 (both single upright curtain, cane pruned) with the third in southern Oregon's Rogue River Valley, Rogue (Lyre trellis, cane pruned). At each site seven rootstocks (own-rooted (not grafted), 5C, 44-53, 101-14, 3309, 420A, or Harmony) under Pinot noir (clone UCD2A syn. Wädenswil clone)) were planted in a completely randomized block design with five replications of five vines per rootstock. Management of the trials was determined by the individual vineyard operators.

Experimental Material - Clone: Plant material came from a block of 20 Pinot noir clones on own roots planted in 1989 (completely randomized block design) at the OSU Woodhall III vineyard in Alpine, Oregon. Vines were cane pruned and trained to a single upright trellis. The clones are listed in Table 5.1, according to the broad types: Pinot fin (small clustered, prostrate vine growth habit), Mariafeld (loose clustered), Upright (erect growth habit), and Fertile (large clustered, prostrate growth habit).

Table 5.1. Pinot noir clones planted at Woodhall III Vineyard in Alpine, Oregon. Clones are own-rooted and planted in 1989. Clone sources: UCD (Foundation Plant Materials Service at University of California, Davis), DJN (Dijon, France), ESP (Espigette, France), COL (Colmar, France). See text for explanation of groupings.

Pinot fin	Mariafeld	Upright	Fertile
UCD2A	UCD17	UCD22	UCD31
UCD4	UCD23	ESP374	UCD32
UCD10		DJN60	UCD33
UCD16			COL538
UCD29			ESP236
DJN113			DJN375
DJN114			DJN10/18
DJN115			

Tissue Collection and Processing: Four flower clusters and petioles opposite were collected from each rep/rootstock combination at bloom in 1994 and 1995. Collection from the clonal trial was similar, but the four samples were collected from the five vines in each of four replications per clone in 1995 only.

Tissues were dried in a forced-air oven at 55°C. The four flower clusters per rep of clone or rootstock were pooled and ground in a Wiley mill with a 20-mesh screen. Petioles were crushed by hand, due to the small amount of material collected. NH_4^+ in 0.1 to 0.4g (depending on expected NH_4^+ concentration) tissue was extracted with 10ml 2% acetic acid for 24h on a rotary shaker at 30rpm. Solutions were filtered with plasma/serum separators (Karlan Research Products Corp., Santa Rosa, CA 95403 USA) and NH_4^+ concentration read with a Wescan Model 360 Ammonia Analyzer (Alltech Assoc., Deerfield, IL 60015 USA). Data are presented as mg NH_4^+-N per gram dry weight.

IN Ratings - Rootstocks and Clones: The severity of IN on clusters was surveyed three weeks after fruit set in 1995 only. A visual rating of four clusters per rep of rootstock or clone was made using the following scale: 1= <5% (little or no necrosis); 2= 5 to 20% (moderate); 3=20 to 40% (significant); 4= 40 to 60% (severe); 5= >60% (very severe). For example, a cluster with approximately equal numbers of necrotic flowers and set berries was rated 4.

Data Analysis: ANOVA and means separation (95% Tukey HSD) were calculated using the Statgraphics 7.0 (Manugistic, Rockville, MD 20852) statistical package.

Results

Rootstock trials, flower cluster NH_4^+: There were significant differences in overall NH_4^+ levels between vineyard sites in both years. NH_4^+ concentration was much higher in Rogue clusters in 1994 than in 1995 (Fig. 5.1), while at Willamette-2 it was considerably lower in 1994. NH_4^+ concentrations changed the least at Willamette-1, but

were slightly higher in 1994. Rogue had the highest overall flower cluster NH_4^+ of the three vineyards.

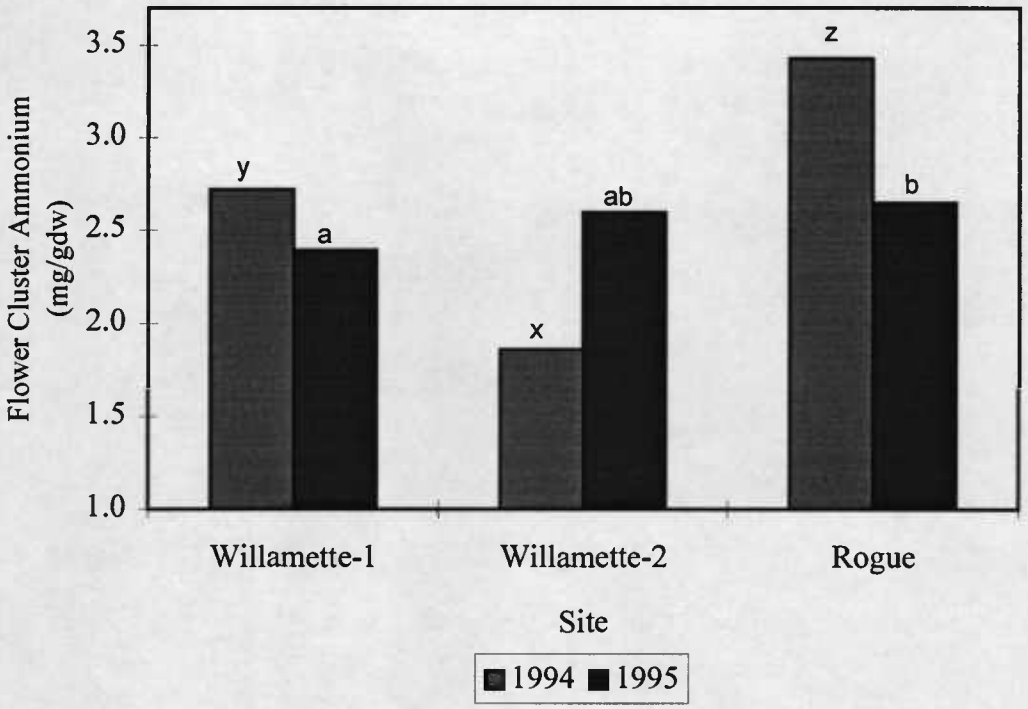

Figure 5.1. Average flower cluster NH_4^+ in 1994 and 1995 in three Oregon rootstock trials. Flower cluster samples were taken at bloom from six rootstocks on and own-rooted vines of Pinot noir in these replicated trials, two in the Willamette Valley and one in the Rogue River Valley. Means separation are within year by 95% Tukey HSD.

Site was an important factor, with relative rankings of rootstocks varying depending on location. In 1994 and 1995, only the site with the highest flower cluster NH_4^+, Rogue, had significantly ($p=0.02$, 1994; $p<0.001$, 1995) different values between rootstocks. 420A and 44-53 were ranked the lowest in both years, while 101-14 and Harmony were the highest in NH_4^+ in 1994 and 3309 and own-rooted the highest in 1995 (Fig. 5.2). In all rootstock combinations, flower cluster NH_4^+ was lower in 1995.

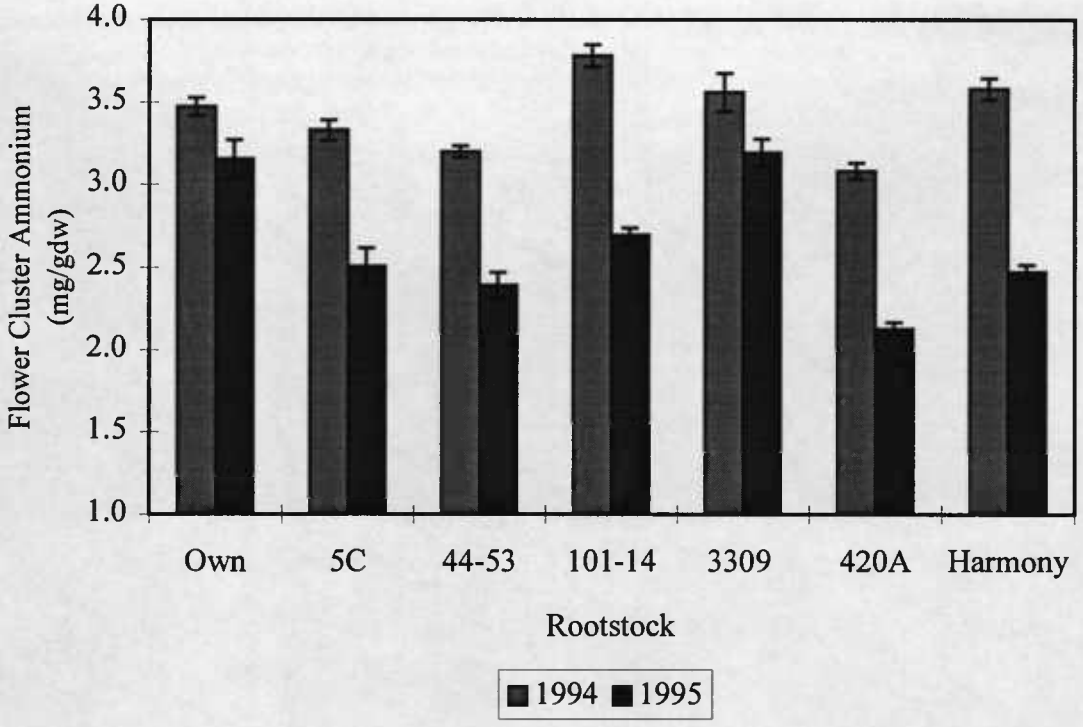

Figure 5.2. Average flower cluster NH_4^+ at the Oregon Rogue River Valley site in 1994 and 1995. Flower cluster samples from the Pinot noir scion were taken at bloom. Bars indicate standard error.

Rootstock trials, petiole NH_4^+: Petiole NH_4^+ levels were much lower than those found in flower clusters, but there were some significant differences between sites and rootstocks. Of the three sites, Rogue had the highest mean petiole NH_4^+ concentration in both years (0.73mg NH_4^+-N/gdw in 1994 and 0.83mg/gdw in 1995). NH_4^+ concentration was generally lower in 1995 than 1994, with the exception of own-rooted vines at Willamette-2 and Rogue, and 5C at Willamette-1 where concentrations increased slightly, but not significantly (data not shown).

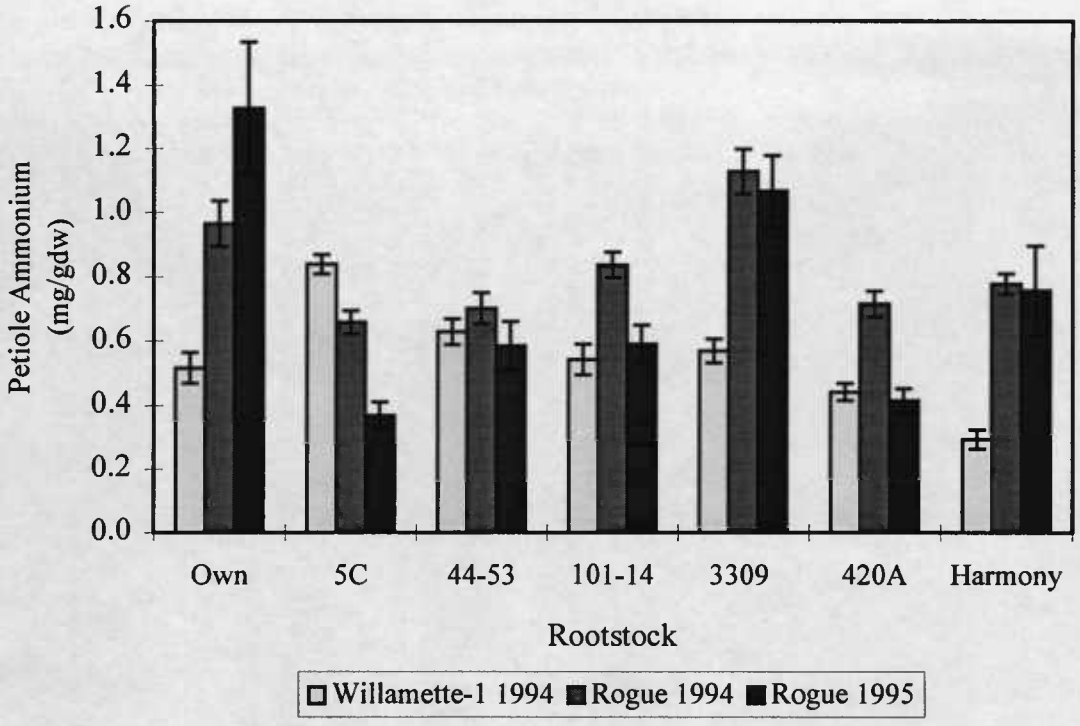

Figure 5.3. Petiole NH_4^+ in two Oregon rootstock trials that showed significant differences between rootstocks over two years. Petioles came from opposite the primary flower cluster and were harvested from the Pinot noir scions at bloom. One trial was in the Willamette Valley and the other in the Rogue River Valley. Bars indicate standard error.

In 1994, there were significant differences between petiole NH_4^+ at the Willamette-1 and Rogue sites, while this was true only at the Rogue site in 1995. Pinot noir on 5C and 420A tended to have the lower and 3309 and own-rooted the higher NH_4^+ concentrations relative to the overall average at the Rogue site, but at Willamette-1 Harmony was the lowest and 5C the highest (Fig. 5.3). Again, as seen in the flower cluster data, NH_4^+ levels tended to be lower at the Rogue site in 1995 than 1994.

Rootstock trials, IN rating: Significant differences in IN severity between rootstocks were found at all vineyard sites in 1995, the only year such data were taken.

Overall severity using the scale for rating clusters in the year surveyed was "moderate" (about 20%).

Willamette-2 had the most severe IN of the three trials (an average rating of about 2.5 vs. 2.0 for Willamette-1 and Rogue). Only vines on own roots at Willamette-2 had scores below 2, with most rootstocks having IN severity rated at or above 2.5. Flower clusters on 3309 and 420A showed the most IN at this site (Fig. 5.4).

At Willamette-1 and Rogue there was a similar range in IN scores between rootstocks, though the least and most susceptible were different at the two sites. Vines on 5C and 44-53 had IN scores of less than 2 at Willamette-1, with vines on Harmony being on the other end of the scale with a severity rating of 2.5. At Rogue, own rooted and vines on 420A had scores of less than 2, 420A having the lowest rating of the entire survey, 1.4. The average scores for 3309 and 101-14 were the highest at 2.4 (Fig. 5.4).

Measures of vine growth taken at the Rogue site (Candolfi-Vasconcelos and Castagnoli, 1996) show that vigor was low at this trial compared to the others; both shoot length and diameter were the lowest of those measured. Flower cluster NH_4^+ and IN severity, along with additional data on measures of growth collected from the Rogue trial (Candolfi-Vasconcelos and Castagnoli, 1996) are presented in Table 5.2. Rootstock 420A consistently ranked the lowest in all four categories, while 3309 and 101-14 ranked among the highest.

Overall, Pinot noir on own roots and 44-53 tended to have lower IN severity and vines on 101-14 tended to have the highest. But since there were considerable ($p=0.06$) site by rootstock interactions, generalizations outside of a particular vineyard should be considered carefully.

On a sample by sample basis, no relationship was found between NH_4^+ in flower clusters or petioles and IN score (data not shown).

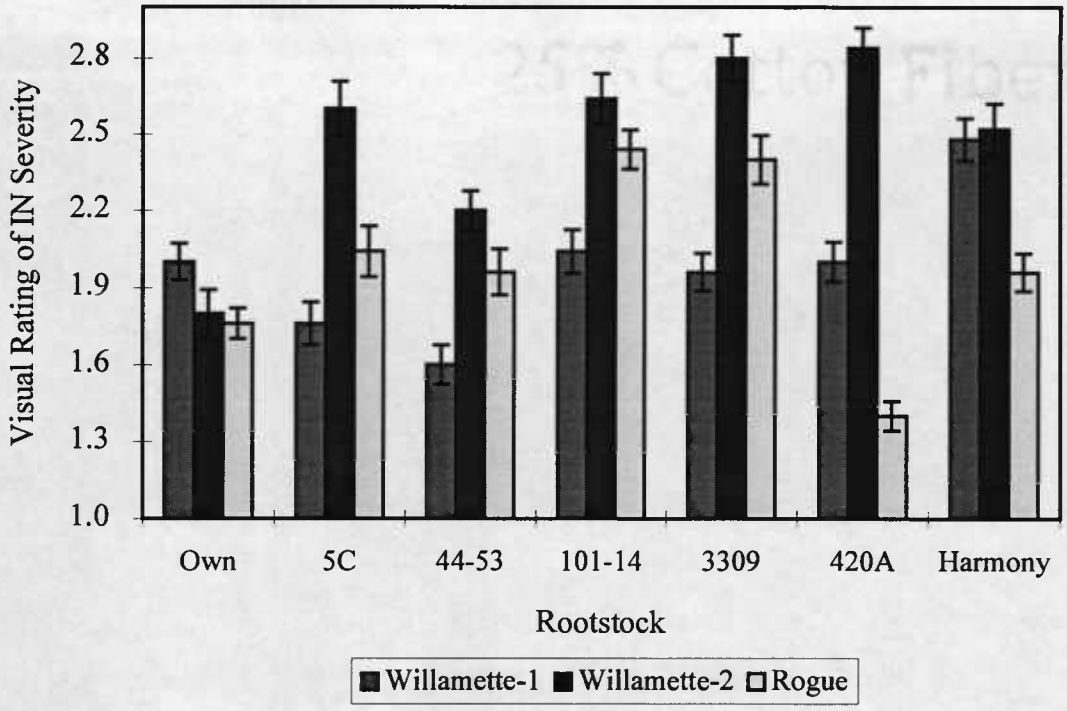

Figure 5.4. Visual ratings of 1995 inflorescence necrosis (IN) severity in three replicated rootstock trials in Oregon, two in the Willamette Valley (Willamette-1 and Willamette-2) and one in the Rogue River Valley (Rogue). Data on the Pinot noir scion were taken three weeks after fruit set in according to a visual rating: 1= <5% (little or no necrosis); 2= 5 to 20% (moderate); 3=20 to 40% (significant); 4= 40 to 60% (severe); 5= >60% (very severe). Bars indicate standard error.

Table 5.2. Rankings of rootstocks according to vine characteristics at the Rogue rootstock trial site in Oregon. Shoot length and pruning weight data from Candolfi-Vasconcelos and Castagnoli (1996). Scion is Pinot noir.

Rank	Shoot length	Pruning wt	Flower Cluster NH_4^+	IN Severity
Low	420A	420A	420A	420A
	Harmony	5C	44-53	Own
	5C	Own	Harmony	Harmony
Medium	44-53	44-53	5C	44-53
	Own	Harmony	101-14	5C
	101-14	101-14	Own	3309
High	3309	3309	3309	101-14

Clonal trial, flower cluster NH_4^+: There was a significant (p=0.05) difference in flower cluster NH_4^+ between the 20 clones of Pinot noir. Pinot fin types had very similar averages, with UCD4 having the highest concentration of all the clones, while UCD23 (a Mariafeld type) and UCD32 (Fertile type) had the lowest flower cluster NH_4^+ (Fig. 5.5).

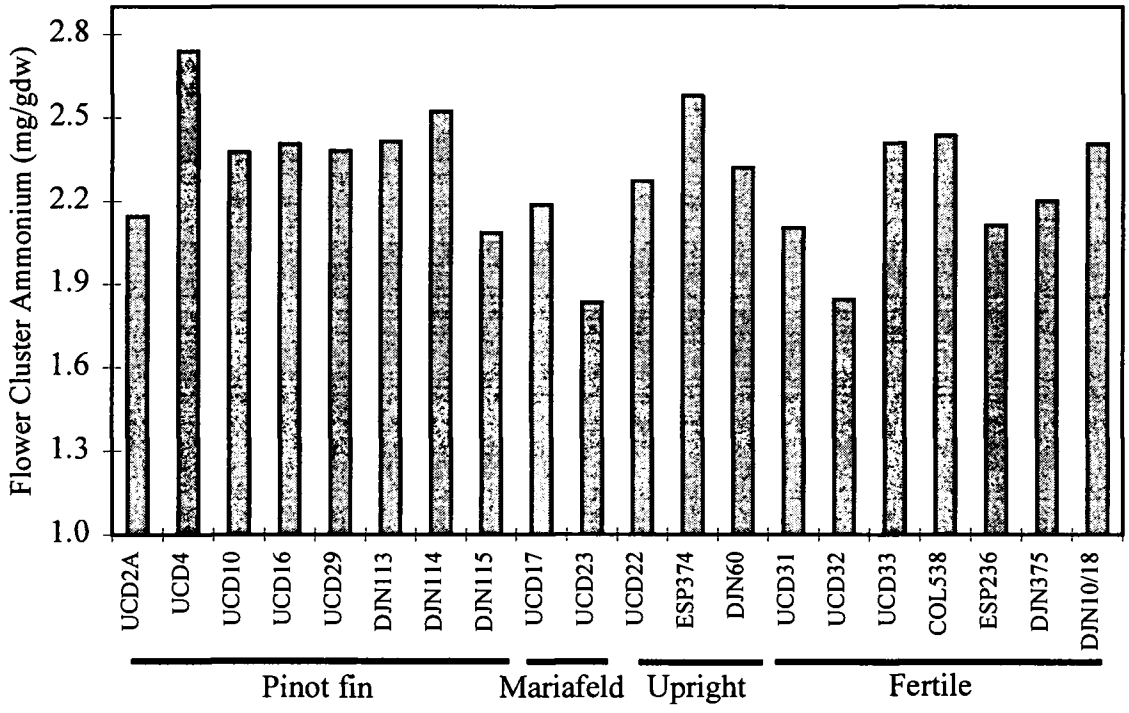

Figure 5.5. Flower cluster NH_4^+ from twenty Pinot noir clones planted at Woodhall III vineyard, Alpine, Oregon. Tissues were sampled at bloom. Significant difference between means at p=0.05. See text for explanation of groupings.

In a less comprehensive survey taken in 1994 results were similar, however, the DJN clones (with the exception of DJN60), had NH_4^+ levels of about 70% that found in 1995 (unpublished data).

Clonal trial, petiole NH_4^+: No significant differences were found between petiole NH_4^+ concentration means, though UCD10 (0.12mg NH_4^+-N/gdw) was quite low relative to the overall mean (0.19mg/gdw).

As was found in the rootstock trials, the concentration of NH_4^+ in petioles was much lower than that found in the flower clusters. In the clonal trial, there was a positive relationship between petiole and flower cluster NH_4^+ ($p=0.004$).

Clonal trial, IN ratings: There were significant ($p=0.02$) differences between clones and their IN severity. No clone had a rating of less than 2.2 in 1995.

DJN115, UCD4, and UCD29 had the lowest ratings, with UCD23, 33, and DJN10/18 having the highest (Fig. 5.6). Overall, Pinot fin types had lower ratings that the other types, and Mariafeld types had above average IN scores. In a separate survey taken in 1994, the greatest IN was found in UCD17, 23, 31, 32, 33, ESP236, and DJN375 (S.F. Price, unpublished data). Note that UCD4, while having the lowest IN severity rating, had the highest flower cluster NH_4^+ recorded (Fig. 5.5).

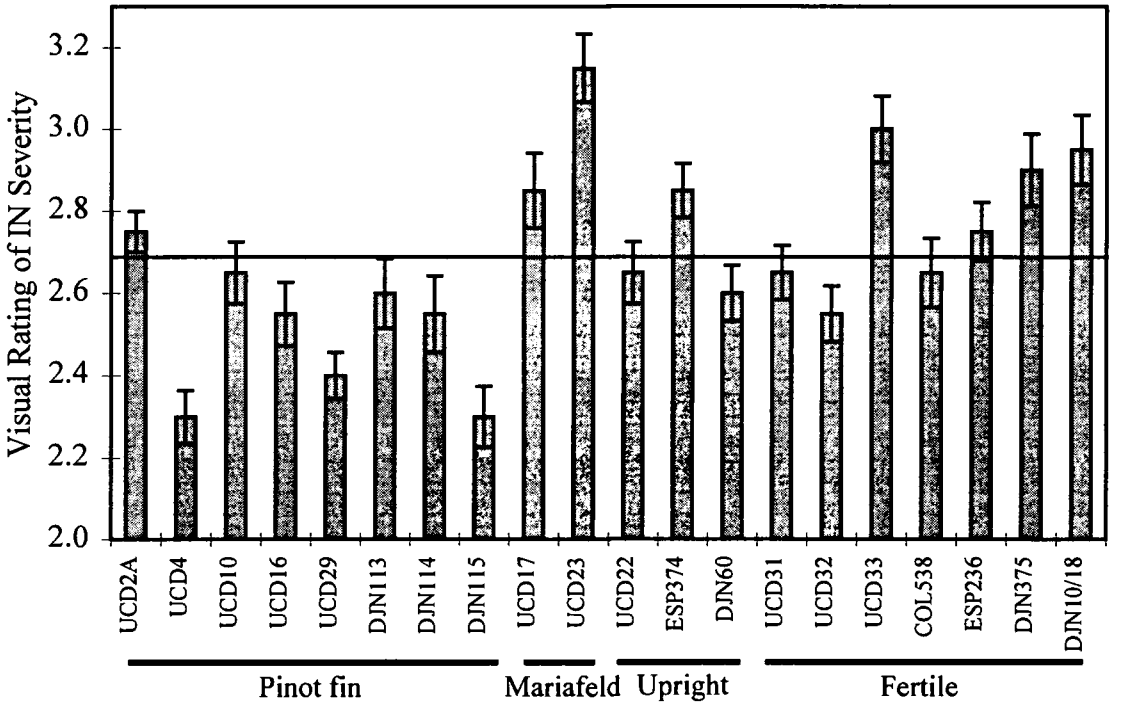

Figure 5.6. Visual ratings of inflorescence necrosis (IN) in the Pinot noir clonal trial, Woodhall III vineyard, Alpine, Oregon. Data were taken three weeks after fruit set in 1995. Line indicates overall average (2.7). Rating scale was as for Fig. 5.4. Bars indicate standard error.

Discussion

Both site and year affected NH_4^+ levels in the rootstock trials. Rogue vines had the highest flower cluster and petiole NH_4^+ in both years surveyed. While soil and climate are likely involved in this result, vine management may have also been a significant factor. Rogue vines were trained to a Lyre trellis and pruned to a large number of buds even when young. Vigor was low (Candolfi-Vasconcelos and Castagnoli, 1996) and vines were further stressed by severe leaf pulling done at bloom, probably reducing canopy photosynthesis and carbohydrate supply. Leaf removal before bloom can raise flower cluster NH_4^+ levels (Chapter 3) and the presence of a leaf opposite the flower cluster can delay the appearance of IN symptoms (in a model system, Keller and Koblet, 1995). Several studies have suggested that the availability of photosynthates/carbohydrates are important to NH_4^+ assimilation (Barker and Mills, 1980; Givan, 1979) and IN severity (Gu *et al.*, 1994; Jordan, 1989; Jordan *et al.*, 1991; Keller and Koblet, 1994), so vine management and choice of trellis may have contributed to the high NH_4^+ concentration at the Rogue site. Though Lombard *et al.* (1993) suggested that vines trained to divided canopies had lower flower cluster NH_4^+ than vines with undivided canopies, the meager shoot growth and severe leaf pulling at bloom at the Rogue vineyard might suggest that vines were under considerable stress, leading to NH_4^+ accumulation.

Soil type, macro climate, and water availability are also factors that influence vine growth. Though the form of nitrogen plant roots take up from the soil does not affect the appearance of IN (Gu *et al.*, 1996), it has been associated with overly vigorous vines, excessively weak vines, cool weather leading up to bloom, and water stress (Jackson, 1991; Jackson and Coombe, 1988; Jordan, 1989; Jordan *et al.*, 1991; Lombard *et al.*, 1993). Rootstocks may have a moderating or intensifying effect on vine growth response to some or all of these factors, thus indirectly affecting IN severity (Lombard *et al.*, 1993). At Rogue, less vigorous rootstocks (in terms of pruning weights (1994/1995) or shoot length (in 1995), Candolfi-Vasconcelos and Castagnoli, 1996) tended to have less

NH_4^+ and less IN (Table 5.2.). Yet at Willamette-2, despite moderate flower cluster NH_4^+, IN was the most severe, especially on 420A.

The wide disparities between sites should only emphasize what has been demonstrated here and elsewhere (Candolfi-Vasconcelos and Castagnoli, 1996). Rootstocks affect scion growth to differing degrees depending on where they are being grown, what soils they are in, and what scion they are under.

Within a given site, however, rootstock does play a role in flower cluster and petiole NH_4^+ levels, especially at a site where NH_4^+ is accumulated to relatively high concentrations. It is possible that differences in NH_4^+ levels and even IN severity may only become apparent when conditions are favorable for development of the necrosis, *i.e.* when there are severe stresses on vine growth.

Visual ratings of IN also showed differences between rootstocks, with losses in potential fruit in the 5 to 20% range in 1995. It is possible that a cluster that has a significant amount of IN may set a greater percentage of the remaining flowers, mitigating the effect of the disorder. Several researchers (Coombe, 1962; Rai, 1950; Yatomi and Harako, 1937) have found that vines were able to compensate for reduced flower number (through manual trimming) by setting a greater proportion of the remaining flowers. However, if a large enough number of flowers become necrotic (*e.g.* a visual rating of 3 or more, equal to >20% necrotic flowers) full compensation through increasing percent set may not be achievable, resulting in a real reduction in yield. A rootstock that influences its scion to less susceptibility to IN would have a better chance at obtaining optimum fruit set and having consistent yield from year to year.

The data collected suggest that the relationship between IN and NH_4^+ in flower cluster (or other) tissues is not straightforward. The site with the highest IN severity ratings (Willamette-2) had NH_4^+ levels very close to those found at the other sites. Stock 420A, for example, tended to have lower than average flower cluster NH_4^+ levels, yet at one vineyard had the most severe IN, and at another, the least severe IN in any of the surveyed sites (Fig. 5.4). NH_4^+ will commonly build up in plant tissues that are under stress (Barker *et al.*, 1991; Feng and Barker, 1992; Lovatt, 1990; Rabe, 1990), and yet

stress may also promote the activity of glutamate synthase (Berteli *et al.*, 1995), one of the key NH_4^+ assimilation enzymes. Thus, for there to be a net increase in tissue NH_4^+, the assimilation enzymes must be limited in their action.

In grape, shade and defoliation are stresses that are associated with IN (Jackson, 1991; Jackson and Coombe, 1988) and an increase in tissue NH_4^+ (Gu *et al.*, 1996; Ibacache *et al.*, 1991; Jordan, 1989; Jordan *et al.*, 1991; Chapter 3). Several studies have suggested that the NH_4^+ cation is responsible for the appearance of IN symptoms (Gu *et al.*, 1994, Keller and Koblet, 1995), though there is some dispute over these results (Jackson and Coombe, 1995). Shown here is evidence that the severity of IN is not directly linked to NH_4^+ in the tissues under field conditions. There does not seem to be a particular concentration of NH_4^+ that will result in IN, which implies that other factors are important in development of the disorder. Indeed, several researchers have stressed the importance of carbohydrate to ammonium assimilation and the ability of supplied carbohydrates to prevent symptoms (Gu *et al.*, 1994; Jordan, 1989; Jordan *et al.*, 1991; Keller and Koblet, 1994; Keller and Koblet, 1995). Presumably, the lack of readily available carbon substrates would limit the ability of the NH_4^+ assimilation pathway to function.

Significant differences in IN severity between clones were observed, which is somewhat surprising given their genetic similarity. Jordan (1989), reported that UCD2A tended to have less severe IN than clones UCD4 or COL538. Grouping the clones by cluster morphology revealed some interesting trends and brings up possible explanations for cluster types. Pinot fin types, though tending to have above average flower cluster NH_4^+ also had lower than average IN severity ratings. Since these are small clustered clones (which implies a smaller cluster framework), a lesser susceptibility to IN would tend to result in greater fruit set, tighter clusters, and thus an increased susceptibility to botrytis infection. Additionally, the loose clustered Mariafeld types (UCD17 and 23) had above average IN, leading to the question, does a chronic susceptibility to IN reduce fruit set and contribute to the loose cluster characteristic? UCD33, a fertile type that also had high IN, had clusters that were more similar to Mariafeld types in berry number per

cluster and cluster weight than to the fertile types in 1995 (Candolfi-Vasconcelos and Castagnoli, 1996). Certainly, genetically determined factors like the length of the cluster framework are major contributors, but a consistent reduction in berry number per cluster through IN susceptibility may play a small part in distinguishing a clone. However, these results are for one year only; IN would need to be tracked over the course of several years to see if this is a consistent trend.

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Chapter 6: Evidence for Fd-Glutamate Synthase Activity in Grape Shoot Tissues

Abstract

Ferredoxin glutamate synthase (ferredoxin glutamine: oxoglutarate aminotransferase, or Fd-GOGAT, EC 1.4.7.1) activity was detected in grapevine (*Vitis vinifera* L.) shoot tissues. Protein in extracts of Pinot noir lamina, petiole, tendril, rachis, flower, and pedicel tissue were partially purified with ammonium sulfate fractionation and used in an assay specific for Fd-GOGAT. Highest Fd-GOGAT activity per unit fresh weight and by specific activity was found in lamina tissue. Moderate, and similar, levels of activity were found in petiole, tendril, rachis, and flower tissue. No activity was detected in extracts from pedicel tissue, though this may be an artifact of tissue collection methods. Azaserine effectively blocked activity of Fd-GOGAT. Efforts to increase levels of α-KG and glutamine above 1m*M* while retaining protein in the assay solution were unsuccessful. Crude protein extracts from the same tissues used above were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with rice Fd-GOGAT antibodies. This experiment resulted in a positive reaction with single grape protein band of 73kDa. Eluates from gel filtration of partially purified protein extracts showed that most Fd-GOGAT activity was in the 150kDa to 250kDa range, which suggests that this enzyme in a dimer or trimer *in vitro*.

Introduction

The ammonium (NH_4^+) assimilation pathway in grapevines has taken on new importance with the recent implication of NH_4^+ in a variety of disorders (Gil, 1993), such

as false potassium deficiency, (Adams, 1991; Christensen *et al.*, 1991), waterberry (Christensen and Boggero, 1985; Silva *et al.*, 1986) and inflorescence necrosis (Ibacache *et al.*, 1991; Jackson and Coombe, 1988; Jordan *et al.*, 1991; Lombard *et al.*, 1993). The primary NH_4^+ assimilation pathway in plants is considered to be catalyzed by the enzymes glutamine synthetase [GS, EC 6.3.1.2] and glutamate synthase (glutamine: oxoglutarate aminotransferase, or GOGAT), which make up the GS/GOGAT cycle (Lea and Miflin, 1974).

GS, which aminates glutamate through the use of NH_4^+ and ATP forming glutamine, has been widely studied in many crops (see Lea and Ridley, 1989) and to some extent in grapevines (Ghisi *et al.*, 1984; Roubelakis-Angelakis and Kliewer, 1983).

GOGAT transfers the amido-group of glutamine to α-keto glutarate (α-KG) with the aid of a reductant, forming two molecules of glutamate. GOGAT is found in two forms, characterized by the reductant used, NAD(P)H [NAD(P)H-GOGAT, EC 1.4.1.13 and 1.4.1.14] or ferredoxin [Fd-GOGAT, EC 1.4.7.1]. These are distinct isoforms of the enzyme and are not interchangeable, *i.e.* the enzyme is specific to one form of reductant and polyclonal antibodies raised to one form are not compatible with enzymes that utilize the other form (García-Gutiérrez *et al.*, 1995; Hawakawa *et al.*, 1992; Sakakibara *et al.*, 1991; Suzuki *et al.*, 1982; Suzuki *et al.*, 1987). While each form of GOGAT is present in root and shoot tissues (Suzuki *et al.*, 1982), their amount varies widely, with the NAD(P)H form more prevalent in the root tissues and the Fd form present primarily in photosynthetically active tissues (Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992).

GOGAT has also been characterized in many herbaceous plants (see Suzuki and Gadal, 1984; Wallsgrove *et al.*, 1977), but little research in woody perennials has been reported (Elmlinger and Mohr, 1991; García-Gutiérrez *et al.*, 1995; Mohanty and Fletcher, 1980; Vézina, *et al.*, 1989). However, to avoid high levels of phenolics prevalent in woody tissues, material used in earlier studies were either from seedlings or dark-grown suspension cultures.

In grape, Roubelakis-Angelakis and Kliewer (1983) were unable to detect GOGAT activity in Chenin blanc root or leaf extracts, but this was probably due to difficulty in

extracting active enzyme. Jordan *et al.* (1992) reported NADH-GOGAT activity in crude grape leaf extracts from Cabernet Sauvignon, supported by experiments showing that an aminotranferase inhibitor (azaserine, a glutamine analog) decreased the enzyme's activity in their assay mixtures. Gu *et al.* (1994) developed a model system that utilized green shoot cuttings having both a leaf and a cluster attached to study NH_4^+ toxicity, which is associated with a fruit set disorder known as inflorescence necrosis (IN, syn. early bunchstem necrosis). Working with Pinot noir shoot cuttings, they used azaserine and methionine sulfoximine (MSO), an irreversible inhibitor of GS that does not affect the action of GOGAT (Stewart and Rhodes, 1976), to infer that certain grape tissues had less GS/GOGAT activity than others. The flower cluster rachis, one of the tissues affected by IN, did not exhibit evidence for GS/GOGAT activity.

Activity of the most important form of GOGAT in photosynthetic tissues, that utilizing Fd as the reductant, has not been demonstrated directly in grape. In this report 1) a Fd-GOGAT-like protein was detected in Pinot noir shoot tissue extracts using antibody raised against rice leaf Fd-GOGAT, 2) an assay for Fd-GOGAT activity from partially purified grape tissue extract was developed, which detected Fd-GOGAT activity in lamina, petiole, tendril, rachis, and flower tissue, and 3) gel filtration of partially purified grape leaf protein and assay for Fd-GOGAT activity showed glutamate production associated with a protein of 150kDa to 250kDa size.

Materials & Methods

Plant material: Grapevine tissue was obtained from both greenhouse and field-grown Pinot noir vines. Rice seeds (M202) obtained from the Rice Experiment Station in Biggs, California, USA were grown in the greenhouse. Grape and rice tissues from the greenhouse was brought to the lab on ice, used immediately in the protein extraction procedure or frozen in liquid nitrogen and stored at -80°C. Material from the field was brought to the laboratory on ice, frozen in liquid nitrogen, and stored at -80°C until use.

Flower clusters were separated into flowers, pedicels, and rachis prior to freezing. Separation was done over ice in a cold room (4°C). The procedure for collecting flower cluster tissues used fine-tipped scissors to clip the flowers from the inflorescence, which were then frozen in liquid nitrogen. Pedicels were clipped from the rachis and put into a microfuge tube sitting in ice. When the tube was nearly full, it was capped, plunged into liquid nitrogen, and stored at -80°C until use. Rachis was then similarly frozen and stored. The protein extraction procedures developed for grape leaves were also used for flower cluster tissues, petioles, tendrils, and rice leaves.

Protein extraction for SDS-PAGE: Tissues collected from the field and rice leaves were frozen in liquid nitrogen, ground in a mortar with 25% (w/w) polyvinylpolypyrrolidone (PVPP) added to the frozen powder, then mixed with 5 volumes of extraction buffer (50m*M* phosphate buffer, pH7.6, 5m*M* ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 0.1m*M* phenylmethylsulfonyl fluoride (PMSF), and 14m*M* β-mercapto-ethanol, the latter two added just before use). The slurry was strained through four layers of cheesecloth and then centrifuged at 10,000*g*/20min/1°C. The supernatant was mixed with five volumes of 0.1*M* ammonium acetate in MeOH at -20°C and kept at -20°C overnight. The suspension was then centrifuged (10,000*g*/20min/3°C), the precipitate washed twice with fresh 0.1*M* ammonium acetate in MeOH, then twice with acetone (at -20°C), centrifuging 10min each between steps. The acetone supernatant was poured off and the samples stored at -20°C until use.

Approximately 0.04g of each tissue's precipitate was resuspended in 500µl sample buffer (64m*M* Tris-HCl, pH6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and 735m*M* β-mercapto-ethanol) with boiling and agitation. Samples were centrifuged (10,000*g*/10min/room temperature, RT) and the protein concentration determined by the Bradford assay (Bradford, 1976), using bovine serum albumin (BSA) as a standard. These data were used to calculate the volumes needed to load equal amounts of protein in the SDS gels.

SDS-PAGE and western blot: Bromophenol blue (1μl of 0.05% (w/v) solution added per 20μl sample) was mixed with the samples and standards (unless prestained) prior to loading on duplicate 7.5% acrylamide, 1% SDS mini gels (Mini-PROTEAN II apparatus, Bio-Rad Laboratories, Richmond, CA 94804 USA). Each sample lane was loaded with 13μg of protein, as determined by the Bradford assay. The following Bio-Rad molecular weight standards were used: myosin, (200kDa), β-galactosidase (116kDa), phosphorylase b (97kDa), BSA (66kDa), and ovalbumin (45kDa) as well as the Bio-Rad prestained standards: myosin (205kDa), β-galactosidase (116kDa), BSA (80kDa), and ovalbumin (49kDa). After electrophoresis at a constant 200V, one gel was stained with Coomassie dye (0.1% (w/v) R-250 in 40% (v/v) MeOH 10% acetic acid) and one used in the western blot procedure.

Proteins were transferred from the SDS gel to supported 0.45μm nitrocellulose membrane (Bio-Rad Trans-Blot® transfer medium) using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. Transfer was done overnight at a constant 30V. The lane containing the high molecular weight standards was cut from the gel and stained with Ponceau red (0.5% (w/v) Ponceau S, 1.0% (v/v) acetic acid) and the color fixed with a 10% (v/v) isopropyl alcohol/10% (v/v) acetic acid mix. Relative migration distances from this portion of the blot were used to estimate molecular weights of proteins visualized on the western blot.

Membranes were probed with IgG anti-rice Fd-GOGAT, supplied by Dr. Akira Suzuki (Suzuki *et al.*, 1982) and diluted 1:3000 with buffer (20m*M* Tris, pH7.5, 500m*M* NaCl, 0.05% (v/v) Tween-20, 1% (w/v) gelatin), for 12h at room temperature with 25rpm agitation. A Bio-Rad Immun-Blot Assay Kit (alkaline phosphatase goat anti-rabbit IgG) was used to visualize positive antibody-protein reactions.

Enzyme assay: Leaves and other tissues were frozen in liquid nitrogen and ground with 75% (w/w) PVPP. The frozen powder was added to 12 volumes or less, depending on tissue type, of 200m*M* phosphate buffer, pH7.6, containing 2.5m*M* EDTA, 75m*M* borax decahydrate, 28m*M* β-mercapto-ethanol, 5m*M* dithiothreitol (DTT), and 0.2m*M* PMSF (the latter three added immediately before use) including another 75% (w/w)

PVPP. The slurry was strained through four layers of cheesecloth and then centrifuged at 15,000*g*/25min/3°C. Ammonium sulfate was added to the supernatant to 25% saturation (at 3°C, equal to 0.134g $(NH_4)_2SO_4$/ml supernatant), allowing 15min equilibration time, followed by centrifugation (15,000*g*/25min/3°C). The supernatant was then brought to 85% saturation (0.395g/ml) and centrifuged as just described. The resulting precipitate was resuspended in 2.0ml resuspension buffer (10m*M* phosphate buffer, pH 7.6 with 2.5m*M* DTT), transferred to dialysis tubing (Spectra/Por MWCO: 6-8000, Spectrum Medical Industries, Inc., Los Angeles, CA 90054 USA), and dialyzed versus >200 volumes of resuspension buffer for 15h at 4°C. The resulting protein solution was used for the assays. Protein concentration was determined by the Bradford method.

Assays were carried out in 16x100mm glass tubes sitting in a 30°C rotating water bath (60rpm) for a reaction time of 2min. The reaction mixture volume was 1.2ml and contained reaction buffer (0.5m*M* α-KG, 1.0m*M* glutamine, 0.48m*M* DTT, 20m*M* KCl, 0.4m*M* EDTA in 10m*M* phosphate buffer, pH7.6), 0.05mg ferredoxin, and 150µl protein solution. The reaction was started with the addition of 50µl 7.4m*M* freshly mixed sodium hydrosulfite (0.031g $Na_2S_2O_4$ in 1.0ml 190m*M* $NaHCO_3$) solution. Reactions were stopped by immersion of tubes in boiling water for 1min with agitation (precipitation was seen by 7sec). Controls were identical except hydrosulfite solution was added just after tubes were immersed in boiling water.

Boiled reaction solutions were centrifuged 10,000*g*/10min/RT and 1.1ml of the supernatant loaded onto a Dowex® column (1x8 200-400 mesh, poured into 8mm diameter glass tubing for a height of 5cm, Matoh *et al.*, 1980). After entry of the sample into the column, it was flushed with 6.0ml H_2O, and then with 0.3*M* acetic acid. Eluate from 1.5ml to 5.0ml after the addition of acetic acid to the top of the column was collected and analyzed using ninhydrin. A 0.5ml aliquot of the eluate was combined with 1.0ml ninhydrin reagent (Sigma Chemical Company, St. Louis, MO 63178 USA), the tubes loosely capped, vortexed for 1sec, placed in a rotating water bath at 60rpm and 80°C for 10 minutes, cooled, and absorbance read at 570nm (see Matoh *et al.*, 1980).

Results were calculated as μmol amino acids produced per minute of reaction time (U) and are presented in terms of U per mg of crude protein and per gram fresh weight.

Some characteristics of the enzyme were determined by the addition of azaserine at concentrations from 0 to 1*mM*, MSO at 5m*M*, or elimination of reaction components from the reaction solution. Reaction buffers with varying concentrations of α-KG or glutamine were used to estimate lamina Fd-GOGAT affinity for those substrates. A Lineweaver-Burke plot was used to estimate K_m. Additionally, a set of eluates from grape lamina complete, control, and 1m*M* azaserine treated reaction solutions were analyzed by an independent lab (AAA Laboratories, Mercer Island, WA 98040 USA) for free amino acids to verify the contents.

Gel filtration: Proteins from a partially purified extract of leaf tissue as used in the enzyme assay were separated on a Superose® 12 HR 10/30 column (Pharmacia Biotech Inc., Piscataway, NJ 08855 USA) using an FPLC system. To estimate size of the proteins coming off the column, aprotinin (6.5kDa), carbonic anhydrase (29kDa), BSA (66kDa), alcohol dehydrogenase (150kDa), and dextran blue (2,000kDa) were used as standards. A total of 6.5mg of the partially purified protein was loaded onto the column and 0.5ml fractions collected at a rate of 0.25ml/min. The eluate was used in an enzyme assay described above, with 380μl of it reacting in the total 1.2ml volume for 10min at 30°C. Reaction solutions were evaluated as described in the Enzyme assay section. Data are presented in terms of glutamate equivalents produced (μmol) and protein concentration (μg/ml, by Bradford method) in the eluates. Protein molecular weights were estimated using a linear regression equation from a plot of the retention time vs. the natural log of the standards molecular weights.

Results

SDS-PAGE and western blotting: Rachis and petiole tissue yielded the least amount of protein, which hindered efforts to load desired quantities of protein in each

lane of the gels. Fig. 6.1 shows a Coomassie-stained gel following electrophoresis. Rachis tissue especially seemed to contain less protein, but protein there and for petiole diffused more than in other lanes. Extracts from young leaf appeared to contain a greater amount of higher molecular weight proteins, possibly due to fewer phenolic compounds, which would bind to proteins and precipitate them out of solution. Both rice and grape lamina have a dense band at 46kDa, which could be a breakdown product of ribulose bisphosphate carboxylase/oxygenase (Holdbrad *et al.*, 1994). A band corresponding to an approximately 75kDa unknown protein was common to all tissue types, including rice leaf.

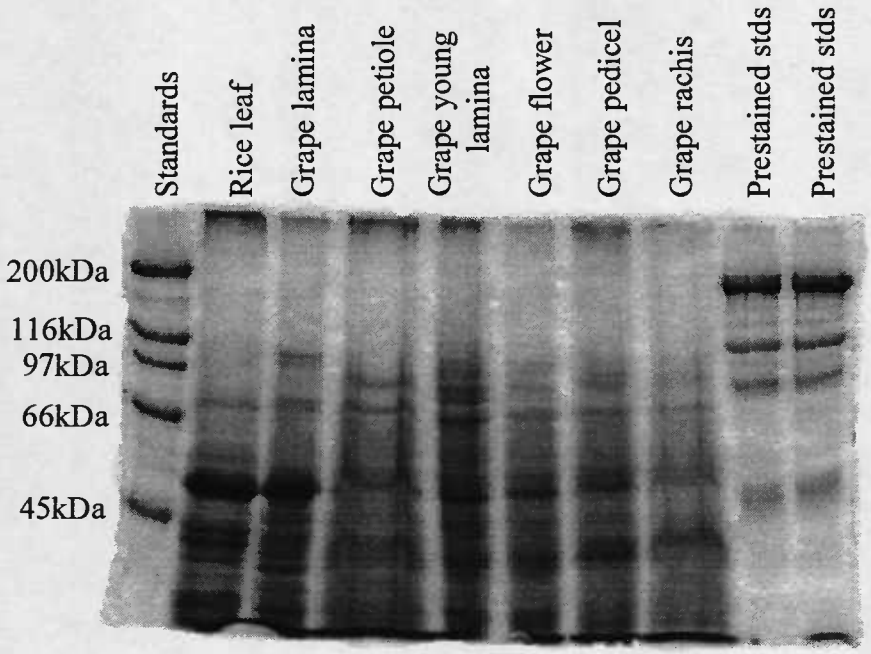

Figure 6.1. Example of an SDS-PAGE mini gel stained with Coomassie brilliant blue. Molecular weight standards used: 200kDa (myosin), 116kDa (β-galactosidase), 97kDa (phosphorylase b), 66kDa (BSA), and 45kDa (ovalbumin); prestained standards, 205kDa (myosin), 116kDa (β-galactosidase), 80kDa (BSA), and 49kDa (ovalbumin). Sample lanes were loaded with 13μg protein each.

The IgG anti-rice Fd-GOGAT bound to proteins blotted from the SDS-PAGE gels onto nitrocellulose membranes (Fig 6.2). Distinct positive reactions occurred in lanes containing each grape tissue tested, though response was weaker for grape lamina and rachis. The major band for rice leaf corresponded to a molecular weight of 177kDa, and that for grape tissues was approximately 73kDa. Numerous smaller molecular weight proteins reacting with the antibody are visible in the rice lane.

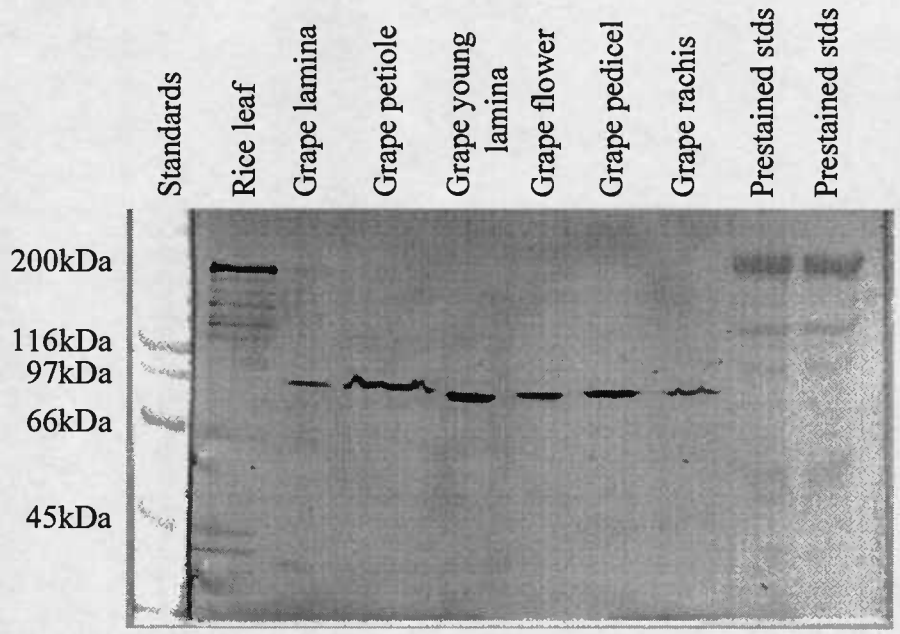

Figure 6.2. Example of western blot of grape shoot tissue proteins transferred from SDS-PAGE gel shown in Fig 6.1. Gel description as for Fig. 6.1. An anti-rice Fd-GOGAT antibody (Suzuki *et al.*, 1982) was used to probe the nitrocellulose membrane after transfer. Color on the western blot was developed using an alkaline-phosphatase-based kit (Bio-Rad Laboratories). Color on lane 1 was developed separately using Ponceau red.

Protein extraction for enzyme assay - Observations: The use of borax decahydrate in the extraction buffer could not be substituted for with anhydrous borax, and was necessary to isolate protein from grape tissues. The protein solution obtained

after dialysis was light tan, but browned considerably and lost virtually all activity over the course of 4 days when stored at 4°C (data not shown). For all data reported here, enzyme assays were done immediately following dialysis to ensure maximum activity. Substrate concentrations greater than those used for the assay (*i.e.* 0.5m*M* α-KG, 1.0m*M* glutamine) resulted in precipitation upon addition of the protein solution, reducing glutamate formation in the assay (data not shown).

Gel filtration: Fig. 6.3 shows the relationship between the natural log of standard protein molecular weight (in Da) and retention time in the column.

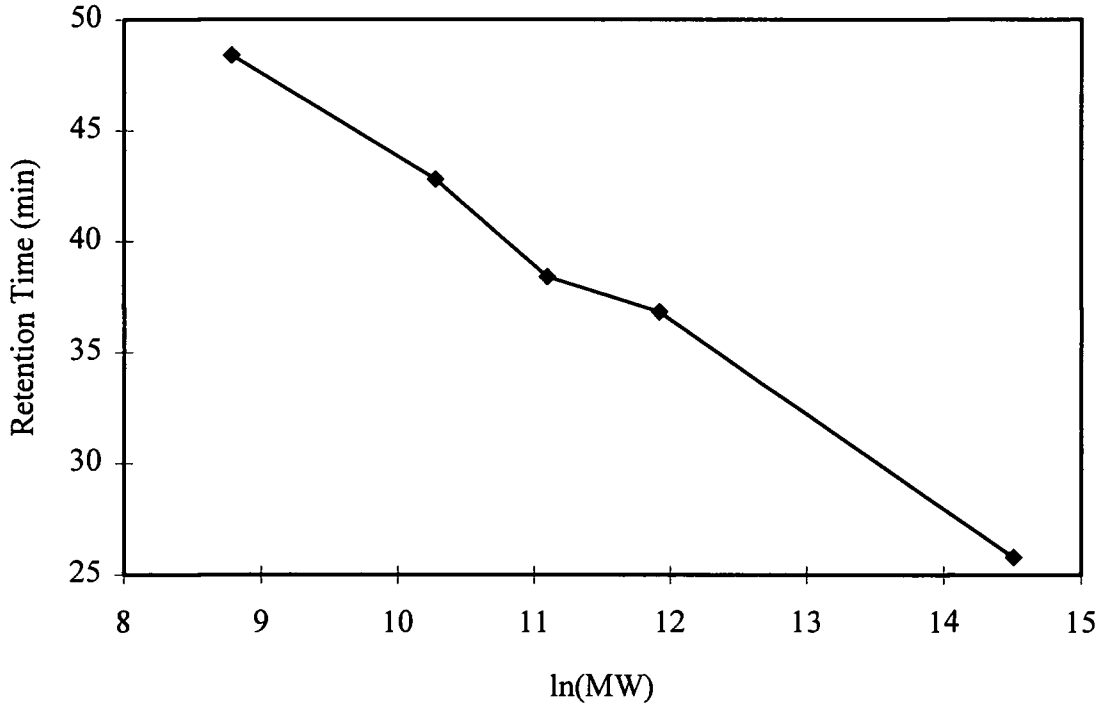

Figure 6.3. Gel filtration of protein standards through a Superose® 12 HR 10/30 column. Standards were aprotinin (6.5kDa), carbonic anhydrase (29kDa), BSA (66kDa), alcohol dehydrogenase (150kDa), and dextran blue (2,000kDa). Molecular weights (in Da) were transformed using natural log to calculate the best-fit linear regression: ln(MW) = 21.1 - 0.253(retention time in min). r^2=0.995. Time = 0 was when the sample was injected into the column.

The linear regression of these data was used to calculate the approximate molecular weight values contained in each of the fractions collected. Because the ninhydrin assay used to determine GOGAT activity in the ion-exchange chromatography eluates also responds to other amino acids and proteins, data for both glutamate produced in the 10min reaction time and protein concentration in the eluate are presented (Fig. 6.4).

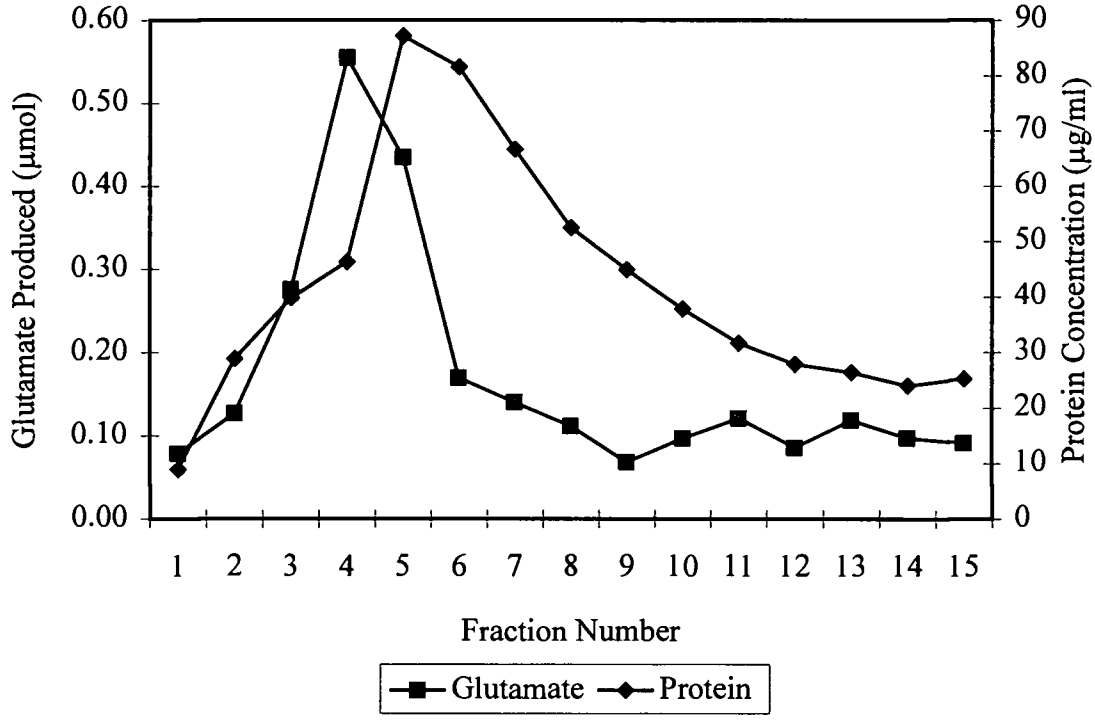

Figure 6.4. Results of gel filtration of partially purified grape lamina protein. Fractions were collected at 2min intervals (0.25ml/min) after one bed volume had passed through the column from addition of the protein. Glutamate equivalents were those produced in an assay for GOGAT activity that used 380µl filtrate and a reaction time of 10min; otherwise reaction conditions and ninhydrin color development were as described in the text. Protein concentration in the column eluate was determined by Bradford assay.

Maximal protein concentration was found in the fifth (90 to 150kDa) fraction, while the most amino acids were produced from the fourth (150 to 250kDa) fraction. The latter

fraction also had the most activity relative to protein, though a high ratio was also found for proteins in the first (>700kDa) fraction.

Enzyme activity: The assay procedure developed for grape tissue detected GOGAT activity. The ion-exchange (Dowex®) column was able to separate glutamine and glutamate (Fig. 6.5). Additional tests showed that the first peak was associated with glutamine and the later peak, eluted with acetic acid, was associated with glutamate (data

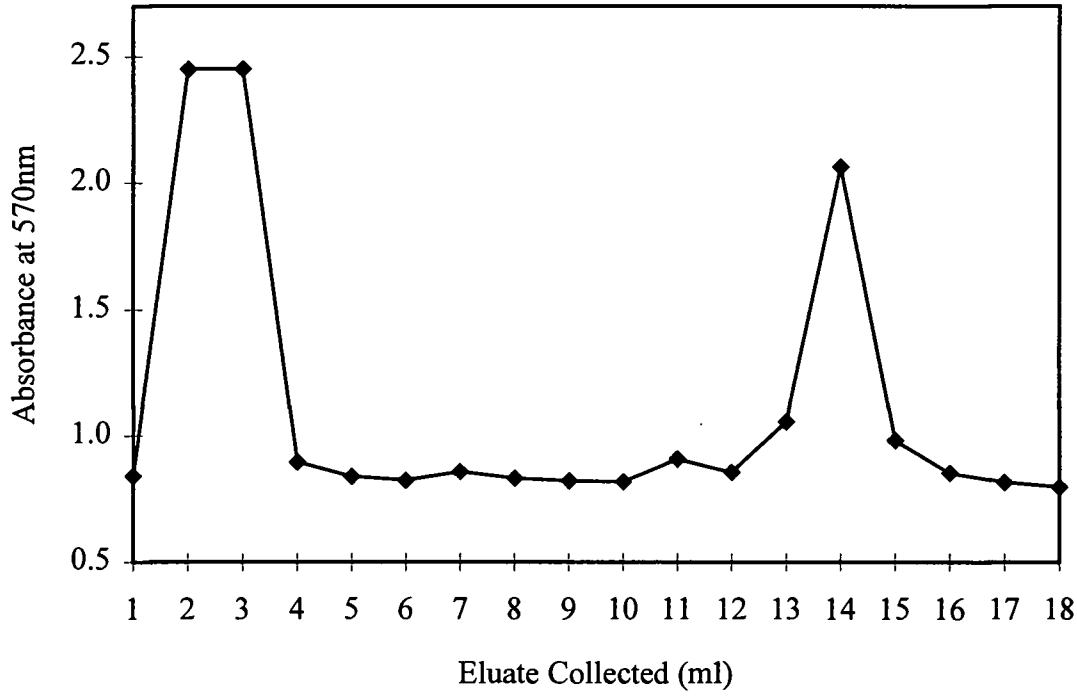

Figure 6.5. Ability of the ion-exchange column to separate glutamine from glutamate. Reaction buffer (see text) and glutamate solution were loaded onto the column, washed in with 9.0ml H_2O, then glutamate eluted with 0.3M acetic acid. A total of 1.10μmol glutamine and α-KG, plus 0.26μmol glutamate were loaded onto the column. Fractions were collected in 1ml intervals from when sample was first loaded. Acetic acid was added after all H_2O had entered the column (*i.e.* at 10.7ml eluate collected). A 0.5ml aliquot of eluate was reacted with ninhydrin reagent as described in the text and absorbance read at 570nm.

not shown). From these data it was determined that 6.0ml of H_2O would be sufficient to clear glutamine from the column and that collecting eluate between 1.5 and 5.0ml after the addition of acetic acid would contain all glutamate flushed from the column. The ninhydrin assay was able to detect low amounts of glutamate equivalents, but the maximum A_{570} achievable with it was 2.5. Production of glutamate in the enzyme assay peaked at 2min reaction time, but continued at a relatively constant rate for 10min (Fig. 6.6).

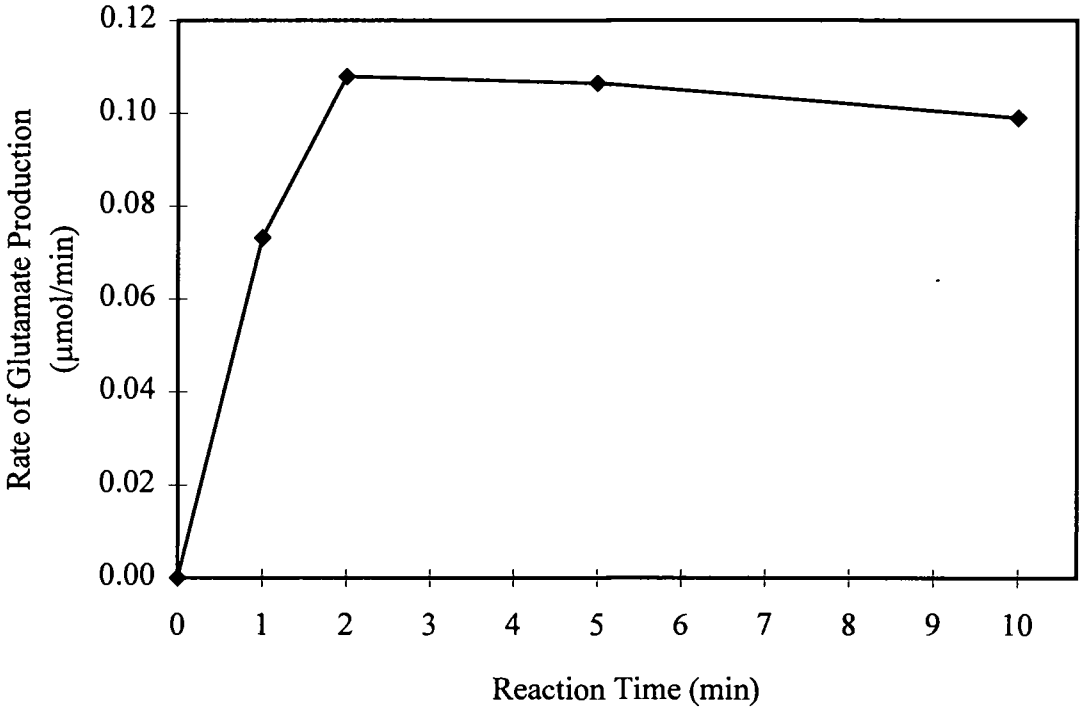

Figure 6.6. Rate of glutamate equivalents produced versus reaction time. Fd-GOGAT activity was from grape lamina tissue and assayed as described in the text.

The validity of the assay was also verified by having the free amino acid content of eluates determined by an independent lab. Analysis of the eluate from an assay for Fd-

GOGAT activity in grape lamina tissue showed glutamate as the only significant free amino acid present, making up 85% of total amino acids. Three amino acids, phenylalanine, cysteine, and valine, accounted for half of the remaining 15%. Analysis of eluate from control reactions showed that only 2nmol of glutamic acid was produced versus almost 72nmol in the sample assay. Addition of 1m*M* azaserine to the assay mixture dropped production of glutamic acid to 5nmol.

Fd-GOGAT activity was detected in grape leaf, petiole, tendril, rachis, and flower tissue (Table 6.1). Azaserine at 1m*M* effectively blocked formation of glutamate equivalents in lamina extracts (Table 6.2), while MSO had virtually no effect on its production (data not shown). Removal of any one of the reaction volume substrates (α-KG, glutamine, protein solution, or Fd) reduced glutamate equivalent production to near zero (data not shown).

Table 6.1. Summary of GOGAT activity data from a variety of shoot tissues from Pinot noir grapevine. Assay conditions were as described in the text. The unit of enzyme activity (U) is defined as 1μmol glutamate equivalent (additional amino acids) produced per minute of reaction time.

Tissue	Source	Activity	
		U/mg protein	U/g fresh weight
Lamina	Greenhouse	0.431	0.921
Petiole	Field	0.176	0.105
		0.165	0.053
Flower	Field	0.152	0.125
		0.168	0.234
Pedicel	Field	nd	nd
Rachis	Greenhouse	0.120	0.162
Tendril	Field	0.171	0.132

Extracts from laminas showed the greatest activity of all tissues tested, with no activity being found in the extract from pedicel tissue. Other tissues exhibited moderate, and similar, levels of activity. Specific activity (U/mg protein) showed less variation than activity on a per gram fresh weight basis, but the value for leaf tissue was more than twice that for any other tissue.

Table 6.2. Effect of azaserine on grape lamina Fd-GOGAT production of glutamate (the major amino acid in the assay eluate). Assay conditions as described in the text.

Azaserine (m*M*)	Amino acids (μmol/min)	Percent Inhibition
0.0	0.248	0%
0.1	0.093	63%
0.5	0.039	84%
1.0	0.030	88%

The K_m of Fd-GOGAT from grape lamina was estimated using buffers of varying α-KG and glutamine concentration. Selected data are presented on a Lineweaver-Burke plot (Fig. 6.7), which showed the enzyme's affinity for α-KG and glutamine to be approximately 110μ*M* and 200μ*M*, respectively.

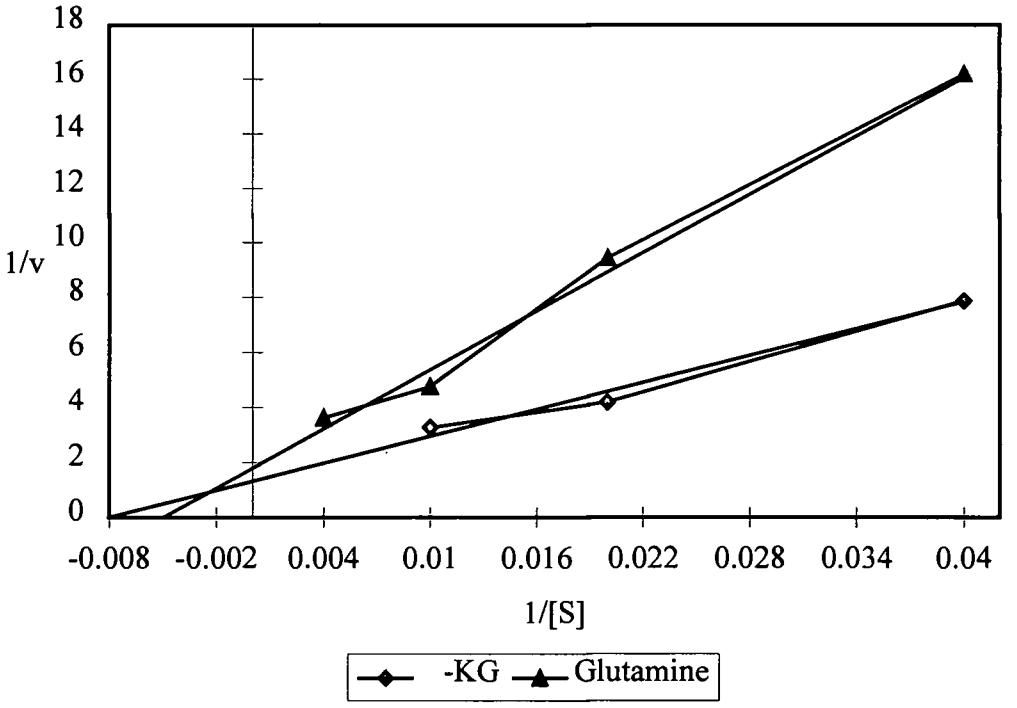

Figure 6.7. Lineweaver-Burke plot of grape lamina Fd-GOGAT response to different concentrations of α-KG or glutamine. Values of S are in μmol and those for v are μmol/min. Low values of 1/[S] (*i.e.* higher concentrations of the substrates) were unattainable in the present assay.

Discussion

Western blots using anti-Fd-GOGAT IgG from rice leaf showed a positive reaction in grape tissues corresponding to a protein of approximately 73kDa. This is a much smaller Fd-GOGAT protein than has been reported in other plant species. In maize leaf, sizes of 145kDa (Commere *et al.*, 1986), and 160kDa (Matoh *et al.*, 1979) have been reported, and rice leaf Fd-GOGAT was associated with a protein of 150kDa (Suzuki *et al.*, 1987). In dicots, sizes of 180kDa, 145kDa have been reported in spinach and bean leaves, respectively (Tamura *et al.*, 1980; Wallsgrove *et al.*, 1977), and 165kDa in pine

seedling cotyledons (García-Gutiérrez *et al.*, 1995). However, Suzuki and Gadal (1984) noted that the number of subunits, molecular weight, and association of prosthetic groups such as flavin varies between species. In this study, rice antibody associated with a rice protein of approximately 177kDa molecular weight, whereas in the study where the antibody was developed the authors reported that the subunit of rice Fd-GOGAT was a 115kDa protein, with a native enzyme having a molecular weight of 224kDa (Suzuki *et al.*, 1982).

There are several possible reasons for the discrepancy in estimates of rice leaf Fd-GOGAT molecular weights. Suzuki and Gadal (1982) reported that no iron-sulfide or flavin was associated with the rice leaf protein, yet one report suggests that spinach leaf Fd-GOGAT does have prosthetic groups associated with it (Hirasawa and Tamura, 1984). It is possible that under the conditions in these experiments that one or more prosthetic groups were still associated with the rice protein subunit during the SDS-PAGE process. The positive reaction of the antibody with smaller molecular weight rice proteins suggests that there was partial degradation of Fd-GOGAT during the extraction process. Other factors that may affect the estimated molecular weight are gel composition, standards used, rice variety used, and extraction method.

That grape Fd-GOGAT protein appears to have a very low molecular weight could be due to degradation of the protein during the extraction process, or grape Fd-GOGAT could be composed of two or more subunits, with or without associations of prosthetic groups. Hirasawa and Tamura (1984) reported that the minimum molecular weight of spinach Fd-GOGAT without any flavin groups was 77kDa, suggesting that grape Fd-GOGAT has a similar make up. Extraction conditions may also have been conducive to protein catabolism, resulting in the antibody reacting with incomplete protein. García-Gutiérrez *et al.* (1995) found 90 and 70kDa proteins that co-purified with Fd-GOGAT and suggested they were degradation products formed during the extraction procedure. Sakakibara et al. (1991) also found co-immuno-reacting bands of 73 and 88kDa on their blots of rice Fd-GOGAT and attributed them to limited cleavage of the whole 160kDa polypeptide. The authors speculated that the cleavage probably occurred during

overnight dialysis, and found that removal of β-mercapto-ethanol from the dialysis buffer partially protected the protein from proteolysis. However, in the present study, only one band was observed in grape protein lanes, suggesting that the grape polypeptide is 73kDa or that complete degradation to a single, but smaller, protein took place.

Grape lamina and rachis tissue had a lesser response in reaction to the antibody. This result could be explained by inaccuracies in determination of the protein concentration, resulting in less protein being loaded into the lanes than was calculated. Alternatively, the amount of Fd-GOGAT protein in lamina tissue may be low due to the age of the leaf, which, unlike leaf tissue used in the activity assays, was taken from the cluster area of the shoot, shaded, and not as photosynthetically active as the younger leaves. In tobacco, high amounts of Fd-GOGAT protein were found in leaves, lesser amounts in pistils and anthers, and none in the corollas or stems (Zehnacker *et al.*, 1992). This enzyme may be expressed preferentially in different tissues.

Through gel filtration, activity in the partially purified proteins was found to be in the 150 to 250kDa range, suggesting that the native form of grape Fd-GOGAT is a dimer or a trimer of the 73kDa protein identified with the polyclonal antibody, or has other subunits associated with it.

Direct comparison between the grape Fd-GOGAT protein's putative molecular weight as determined by SDS-PAGE and gel filtration may not be valid. Discrepancies between these two methods have been reported (Hirasawa and Tamura, 1984), possibly due to conformational changes brought on by the action of SDS (Reynolds and Tanford, 1970). Also, because polyclonal antibodies are not entirely specific to the antigen to which they were raised, there is the possibility that positive reactions seen in the western blots may be due cross-reactivity with unrelated proteins that have similar antigenic sites. Fd-GOGAT from a variety of plant species have been sequenced, and are fairly highly conserved (Avila *et al.*, 1993; García-Gutiérrez *et al.*, 1995; Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992). Fd-GOGAT antibody from one species has successfully been used to probe other species, including monocot to dicot probes (Becker *et al.*, 1993;

Commere, *et al.*, 1986; García-Gutiérrez *et al.*, 1995), so it is likely that the positive reaction reported here indicates some protein related to Fd-GOGAT in grape.

Roubelakis-Angelakis and Kliewer (1983) have demonstrated GS activity in grape lamina and root tissues, and Ghisi *et al.* (1984) showed GS operating in lamina and berry extracts. NADH-GOGAT activity in grape was reported in partially purified (by ammonium sulfate precipitation) extracts of lamina tissue by Jordan *et al.* (1992). Here, Fd-GOGAT activity was found in grape lamina, petiole, tendril, rachis, and flower, but not pedicel tissue. The assay for Fd-GOGAT appeared to be specific, given that azaserine stopped production of glutamate. Studies of Fd-GOGAT from rice (Suzuki and Gadal, 1982) have also demonstrated the effectiveness of azaserine in inhibiting glutamate production by it. Elimination of Fd, α-KG, or glutamine from the assay volume reduced glutamate production to near zero. Analysis of free amino acids after the assay showed that glutamate was the only significant amino acid eluted from the column, that azaserine inhibited the production of glutamate, and that virtually no glutamate was detectable at zero reaction time. A limitation of the assay as described here is the inability to achieve substrate concentrations of above 0.5m*M* α-KG and 1m*M* glutamine without precipitation of proteins from the mixture. Reducing the total solute concentration in the assay mixture was unsuccessful in preventing the precipitation. Phenolic or other compounds still present in the extract may be interacting with proteins and causing their precipitation.

Further experimentation would be needed to fully explore the characteristics of grape Fd-GOGAT. From data gathered with the present method, a K_m of 110μ*M* and 200μ*M* for α-KG and glutamine, respectively, were estimated for grape leaf Fd-GOGAT (Fig 6.7). These values are somewhat lower than those reported for Fd-GOGAT from bean, rice, or maize (Matoh *et al.*, 1979; Suzuki and Gadal, 1982; Wallsgrove *et al.*, 1977), which ranged from 150 to 330μ*M* for α-KG, and 270 to 1100μ*M* for glutamine. This assay was limited to substrate concentrations lower than 1m*M*, and estimated K_ms are close to substrate concentrations at which there was no reduction in the rate of glutamate production (*i.e.* 100mM for α-KG and 250mM for glutamine, Fig. 6.7).

Nevertheless, all reports indicate that the affinity of Fd-GOGAT for α-KG is greater than that for glutamine.

It is intriguing that pedicel tissue, which is the first part of the flower cluster to show necrosis associated with IN (Ibacache, 1990; Keller and Koblet, 1995), did not display any Fd-GOGAT activity despite the protein being present (Fig. 6.2). Speculation as to the cause of IN has focused on the ability of flower cluster tissues to assimilate NH_4^+ (Jordan *et al.*, 1991). Gu *et al.* (1994) showed that MSO and azaserine raised NH_4^+ levels in all leaf and cluster tissues near bloom, with the exception of the rachis, and suggested that the GS/GOGAT pathway was not functioning in that tissue. There were complications associated with this two year study using green shoot cuttings incubating in various solutions, however. Azaserine was included in the study for only one stage (one week post bloom) in one year (1990). Rachis tissue had no response to inhibitors in either year tested. Pedicel tissue respond to the inhibitors, but at only one of the three stages (one week pre-anthesis, anthesis, and one week post-anthesis) tested in each year. In 1990, pedicel tissue was responsive only at one week post-anthesis, while in 1991 the significant response occurred at one week pre-anthesis. It is possible that there are unknown factors that trigger the activity of the GS-GOGAT pathway *in vivo*, and that pedicel tissue in this study was collected at a stage where Fd-GOGAT was not active, despite it being present in the tissue (Fig. 6.2).

There may be other reasons that pedicel tissue failed to show Fd-GOGAT activity. Because of the small size of the pedicels (ca. 3mm by 1mm) considerable mechanical damage was done in comparison to the volume of tissue collected. This, in combination with the time required to clip the pedicels from the rachis and freeze them in liquid nitrogen, would have allowed enzymatic and oxidative processes to inactivate enzymes within the tissues.

Leaf tissue had the highest level of Fd-GOGAT activity, which is not surprising considering that photosynthetically active tissue has a high turnover rate in NH_4^+ due to photorespiration (Wallsgrove *et al.*, 1983). Hirasawa and Tamura (1984) reported that the specific activity of Fd-GOGAT in partially purified (by acetone precipitation) extract

from spinach leaf tissue was 1.17U/mg protein, over twice the value reported here. From rice and tobacco leaves, Fd-GOGAT was reported to have a specific activity of 0.195 and 2.64U/mg protein, respectively (Suzuki and Gadal, 1982; Zehnacker *et al.*, 1992), after an ammonium sulfate precipitation similar to that used here. It is possible that phenolic compounds are still tying up proteins in the extraction process, thus resulting in a lower yield and activity.

This is a preliminary study into the action of Fd-GOGAT in grape tissues. Further experimentation with assay conditions is needed to improve the range of substrate concentrations that can be used, and the enzyme should be isolated to better characterize it versus GOGATs from other species. The question as to the *in vivo* activity of the full GS-GOGAT cycle in relation to IN in grape, especially cluster tissues, still remains to be answered, but the experiments reported here have shown that Fd-GOGAT is present in shoot tissues, and active in most.

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Chapter 7: Epilogue

The preceding four chapters are diverse in their scope, but all revolve around one thing, a fruit set problem of grape known as inflorescence necrosis (IN). Through defoliation, shade, and tipping experiments, the ability to increase flower cluster NH_4^+ levels (though in one case there was a significant reduction) was demonstrated, but these treatments did not affect IN severity. Curiously, significant treatment differences did appear on secondary clusters of shoots whose primary cluster had been removed at bloom (for NH_4^+ analysis). Whether the removal of the primary cluster constituted an additional stress that aggravated NH_4^+ levels, resulting in more severe IN isn't known. Ideally, the NH_4^+ in secondary clusters should also be investigated, to see if the NH_4^+ levels within respond differently than those in primary clusters. It has already been noted that secondary clusters appear to have less severe IN than their primary counterparts (Keller and Koblet, 1994; Chapter 3), and usually only the primary cluster is the subject of experiments.

Whole vine shade increased shoot NH_4^+ levels, as has been reported previously (Gu *et al.*, 1996; Ibacache, 1990; Jordan, 1989; Smart *et al.*, 1988). This thesis has shown that individual shoot shade also has an effect on shoot tissue NH_4^+, including that of the flower cluster. However, reducing the scope of the shade to include just the clusters had no effect on its NH_4^+ status. Individual shoot shade, like in the defoliation treatments, had no effect on IN severity, despite raising flower cluster NH_4^+.

Another series of experiments was aimed at determining if there is a link between NH_4^+ and IN in field grown vines. The clusters were subjected to treatments that have increased IN severity in previous research (Gu *et al.*, 1994; Ibacache, 1990; Jackson, 1991; Jackson and Coombe, 1988), or to treatments that, given the current understanding of the disorder and its physiology, should increase or decrease IN. Again, it turned out that there wasn't a discernible link between NH_4^+ in the flower cluster and IN in the field, even though this has been established under more controlled environments (Gu *et al.*, 1994; Ibacache *et al.*, 1991). For example, in Chapter 4 the ethephon treatment raised

flower cluster NH_4^+ by only 20% over controls (a non-significant increase), but had the most severe IN of any of the treatments. However, very little necrosis was observed in MSO treated clusters, despite the NH_4^+ concentration inside them being twice as high as the controls.

Since rootstocks are often utilized on the basis of their effects on the growth of the scion grafted to it, it seemed a logical step that IN may also be one of the factors influenced. This and other observations by viticulturists at OSU led to the investigation of rootstock and clonal effect on petiole and flower cluster NH_4^+ and IN severity. Perhaps not so surprisingly, significant differences in these parameters were found, though rootstocks in different vineyards responded uniquely, making blanket recommendations as to *the* rootstock to use impractical. Factors involved in site differences, such as weather, soil, vineyard management, *etc.* have an effect on rootstocks in differing ways (Candolfi-Vasconcelos and Castagnoli, 1996). Are the effects of rootstock on IN or NH_4^+ in the scion direct (*e.g.* increased supply of NH_4^+ from the roots) or indirect (*e.g.* imparting greater vigor, which increases shading, which leads to more severe IN)? Since NH_4^+ in the flower clusters or petioles doesn't seem to correspond to IN, it would appear that the latter case is more defendable. Continued monitoring of IN in rootstock trials would be of value to chart trends from site to site and year to year.

There are many clones of Pinot noir, having been chosen and propagated because of some desirable viticultural characteristics such as growth habit, cluster size, or cluster morphology. Of the Pinot noir clones studied in this thesis, significant differences in flower cluster NH_4^+ and IN severity were found in 1995. Since differences in IN between these same clones were found in 1994 (S.F. Price, unpublished data), it appears that consistent year to year differences occur. If a clone is particularly and chronically susceptible to IN, fruit set would then be reduced on a regular basis compared to other clones. This would tend to loosen clusters, which is a characteristic of the Mariafeld types, such as UCD17 and 23. In 1994 (S.F. Price, unpublished data) and 1995 (Chapter 5) surveys of IN severity in the clonal block, both UCD17 and 23 scored above average. It would be worthwhile to continue following IN severity in select clones such as 17 and

23 in this block (and elsewhere). The possibility that a clone selected for its loose-cluster characteristic was, unknowingly, being selected for chronic IN susceptibility leads to the notion that a desirable clone was picked out due to what has been described as a "disorder!"

The final experiments of this thesis made the jump from the field to the laboratory. Earlier research had suggested that the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle wasn't functioning in petiole and rachis tissue thus leading to necrosis (Gu, 1992). These experiments used inhibitors of those enzymes to determine "activity," however. GS has been extracted and its activity measured in grape roots, leaves, and berries (Ghisi *et al.*, 1984; Roubelakis-Angelakis and Kliewer, 1983), so it should be relatively easy to adapt a technique to measure GS activity in flower cluster tissues. Fd-GOGAT activity, which is the principal form of GOGAT in green tissues, however, had not been demonstrated in grape. So the question chosen to answer here was: is Fd-GOGAT present and functioning in the flower cluster?

The answer is a qualified yes. Antibody raised against rice leaf Fd-GOGAT reacted with a single 73kDa grape protein from lamina, petiole, rachis, flower, and pedicel tissue. Through gel filtration and an assay for Fd-GOGAT activity, the native grape enzyme (from lamina tissue) must have a molecular weight between 150 and 225kDa, which means that it is possibly a dimer or trimer of the 73kDa protein identified by the antibody.

Using the assay developed for this thesis, Fd-GOGAT activity was found in extracts from lamina, petiole, flower, rachis, and tendril tissues, but not from those of pedicel. There are two sides to this finding: 1) the absence of Fd-GOGAT activity in pedicel tissue corresponds well with the observations that in IN affected clusters, the pedicel tissue is the first to become necrotic (Ibacache, 1990; Keller and Koblet, 1995). If GOGAT (and/or GS) are not functioning in this tissue, NH_4^+ concentration could build to toxic levels, possibly resulting in necrosis and IN. But the other side to this finding is 2) the absence of Fd-GOGAT activity may be due to an artifact, specifically, due to difficulties in collecting and processing this very tiny part of the flower cluster. Further

experiments to try to answer the question of whether there really is or isn't activity in the pedicel must be designed.

One possibility is that too much of the tissue was mechanically damaged, which caused the elimination of activity in the tissue. The other is that Fd-GOGAT is present in the tissue, but not active (Chapter 6). A series of experiments may help answer the question of the level of Fd-GOGAT activity in pedicels, barring the development of some more efficient way of collecting the tissue. Leaves, which contain generous amounts of activity, could be mixed with pedicel tissue and extracted by the procedure described in Chapter 6 (alternatively, leaf extract that has a known amount of activity could be mixed with pedicel extract). If there is no activity, then something in the pedicels prevented it, perhaps a specific or general (*e.g.* phenolic) inhibitory compound. If there is activity in the extract, then further experiments need to be designed, perhaps going back to the beginning and trying to eliminate more of the phenolic compounds at the start (either by modifying the extraction buffer/procedure, or by growing tissue with fewer phenolics).

The inability to assay Fd-GOGAT activity under relatively high substrate concentrations is a problem that must be resolved. If the subunit of grape Fd-GOGAT is indeed 73kDa, then it is an enzyme that deserves further research, in order to compare it to the larger enzymes reported in other plant species. To find out if flavins, iron sulfides, or other groups are associated with the enzyme, it must be purified, which will require a different extraction technique than that used here. It seems likely, given the browning and loss of activity in the partially purified enzyme solution, that phenolic or other interfering compounds remain in the extract. Since removing phenolics from the grape extracts remains problematic, perhaps it would be better to characterize Fd-GOGAT from grapevine callus tissue, which should be low in phenolic content. The effect of light on Fd-GOGAT production and activity could then also be elucidated. Does tissue well exposed to light lose GOGAT activity or protein if shaded? Does tissue grown under low light produce more or activate existing Fd-GOGAT protein when the light level is increased?

Much research in determining the role of GS in the grape flower cluster remains as well. An assay capable of detecting GS activity in individual cluster parts should be developed and used in conjunction with Fd-GOGAT assay to explore the GS/GOGAT pathway of NH_4^+ assimilation in grape and its possible connection with IN.

The work of Gu *et al.* (1994) suggested that GS and/or GOGAT activity in flower cluster parts may vary before and after bloom, with rachis tissue never being affected by the addition of a GS inhibitor, and other tissues being affected at only certain stages. Is it possible that the GS/GOGAT pathway is switched on and off as bloom approaches? Does the yearly variation in spring weather affect the activity of the pathway? Careful collection of tissues combined with assays for GS and GOGAT may help answer these questions.

Ibacache (1990) reported that shading field vines tended to lower the α-KG in the flower cluster rachis, which supported the hypothesis that α-KG is limiting to NH_4^+ assimilation in the flower cluster, thus leading to an accumulation of NH_4^+ and necrosis (Jordan, 1989; Jordan *et al.*, 1991). Additionally, in comparison to other amino acids present in the rachis, the concentration of α-KG is fairly low (Ibacache, 1990). Plants with a high carbohydrate availability, especially of α-KG, are better able to utilize NH_4^+ and obviate toxicity symptoms (see Barker and Mills, 1980). Further research into substrate availability for NH_4^+ assimilation, and the rest of the nitrogen and carbon economy of the grape inflorescence, would be invaluable in determining how IN develops.

Jackson and Coombe (1988), Jordan (1989), and Lombard *et al.* (1993) have hypothesized that IN is worsened by cool, wet, and cloudy weather preceding grape bloom. If there is, as these researchers suggest, a connection between NH_4^+ and IN, the weather may well have an effect on NH_4^+ concentration in shoot tissues. The differences in NH_4^+ concentrations between years are then likely due to varying weather leading up to bloom. The spring of 1995 was generally mild, but had above average rainfall. Temperatures rose to near 30°C immediately before bloom, but there was significant rainfall near bloom (source, Oregon Climate Service). The spring of 1994 was largely a

dry and warm pre-bloom season, but it became cooler and wetter in the few weeks before and during bloom, which hindered fruit set (Watson and Price, 1995). The weather leading up to bloom in 1993 was very wet, with well above average rainfall from March through June (Watson and Price, 1994). The favorable weather in the months before bloom in 1994 may be responsible for the lower NH_4^+ concentrations found in all tissues surveyed in that year compared to the others. The hypothesis that shade, or generally overcast skies, increases stress levels in the vine, leading to NH_4^+ accumulation and the appearance of IN symptoms is one theory of the mechanism behind IN (Gu *et al.*, 1996; Jordan *et al.*, 1991; Lombard *et al.*, 1993). Keller and Koblet (1994) suggest that overcast conditions result in a decrease in net photosynthesis that reduces the availability of carbon compounds, leading to the appearance of IN symptoms.

Further observations as to how weather, and soil temperature, affect IN should be noted in vineyards. Perhaps a study using potted vines growing in growth chambers could be utilized. The more controlled conditions would allow the variation of a single factor, such as light, temperature, and moisture.

A very important question that remains to be answered is: What is the source of the NH_4^+ that accumulates? NH_4^+ concentrations approached 4mg NH_4^+-N per gram dry weight of flower cluster tissue in the rootstock survey (Chapter 5). The florets themselves are relatively low and the rachis high in NH_4^+ (Gu *et al.*, 1996), so if the florets are separated from the cluster and the NH_4^+ concentration in the rachis measured, levels of up to 10mg NH_4^+/gdw have been reported in field vines (Ibacache *et al.*, 1991)! Where could all this NH_4^+ come from?

Photorespiration is commonly cited as a significant contributor to NH_4^+ turnover in photosynthetic tissue (Frantz *et al.*, 1982), but in comparison to leaves the rachis is not a rapidly photosynthesizing tissue, and Gu (1992) reported evidence that photorespiration was not a contributor to NH_4^+ accumulation in the flower cluster.

Since IN is associated with high-vigor vines, and excess supply of nitrogen contributes to rapid growth, perhaps the NH_4^+ derives from the soil? In potted vines of Pinot noir, Gu *et al.* (1996) showed that nitrogen source (NH_4^+ or NO_3^-) had no effect on

flower cluster NH_4^+ before bloom or IN severity. IN has also been observed on very weak, as well as vigorous vines, so a direct relationship with soil nitrogen seems unlikely. ^{15}N might be used to trace the source of NH_4^+ within grape tissue, if a suitable system could be developed. ^{15}N has been used to determine where shoot tissues derive their early season nitrogen (Glad *et al.*, 1994), showing that stored nitrogen is an important factor in the early development of the shoots and flower clusters up until bloom. ^{15}N, in combination with NMR, may also allow *in vivo* observation of when NH_4^+ is appearing. This technique was used to show that the GS/GOGAT pathway, rather than the GDH, was responsible for the bulk of NH_4^+ assimilation in corn seedlings (Amancio and Santos, 1992). This real-time, *in vivo* method of measuring the appearance of labeled substances could help identify the source of NH_4^+ accumulation in the cluster tissue.

Protein degradation is also a source of NH_4^+, as the amino acids that made up the proteins are catabolized (Cullimore and Sims, 1980). As noted earlier, there are a great many abscission zones forming on the flower cluster at bloom, since for each calyptera to come off of the floret its base must separate from the ovary. NH_4^+ concentration peaks at bloom (Ibacache, 1990), as does respiration in the flower cluster (Blanke and Leyhe, 1989). Respiration increases with increasing NH_4^+ levels (Matsumoto and Wakiuchi, 1974) in order to supply more substrates needed to re-assimilate the NH_4^+. If substrates are limiting in the grape flower cluster (a question that still needs to be resolved), however, then perhaps there is a fault in this proposed chain of events, or there is a fault in the flower tissues that doesn't allow the chain to come full circle, and the NH_4^+ to be assimilated. Addition of MSO to plant tissues blocked NH_4^+ assimilation and also resulted in a rise in the protein breakdown rate (Cullimore and Sims, 1980). Could the protein degradation associated with calyptera senescence combine with a blockage of GS or GOGAT in grape tissues lead to NH_4^+ release that cannot be re-assimilated into amino acids? If proteases are active in the florets at bloom, could PMSF be used somehow to show that proteases are or are not involved in the formation of IN?

Another idea worth following up on is that NO_3^- may reduce or prevent toxicity symptoms at NH_4^+ concentrations that would otherwise kill tissues (see Barker and Mills,

1980). Gu *et al.* (1994) reported that NO_3^- had some protective effect against the appearance of IN in green shoot cuttings after, but not before, bloom. Shade, known to induce IN symptoms increases shoot tissue NO_3^- as well as NH_4^+ concentrations (Gu, 1992), though NO_3^- itself does not induce symptoms. The true involvement of NO_3^- and IN is still not known.

It is also curious that IN-affected florets often remain on the cluster through the season, whereas other unfertilized flowers fall from the cluster during the shatter stage. It is possible that death of the tissue due to IN is not a normal part of floret senescence, thus an abscission zone does not form. Treatment of vines with ethephon resulted in necrosis very much like that of IN, and similarly persistent dead florets (Chapter 4). Flower clusters should be collected near bloom, embedded in a sectioning material, and examined for the appearance of abscission zones at the base of the pedicel. If enough samples are collected, the very early stages of IN should be detected, which may help determine exactly where the necrosis starts first, and if an abscission zone is associated with the disorder.

Examination of Fig. 2.1 reveals that on the particular cluster pictured, death of the florets occurred just as the calypteras were separating from the ovaries. Examination of other photos (Ibacache, 1990; Jackson and Coombe, 1988; Jordan *et al.*, 1991) shows that death of the florets appears to occur just before or after the calyptera separates from the ovary. Ibacache (1990) noted that symptoms of IN first appeared at beginning bloom and increased until full bloom. Since NH_4^+ concentration in the clusters peaks at bloom, is it a coincidence that the florets became necrotic at this stage? Farquhar *et al.* (1979) note that senescing leaves emit NH_3 gas, so perhaps emission of this could be measured through bloom and associated with IN severity?

In summary, considerable mystery still surrounds IN and its physiology. Research that has been reported within the last few years is strengthening the argument that carbohydrates play a role in the appearance of IN, first proposed by Jordan (1989). It would seem that interaction between carbohydrate availability and perhaps some trigger for NH_4^+ accumulation leads to IN symptoms in the grape flower cluster. Clearly, further

investigation into the nitrogen and carbon economy of the grape flower cluster is necessary to elucidate this problem.

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