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Inflorescence necrosis (IN) is a recently described disorder in grapes characterized by partial or complete deterioration of the pedicel and rachis near bloom. Elevated levels of ammonium in the rachis are associated with the incidence of IN and hypothesized as the cause of the disorder. An incubation system with single-node cuttings was developed to test this hypothesis, to determine the presence of the primary ammonium assimilation pathways, and to investigate the source of ammonium accumulation. Shading and various sources of nitrogen were applied to grapevines to determine their effects on IN severity, tissue ammonium, and nitrate status.

Symptoms of IN were induced by incubating the base of single-node 'Pinot noir' cuttings, with a single leaf and cluster, in solutions of 120 mM or higher NH_4^+. The addition of α-keto-glutarate prevented IN and reduced the concentration of ammonium in flowers and fruit to that of the control.

Incubation of cuttings with inhibitors of the glutamine synthetase/ glutamate synthase (GS/GOGAT) pathway, 10 mM methionine sulfoxinine (MSX) or 5 mM azaserine (AS), increased the ammonium concentration in the lamina, pedicel, and flower or fruit, suggesting the presence of this pathway. Since MSX and AS had no effect on the concentration of ammonium in the petiole and rachis, GS/GOGAT activity may be low or absent in these tissues. Studies with cluster tissues indicated that glutamate dehydrogenase (GDH) was not an important pathway for ammonium detoxification nor where nitrate reduction or photorespiration major sources of ammonium.

Shading (60%) two-year-old vines increased the concentration of ammonium and nitrate in the lamina, petiole, and rachis and enhanced the severity of IN.

At one week post-anthesis, vines fed either NH_4^+ or NO_3^- had higher levels of ammonium in the rachis, petiole, fruit, and tendril than vines receiving no additional nitrogen. Neither nitrogen treatment caused an increase in IN. At all samplings, new roots of NH_4^+-fed vines had higher ammonium levels than those fed NO_3^-, whereas levels in shoots were elevated only after anthesis. Nitrate concentration in all sampled tissues was proportional to soil NO_3^- except in the rachis, flowers, or fruit.

This study suggests that 1) ammonium is the causal agent of IN, 2) the rachis and pedicel have a low capacity for ammonium assimilation and detoxification, and 3) nitrate reduction, photorespiration, and soil supplied nitrogen are not major sources of the elevated ammonium responsible for IN.

Relationships of Ammonium and Nitrate Status with Inflorescence Necrosis in 'Pinot noir' Grapevines

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TO

My Parents: Qiyu and Guizhi
My Wife: Xiumei
My Sons: Fei and Kevin

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Chapter 1

Introduction

Inflorescence necrosis (IN), or early bunchstem necrosis (Ibacache, 1990; Jackson and Coombe, 1988a, b; Jordan, 1989) is described as a partial or complete breakdown of the rachis and pedicels near bloom in grapes (Ibacache, 1990). The cause of IN is unknown. No pathogens have been found on the affected tissue and the disorder appears to have a physiological cause (Jackson and Coombe, 1988a, b). It is believed that IN is associated with inorganic nitrogen. Stresses such as shading and drought can increase its incidence (Jackson and Coombe, 1988a, b). Elevated ammonium levels in rachis tissue have been related to greater incidence of IN (Ibacache, 1990; Jordan, 1989). Also, diammonium phosphate applied to inflorescences one week before flowering caused typical early bunchstem necrosis symptoms (Jackson and Coombe, 1988a, b). However, tissue sampling always occurred after symptoms had developed, leaving the question of cause or effect unanswered.

The glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is the primary route for ammonium assimilation in leaves of higher plants (Miflin and Lea, 1976; Wallsgrove *et al.*, 1980). Several researchers have suggested an assimilatory role for glutamate dehydrogenase (GDH) in some plant tissues (Loyola-Vargas and Sanchez de Jimenez, 1984; Pahlich and Hoffman, 1975; Singh and Srivastava, 1986; Zeleneva and Khavkin, 1980). GS and GDH activities have been detected in grape berries, roots, and leaves. (Ghisi *et al.*, 1984; Roubelakis-Angelakis and Kliewer, 1983a, b). However, Roubelakis-Angelakis and Kliewer (1983b) were unable to detect GOGAT activity in berries. To understand the factors which affect the buildup of ammonium in grape tissues it is necessary to understand the enzyme systems for ammonium assimilation and detoxification and sources of accumulated ammonium. The activity of ammonium assimilation pathways and sources of ammonium in grapes, especially in pedicel and rachis tissues, may be critical to the development of IN.

Inhibitors of the GS/GOGAT pathway, such as L-methionine D,L-sulfoximine (MSX) and azaserine (AS), have been widely used in studies of plant carbon-nitrogen metabolic pathways. Several workers found that treatment of photosynthesizing plants with MSX leads to ammonium accumulation in various species (Anderson and Done, 1977; Arima and Kumazawa, 1977; Frantz *et al.*, 1982; Platt and Anthon, 1981; Singh and Widholm, 1975). It is also found that MSX did not affect the CO_2 fixation process (Romero *et al.*, 1985), the activity of nitrate reductase (Frantz *et al.*, 1982), or GDH (Arima and Kumazawa, 1977; Brenchley, 1973). AS, an analog of glutamine, inhibits all glutamine amide transfer reactions, including that catalyzed by GOGAT, but does not affect GDH activity (Dunn and Klucas, 1973; Miflin and Lea, 1976).

Ammonium accumulation in plant tissues can be a consequence of low carbon substrates which are essential for ammonium assimilation (Barker and Mills, 1980; Givan, 1979). Assimilation is the main defensive mechanism for plants to prevent ammonium accumulation (Givan, 1979). Jordan (1989) reduced the ammonium concentration in grape tendrils with α-keto-glutarate after ammonium incubation. Low carbohydrate status due to shading may induce ammonium accumulation and lead to necrosis of the flowers. Shaded clusters also grow weakly and could be more sensitive to any toxic element including ammonium (Ibacache, 1990).

Tissue ammonium levels are dynamic pools influenced by input and output components. Ammonium input to a given tissue can occur directly by xylem translocation. Ammonium-fed vines accumulated more free ammonium in petioles at veraison and in the petioles and rachis at harvest than NO_3^--fed plants (Chang and Kliewer, 1991). However, there is no information about the effect of nitrogen feeding and its source on ammonium and nitrate status around anthesis, the critical time for development of IN.

The objectives of this study were: 1) to investigate ammonium and/or nitrate as the possible cause of IN and the effect of α-keto-glutarate, a carbon substrate for ammonium assimilation, on IN and ammonium accumulation; 2) to verify the presence of the GS/GOGAT and GDH pathways and determine the contribution of nitrate reduction and photorespiration to ammonium accumulation; and 3) to establish dynamic patterns of ammonium and nitrate status of various tissues near anthesis and determine the effect of shading and nitrogen source on tissue ammonium, nitrate status, and IN in 'Pinot noir' grapevines.

Chapter 2

Literature Review

I. Ammonium Assimilation and Detoxification in Higher Plants

Ammonium assimilation plays a central role in plant nitrogen metabolism since, in addition to ammonium being absorbed directly by roots, ammonium is also the product of nitrate and urea assimilation as well as molecular nitrogen fixation (Haynes, 1986). The inorganic nitrogen acquired by plants is ultimately converted to ammonium before incorporation into organic molecules. The pathways of ammonium assimilation and detoxification have been reviewed in detail by several authors (Fowler and Barker, 1979; Givan, 1979; Miflin and Lea, 1976, 1977, 1980, 1982; Stewart *et al.*, 1980). Ammonium detoxification is mainly by assimilation. Plants can detoxify ammonium as long as they can convert ammonium into amino acids (Givan, 1979), therefore ammonium assimilation and detoxification are discussed together.

The reactions which are considered entry points for conversion of ammonium into the organic form are listed in Table 2-1. Prior to 1970, it was generally considered that glutamate dehydrogenase (GDH) was the major route of ammonium assimilation. However, the entrance of ammonium *via* GDH in higher plants is questionable since GDH has a K_m for ammonium that is above the normal cellular concentration of this ion. This led to the conclusion that GDH either has a detoxifying role at high ammonium concentrations or that it is mainly a deaminating enzyme (Givan, 1979; Miflin and Lea, 1980). Recent work, however, suggests an assimilatory role for GDH in some plant tissues (Loyola-Vargas and Sanchez de Jimenez, 1984; Singh and Srivastava, 1982; Zeleneva and Khavkin, 1980).

Glutamine synthetase (GS) was the other major candidate of ammonium assimilation in terms of its universal distribution and great activity. Work by Tempest *et al.* (1970a, b) showed that glutamate synthase (GOGAT), coupled with GS provided a means whereby bacteria could assimilate ammonium into amino acids when grown on limited quantities of inorganic nitrogen. Subsequently, GOGAT was found in higher plants, *i.e.* an NAD(P)H-dependent enzyme in non-

green tissues (Dougall, 1974) and a novel ferredoxin (Fd)-dependent enzyme in green leaves (Lea and Miflin, 1974). It is well established that GS/GOGAT is widely distributed and therefore most organisms have the potential for ammonium assimilation through this route. GS has lower K_m for ammonium ions than GDH and is coupled with GOGAT in most plant tissues (Arima and Kumazawa, 1977; Lea and Miflin, 1974; Stewart and Rhodes, 1978; Yoneyama and Kumazawa, 1974). Rhodes *et al.* (1980) have termed the GS/GOGAT pathway the 'glutamate synthase cycle'.

A. Primary Pathway -- Glutamate Synthase Cycle

Much evidence shows that the GS/GOGAT pathway is the primary route for the assimilation of ammonium in low (normal) intercellular concentrations (Anderson and Done, 1977; Dry and Wiskich, 1983; Miflin and Lea, 1974; Mitchell and Stocking, 1975; Somerville and Ogren, 1980; Wallsgrove *et al.*, 1980; Woo, 1983; Woo and Osmond, 1982, Woo *et al.*, 1987). The key characteristic of this route is its cyclical nature, in which glutamate acts as both the acceptor of ammonium and product of ammonium assimilation; one molecule of glutamate continuously recycles while the second may be transmitted to other amino acids or converted to proline and arginine (Miflin and Lea, 1982).

Within the cycle (eq. 1 and 2, Table 2-1), ammonium is incorporated into glutamate as an amide group by GS to form glutamine. Subsequently, GOGAT catalyses the reductive transfer of the amide group to α-keto-glutarate, yielding two molecules of glutamate (Stewart *et al.*, 1980). Operation of this pathway for the net synthesis of glutamate requires the export of α-keto-glutarate from mitochondria for use in the GOGAT reaction (Miflin and Lea, 1982; Weger *et al.*, 1988).

The distribution of these enzymes within the cell has also been studied. GS has been found in the chloroplasts of many species (Haystead, 1973; O'Neal and Joy, 1974). Although it has been suggested that GS/GOGAT is also present in mitochondria (Jackson *et al.*, 1979), this has not been substantiated (Keys *et al.*, 1978). GS is also present in the cytoplasm (Miflin, 1979b). The dual location of GS and GOGAT probably means that there is more than one form of the enzymes present. The two isoforms of GS known to exist in leaves of many species are GS_1 localized in the cytosol, and GS_2 in the chloroplast (Hirel and Gadal, 1980;

Nishimura *et al.*, 1982). Research suggests that GS_1 is present in leaves, roots, and seeds, whereas GS_2 is restricted to green tissues (Hirel *et al.*, 1982). By virtue of its subcellular localization, cytosolic GS_1 may function to assimilate ammonium ions produced in photorespiration (Cullimore *et al.*, 1983; Hirel and Gadal, 1980; McNally and Hirel, 1983; Wallsgrove *et al.*, 1977) while chloroplastic GS_2 which has a low K_m for ammonium, is thought to be involved in the primary assimilation of ammonium produced by nitrate reduction (O'Neal and Joy, 1974; Stewart *et al.*, 1980). Since the chloroplastic isoform of GS appears to be predominant in leaves of C_3 plants (McNally and Hirel, 1983), assimilation of ammonium during photorespiration (Keys *et al.*, 1978; Wallsgrove *et al.*, 1980; Woo *et al.*, 1987, Woo and Osmond, 1982) likely takes place predominantly in the chloroplast (Bergman *et al.*, 1981; Woo and Osmond, 1982). McNally and Hirel (1983) divided plants into four categories according to the isoenzymes of GS: 1) achlorophyllous higher plant parasites nutritionally dependent on their hosts (e.g. *Orobache* species) and containing only cytosolic form GS_1; 2) a group containing a range of plants including spinach, tobacco, and lupin which have only the chloroplastic form GS_2, 3) A group of C_3 grasses and temperate legumes which have a minor GS_1 (5-30%) and a major GS_2 (70-95%) form; and 4) a group of C_4 and CAM plants and tropical legumes that have a high proportion of cytosolic GS_1, as much as 80% of the total.

GS has a molecular weight in the range of 350 kD to 400 kD and consists of eight apparently identical subunits (McParland *et al.*, 1976; Stewart *et al.*, 1980). It exhibits an absolute requirement for divalent cations, especially Mg^{2+} and to a lesser degree Mn^{2+} and Co^{2+} (O'Neal and Joy, 1974). Its reaction mechanism probably involves the binding of substrates in an ordered sequence (Stewart *et al.*, 1980).

Two forms of GOGAT are known to exist in plants. The NAD(P)H-dependent form is found in nonphotosynthetic leaf and root tissues (Fowler *et al.*, 1974; Suzuki *et al.*, 1982), while the Fd-dependent enzyme is found in photosynthetic tissues (Stewart and Rhodes, 1978; Suzuki and Gadal, 1982) and in roots (Miflin and Lea, 1975; Suzuki *et al.*, 1982). The enzyme from both roots and leaves is located in the plastids (Suzuki *et al.*, 1982; Suzuki and Gadal, 1982). The Fd-dependent GOGAT represents the major GOGAT isoform in light-grown leaves (Keys *et al.*, 1978; Lea and Miflin, 1974; Somerville and Ogren, 1980; Suzuki *et al.*, 1982; Suzuki and Gadal, 1982). It is active in the reassimilation of ammonium

in photorespiration (Keys *et al.*, 1978; Lea and Miflin, 1974; Ogren, 1984) and may also be involved in the incorporation of ammonium derived from the reduction of nitrate or the degradation of nitrogen-containing compounds (Cullimore and Sims, 1980; Platt and Anthon, 1981). It has also been demonstrated that the two GOGAT isoenzymes do not have the same function in higher plants. NAD(P)H-dependent GOGAT functions in nitrogen assimilation. However, Fd-dependent GOGAT may serve primarily in exporting reducing equivalents from chloroplasts (Loyola-Vargas and Sanches de Jimenez, 1986; Tanabe and Kawashima, 1982). However, Fd-dependent GOGAT may also play a role in leaf ammonium recycling, as has suggested by Platt and Rand (1982).

Most reports indicate the enzyme is a single polypeptide chain with a molecular weight in the range of 140 kD to 180 kD (Matoh *et al.*, 1980; Tamura *et al.*, 1980; Wallsgrove *et al.*, 1977). Very little is known of the reaction mechanism of the enzyme (Stewart *et al.*, 1980), although it may be sequential with respect to α-keto-glutarate and glutamine, the former binding first (Haynes, 1986).

B. Alternative Pathways

Ammonium accumulation in plant tissues has a particularly deleterious effects on plant growth and metabolism. In addition to the GS/GOGAT cycle there are other potential routes of ammonium assimilation (eq. 3-6, Table 2-1) that might operate at high levels of tissue ammonium and have detoxification roles (Givan, 1979). Reactions in this category involve enzymes with a relatively low affinity for ammonium.

1. Glutamate Dehydrogenase

The role and regulation of GDH in higher plants have been reviewed by Srivastava and Singh (1987). It catalyzes the combining of α-keto-glutarate with ammonium to yield glutamate, and the reverse reaction which deaminates glutamate (eq. 3, Table 2-1). Because of its high K_m for ammonium, GDH has been suggested as an alternative means of ammonium assimilation when the intercellular ammonium concentration is unusually high (Barash *et al.*, 1973, 1975; Given, 1979; Rhodes *et al.*, 1976; Shepard and Thurman, 1973; Taylor

and Havill, 1981). Recent data, however, suggest an assimilatory role for GDH in some plant tissues (Loyola-Vargas and Sanchez de Jimenez, 1984; Pahlich and Hoffman, 1975; Singh and Srivastava, 1982; Zeleneva and Khavkin, 1980). The magnitude of GDH contribution to ammonium assimilation in plants is controversial, as ammonium concentration in mitochondria ranges from 5 to 10 mM, which is close to the K_m values of GDH (Loulakakis and Roubelakis-Angelakis, 1990a; Yamada and Matsumoto, 1985). A relatively prominent role of GDH at high ammonium levels is further suggested by the considerable increase in GDH activity when plants are exposed to excessive concentrations of ammonium (Barash *et al.*, 1975; Shepard and Thurman, 1973). Since roots are the major site of assimilation of absorbed ammonium, there is a greater increase in GDH activity in roots than shoots when plants are supplied with high levels of ammonium (Taylor and Havill, 1981).

Isoenzymes of GDH are often present, seven being the most common number (Lee, 1973; Hartman and Ehmke, 1980; Scheid *et al.*, 1980). Their number can be increased by adding ammonium to the plants (Barash *et al.*, 1975; Nauen and Hartman, 1980). All the isoenzymes of GDH have the same molecular weight but different charge characteristics (Nagel and Hartman, 1980; Pahlich, 1972). Divalent cations are necessary for optimum activity: Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+} all activate GDH (King and Wu, 1971; Scheid *et al.*, 1980).

The apparently high K_m for ammonium is the evidence cited by Miflin and Lea (1976) to suggest that GDH is unlikely to have a role in ammonium assimilation *in vivo*. A table prepared by Stewart *et al.* (1980) indicates that K_m values in higher plants lie between 5.2 and 70 mM. However, care must be taken in quoting one value of K_m for ammonium as it may be influenced by pH and the concentration of the substrates (Miflin and Lea, 1982).

The molecular weight of NAD(P)H-dependent GDH varies from 208 kD (Pahlich and Joy, 1971) to 270 kD (Stone *et al.*, 1980) with subunits of 46 kD (Stewart *et al.*, 1980) to 58 kD (Scheid *et al.*, 1980). There is, therefore, some discrepancy in the literature as to whether the enzyme exists as a tetramer or hexamer (Miflin and Lea, 1982).

The reaction mechanism of plant GDH, which was initially proposed by King and Wu (1971) and confirmed by Stone *et al.* (1980) and Nagel and Hartman (1980), is similar to that of microbial enzymes, *i.e.* the compulsory ordered binding of NAD(P)H, α-keto-glutarate, and ammonium, followed by the ordered

release of glutamate and NAD(P). However, two reports published in 1977 suggested that the reaction may be fully (Groat and Soulen, 1977) or partially (Garland and Dennis, 1977) random.

2. Asparagine Synthetase

Givan (1979) has suggested that, at high levels of tissue ammonium, asparagine synthetase could also become an assimilating enzyme (eq. 5, Table 2-1), but there is no direct evidence (Givan, 1979). This enzyme normally transfers the amide nitrogen from glutamine to aspartate to form asparagine (Rognes, 1975). It can also react with ammonium directly although its K_m for ammonium is at least an order of magnitude higher than its K_m for glutamine (Givan, 1979). Hence, this pathway is likely to be a primary assimilation pathway at high ammonium levels only (Givan, 1979).

3. Other Enzymes

Although the other enzymes in Table 2-1 have been shown to be present in various plants there is little or no reason to believe that they play any significant role in ammonium assimilation (Miflin and Lea, 1980).

C. Ammonium Assimilation in Grapes

Little is known about ammonium assimilation in grapes. GDH and GS activities in *Vitis vinifera* L. *cv.* 'Chenin Blanc' leaf and root tissues were found to be associated with both particulate and soluble fractions (Roubelakis-Angelakis and Kliewer, 1983a, b, 1984). GDH from both leaves and roots was inhibited by EDTA and glutamate. Activation with Ca^{2+} was more pronounced in GDH from roots than leaves (Roubelakis-Angelakis and Kliewer, 1983a). Leaf GS activity was always greater than root activity (Roubelakis-Angelakis and Kliewer, 1983b). GS and GDH are also present in berries (Ghisi *et al.*, 1984). However, Roubelakis-Angelakis and Kliewer (1984) were unable to detect GOGAT activity in grape tissues.

Recently, detailed studies on GDH from grapevine tissues were reported by Loulakakis and Roubelakis-Angelakis (1990a, b, c, d). The majority of seven

NAD(H)-GDH isoenzymes were purified to homogeneity. The amination reaction was fully activated by the presence of Ca^{2+}. The molecular weight of the native enzyme was estimated to be 512 kD. At optimum pH, the apparent K_m values for ammonium, as ammonium chloride and ammonium sulfate, α-keto-glutarate, NADH, glutamate, and NAD^+ were 42.0, 13.0, 2.1, 0.069 and 0.195 mM, respectively (Loulakakis and Roubelakis-Angelakis, 1990a, b). Furthermore, study of the structure and function of NAD(P)H-dependent GDH using grapevine *cv.* 'Sultanina' callus grown under different nitrogen sources revealed that the enzyme consists of two subunits, α and β, which have similar antigenic properties but different molecular weight and charge (Loulakakis and Roubelakis-Angelakis, 1990c). The two subunits have molecular weights of 43.0 and 42.5 kD, respectively. The holoenzyme is hexametic; the isoenzymes 1 and 7 are homoexamers, and the isoenzymes 2 and 6 are hybrids. The α subunit is characterized as anabolic and the β subunit as catabolic (Loulakakis and Roubelakis-Angelakis, 1990c). From the profile of GDH isoenzymes it was evidenced that increases in ammonium concentration resulted in decreases in the protein of more cathodal isoenzymes and increases in the more anodal ones. ^{35}S-methionine labeling of GDH suggested that the increase was due to *de novo* protein synthesis rather than the activation of pre-existing protein, and/or decrease in the degradation rate of GDH (Roubelakis-Angelakis, 1991).

GDH from grapevine *cv.* 'Merlot' leaves showed maximum activity at anthesis and 5 weeks thereafter (Ghisi *et al.*, 1984). In berries, GDH activity showed a maximum approximately 4 weeks following anthesis, then decreased and peaked again 4 weeks following veraison. GDH activity of the berries was always higher than that of leaves (Ghisi *et al.*, 1984).

GS was also partially purified from leaf and root tissues of *cv.* 'Chenin blanc' (Roubelakis-Angelakis and Kliewer, 1983a). Enzyme activity was 50% higher in leaves than roots. K_m values were 3.2, 0.75, and 0.8 for glutamate, ATP and hydroxylamine, respectively. Enzyme activity in developing leaves and berries showed a parallel pattern of change. Maximum activity was found at veraison (Roubelakis-Angelakis and Kliewer, 1983b).

The addition of 10 mM MSX to grape tendrils did not prevent the reduction in ammonium concentration of those treated with α-keto-glutarate, compared to tendrils treated with ammonium alone (Jordan, 1989). The failure of MSX to prevent α-keto-glutarate from lowering the ammonium concentration in tendrils

supplied with ammonium suggests that the assimilation stimulated by α-keto-glutarate did not involve GS. Another assimilation pathway, most likely that catalyzed by GDH, must have involved.

D. Inhibitors Used in Research of Ammonium Assimilation

Inhibitors of GS/GOGAT pathway, such as L-methionine D,L-sulfoximine (MSX), tabtoxin and O-diazoacetyl-L-serine (azaserine, AS), have been widely used in studies of plant carbon-nitrogen metabolic pathways. No specific inhibitors for GDH or other ammonium assimilation enzymes have been found so far.

1. Inhibitors of Glutamine Synthetase

MSX is an irreversible inhibitor of GS (Flores *et al.*, 1980; Meister, 1980; Tate and Meister, 1973). In the presence of ATP and metal ions, MSX becomes tightly bound to the enzyme in the form of MSX-phosphate, and this is analogous to the formation of γ-glutamyl phosphate by the enzyme. However, MSX-phosphate bound to the enzyme can only be removed by heating to 100°C. MSX is therefore an extremely powerful tool in *in vivo* studies since upon binding the enzyme it is not easily released, but binding may be hindered by high concentrations of glutamate and prevented by combined presence of ammonium and glutamate (Miflin and Lea, 1980).

Treatment of photosynthesizing plants with MSX leads to large scale ammonium accumulation in various species (Anderson and Done, 1977; Arima and Kumazawa, 1977; Frantz *et al.*, 1982; Ikeda, 1985; Lara and Romero, 1986; Ohyama and Kumazawa, 1980; Platt and Anthon, 1981; Singh and Widholm, 1975) without affecting the CO_2 fixation process (Romero *et al.*, 1985), nitrate reductase (Frantz *et al.*, 1982), and GDH (Arima and Kumazawa, 1977; Brenchley, 1973; Probyn and Lewis, 1979).

Tabtoxin, produced by certain phytopathogenic pathovars of *Pseudomonas syringae* and readily taken up by the roots (Knight *et al.*, 1986), leads to the chlorotic halo that characteristically surrounds the infection site. In the infected plant, tabtoxin is hydrolyzed to yield its biologically active form, tabtoxinine-β-lactum [2-amino-4(3-hydroxy-2-oxo-azacyclobutan-3-yl)-butanoic acid] (Durbin *et al.*, 1978), which is an inhibitor of GS. The inhibition of GS by tabtoxinine-β-

lactum appears to be similar to that caused by MSX (Meister, 1980). Glutamate and tabtoxinine-β-lactum may compete for the same site on the enzyme and the enzyme is irreversibly inactivated. High tissue ammonium accumulation was obtained in several species after tabtoxin treatments (Frantz *et al.*, 1982; Knight and Langston-Unkefer, 1988; Langston-Unkefer *et al.*, 1984) without affecting nitrate reductase activities (Frantz *et al.*, 1982).

MSX reportedly has other effects on plant tissues, e.g. inhibition of methionine uptake (Meins and Abrams, 1972) and GOGAT at relatively high concentrations (Brenchley, 1973; Miflin and Lea, 1975). Durbin (1971) reported that the activities of other major enzymes were unaffected by tabtoxin *in vitro*. These data support the suggestion that tabtoxin specifically affects GS.

Both tabtoxin and MSX inhibit photosynthesis. Platt and Anthon (1981) reported a significant decrease in CO_2 uptake by spinach leaves within 1 hour of incubation in 5 to 10 mM MSX. However, at 1 mM, MSX had only a slight inhibitory effect on photosynthesis while still inhibiting GS activity after 3 hours of treatment (Frantz *et al.*, 1982). Tabtoxin was shown to inhibit RuBPCase *in vitro* and the effect was inversely related to tabtoxin concentration (Crosthwaite and Sheen, 1975).

In view of the present knowledge of photosynthesis and photorespiration in C_3 plants, tabtoxin and MSX inhibit GS with only a slight disturbance of RuBPCase and photorespiration. Significant amounts of ammonium accumulated after treatment with MSX and tabtoxin because further assimilation by GS/GOGAT cycle was blocked (Frantz *et al.*, 1982).

2. Inhibitors of Glutamate Synthase

Analogs of glutamine, such as AS, 6-diazo-5-oxo-L-norleucine (DON), and L-2-amino-3-ureidoproprionic acid (albizziine), are inhibitors of a wide range of glutamine using enzymes in which the amide-amino group is transferred (Dunn and Klucas, 1973; Ohyama and Kumazawa, 1980; Weger *et al.*, 1988). Both AS and DON show a mixed type of inhibition with glutamine enzymes. Because of the structural similarity between them and glutamine, they compete for binding to the active site. However, once bound, the diazo-actyl group of the analogs reacts with the enzyme and becomes irreversibly attached and can not be competed away. Although there have been relatively few studies done with GOGAT, it is

known that all of these analogs inhibit the enzyme. For example, half of the pea root enzyme is inhibited by 0.2 mM AS and 2 mM albizziine (Miflin and Lea, 1975) and similar values were also obtained with the leaf enzyme (Wallsgrove *et al.*, 1977). Neither AS nor DON have any known effect on GDH (Dunn and Klucas, 1973; Miflin and Lea, 1975).

Because of their reasonably specific nature, these inhibitors are useful in determining the path of ammonium assimilation and detoxification. If it occurs *via* GS then MSX or tabtoxin should block the process, causing ammonium to accumulate. Similarly, if the route from ammonium to amino nitrogen is *via* the amide of glutamine, then addition of AS or DON should block the production of glutamate and amino nitrogen and lead to the accumulation of glutamine and ammonium. In such an experiment it is necessary that inhibitors be shown to enter the cells and to have inactivated GS or GOGAT. It is also advisable to limit the use of these inhibitors to short-term experiments because of the possibility of secondary, indirect effects, such as the inhibition of photorespiration and protein synthesis (Miflin and Lea, 1980).

II. Ammonium Toxicity in Higher Plants

Theoretically, ammonium should be the preferred form of nitrogen for plants (Reisenauer, 1978). It should be used more efficiently than nitrate, for it need not be reduced before incorporation into organic matter. In the soil, ammonium is less subject to leaching and to denitrification losses than nitrate (Barker and Mills, 1980). However, ammonium is probably the most toxic to plants of all the forms of inorganic nitrogen that roots might encounter in a cropping system. It seems that most plants have evolved under conditions of a very low ammonium supply and where nitrate is the predominant form of available nitrogen. Thus, these plants are not adapted for the use of ammonium as their major or sole source of nitrogen. They have little tolerance to high levels of ammonium nitrogen in their tissues and root environment (Barker and Mills, 1980; Haynes, 1986).

Ammonium toxicity to higher plants has been shown repeatedly. However, the exact biochemical cause and toxic concentration are not well defined (Givan, 1979). Reduced growth, chlorosis, and/or necrosis are common ammonium toxicity symptoms. Many physiological and biochemical effects of ammonium toxicity have been reported.

A. Symptoms of Ammonium Toxicity

In general, ammonium toxicity is characterized by an immediate restriction in plant growth and morphological disorders (Barker *et al.*, 1966a, b; Maynard *et al.*, 1966, 1968; Maynard and Barker, 1969).

1. Reduced Growth

Plant species differ greatly in growth in response to ammonium nutrition. Most crop species react adversely by exhibiting reduced growth of almost all types of tissues.

Growth of tomato and maize is reduced when ammonium is the sole form of nitrogen in a solution culture (Magalhaes and Huber, 1989). Ammonium decreased growth of tomato shoots and roots, compared with nitrate (Torres de Classen and Wilcox, 1974). High ammonium reduces leaf and root growth and produces symptoms of ammonium toxicity in tomato (Carpena-Artes *et al.*, 1983; English

and Barker, 1983; Ikeda and Yamada, 1984). An inhibitory effect of ammonium was found on growth of radish plants (Goyal, 1974; Goyal *et al.*, 1982a, b). Ammonium was injurious to the growth of bean, sweet corn, cucumber, and pea (Maynard and Barker, 1969). With ammonium alone in the solution, plant growth of several vegetable crops was poorer than with nitrate or nitrate/ammonium (Ikeda and Osawa, 1982). A reduction in growth rate, root growth, and eventual death was reported as a result of ammonium toxicity in chrysanthemum plants (Nelson and Kuo Hsien Hsieh, 1971). Toxicity of ammonium is also characterized by greatly restricted root growth with the production of short, thick, less branched, and darkly colored roots (Maynard and Barker, 1969; Warncke and Barker, 1973). Warncke and Barker (1973) observed that root growth was restricted more than top growth so that the shoot/root ratio increases.

Ammonium nutrition significantly decreased dry weight of fruits, leaves and roots in cucumber (Alan, 1989). Dry weight of lima beans grown in solution culture was consistently lower when >25% of nitrogen was supplied as ammonium (McElthannon and Mills, 1978). The dry weights of southern pea roots, stems, leaves, pods, seeds, and total plants were significantly reduced at three different stages of development when grown in solution culture in which ammonium was the sole source of nitrogen (Sasseville and Mills, 1979a, b). Fresh and dry shoot weights of pea and cucumber plants grown in ammonium were significantly lower than those grown in equivalent concentrations of nitrate (Barker and Maynard, 1972). Ammonium toxicity caused reduction in plant fresh weight of maize (Soyer *et al.*, 1974), *Impatiens platypetala* (Weigle *et al.*, 1982), and excised cotyledons of cucumber (Patnaik *et al.*, 1972). Lowest shoot and root dry weight, accompanied by the highest solution acidity and initial ammonium absorption, occurred with 75 and 100% ammonium of nitrogen supplied in *poinsettia* (Cox and Seeley, 1984).

Dirr *et al.* (1973) reported that the number and length of *Leucotheo catesbaei* shoots were, respectively, lower and shorter when cultured on ammonium, compared to nitrate. Cell division is reportedly inhibited at toxic ammonium levels (Okamura *et al.*, 1984; Sauer *et al.*, 1987).

2. Morphological Changes

Besides reduced plant growth, other symptoms of ammonium toxicity have been reported repeatedly, including leaf chlorosis, leaf epinasty, marginal necrosis, necrotic spots, stem lesions, and eventual death of the plants.

Chloroplast structure deteriorates during ammonium toxicity, resulting in chlorosis of the leaves (Puritch and Barker, 1967). Tomato plants grown in soil with nitrogen supplied from $(NH)_2SO_4$ showed a morphological disorder characterized by leaf epinasty (Corey *et al.*, 1987). Tomato plants supplied with ammonium nutrition under high light intensity showed symptoms of stunting, leaf-roll, wilting, and interveinal chlorosis of the older leaves (Magalhaes and Wilcox, 1984). Toxic ammonium levels induced by water stress caused leaf necrosis in avocados (Nevin and Lovatt, 1987). Incubation of excised cotyledons of cucumber in NH_4Cl solutions exceeding 1 mM inhibited greening (Patnaik *et al.*, 1972). Symptoms of ammonium toxicity in chrysanthemum include necrotic spots, thickened leathery leaves, and eventual death of the plants (Nelson and Kuo Hsien Hsieh, 1971). Apple trees showed a higher susceptibility to bitter pit when supplied with ammonium than nitrate (Fukumoto and Nagai, 1983). Inflorescence necrosis and 'waterberry' in grapes are associated with ammonium toxicity (Christensen and Boggero, 1985; Jordan, 1989).

B. Physiological and Biochemical Changes

Numerous reports have contrasted the effects of added ammonium rather than nitrate on the physiology and biochemistry of plants. Increased concentration of ammonium in plant tissue is one of the first and basic elements of ammonium toxicity (Barker *et al.*, 1966a; Goyal, 1974; Maynard and Barker, 1969). The consequences of this can be manifold.

1. Photosynthesis and Carbohydrates

In general, photosynthesis is reduced by ammonium toxicity (Hageman, 1984; Magalhaes and Wilcox, 1984). Gibbs and Calo (1959) reported that ammonium inhibited CO_2 fixation in isolated spinach chloroplasts by 81% at a concentration of 1 mM and by 99% at 10 mM. Photosynthesis of intact radish leaves of NH_4^+-

fed plants was only 50% of leaves on NO_3^--fed plants (Goyal, 1974). Plants grown with nitrate fixed at least twice as much $^{14}CO_2$ *in vitro* into acid stable products using ribulose-1,5-biphosphate, ribose-5-phosphate, 3-phosphoglyceric, and phosphoenlpyruvic acids as substrates. CO_2 fixation both in light and darkness was decreased by ammonium nutrition, indicating that ammonium toxicity in radish plants is closely related to inhibition of photosynthesis (Goyal *et al.*, 1982a, b).

Other authors, however, showed that ammonium increases photosynthetic CO_2 fixation in isolated cells of spinach (Woo and Canvin, 1980a b) and in intact spinach chloroplasts (Bennedetti *et al.*, 1976; Heath and Leech, 1978;). The stimulation of photosynthesis by ammonium was described as an activation of RuBPCase (Bennedetti *et al.*, 1976). Heath and Leech (1978), however, concluded that this was caused by altered stromal pH, since raising the medium pH from 7.6 to 8.2 lowered the ammonium-induced stimulation of photosynthesis. As external pH was increased further, ammonium inhibited CO_2 fixation.

Uncoupled photophosphorylation is cited as a main toxic effect of ammonium on photosynthesis (Avron, 1960; Hageman, 1984; Magalhaes and Wilcox, 1984; Sauer *et al.*, 1987). Ammonium ions act as an uncoupler of photophosphorylation in isolated chloroplasts (Avron, 1960; Gibbs and Calo, 1959; Krogmann *et al.*, 1959), resulting in concentration increases of AMP and ADP, and decreases in ATP (Krogmann *et al.*, 1959; Losada *et al.*, 1973; Trebst *et al.*, 1960). $NADP^+$ reduction is also inhibited by ammonium toxicity (Magalhaes and Wilcox, 1984; Sauer *et al.*, 1987).

Ammonium may also affect carbohydrate synthesis, although reports are inconsistent. Platt *et al.* (1977) showed that ammonium increased transfer of photosynthetically incorporated carbon into synthesis of amino acid skeletons at the expense of sucrose synthesis by activating pyruvate kinase. Kanazawa *et al.* (1972) reported similar findings. Cucumber leaves have higher levels of glucose, fructose, uridine diphosphoglucose (UDPG), G-1-P, and F-6-P and lower levels of starch, an uridine compound containing sugar or sugar derivative (UDPX), and G-6-P during ammonium toxicity (Matsumoto *et al.*, 1968, 1969). The accumulated glucose and fructose were the products of newly fixed carbon and not degradative products of a glucose polymer. Glucose transformation after its synthesis, at least up to starch synthesis, may have been inhibited, resulting in a

disorder of carbohydrate metabolism during ammonium toxicity (Goyal and Huffaker, 1984; Matsumoto *et al.*, 1968).

Carbohydrates, including reducing sugars, non-reducing sugars and crude starch, accumulated to higher levels in leaves and roots of NH_4^+-fed tomato plants than in those fed nitrate. Invertase activity was higher in leaves of NH_4^+-fed plants, probably due to the accumulation of reducing sugar. Results of experiments involving feeding with glucose-1-^{14}C and glucose-6-^{14}C suggest that the hexose monophosphate shunt plays a greater part in glucose metabolism than the EMP pathway in NH_4^+-fed plants (Wakimoto and Yamada, 1985c).

The incorporation of photosynthesized ^{14}C into starch and other higher polymers is suppressed by ammonium toxicity in cucumber leaves. The quantity of starch granules of injured cucumber plants was less than that of normal plants (Matsumoto *et al.*, 1971b). Blocked starch synthesis was also reported in tomato plants (Magalhaes and Wilcox, 1984).

2. Respiration

There are indications that ammonium inhibits respiration. Burkhart (1938) concluded that etiolated seedlings of several species absorb and utilize ammonium until available carbohydrates are exhausted. Breakdown of carbohydrates was accelerated due to enhanced respiration during rapid ammonium assimilation (Willis and Yemm, 1955; Syrett, 1956). Wakiuchi *et al.* (1971) reported respiration of cucumber leaves was higher in plants grown in higher NH_4Cl solution, which was not due to uncoupling in mitochondria (Matsumoto *et al.*, 1971b). Ammonium also slightly increased respiration of detached barley leaves (Berner, 1971). Detached roots treated with a high concentration ammonium solution for several hours had a higher subsequent O_2 uptake (Wakimoto and Yamada, 1985b). Ammonium accelerated respiration in both leaves and roots. Respiratory quotient (RQ) values were lower in leaves of NH_4^+-fed tomato plants than in leaves of those fed NO_3^- (Wakimoto and Yamada, 1985b).

By contrast, the dark respiration rates of bean (Barker *et al.*, 1965) and radish leaves (Goyal, 1974; Goyal *et al.*, 1982a, b) from plants grown in nitrate or ammonium were the same. There was no difference in the RQ values for roots of plants fed with different nitrogen sources (Wakimoto and Yamada, 1985b).

3. Enzyme Activities

a. Ammonium Assimilation Enzymes: GDH activity in roots of tomato, maize, and rice plants is higher in the presence of ammonium than nitrate nitrogen (Magalhaes and Huber, 1989). *In vitro* activities of GDH were higher in radish plants cultured with ammonium than those with nitrate (Goyal *et al.*, 1982a, b). GDH activity was increased by ammonium toxicity in the mitochondria of cucumber leaves (Matsumoto *et al.*, 1971a). Roots and shoots of rice had much higher GS activity than tomato and maize. GS activity in rice increased sharply in the presence of ammonium, and much less free ammonium was detected in green tissues, compared to tomato plants. Therefore, GS appears to be a key factor to detoxify ammonium in the leaves of rice (Magalhaes and Huber, 1989).

b. Other Enzymes: Starch synthetase activity of ammonium injured plants was shown to be less than that of normal plants (Matsumoto *et al.*, 1969). Carboxylase activity was reduced by ammonium toxicity (Magalhaes and Wilcox, 1984). Photodependent activation of ATP synthetase was inhibited by ammonium toxicity (Sauer *et al.*, 1987). The adenosine triphosphatase activity of injured plants was lower than that of normal plants (Matsumoto *et al.*, 1971a).

It was reported that the activities of malate dehydrogenase, succinate dehydrogenase, cytochrome c oxidase, NADH diaphorase, NADH oxidase, succinate:cytochrome c oxidoreductase, and NADH:cytochrome c oxidoreductase were higher in mitochondria of cucumber leaves from NH_4^+-fed plants (Matsumoto *et al.*, 1971a). Catalase activities were higher in leaves and roots of NH_4^+-fed plants, compared to those fed NO_3^- (Wakimoto and Yamada, 1985a).

Triphenyl tetrazolium chloride (TTC) reducing activity of roots was lower in NH_4^+-fed than NO_3^--fed plants, suggesting a decrease in dehydrogenase activity (Wakimoto and Yamada, 1985a).

Polyphenol oxidase activities were higher in leaves and roots of NH_4^+-fed plants, suggesting that the root browning due to lignification observed under ammonium excess is related to enhanced polyphenol oxidase activity (Wakimoto and Yamada, 1985a).

4. Organic Acids

Ammonium nutrition lowered levels of organic acid anions (fumaric, succinic, malonic. malic, citric, oxalate, and total) in leaves, petioles, stems, and roots of tomato (Kirkby and Mengel, 1967) and sugar beet plants (Bretler, 1973). The effect was more pronounced in tops than in roots. Depleted organic acid levels were also found by Hageman (1984). Accumulation of organic acids with nitrate nutrition has been explained as a cellular response to maintain intercellular pH and counteract OH^- produced during nitrate reduction (Raven and Smith, 1976).

The addition of organic acids to the culture solution suppressed ammonium toxicity in mitochondria of cucumber leaves (Matsumoto *et al.*, 1971a). The accumulation of free ammonium in leaves was also repressed by addition of organic acids. The results suggest that increased respiration due to ammonium toxicity is required for enhanced supply of organic acids, especially α-keto-glutaric acid, for ammonium assimilation. Uncoupling in mitochondria resulting in the increase of respiration does not seem to occur during ammonium toxicity (Matsumoto *et al.*, 1971a).

5. Mineral Compositions

a. **Ammonium and Total Nitrogen**: Higher levels of free ammonium accumulated as a result of ammonium nutrition in radish plants (Goyal *et al.*, 1982a). Ammonium nutrition increased the ammonium content of the tissue in tomato plants as compared to nitrate nutrition (Magalhaes and Wilcox, 1984). Accumulation of ammonium was accompanied by an increase in the content of amino acids (Kondrat'ev, 1977). In plants showing toxicity, ammonium content was increased in expressed cell sap and in leaf tissue (Soyer *et al.*, 1974). In tomato, the nitrogen content of the fruits, leaves, stems, and roots was significantly increased when 50% or more of the nitrogen source was ammonium (Alan, 1989).

b. Phosphorus: The P content of fruits, leaves, stems and roots was significantly increased in tomato when 50% or more of the nitrogen source was ammonium (Alan, 1989). Phosphorus uptake increased when ammonium was supplied as the nitrogen source (Mattson, 1966). In plants showing toxicity, the contents of mineral P in expressed cell sap and in leaf tissue were increased

(Soyer *et al.*, 1974). The effect of nitrogen source on P uptake has been attributed to changes in pH of the rhizosphere, which was lowered during ammonium absorption (Riley and Barker, 1971; Soon and Miller, 1977). Lowering of growth medium pH, resulting from ammonium absorption, may increase the concentration of P in the $H_2PO_4^-$ form, which is absorbed several times faster than HPO_4^{2-} (Miller *et al.*, 1970; Soon and Miller, 1977). Moreover, HPO_4^{2-} can precipitate as $CaHPO_4.2H_2O$ at the root surface in the absence of ammonium (Miller *et al.*, 1970).

c. Cations: Numerous reports have shown that ammonium reduces the uptake of other cations; Ca^{2+}, Mg^{2+}, and K^+ are affected most (Arnon, 1939; Barker *et al.*, 1967; Holley *et al.*, 1931; Kirkby, 1968; Magalhaes and Wilcox, 1984; Wander and Sites, 1956). However, there is insufficient evidence for concluding that reduced uptake of cations is the sole cause of ammonium toxicity to plants. Cox and Reisenauer (1973) reported that cation uptake decreased as ammonium levels increased in the root medium, and explained it as ionic competition at the site of intake, either with ammonium *per se* or with H^+ produced during ammonium uptake.

Potassium: Reports suggest that ammonium toxicity in plants may result from reduced uptake of K^+. Arnon (1939) postulated that K^+ and ammonium competed for uptake since ammonium solutions contain two cations (NH_4^+ and K^+), whereas nitrate solutions contain K^+ as the only rapidly absorbed cation. Tromp (1962) reported that K^+ uptake by wheat roots was reduced by half or more by the concurrent uptake of ammonium. Plants grown in both soil and solution culture with ammonium contained lower levels of K^+ than those grown in nitrate, even though the amount of K^+ supplied was the same in both cases (Ajayi *et al.*, 1970; Maynard *et al.*, 1968). Ammonium as a nitrogen form decreased the K^+ content of all tissues of tomato plants (Alan, 1989). Smith and Epstein (1964) reported that ammonium uptake was strictly competitive with K^+ and Rb^+. Epstein (1972) found that the uptake of ammonium resembles that of K^+, indicating that the two ions are transported by the same carrier. Deficient K^+ was also reported by others (Magalhaes and Wilcox, 1984; Goyal *et al.*, 1982a, b).

Ajayi *et al.* (1970) and Maynard *et al.* (1968) were able to correct ammonium toxicity symptoms by using excessive amounts of K^+. However, a greatly increased K^+/NH_4^+ and Ca^{2+}/NH_4^+ ratio in solution culture did not help alleviate ammonium toxicity in radish plants (Goyal, 1974).

Treatment with KCl solutions of the same concentrations as NH_4Cl stimulated greening, fresh weight increase, and the absorption and incorporation of ^{14}C-leucine. The toxicity of NH_4Cl was alleviated by KCl solutions at low concentrations (Patnaik *et al.*, 1972).

Calcium: The reduction of Ca^{2+} due to ammonium has also been studied (Alan, 1989, Carpena-Artes *et al.*, 1983; Goyal *et al.*, 1982a, b; Magalhaes and Wilcox, 1984; Soyer *et al.*, 1974). Lower Ca^{2+}, as well as higher nitrate and ammonium contents, was observed in the fruit flesh near the calyx end in bitter pit-affected fruit of apples (Fukumoto and Nagai, 1983). Adams (1966) suggested, based on root growth experiments in soil, that Ca^{2+} deficiency was the mechanism of $(NH_4)_2HPO_4$ injury to cotton seedlings. Hoff *et al.* (1974) also suggested that ammonium toxicity was a manifestation of intercellular Ca^{2+} deficiency. However, Bennett and Adams (1970a), using sterilized soil culture, demonstrated that Ca^{2+} deficiency and ammonium toxicity were separate causal factors of $(NH_4)_2HPO_4$ injury to seedlings.

Manganese: Reduced Mg^{2+} is also found in tissues during ammonium toxicity. Mg^{2+} was decreased by ammonium nutrition in tomato plants (Carpena-Artes *et al.*, 1983; Magalhaes and Wilcox, 1984). The Mg^{2+} content was lower in corn plants showing ammonium toxicity symptoms (Soyer *et al.*, 1974).

6. Amino Acids and Other N-Compounds

In tomato, the addition of ammonium brings about a several-fold increase in free amino acids in roots, while smaller increases are found in mature, fully expanded leaves, in young growing leaves, and in fruit (Patnaik *et al.*, 1972). The aspartic and glutamic acids, as well as their amides, were predominantly synthesized in the roots when the nitrogen source was ammonium. Ammonium increased the free amino acid content of the tissue of tomato plants as compared to nitrate nutrition (Magalhaes and Wilcox, 1984). Ammonium toxicity was accompanied by a large increase in the levels of gamma-aminobutyric acid (GABA) and serine in radish leaves (Goyal *et al.*, 1982a, b).

Chlorophyll and total soluble protein concentration of radish plants cultured with ammonium were about 33% and 70%, respectively, of those cultured with nitrate (Goyal *et al.*, 1982a, b). Incubation of excised cotyledons of cucumber

with NH_4Cl solutions exceeding 1 mM inhibited incorporation of ^{14}C-leucine into insoluble nitrogen compounds (Patnaik *et al.*, 1972).

7. Water Relations

Ammonium reduced water permeability of potato tuber slices (Stuart, 1973). Tomato (Quebedaux and Ozbun, 1973) and sugar beet (Stuart and Haddock, 1968) plants grown in ammonium had a lower water uptake than those in nitrate. Radish plants absorbed similar amounts of water whether grown in ammonium or nitrate (Goyal, 1974).

8. Ethylene Evolution

Ammonium nutrition of tomato, compared to nitrate, elevates rates of ethylene evolution and induces toxicity symptoms, characterized by the morphological disorder, leaf epinasty (Corey *et al.*, 1987; Corey and Barker, 1989). An inhibitor of ethylene synthesis, amino-oxyacetic acid (AOA), and an inhibitor of ethylene action, Ag^+, were effective in reducing ammonium toxicity symptoms (Barker and Corey, 1991). Barker and Corey (1991) proposed that ammonium accumulation, ethylene evolution, and appearance of the symptoms of environmental stresses are interconnected events. Ammonium toxicity stimulates ethylene biosynthesis or, conversely, ethylene biosynthesis may stimulate ammonium accumulation, and these events then initiate symptom development.

C. pH and Ammonium Toxicity

In *poinsettia*, the lowest subsequent ammonium absorption, accompanied by the highest solution acidity and initial ammonium absorption, occurred when 75 and 100% of the nitrogen was supplied as ammonium in *poinsettia* (Cox and Seeley, 1984). The detrimental effects of ammonium on *poinsettia* appear to be dependent on the degree of pH control in the root medium and its effects on root growth and ammonium absorption and utilization (Cox and Seeley, 1984).

Of 14 species, including barley, maize, oats, sorghum, yellow and white lupin, pea, soybean, carrot, flax, castor-oil, spinach, sugar beet, and sunflower, most seedlings grew optimally at pH 6.0 with slight reductions at pH 5.0 (Magalhaes

and Huber, 1989). Growth of many species was severely inhibited at pH 4.0, but this inhibition was not observed with legume and cereal species. Yield depressions at pH 4.0 relative to pH 6.0 were well correlated with the relative decreases of the K^+ concentration in roots. In the roots of sunflower and flax, total nitrogen concentrations were also strongly reduced at pH 4.0. Apparently, the interactions between uptake of K^+, NH_4^+, and H^+ ions become the prevalent problem at suboptimal pH. Yields were also considerably decreased at pH 7.0, with the exception of the lupins. At this pH, the roots of the growth-inhibited plants were characterized by increased levels of total nitrogen and free ammonium. It is thought that the binding capacity of the roots for ammonium is an important factor in preventing ammonium toxicity at supraoptimal pH (Magalhaes and Huber, 1989). So, ammonium can inhibit plant growth at all pH's except at or near neutrality, but the reasons are unclear.

According to Bennett and Adams (1970b), the toxicity of ammonium to plants depends on the substrate-solution concentration of NH_3. Since the NH_3 concentration increases as pH increases (Weir *et al.*, 1972), ammonium should be more toxic when pH is higher. But numerous reports show that a decrease in solution pH, resulting from ammonium absorption, limits ammonium utilization.

Calcium carbonate: Inhibition of plant growth by ammonium can be overcome by using $CaCO_3$ in the root medium as a buffer (Barker, 1967a; Barker *et al.*, 1966a, b). Maynard and Barker (1969) showed that bean, sweet corn, cucumber, and pea plants, which are inhibited by ammonium, grew normally when the pH of the sand culture was maintained near neutrality by adding 1% $CaCO_3$. The inability of ammonium to serve as a source of nitrogen for the growth of excised roots was altered when the medium was buffered at pH 7.0 using $CaCO_3$ (Hanny *et al.*, 1959; Sheat *et al.*, 1959). The addition of $CaCO_3$ as a buffer permitted plants otherwise highly susceptible to injury to have near-normal growth in ammonium (Barker, 1967a, b; Barker *et al.*, 1966a, b; Prianishnikov, 1951). The reduction in plant fresh weight of maize due to ammonium toxicity was decreased by application of $CaCO_3$ (Soyer *et al.*, 1974).

$CaCO_3$ at all rates of NO_3^-/NH_4^+ greatly increased ammonium absorption and increased total reduced nitrogen in the leaves and roots of *poinsettia* (Cox and Seeley, 1984). Even with $CaCO_3$, which increased the solution pH, the ammonium toxicity was not reduced and none of several vegetable crops grew as well as with nitrate nitrogen (Ikeda and Osawa, 1982).

D. Nitrate and Ammonium Toxicity

Some evidence suggests that nitrate facilitates ammonium assimilation in radish plants (Goyal *et al.*, 1982a, b). In isolated cells of cotton leaves, ammonium in the presence of 5 mM $Ca(NO_3)_2$ for up to 7.5 hours did not affect total photosynthetic CO_2 fixation (Rehfeld and Jensen, 1973). Platt *et al.* (1977) found that ammonium did not affect total CO_2 fixation in leaf disc from alfalfa plants grown on Hoagland's solution, a source of nitrate. At equimolar concentrations, added $(NH_4)SO_2$ was generally more injurious than added NH_4NO_3 (Ormrod, 1979). Nitrate equivalent to 10% or more of ammonium concentration alleviated the inhibitory effect of ammonium on growth of radish plants but did not reverse the inhibitory growth effects on etiolated seedlings (Goyal *et al.*, 1982a, b).

E. Stresses and Ammonium Toxicity

Stresses such as temperature, water, light, salt, and herbicide application can increase free ammonium levels and cause ammonium toxicity in plant tissues (Navin and Lovatt, 1988; Puritch and Barker, 1967).

1. Water Stress

Preliminary experiments with 'Hass' avocado on clonal 'Duke 7' rootstock provided evidence that NH_3-NH_4^+ accumulated in leaves in response to water deficit and low temperature stress (Nevin and Lovatt, 1987). Apparent ammonium toxicity in beans, in terms of visible injury and reduced plant height and weight, was generally accentuated by high water stress prior to fertilizer application, and to a lesser extent, by lower night temperature (Ormrod, 1979).

Ammonium increases in leaves of various woody perennials and herbaceous annuals in response to water stress. In addition, Nevin and Lovatt (1987) found that ammonium accumulates to toxic levels, resulting in leaf necrosis in stress-sensitive plants, while stress-tolerant plants detoxify their cells of ammonia through the *de novo* synthesis of arginine. It is suggested that the photorespiratory nitrogen cycle is the major source of ammonia that is produced

during stress, and that carbohydrate depletion is not a prerequisite for, but a consequence of, ammonia accumulation (Nevin and Lovatt, 1987).

2. Light

Tomato plants supplied with ammonium under high light intensity showed symptoms of stunting, leaf-roll, wilting, interveinal chlorosis of the older leaves, and produced one-third the dry weight of NO_3^--fed tomato plants (Magalhaes and Wilcox, 1984). In contrast, plants receiving ammonium with light reduced over 50% appeared normal. Shading greatly decreased ammonium levels and increased the level of free amino acids, mainly asparagine and glutamine, in shoots of ammonium-treated plants. The nitrate content increased sharply with shade. The result suggested that the increased ammonium concentration in leaves from photorespiration can be a major factor in the ammonium toxicity expression under high light intensity (Magalhaes and Wilcox, 1984).

3. Herbicide

Free ammonia content in *Echinochloa utilis*, *Digitaria adscendens* (*D. ciliaris*), *Amaranthus lividus* and *Cyperus rotundus* increased 30- to 100-fold 24-48 h after treatment with bialaphos (L-2-amino-4-((hydroxy)(methyl) phosphinoyl)butyryl-L-alanyl-L-alanine). The increase was more marked in shoots than in roots of *E. utilis* and in leaves than in stems of *A. lividus*. The accumulation persisted until death of the plant. Accumulation of ammonia was not observed in plants treated with 2,4-D, MCPA, simazine, DCMU (diuron), propanil, or amitrole. The close correlation between free ammonia content and herbicidal activity indicated that the toxicity of accumulated ammonia is a primary factor in the herbicidal activity of bialaphos (Tachibaba *et al.*, 1986).

III. Inflorescence Necrosis in Grapevines

Inflorescence necrosis (IN) is a recently described physiological disorder that severely reduces fruit set in grapes (Ibacache, 1990; Ibacache *et al.*, 1991; Jackson and Coombe, 1988a, b; Jackson, 1991; Jordan, 1989; Jordan *et al.*, 1991). Pedicels and/or rachis become necrotic and prevent fruit set. IN is not simply due to poor pollination or fertilization. Apparently, IN has occurred for many years but was observed as 'poor set' or attributed to *Botrytis* infection.

Jackson and Coombe (1988a, b) used the term 'early bunchstem necrosis' (EBSN) for this disorder since its appearance and development is similar to bunchstem necrosis (waterberry) which occurs in late season. Field observations showed pedicels to be the most affected tissue, as well as flowers, berries, and rachis near bloom (Ibacache, 1990; Ibacache *et al.*, 1991; Jordan, 1989; Jordan *et al.*, 1991). Consequently, the term inflorescence is preferred to bunchstem because it botanically describes the affected tissues and indicates the phenological stage of the disorder. Inflorescence necrosis was abbreviated as INec by Jordan (1989) and used by Ibacache (1990) and Ibacache *et al.* (1991). We prefer IN.

Little is known about IN, but researchers have clearly shown it has a physiological rather than a pathological cause (Jackson and Coombe, 1988a, b). The exact cause of IN and its physiology are not well understood.

A. Symptoms

As first described by Jackson and Coombe (1988a, b), IN is a disorder characterized by death of portions of the cluster (bunch) which finally becomes shriveled in appearance. In the most serious cases, all lateral branches of the rachis die and abscise, while in minor situations only a few small branches are affected. The first indicator of IN is shriveled and browning pedicels prior to flowering; and later, portions of the cluster drop, which does not seem to be a consequence of abscission layer formation. The necrosis develops principally between the time the cluster (bunch) is 2 cm long prior to flowering, and continues up to a week or two before bloom (Jackson and Coombe, 1988a, b).

In Oregon, observations provided broader symptoms than those published by Jackson and Coombe. Jordan (1989) noted that IN affects only clusters. Slightly

affected clusters have a few necrotic flowers and pedicels. With increased severity, more pedicels are affected and the necrosis extends into the rachis. In extreme situations, clusters were completely necrotic from extensive peduncle damage. IN can appear before bloom, at bloom, and/or as late as early fruit set. Affected flowers or berries either fall from or remain on the clusters. Necrotic tissues and barren sections of clusters were often still visible at harvest.

Ibacache (1990) confirmed the observations of Jordan and provided more information, emphasizing that flowers and pedicels are the principal tissues affected and that only in severe cases is the rachis also damaged. When the rachis was affected, necrosis began at the apical section and continued toward the peduncle. Affected tissues were characterized by brown or black coloration. Necrosis occurred from the beginning of bloom until full bloom and continued until the end of IN occurrence. When the whole cluster was affected, necrosis was complete by full bloom. No IN occurred after fruit set.

Another typical IN symptom described by Ibacache (1990) is the presence of numerous small undeveloped berries that remain strongly attached to the cluster. These berries were apparent at fruit set, or soon after, and they were smaller, more elongated, and had lighter green color than normal shot berries. In severely damaged clusters, only the small berries remain attached.

B. Geological Distribution

IN was first described and recorded in New Zealand and Australia (Jackson and Coombe, 1988a, b). It was later reported in the Pacific Northwest of the USA (Jordan, 1988; Ibacache, 1990). A crop reduction of 90% in muscat varieties in New Zealand (Jackson and Coombe, 1988a, b) and a crop loss greater than 50% in *cv.* 'Pinot noir' in Oregon Willamette Valley were attributed to IN (Jordan, 1989). IN may be causing fruit set reduction in other grape production regions of the world since it has often gone unnoticed and been confused with 'normal' fruit set problems. However, observation at various locations suggested that IN may be worse in cooler regions (Jackson and Coombe, 1988b).

C. Cultivar and Clone Susceptibility

Differences in susceptibility to IN were observed among cultivars (Jackson and Coombe, 1988a). With test plants grown by Mullins' techniques (Mullins and Rajasekaran, 1981), high IN incidence was found in *cvs.* 'Alicante', 'Brown Muscat', 'Muscat Hamburg', 'Muscat Ottonel', 'Queen of the Vineyard', 'Frankenthalle', and 'Italia', in decreasing order of susceptibility. Low IN incidence was found in 'Schuryler', 'Carbernet Sauvignon', 'Pearl of Csaba', 'Fiesta', 'Chasselas', 'Flora', and 'Cardinal' (Jackson and Coombe, 1988a). In field conditions, the following were found to have moderate levels of IN: 'Malbec', 'Pinot noir', 'Merlot', and 'Meunier'. They observed very little IN on 'Reisling', 'Chardonnay', 'Cabernet Sauvignon', 'Pinotage', and 'Chasselas'.

'Pinot noir' was the most affected cultivar in the Willamette Valley of Oregon in 1988, but 'Gewurztraminer', 'Cabernet Sauvignon', and early muscats were also susceptible (Jordan *et al.*, 1991). 'Riesling' was partially affected in 1988 and 1990 even though this cultivar sets very well.

Clones of some cultivars appeared to vary in IN susceptibility. Clones 'UCD 4' and 'Colmar (538)' were the most severely affected 'Pinot noir' clone, while 'Wadenswil' showed the lowest susceptibility (Jordan, 1989).

D. Vineyard and Vine Variations

Differences in IN incidence have been found among, and/or within, several vineyards (Jackson and Coombe, 1988a, b). Various levels of IN were observed on 'Pinot noir' in three vineyards in Canterberry, New Zealand. In South Australia, more IN was found in cooler sites at higher altitudes in the Adelaide hills than at warmer and lower sites on the Adelaide plain. Large differences in IN were also observed within some vineyards.

IN levels were 23-46% for four of the surveyed 'Pinot noir' vineyards in the Willamette Valley in 1988 (Jordan, 1989). Vines had some of the above described symptoms with different degrees of severity (Ibacache, 1990).

There appeared to be more IN in the central part of the vine above the trunk compared with peripheral areas (Jackson and Coombe, 1988a), suggesting that shaded areas within the vine canopy had greater IN (Jackson and Coombe, 1988b).

E. Pathogen Relationships

No fungi or other obvious pathogens were isolated from IN affected tissues even though they had the appearance of a fungal infection (Jackson and Coombe, 1988a, b). Insects such as *Tortris* may cause similar death of branches by chewing the surface of the pedicel and rachis (bunchstem), but the timing of the damage differs. Apparently, pathogens and pests are not involved, IN most likely has a physiological cause.

F. Association with Shading

IN incidence is increased by dense shade. Areas within the vine canopy which are more shaded seem to have more IN (Jackson and Coombe, 1988a). In a study of 'Reisling' vines growing in four light regimes, all clusters were necrotic when the light exposure on clusters was 10% of normal, compared with unshaded plants. The incidence of the disorder was doubled when the test plants were shaded to 50% incident light (Jackson and Coombe, 1988a, b). Shading with 60% shade cloth increased the percentage of necrotic flowers 2.3-2.7 times in field grown 'Pinot noir' grapes (Ibacache, 1990, Ibacache *et al.*, 1991). The low carbohydrate status due to shading may induce ammonium accumulation and lead to necrosis (Ibacache, 1990).

G. Association with Ammonium Toxicity

Jordan (1989) showed that clusters with high (>2.0 mg NH_4^+ g^{-1} DW) ammonium concentrations in the rachis 2 weeks post-anthesis had greater severity of IN. There was a positive correlation between the percentage of necrotic flowers and rachis ammonium concentration in five-year old 'Riesling' vines.

In field vines of 'Pinot noir', 60% shade increased the rachis ammonium concentration 23% at the beginning of bloom and increased IN about 2.5 times (Ibacache, 1990; Ibacache *et al.*, 1991). Positive correlation existed between the percentage of necrotic flowers and rachis and petiole ammonium concentrations at beginning bloom. The increase in IN and tissue ammonium when vines were shaded suggests a role of photosynthetically produced assimilates, especially

those critical substrates for ammonium assimilation (Jordan *et al.*, 1991). When carbon supply is reduced by shading, ammonium may accumulate to toxic levels and cause IN symptoms.

Dipping clusters (bunches) into $CaCl_2$ (1%) and $(NH_4)_2HPO_4$ (2%) caused symptoms indistinguishable from IN in *cvs.* 'Monukka' and 'Ruby Seedless'. Further work with other salts showed that the cation Ca^{2+} and NH_4^+ were the causal agents (Jackson and Coombe, 1988a, b). Other cations, K^+ and Mg^{2+} and anions, Cl^-, NO_3^-, and HPO_4^{2-}, had no effect (Jackson and Coombe, 1988a, b). The symptom could be due to a toxic effect of these chemicals on the surface of the tissues.

These associations of high ammonium concentrations in tissue affected by IN suggest that toxic levels of ammonium could be the cause. However, tissue sampling for ammonium occurred after symptoms had developed, leaving the question of cause or effect unanswered.

H. Control and Protection

No successful control or protective procedures for IN have yet been found. Girdling or lower shoot density did not affect the percentage of necrotic flowers (Ibacache, 1990). The lack of effect of lowering shoot density on IN may be attributed to excessive shoot vigor, which in turn prevented a difference in radiant energy absorption at cluster level (Ibacache, 1990). Magnesium sprays also failed to prevent IN (Jackson and Coombe, 1988a). Further research needs to be done to understand and achieve control of IN.

Table 2-1. Enzymes potentially capable of incorporating ammonium into organic compounds (after Miflin and Lea, 1980)

Enzymes and Reactions	E.C.N.	Eq.
Glutamate synthetase		
L-Glutamate:ammonia ligase (AMP forming)	6.3.1.2	
L-Glutamate + NH_4^+ + ATP =		
L-Glutamine + Orthophosphate + ADP		(1)
Glutamate synthase		
L-Glutamate:$NADP^+$ oxidoreductase (transaminating)	1.4.1.13	
L-Glutamate:ferredoxin oxidoreductase (transaminating)	1.4.7.1	
L-Glutamine + α-keto-glutarate		
+ NADPH (or NADH or ferredoxin reduced) = 2 L-Glutamate		
+ $NADP^+$ (or NAD^+ or ferredoxin oxidized)		(2)
Glutamate dehydrogenase		
L-Glutamate:NAD^+ oxidoreductase (deaminating)	1.4.1.2	
L-Glutamate:NAD(P)+ oxidoreductase (deaminating)	1.4.1.3	
L-Glutamate:$NADP^+$ oxidoreductase (deaminating)	1.4.1.4	
L-Glutamate + H_2O + NAD^+ (or $NADP^+$) =		
α-Keto-Glutarate + NH_4^+ + NADH (or (NADPH)		(3)
Alanine dehydrogenase		
L-Alanine:NAD^+ oxidoreductase (deaminating)	1.4.1.1	
L-Alanine + H_2O + NAD^+ = Pyruvate + NH_4^+ + NADH		(4)
Aspartate dehydrogenase		
L-Aspartate:NAD^+ oxidoreductase (deaminating)		
L-Aspartate + H_2O + NAD^+ = Oxaloacetate + NH_4^+ + NADH		(5)
Asparagine synthetase		
L-Asparate:ammonia ligase (AMP forming)	6.3.1.1	
L-Asparate + NH_4^+ + ATP =		
Pyrophosphate + L-Asparagine + AMP		(6)

Chapter 3

Inflorescence Necrosis Induced from Ammonium Incubation and Prevented by α-Keto-Glutarate in Clusters of 'Pinot noir' Grapevines

Abstract

Inflorescence necrosis (IN) was successfully induced by incubating single-node, field-grown, 'Pinot noir' (*Vitis vinifera* L.) cuttings with one leaf and cluster in 120 mM or higher NH_4^+ solutions of NH_4NO_3 or $(NH)_2SO_4$. Incubation with solutions of NO_3^-, $SO_4^=$, and K^+ did not induce IN at an equimolar concentration. The addition of 80 mM α-keto-glutarate, a substrate for ammonium assimilation, to an incubation solution containing 160 mM NH_4^+ prevented IN and lowered flower or fruit ammonium levels to near control levels. Significant correlations were found between the incidence of IN and ammonium concentration in cluster tissues.

Introduction

Inflorescence necrosis (IN), or early bunchstem necrosis (Ibacache, 1990; Jackson and Coombe, 1988; Jordan, 1989), in grapevines is described as a partial or complete breakdown of the rachis or pedicel near bloom (Ibacache, 1990). The cause of IN is unknown. No pathogens have been associated with the affected tissue and the disorder appears to have a physiological cause (Jackson and Coombe, 1988). It is believed that IN is associated with inorganic nitrogen. Stresses such as shading and drought can increase its incidence (Jackson and Coombe, 1988). Elevated ammonium levels in rachis tissue is linked with a higher incidence of IN (Ibacache, 1990; Jordan, 1989). Also, diammonium phosphate applied to inflorescences one week before flowering caused typical symptoms of early bunchstem necrosis (Jackson and Coombe, 1988). However, tissue sampling always occurred after symptoms had developed, leaving the question of cause or effect unanswered.

Ammonium accumulation in plant tissues could be a consequence of low carbon substrates essential for ammonium assimilation (Barker and Mills, 1980; Givan, 1979). Assimilation is the main defensive mechanism for plants to prevent ammonium accumulation (Givan, 1979). Jordan (1989) reduced the ammonium concentration in tendrils with α-keto-glutarate after ammonium incubation.

The objective of this study was to investigate ammonium and/or nitrate as the possible cause of IN and to determine the effect of α-keto-glutarate on IN and ammonium accumulation in 'Pinot noir' grapevines.

Materials and Methods

Experimental plant material came from mature 'Pinot Noir' vines (*Vitis vinifera* L.) grown at Beaver Creek Vineyard near Corvallis, Oregon, with a history of severe IN.

Incubation trials were conducted at one week pre-anthesis, anthesis, and one week post-anthesis in 1990 and 1991. Shoots with clusters were selected for uniformity and then excised at the base. Single node cuttings with a cluster and opposing leaf were cut from the shoots under water. The basal end of the cuttings was immersed in various solutions in 20 ml plastic vials, additional solution was added as necessary. Incubation was continued for 48 hours at temperatures of 25°C/21°C (day/night) in a greenhouse.

Experiment 1 (1990 and 1991): Clusters were incubated in: a) 160 mM NH_4NO_3 (160 mM NH_4^+ and 160 mM NO_3^-), b) 80 mM $(NH)_2SO_4$ (160 mM NH_4^+), and c) 80 mM $(NH)_2SO_4$ plus 80 mM α-keto-glutarate to investigate the effect of NH_4^+, NO_3^- and α-keto-glutarate on IN. Distilled water was used as a control. All the experimental solutions except distilled water were adjusted to pH 6.5 with KOH. Equal concentrations of KNO_3 and K_2SO_4 were used to test the effect of K^+ and $SO_4^=$, contained in major incubation solutions.

Experiment 2 (1991): Clusters were incubated in 0, 60, 80, 120, 160 and 200 mM NH_4^+ solutions of $(NH_4)_2SO_4$ to establish the relationship between IN and tissue ammonium concentration.

Necrosis was expressed as a percentage of necrotic tissue on the rachis and pedicel. Flowers (one week pre-anthesis and anthesis) or fruit (one week post-anthesis), both with the pedicels, and the rachis were sampled after 48 hours of incubation. Samples were dried at 55°C for 48 hours and ground through a 20 mesh screen. A sample of 100 mg dried tissue was extracted in 10 ml of 2% (v:v) acetic acid for 1 hour on a shaker and then allowed to stand for 30 minutes at room temperature. Extracts were filtered through in-tube Plasma/Serum separators (Karlan Chem. Corp., Torrance, California). Ammonium concentration was determined with a Wescan Model 360 ammonia analyzer (Alltech Assoc., Inc./Wescan Instruments, San Jose, California) expressed as mg N g^{-1} dry weight.

A completely randomized experimental design with 5 replications was used for both experiments in both years. Since the main effects of year and developmental stage were both significant, data were analyzed with ANOVA and

correlation procedures and presented separately for each stage in both years. Means were separated by Duncan's multiple range test.

Results

Experiment 1: IN symptoms (both rachis and pedicel necrosis) occurred only when cuttings were incubated in NH_4NO_3 and $(NH)_2SO_4$ (Fig. 3-1A, B). Pedicels had a higher incidence of necrosis than rachis at all stages in both years. The addition of α-keto-glutarate, a substrate for ammonium assimilation, to the $(NH)_2SO_4$ solution prevented IN. No IN was found on clusters incubated with KNO_3 and K_2SO_4. The $(NH)_2SO_4$ solution induced higher rachis necrosis than NH_4NO_3 at anthesis or one week post-anthesis, while NH_4NO_3 induced similar rachis necrosis at one week pre-anthesis in 1990 but higher rachis necrosis one week pre-anthesis in 1991. In severity was higher one week pre-anthesis for NH_4NO_3 but it was reversed one week post-anthesis, that is, higher for $(NH)_2SO_4$ (Fig. 3-1A, B).

Tissue ammonium concentrations increased several-fold with NH_4NO_3 and $(NH)_2SO_4$ incubation (Fig. 3-1C, D). Flowers and fruit had lower ammonium concentrations than the rachis. Rachis ammonium was generally higher when cuttings were incubated in NH_4NO_3 than $(NH)_2SO_4$. Flower and fruit ammonium varied but was similar to that for NH_4NO_3 and $(NH)_2SO_4$ incubation, only at post-anthesis. The addition of α-keto-glutarate reduced rachis ammonium concentration one week pre-anthesis in 1990 and flower and fruit ammonium at all three stages in 1991.

Experiment 2: Tissue ammonium increased with the ammonium concentration of incubation solutions (Table 3-1). Necrosis was associated with a solution threshold level of 120 mM ammonium. The strongest correlation between IN severity and tissue ammonium was found at the earliest stage (Table 3-2). Flower ammonium correlated better with IN severity than rachis ammonium. Correlation between flower ammonium concentration and pedicel or rachis necrosis at pre-anthesis is presented in Fig. 3-2.

Discussion

Incubation of grape cuttings with distilled water, 160 mM KNO_3, or K_2SO_4 did not induce IN, suggesting that H_2O, NO_3^-, $SO_4^=$, or K^+ ions were not associated with observed necrosis. Therefore, only NH_4^+ from NH_4NO_3 and $(NH)_2SO_4$ incubation was probably the causal agent for IN. The high correlation between necrosis incidence and ammonium concentration also indicated that ammonium can cause IN. However, ammonium levels of flowers or fruit were better indicators for IN because of stronger correlations with IN, especially pedicel necrosis.

Among the three important assimilation pathways, glutamine synthetase/glutamate synthase (GS/GOGAT) is considered to be the primary ammonium detoxification mechanism since it has the highest ammonium affinity (Givan, 1979). Glutamate dehydrogenase (GDH) and asparagine synthetase generally have 3 to 4 orders of magnitude less affinity than GS/GOGAT, so Givan (1979) considered these pathways to be important only at high ammonium levels. The carbon substrate of ammonium assimilation, α-keto-glutarate, eliminated IN and lowered tissue ammonium, especially in flowers or fruit. This suggests that enzymes utilizing α-keto-glutarate as a substrate for ammonium assimilation such as GS/GOGAT and/or GDH were operating and are very important in ammonium detoxification. The role of GS/GOGAT (Gu *et al.*, 1991; Miflin and Lea, 1976; Roubelakis-Angelakis and Kliewer, 1983b; Wallsgrove *et al.*, 1980) and GDH (Jordan, 1989; Loyola-Vargas and Sanchez de Jimenez 1984, 1986; Pahlich and Hoffman, 1975; Roubelakis-Angelakis and Kliewer, 1983a; Singh and Widholm, 1975; Zeleneva and Khavkin, 1980) in ammonium assimilation and/or detoxification has been demonstrated in several species.

Pedicels were more sensitive to ammonium than the rachis since they had lower ammonium levels and more necrosis. The rachis had greater ammonium accumulation, while flowers or fruit showed less or no ammonium increases when incubated with NH_4NO_3 (Fig. 3-1C, D). The high nitrate reduction and low or absent GS/GOGAT activity in the rachis (Gu *et al.* 1991) explain the high ammonium levels from NO_3^- incubation. Carbon substrates are critical for ammonium assimilation and detoxification, which could explain why flowers or fruit are less sensitive to necrosis since α-keto-glutarate reduced ammonium concentration in these tissues. These varied tissue responses could be due to

differences in the levels of ammonium assimilation, nitrate reduction enzyme systems, and/or the availability of their substrates in various grape tissues (Gu *et al.*, 1991).

Nitrate incubation had some protective effect on susceptibility of cluster tissues to IN at later stages since lower IN levels were noted with NH_4NO_3, compared to $(NH)_2SO_4$ at equimolar concentrations. This was consistent with previous reports for various species (Ormrod, 1979; Goyal *et al.*, 1982a, b).

Summary

This research suggests that elevated ammonium concentrations in rachis and pedicel tissue are the cause, not the result, of IN. IN was successfully induced by incubation of single-node cuttings with ammonium solutions and significantly correlated with tissue ammonium concentration. Nitrate did not induce IN. Pedicels were more sensitive to high ammonium concentrations than the rachis but the rachis accumulated higher concentrations of ammonium. A carbon substrate for ammonium assimilation, α-keto-glutarate, prevented IN induced by ammonium incubation and lowered the ammonium concentration in flower and fruit.

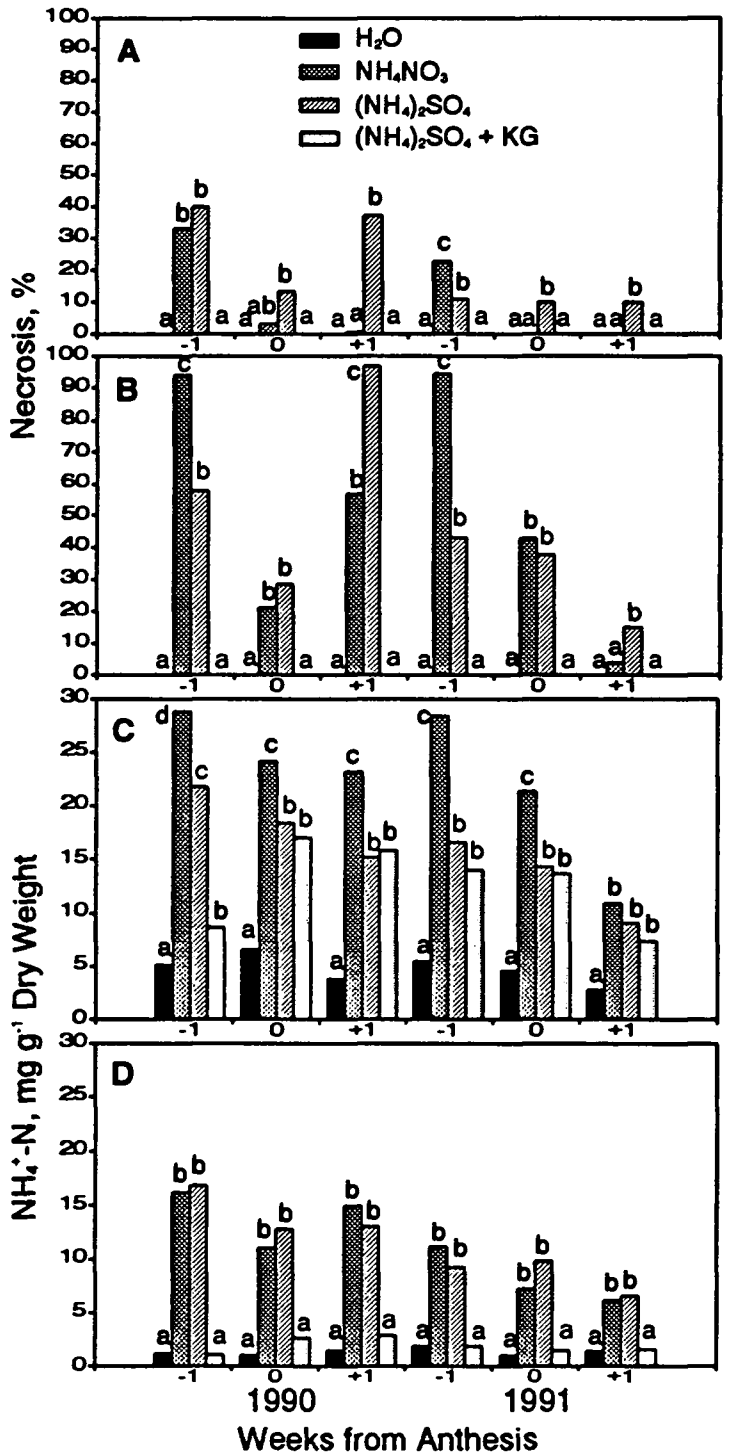

Fig. 3-1. Effect of incubating single-node cuttings with NH_4NO_3, $(NH)_2SO_4$, and α-keto-glutarate (KG) solutions on inflorescence necrosis of rachis (A) and pedicel (B) and tissue ammonium of rachis (C) and flowers/fruit (D) in 'Pinot noir' grapevines (Expt. 1, 1990 and 1991). All solutions were adjusted to a pH of 6.5 with KOH except H_2O. Mean separation for each sampling by Duncan's multiple range test at P=0.05 level.

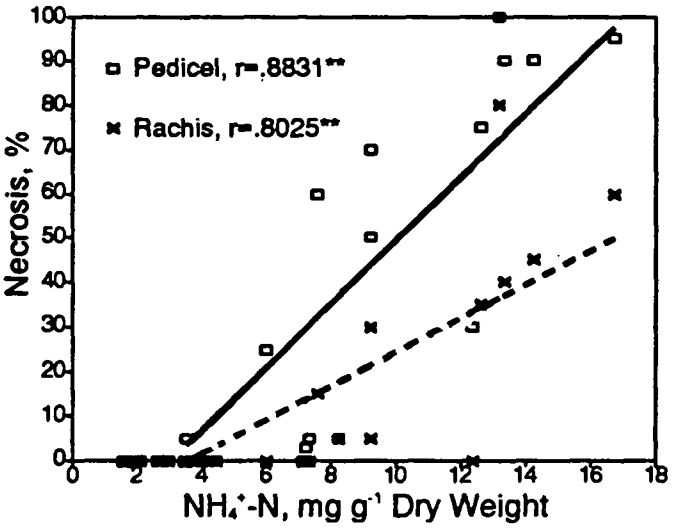

Fig. 3-2. Relationship between flower ammonium concentration and rachis or pedicel necrosis of 'Pinot noir' clusters incubated with $(NH)_2SO_4$ (0 to 200 mM NH_4^+) solutions one week pre-anthesis (Expt. 2, 1991). Correlation coefficients significant at P=0.05 (**) level.

Table 3-1. Effect of incubating single-node cuttings with $(NH)_2SO_4$ on inflorescence necrosis and ammonium concentration of rachis and flowers/fruit in 'Pinot noir' grapevines (Expt. 2, 1991)

NH_4^+-N Concentration (mM)	Necrosis %		NH_4^+-N, mg g^{-1} Dry Weight	
	Pedicel	Rachis	Rachis	Flower/Fruit
		Pre-anthesis		
0	0 a[z]	0 a	5.43 a	1.97 a
60	0 a	0 a	11.64 b	2.80 ab
80	0 a	0 a	13.20 b	3.85 b
120	8 a	2 a	16.53 c	6.24 c
160	43 b	10 a	16.65 c	9.33 d
200	90 c	56 b	19.42 c	14.03 d
		Anthesis		
0	0 a	0 a	4.56 a	1.00 a
60	0 a	0 a	8.34 b	1.67 a
80	0 a	0 a	10.84 bc	2.53 a
120	35 b	0 a	10.58 bc	6.50 b
160	38 b	11 b	14.40 d	9.91 c
200	72 c	52 c	12.97 cd	13.44 d
		Post-anthesis		
0	0 a	0 a	2.81 a	1.42 a
60	0 a	0 a	3.55 a	1.87 a
80	0 a	0 a	5.35 ab	4.44 b
120	11 a	13 ab	7.96 bc	5.24 b
160	15 a	10 ab	9.06 c	6.56 bc
200	42 b	24 b	8.90 c	7.56 c

[z] Mean separation in columns for each sampling by Duncan's multiple range test at P=0.05 level

Table 3-2. Correlation coefficients between inflorescence necrosis and tissue ammonium concentrations in 'Pinot noir' clustersincubated with 0 to 200 mM NH_4^+ (Expt. 2, 1991)

Tissue Ammonium	Necrosis	
	Pedicel	Rachis
	Pre-anthesis	
Rachis	.6378**[z]	.5043**
Flower	.8832**	.8025**
	Anthesis	
Rachis	NS	NS
Flower	.7566**	.6888**
	Post-anthesis	
Rachis	NS	NS
Fruit	.4586*	NS

[z] *, ** significant at P=0.05 and P=0.01 levels, respectively.

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Chapter 4

Effect of Glutamine Synthetase/Glutamate Synthase (GS/ GOGAT) Inhibitors, α-Keto-Glutarate, Nitrate, and Low Oxygen Atmosphere on Ammonium Accumulation in 'Pinot noir' Leaf and Cluster Tissues

Abstract

Shoot cuttings of 'Pinot noir' grapevines (*Vitis vinifera* L.) with a leaf and cluster attached were taken from field-grown plants at one week pre-anthesis, anthesis, and one week post-anthesis. The base of the cuttings were placed in solutions containing glutamine synthetase/glutamate synthase (GS/GOGAT) inhibitors to determine the presence of the primary ammonium assimilation pathways and investigate sources of ammonium accumulation. The inhibitors were 10 mM methionine sulfoximine (MSX, a GS inhibitor) and 5 mM azaserine (AS, a GOGAT inhibitor). Elevated ammonium concentrations were found in laminas, flowers or fruit, and pedicels of cuttings treated with MSX or AS, indicating the presence of a GS/GOGAT pathway for ammonium assimilation. MSX and AS had no effect on the ammonium concentrations in petiole and rachis, suggesting a lack of effective ammonium assimilation by GS/GOGAT in these tissues. Ammonium accumulation occurred only in laminas and pedicels when cuttings were incubated in 40 mM NO_3^- plus MSX solution. Incubation with α-keto-glutarate reduced ammonium concentration in laminas and petioles at anthesis, but no reduction in ammonium occurred when incubated with α-keto-glutarate and MSX, indicating that glutamate dehydrogenase (GDH) is not a primary pathway for ammonium detoxification. Low O_2 (2%) resulted in a 72% decrease in the amount of ammonium induced by MSX in laminas one week post-anthesis but had no effect on ammonium accumulation in cluster tissues, suggesting that photorespiration was not a major source of ammonium in cluster tissues.

Introduction

Nitrogen metabolism in grapes has received increased attention since high levels of ammonium in tissues was associated with two important disorders: inflorescence necrosis (IN), or early bunchstem necrosis (Ibacache, 1990; Jackson and Coombe,1988; Jordan, 1989), and bunchstem necrosis, or waterberry (Christensen and Boggero, 1985; Jordan, 1989). Both of these disorders can cause economic crop losses. To understand the factors which affect the buildup of ammonium in grape tissue it is necessary to understand the enzyme systems for ammonium assimilation and detoxification and sources of the accumulated ammonium. The activity of ammonium assimilation pathways and sources of ammonium in grape, especially in pedicel and rachis tissues, may be critical to the development of these two disorders.

Glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is the primary route for ammonium assimilation in leaves of higher plants (Miflin and Lea, 1976; Wallsgrove *et al.*, 1980). Several researchers have suggested an assimilatory role for glutamate dehydrogenase (GDH) in some plant tissues (Loyola-Vargas and Sanches de Jimenez, 1984; Pahlich and Hoffman, 1975; Singh and Srivastava, 1986; Zeleneva and Khavkin, 1980). GS and GDH activities have been detected in grape berries, roots, and leaves (Ghisi *et al.*, 1984; Roubelakis-Angelakis and Kliewer, 1983a, b). However, Roubelakis-Angelakis and Kliewer (1983b) were unable to detect GOGAT activity in berries.

Inhibitors of the GS/GOGAT pathway, such as L-methionine D,L-sulfoximine (MSX) and azaserine (AS), have been widely used in studies of plant carbon-nitrogen metabolic pathways. Treatment of photosynthesizing plants with MSX leads to a large-scale ammonium accumulation in various species (Anderson and Done, 1977; Arima and Kumazawa, 1977; Frantz *et al.*, 1982; Platt and Anthon, 1981; Singh and Widholm, 1975), but without affecting the CO_2 fixation process (Romero *et al.*, 1985), nitrate reductase (Frantz *et al.*, 1982), or GDH activity (Arima and Kumazawa, 1977; Brenchley, 1973). AS, an analog of glutamine, inhibits all glutamine amide transfer reactions, including GOGAT, but does not affect GDH activity (Dunn and Klucas, 1973; Miflin and Lea 1976).

The objective of this research was to verify the presence of the GS/GOGAT and GDH pathways and determine the contribution of nitrate reduction and

photorespiration to ammonium accumulation in leaf and cluster tissues of 'Pinot noir' grapevines.

Materials and Methods

Plant material came from mature 'Pinot noir' vines (*Vitis vinifera* L.) grown at the Beaver Creek Vineyard near Corvallis, Oregon. The vineyard is excessively vigorous and has a history of severe inflorescence necrosis.

Incubation trials were conducted at one week pre-anthesis, anthesis and one week post-anthesis in 1990 and 1991. Shoots with clusters were selected for uniformity and then excised at the base. Single-node cuttings with a cluster and opposing leaf were cut from the shoots under water. The basal end of the cuttings was immersed in various solutions in 20 ml plastic vials. Incubation was continued for 6 hours at 25°C in a greenhouse.

Experiment 1: Cuttings were incubated in 10 mM KNO_3 solutions with or without GS/GOGAT inhibitors, *i.e.* 10 mM MSX or 5 mM AS in 1990 and 1991 (AS treatments were applied only one week post-anthesis in 1990). A completely randomized experimental design with 5 replications was used.

Experiment 2: In 1991, cuttings were incubated in 20 mM $(NH)_2SO_4$ (40 mM NH_4^+) solutions with or without 10 mM MSX and/or 20 mM α-keto-glutarate to test the presence of the GDH pathway. The factorial experiment consisted of 4 treatments with 5 replications in a completely randomized design.

Experiment 3: In 1991, cuttings were incubated in 0, 10 and 40 mM KNO_3 solutions with or without 10 mM MSX to test the contribution of nitrate reduction to ammonium accumulation. The factorial experiment consisted of 6 treatments with 5 replications in a completely randomized design.

Experiment 4: Cuttings were incubated in 10 mM KNO_3 solutions with or without 10 mM MSX in controlled atmosphere to compare ammonium accumulation with or without inhibition of photorespiration in 1991. Cuttings incubated in 20 ml plastic vials were placed in a 6 L jar containing inlet and outlet ports. Gases were supplied from cylinders containing 2% or 21% O_2, 330 ppm CO_2 and N_2 (Industrial Welding Supply Inc., Corvallis, Oregon). The gases which were humidified by bubbling through H_2O were regulated to flow at 2 L/minute. The factorial experiment consisted of 4 treatments with 5 replications in a completely randomized design.

Lamina, petiole, flower or fruit, pedicel, and rachis samples were taken after 6 hours of incubation. Samples were dried at 55°C for 48 hours and ground through a 20 mesh screen. A sample of 50 to 100 mg dried tissue was extracted

in 10 ml 2% (v:v) acetic acid solution for 1 hour on a shaker and then let stand for 30 minutes at room temperature. Extracts were filtered through an in-tube Plasma/Serum separator (Karlan Chem. Corp., Torrance, California). Ammonium concentration was determined on Wescan Model 360 ammonia analyzer (Alltech Assoc., Inc./Wescan Instruments, San Jose, California) and expressed as mg g^{-1} dry weight.

All data were subjected to ANOVA analysis. Treatment levels were subsequently analyzed by Duncan's multiple comparison test. When interactions existed, means for the various treatment combinations were compared. Main and interaction significances are also presented.

Results

Tissue Ammonium Levels: Ammonium concentration was highest in the rachis of all tissues sampled, ranging from 3.0 to 6.5 mg N g^{-1} dry weight, compared with 0.5 to 3.5 for petioles, while laminas, pedicels, flowers and fruit had less (Figs. 4-1, 4-2). The differences in tissue ammonium concentrations varied with years. Tissue ammonium generally peaked at anthesis in 1990, while it was at maximum one week pre-anthesis in 1991. Ammonium concentration in fruit was greater than in flowers in 1990 (Fig. 4-2a).

Experiment 1: GS/GOGAT Inhibition. The GS inhibitor MSX increased tissue ammonium concentration in laminas, flowers, fruit, and pedicels in both years. Ammonium concentration in MSX-treated laminas was nearly 3 times that of the untreated tissue (Fig. 4-1). MSX increased ammonium concentration by 17% in flowers and 80% in fruit in 1990 (Fig. 4-2) and by 69% in flowers one week pre-anthesis in 1991. MSX also increased ammonium in pedicels by about 2 times in both year (Fig. 4-2). MSX did not cause a rise in the ammonium concentration in the petioles or rachis (Fig. 4-1, 4-2). Similar effects were found in other experiments in which the cuttings were incubated in solutions of higher nitrate or ammonium concentrations or under low O_2 atmosphere (Tables 4-1, 4-2, and 4-3).

AS at 5 mM, applied only at one week post-anthesis in 1990, had the same effect on ammonium accumulation as MSX. No significant differences in ammonium accumulation were found between AS and MSX treatments in all sampled tissues except pedicels from AS-treated cuttings which accumulated less ammonium (Figs. 4-1 and Fig. 4-2).

Experiment 2: GDH Pathway. MSX increased lamina ammonium at all samplings and increased flower and pedicel ammonium at the first two samplings with or without the addition of α-keto-glutarate (Table 4-1). Incubation with α-keto-glutarate with ammonium alone generally decreased tissue ammonium but had the greatest reduction in laminas and pedicels at the first sampling and in laminas only at the last two samplings. No significant interactions were found between α-keto-glutarate and MSX treatments in any tissue tested at any time.

Experiment 3: Nitrate Reduction. Incubation with 40 mM NO_3^- increased ammonium in laminas at pre-anthesis and anthesis (Table 4-2). But treatment with 10 mM NO_3^- had no effect on lamina ammonium. However, tissue ammonium increased with 40 mM NO_3^- plus MSX for the pedicels and the rachis in post-

anthesis only. Interaction between the nitrate rate and MSX was significant for pedicels at anthesis and post-anthesis. Nitrate and MSX increased the ammonium concentration in laminas at all samplings and in flowers and pedicels at pre-anthesis.

Experiment 4: Photorespiration. Low (2%) O_2 had little or no effect on ammonium concentration in any tissues except it reduced ammonium levels in laminas at post-anthesis and showed a significant reduction with MSX (Table 4-3). Treatment of cuttings with low O_2 and MSX reduced the ammonium concentration by 28%, compared to ambient O_2.

Discussion

Ammonium Assimilation Pathways: Increases in tissue ammonium accumulation as a result of MSX and AS treatments suggest that ammonium assimilation by the GS/GOGAT cycle was blocked. The elevated ammonium concentrations in laminas, flowers, fruit and pedicels treated with the inhibitors may reflect the importance of GS/GOGAT in nitrogen assimilation in grapevine tissues, and are consistent with previous reports for other species (Anderson and Done, 1977; Arima and Kumazawa, 1977; Frantz *et al.*, 1982; Platt and Anthon, 1981; Singh and Widholm, 1975). In all sampled tissues, AS showed an effect similar to MSX on ammonium accumulation, implying that GOGAT is tightly coupled with GS. Since these inhibitors had no effect on ammonium accumulation in petiole and rachis tissue, we assume that GS/GOGAT are absent or at very low levels in these tissues. Roubelakis-Angelakis and Kliewer (1983b) could not detect GOGAT activity in grape leaf and root extracts, but this may have been more of a problem with enzyme extraction than the absence of the enzyme itself.

An ammonium assimilatory role for GDH in some plant tissues was recently suggested (Loyola-Vargas and Sanches de Jimenez, 1984; Pahlich and Hoffman, 1975; Singh and Srivastava, 1986; Zeleneva and Khavkin, 1980). Jordan (1989) found that the addition of 10 mM MSX did not prevent the reduction in ammonium concentration of tendrils treated with α-keto-glutarate, compared to those treated with ammonium alone. This suggests that the assimilation stimulated by α-keto-glutarate did not involve GS. Yet another assimilation pathway, most likely that catalyzed by GDH, may have operated. This was also suggested by the change in amino acid concentrations (Jordan 1989). However, in our case, when grape cuttings were incubated with MSX, α-keto-glutarate reduced lamina and petiole ammonium only at anthesis without influencing cluster tissue ammonium (Table 4-1), indicating the absence of, or very low, GDH activities in 'Pinot noir' leaf and cluster tissues, even with 40 mM NH_4^+ incubation.

The response to the MSX and AS treatment varied with tissues. Ammonium concentration in laminas was about 2 times greater after treatment with the inhibitors, whereas only slight differences were noted in petioles and rachis. It is reasonable to expect variation in enzyme systems, the levels of enzyme activity, and/or its substrates in these tissues. Differences in tissue ammonium

accumulation with or without inhibitors also varied with developmental stages of the cluster.

Plants can detoxify ammonium by converting it into amino acids. Givan (1979) suggested three assimilation pathways for removal of free ammonium. GS/GOGAT is believed to be the usual pathway for the assimilation of ammonium at low (i.e. normal) intercellular concentration, while GDH and asparagine synthetase reactions probably assimilate ammonium only when the intercellular ammonium concentration is unusually high. We could only show the presence or absence of GS/GOGAT and GDH pathways in leaves and cluster tissues of grapes. If excessive ammonium is the causal agent of IN, our data indicate that the rachis and pedicel would be more subject to the necrosis.

Ammonium Sources: There are three major metabolic sources of the ammonium in plant tissues: nitrate reduction, photorespiration, and protein hydrolysis followed by degradation of amino acids. Photorespiration is the major source of ammonium in leaves of many species (Miflin and Lea, 1980; Chollet and Ogren, 1975; Hartman and Ehmke, 1980; Somerville and Ogren, 1980). This is unlikely to be a major source of ammonium in grape cluster tissues which have low photosynthetic capacity. The ammonium generated by photorespiration is believed to be assimilated by the GS/GOGAT cycle (Hartman and Ehmke, 1980; Somerville and Ogren, 1980; Wallsgrove *et al.*, 1980). Therefore, a reduction of ammonium accumulation induced by MSX in a low O_2 atmosphere, which inhibits photorespiration (Chollet and Ogren, 1975), could indicate the contribution of photorespiration to ammonium. In the plant material we used, only laminas showed that photorespiration may have contributed significantly to ammonium accumulation at post-anthesis, indicating that photorespiration is not an important source for ammonium in other tissues sampled such as the petioles and rachis.

Nitrate normally accumulates in petioles of grapevines (Cook and Lider, 1964). This suggests that nitrate is transported to the aerial part of the vine and reduced in the lamina. Nitrate reduction has been found in grape laminas (Perez and Kliewer, 1978). Our results suggest that nitrate reduction was a significant source of ammonium in laminas and pedicel but not in petioles, flowers or fruit, or rachis.

Summary

Grapevine tissues varied considerably in ammonium accumulation responses to MSX and AS treatments. The presence of GS/GOGAT pathway was suggested by the significant increases of ammonium concentration in laminas, flowers, fruit, and pedicel treated with MSX and AS. Since MSX and AS had no effect on ammonium accumulation in petioles and rachis tissue, it is assumed that GS/GOGAT enzymes are absent or at very low levels in these tissues. If ammonium is the causal agent of IN, the data indicate that the rachis and pedicel would be more subject to the necrosis at pre-anthesis stages. It was also suggested that nitrate reduction and photorespiration are not the major sources of ammonium in cluster tissues.

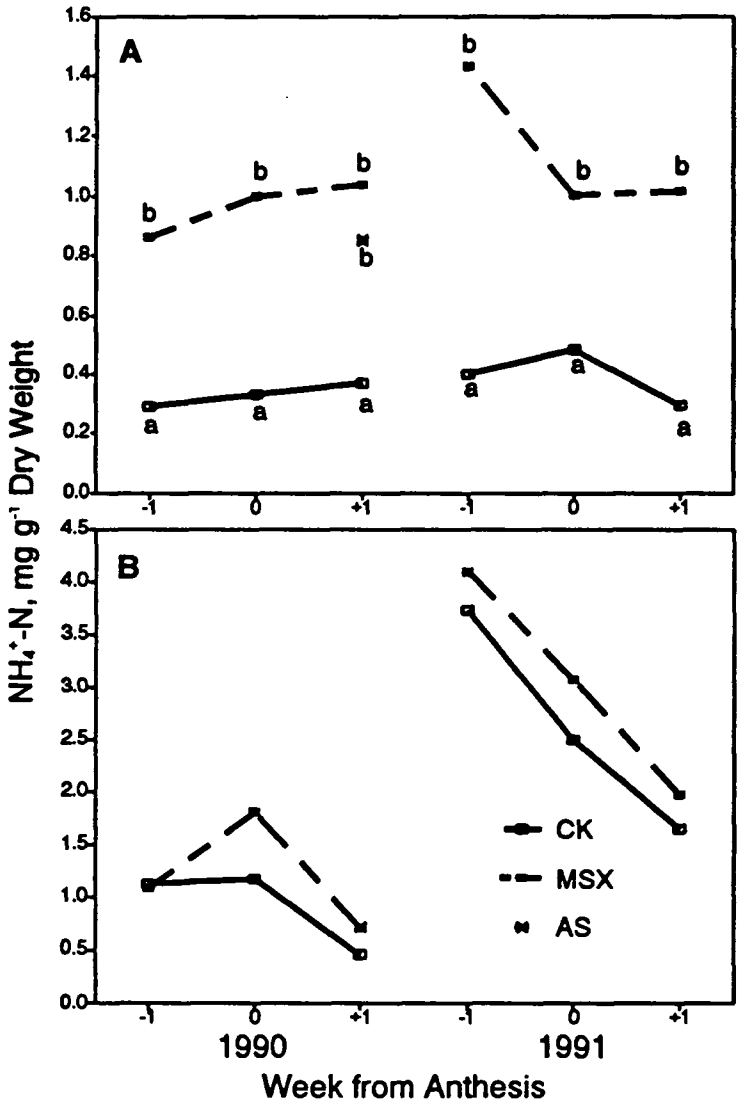

Fig 4-1. Effect of methionine sulfoximine (MSX) and azaserine (AS), compared to control (CK), on ammonium accumulation of laminas (A) and petioles (B) in 'Pinot noir' grapevines (Expt. 1, 1990 and 1991). Mean separation among treatment for each sampling by Duncan's multiple range test at P=0.05 level.

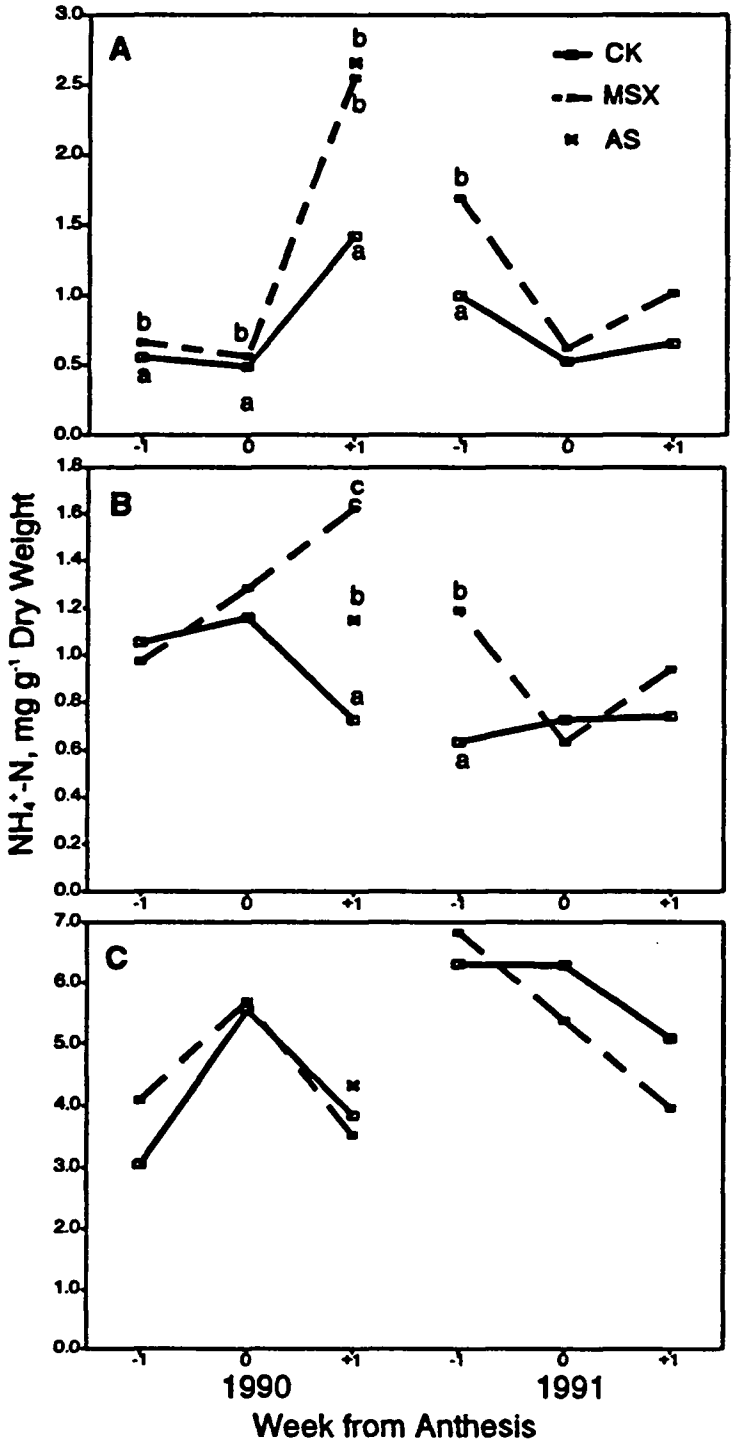

Fig 4-2. Effect of methionine sulfoximine (MSX) and azaserine (AS), compared to control (CK), on ammonium accumulation of flowers/fruit (A), pedicels (B), and rachis (C) in 'Pinot noir' grapevines (Expt. 1, 1990 and 1991). Mean separation among treatment for each sampling by Duncan's multiple range test at P=0.05 level.

Table 4-1. Effect of incubating cuttings with 40 mM NH_4^+ and α-keto- glutarate (KG) or methionine sulfoximine (MSX) on tissue ammonium accumulation of leaves and clusters in 'Pinot noir' grapevines (Expt. 2, 1991)

Treatment	NH_4^+-N, mg g^{-1} Dry Weight				
	Lamina	Petiole	Flower/ Fruit	Pedicel	Rachis
			Pre-anthesis		
NH_4^+	1.35 a[z]	4.99	1.00 a	0.73 a	7.12
NH_4^+ + MSX	2.39 b	4.61	1.71 b	2.07 b	7.72
NH_4^+ + KG	0.96 a	5.47	1.02 a	0.55 a	6.35
NH_4^+ + KG + MSX	2.10 b	4.02	1.60 b	1.69 b	7.58
Significance					
KG	NS[y]	NS	NS	NS	NS
MSX	**	NS	**	**	NS
MSX x KG	NS	NS	NS	NS	NS
			Anthesis		
NH_4^+	1.28 ab	4.50 a	0.46 a	0.63 ab	7.21
NH_4^+ + MSX	1.53 b	5.86 b	0.80 ab	0.78 bc	6.59
NH_4^+ + KG	0.98 a	4.08 a	0.51 ab	0.36 a	5.93
NH_4^+ + KG + MSX	1.28 ab	3.96 a	0.85 b	1.04 c	6.69
Significance					
KG	*	**	NS	NS	NS
MSX	*	NS	**	**	NS
MSX x KG	NS	NS	NS	NS	NS
			Post-anthesis		
NH_4^+	0.87 ab	3.01	1.09	0.69	5.52
NH_4^+ + MSX	1.19 b	2.45	0.77	0.98	5.44
NH_4^+ + KG	0.54 a	2.00	1.27	0.71	5.53
NH_4^+ + KG + MSX	1.09 b	2.22	0.89	1.23	6.12
Significance					
KG	NS	NS	NS	NS	NS
MSX	**	NS	NS	*	NS
MSX x KG	NS	NS	NS	NS	NS

[z] Mean separation in column for each sampling by Duncan's multiple range test at P = 0.05 level.

[y] NS, *, ** non-significant, significant at P = 0.05 and P = 0.01 levels, respectively

Table 4-2. Effect of incubating cuttings with NO_3^- and methionine sulfoximine (MSX) on tissue ammonium accumulation of leaves and clusters in 'Pinot noir' grapevines (Expt. 3, 1991)

Treatment	NH_4^+-N, mg g^{-1} Dry Weight				
	Lamina	Petiole	Flower/ Fruit	Pedicel	Rachis
			Pre-anthesis		
H_2O	0.40 a[z]	3.43 a	1.30 a	0.72 a	6.75
H_2O + MSX	1.79 c	4.86 bc	1.66 b	1.18 b	6.19
10 mM NO_3^-	0.40 a	3.73 ab	1.00 a	0.63 a	6.31
10 mM NO_3^- + MSX	1.43 b	4.10 abc	1.69 b	1.19 b	6.82
40 mM NO_3^-	1.02 b	3.78 abc	0.94 a	0.55 a	5.63
40 mM NO_3^- + MSX	2.37 d	4.96 c	1.45 b	1.61 b	7.23
Significance					
NO_3^-	**[y]	NS	NS	NS	NS
MSX	**	**	**	**	NS
MSX x NO_3^-	NS	NS	NS	NS	NS
			Anthesis		
H_2O	0.25 a	2.21	0.70	0.58 a	6.05
H_2O + MSX	1.09 cd	3.21	0.63	0.74 a	6.03
10 mM NO_3^-	0.49 a	2.49	0.52	0.72 a	6.28
10 mM NO_3^- + MSX	1.00 bc	3.08	0.62	0.63 a	5.38
40 mM NO_3^-	0.63 ab	2.61	0.58	0.69 a	6.05
40 mM NO_3^- + MSX	1.51 d	2.09	0.83	1.15 b	6.10
Significance					
NO_3^-	*	NS	NS	*	NS
MSX	**	NS	NS	*	NS
MSX x NO_3^-	NS	NS	NS	*	NS
			Post-anthesis		
H_2O	0.12 a	1.36	0.96	0.67 a	4.70 ab
H_2O + MSX	0.91 b	2.27	0.94	1.39 b	5.97 bc
10 mM NO_3^-	0.29 a	1.65	0.66	0.74 a	5.09 abc
10 mM NO_3^- + MSX	1.02 b	1.97	1.02	0.94 ab	3.96 a
40 mM NO_3^-	0.26 a	2.24	0.70	0.69 a	4.54 ab
40 mM NO_3^- + MSX	1.12 b	2.51	1.03	2.01 c	6.63 c
Significance					
NO_3^-	NS	NS	NS	*	NS
MSX	**	NS	NS	**	NS
MSX x NO_3^-	NS	NS	NS	*	**

[z] Mean separation in column for each sampling by Duncan's multiple range test at P=0.05 level.
[y] NS, *, ** non-significant, significant at P=0.05 and P=0.01 levels, respectively.

Table 4-3. Effect of incubating cuttings with 10 mM nitrate at 2% O_2 atmosphere and methionine sulfoximine (MSX) on tissue ammonium accumulation of leaves and clusters in 'Pinot noir' grapevines (Expt. 4, 1991)

Treatment	NH_4^+-N, mg g^{-1} Dry Weight				
	Lamina	Petiole	Flower/ fruit	Pedicel	Rachis
			Pre-anthesis		
NO_3^-	0.40 a[z]	3.73	1.00 a	0.63 ab	6.31
NO_3^- + MSX	1.43 b	4.10	1.69 c	1.19 c	6.82
NO_3^- + 2% O_2	0.35 a	3.30	1.03 a	0.39 a	6.09
NO_3^- + 2% O_2 + MSX	0.94 b	3.33	1.33 b	0.84 bc	5.17
Significance					
O_2	NS[y]	NS	NS	NS	NS
MSX	**	NS	*	*	NS
MSX x O_2	NS	NS	NS	NS	NS
			Anthesis		
NO_3^-	0.49 ab	2.49	0.52	0.72	6.28
NO_3^- + MSX	1.00 b	3.08	0.62	0.63	5.38
NO_3^- + 2% O_2	0.36 a	2.55	0.62	0.76	6.32
NO_3^- + 2% O_2 + MSX	0.82 ab	3.10	0.53	0.49	6.23
Significance					
O_2	NS	NS	NS	NS	NS
MSX	*	*	NS	NS	NS
MSX x O_2	NS	NS	NS	NS	NS
			Post-anthesis		
NO_3^-	0.29 a	1.65	0.66	0.74	5.09
NO_3^- + MSX	1.02 b	1.97	1.02	0.94	3.96
NO_3^- + 2% O_2	0.10 a	3.40	0.83	0.92	5.47
NO_3^- + 2% O_2 + MSX	0.29 a	2.91	0.73	0.59	5.09
Significance					
O_2	**	NS	NS	NS	NS
MSX	**	NS	NS	NS	NS
MSX x O_2	*	NS	NS	NS	NS

[z] Mean separation in column for each sampling by Duncan's multiple range test at P = 0.05 level.

[y] NS, *, ** non-significant, significant at P = 0.05 and P = 0.01 levels, respectively.

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Chapter 5

Shading Influenced Plant Growth, Tissue Ammonium, and Nitrate Status, and Induced Inflorescence Necrosis in 'Pinot noir' Grapevines

Abstract

One- and two-year-old potted 'Pinot noir' grapevines (*Vitis vinifera* L.) were irrigated with Hoagland's nutrient solution and covered with 60% shade cloth to investigate the effect of shading on vine growth, inflorescence necrosis (IN), tissue ammonium, and nitrate status. New growth of the vines was significantly reduced by shading. There was 26.6% IN on shaded vines, compared to only 0.6% on unshaded vines. Shading increased tissue ammonium and nitrate concentrations in lamina, petiole, and rachis of two-year-old vines. Cluster development was inadequate for monitoring IN in one-year-old vines. Tissue ammonium and nitrate concentrations were increased by shading in most tissues with few exceptions in one-year-old vines. Tissue ammonium correlated with nitrate concentration at later stages of one-year-old vines and in lamina, petiole, fruit, and rachis of two-year-old vines. Elevated tissue ammonium in rachis of shaded vines and increased incidence of IN suggests that it is the causal agent of the IN.

Introduction

Inflorescence necrosis (IN), or early bunchstem necrosis, is described as a partial or complete breakdown of the rachis or pedicels near bloom in grapevines (Ibacache *et al.*, 1991; Jackson and Coombe, 1988; Jordan *et al.*, 1991). The specific cause of IN is unknown. No pathogens have been found on affected tissue and the disorder appears to have a physiological cause (Jackson and Coombe, 1988). It is believed that IN is associated with inorganic nitrogen (Jordan *et al.*, 1991). Stresses such as shading and drought can increase its incidence (Ibacache *et al.*, 1991; Jackson, 1991; Jackson and Coombe, 1988). Elevated ammonium levels in rachis tissue have been related to IN (Ibacache *et al.*, 1991; Jordan *et al.*, 1991). Also, diammonium phosphate applied one week before flowering to inflorescences caused typical early bunchstem necrosis symptoms (Jackson and Coombe, 1988). However, tissue sampling always occurred after symptoms had developed, therefore it is unclear whether elevated ammonium levels are a cause or effect of the disorder.

Ammonium accumulation in plant tissues could be a consequence of low carbon substrates which are essential for ammonium assimilation (Barker and Mills, 1980; Givan, 1979). Assimilation is the main defensive mechanism for plants to prevent ammonium from accumulating to toxic levels (Givan, 1979). The low carbohydrate status due to shading may induce ammonium accumulation and lead to necrosis of the clusters. Shaded clusters also grow weakly and could be more sensitive to any toxic element, including ammonium (Ibacache, 1990).

The objective of this experiment was to investigate ammonium or nitrate as the possible cause of IN by: 1) establishing dynamic patterns of NH_4^+ and NO_3^- of various tissues near anthesis and 2) determining the effect of shading on vine growth, tissue ammonium, and nitrate status, and IN in 'Pinot noir' grapevines.

Materials and Methods

Experiment 1 (1989): One-year-old 'Pinot noir' grapevines (*Vitis vinifera* L.) were potted with vermiculite and irrigated with full strength Hoagland's nutrient solution containing 16 mM NO_3^-, without NH_4^+, in a greenhouse. Half of the plants were shaded with 60% polyvinyl shading screens from bud break untill two weeks post-anthesis to determine the effects of shading on ammonium and nitrate status and IN. Plants were harvested 5 times at one week intervals before, at and after anthesis. Tendrils, clusters, shoots, young laminas, mature laminas, young petioles, mature petioles, stems, old roots, and new roots were sampled for dry weight, ammonium, and nitrate analyses.

Experiment 2 (1990): Two-year-old plants were cultured as described above; the older plants assured adequate cluster development for IN evaluation. Shading (60%) was applied from bud break untill two weeks post-anthesis. Plant top fresh weight was taken and laminas, petioles, tendrils, rachis, and fruit were sampled for ammonium and nitrate analysis two weeks post-anthesis. IN was expressed as a percentage of necrotic tissue on the rachis and pedicel.

Tissue Ammonium and Nitrate Analysis: Tissue samples were dried at 55°C for 48 hours and ground through a 20 mesh screen. A sample of 100 mg dried tissue was extracted in 10 ml 2% (v:v) acetic acid solution for 1 hour on a shaker and then let stand for 30 minutes at room temperature. Extracts were filtered through an in-tube Plasma/Serum separator (Karlan Chem. Corp., Torrance, California). Ammonium concentration was determined on a Wescan Model 360 ammonia analyzer (Alltech Assoc., Inc./Wescan Instruments, San Jose, California). Nitrate was reduced to ammonium with a continuous flow reduction method developed by Carlson (1986) and analyzed the same way as ammonium. Ammonium and nitrate concentrations were expressed as mg N g^{-1} dry weight.

Experimental Design and Data Analysis: A completely randomized experimental design with 9 (1989) and 7 (1990) replications was used in this study. Means were separated with the F test at $P=0.05$ level.

Results

Growth: Plant dry weight of one-year-old plants in 1989 decreased from bud break to one week pre-anthesis and rapidly increased afterwards. There was a significant reduction of dry weight in shaded plants one week pre-anthesis, and thereafter the difference between treatments increased (Fig. 5-1). Top growth responded significantly to shading near the beginning of the sampling. Shaded vines had only half the top dry weight of unshaded plants two weeks post-anthesis. Stem and old root dry weight decreased about 30% from bud break to one week pre-anthesis and then remained constant thereafter. Shading did not influence the dry weight changes in stems and old roots (Fig. 5-2b). New root dry weight of shaded plants was between 50% and 22% of unshaded ones at one week pre-anthesis and at post-anthesis, respectively. No new root growth occurred in shaded plants during the last 4 weeks near anthesis (Fig. 5-2c).

The shoot, leaf and, cluster growth of two year old plants in 1990 was reduced by shading, also (Table 5-1).

Inflorescence Necrosis: There was inadequate cluster development for monitoring IN on one-year-old vines in 1989, but on two year old vines in 1990 there was 26.6% necrosis on shaded vines compared to 0.6% on unshaded vines (Table 5-1). Shading also significantly reduced berry number per cluster and cluster dry weight, but had no influence on cluster number per plant.

Tissue Ammonium Concentrations: With one-year-old vines in 1989, ammonium decreased during the season in laminas, petioles (Fig. 5-3), shoots, and tendrils (Fig. 5-4a, b), but increased in stem tissue only prior to anthesis (Fig. 5-4c), and fluctuated in roots (Fig. 5-5). Tendrils and mature petioles had the highest tissue ammonium concentration, while stem and root tissues had the lowest.

Shading increased ammonium concentrations in most tissues. Shaded plants had twice as much tissue ammonium in laminas and petioles as unshaded plants. Young petioles and laminas showed lower ammonium concentration than mature petioles and laminas. Tissue ammonium in tendrils and shoots followed a similar pattern to petioles and laminas (Fig. 5-4a, b, c). Shading maintained a greater concentration of tendril ammonium (Fig. 5-4a). Root ammonium was not influenced by shading except at two weeks post-anthesis (Fig. 5-5). Ammonium

concentration in new roots correlated well with that in old roots throughout the experimental period.

Laminas and petioles in two-year-old vines had higher ammonium concentration in shaded plants than unshaded plants two weeks post-anthesis (Table 5-2).

Tissue Nitrate Concentrations: Nitrate in one-year-old vines peaked near anthesis in laminas, petioles (Fig. 5-6), tendrils, shoots and stems (Fig. 5-7), and increased linearly in old roots (Fig. 5-8). Highest tissue nitrate concentrations were found in petioles and new roots and lowest in stems. Nitrate concentration underwent several-fold changes in most tissues and was increased by shading as well.

Nitrate concentration in laminas and petioles was increased more than ammonium by shading, with the greatest difference (7.5 times) found in mature laminas at anthesis (Fig. 5-6). Shading increased nitrate concentration in tendrils and shoots except at two weeks pre-anthesis in tendrils. Stems from shaded plants had greater nitrate concentrations (Fig. 5-7). Shading increased nitrate concentration in new roots but not in old roots (Fig. 5-8).

With two-year-old vines in 1990, laminas, petioles, and rachis had higher nitrate concentrations in shaded plants (Table 5-2).

Correlations between IN, Ammonium, and Nitrate Concentrations: IN was correlated best with ammonium concentrations in rachis of two-year-old vines (Table 5-3). Significant correlations also existed between IN and ammonium concentrations in laminas and petioles, and nitrate concentration in laminas, petioles, rachis, and tendrils. Tissue ammonium concentration was significantly correlated with nitrate in some tissues of one-year-old vines, especially at post-anthesis (Table 5-4). Tissue ammonium was correlated with nitrate in laminas, petioles, fruit, and rachis, but not in tendrils, in two-year-old vines (Table 5-3).

Discussion

Growth: It is well established that shading reduces photosynthesis, carbohydrate status, and plant dry weight in fruit crops (Flore, 1980, 1981; Grant and Ryugo, 1984a, b; Kappel, 1980, 1981; Rom and Ferree, 1986; Sams, 1980). Similar observations were made with greenhouse-grown 'Pinot noir' grapevines in this study. Decreased dry weight of stems and old roots suggested the consumption of the reserved organic matter. All the new growth occurred on newly formed tissues -- shoots, leaves, clusters, tendrils, and new roots.

Considerable differences in new root dry weight between shaded and unshaded plants suggest that the new photosynthates were unavailable for transport for new growth underground. Less top growth in shaded grape plants may have been caused by ammonium toxicity, as observed in other species (Alan, 1989; Sasseville and Mills, 1979a, b; Soyer *et al.*, 1974).

IN and its Relationship with Tissue Ammonium and Nitrate Concentrations: The increase in IN by shading has been reported by others with test plants (Jackson, 1991; Jackson and Coombe, 1988) and field grown grapevines (Ibacache *et al.*, 1991; Jordan *et al.*, 1991). IN was induced by shading greenhouse-grown vines which also had higher ammonium and nitrate concentrations in various tissues, especially the rachis. Shaded clusters may be more sensitive to phytotoxicity, including that of ammonium. Ammonium is probably one of the most toxic forms of nitrogen for plants. A few milligrams of ammonium per gram dry weight are sufficient to kill tissue (Barker *et al.*, 1966; Maynard and Barker, 1969). There were significant correlations between IN and tissue ammonium concentration. Shading increased tissue ammonium at the very beginning of sampling, much earlier than the time of IN occurrence. These results suggest that elevated ammonium may be the causal agent for IN. Lack of sufficient carbohydrates from shading (Ibacache, 1990) could lead to ammonium accumulation and cause IN.

Ammonium assimilation is the only way plants can reduce elevated ammonium levels, and its assimilation requires carbon substrates like α-keto-glutarate. Shading reduces photosynthesis and carbohydrate levels, in turn the total organic acid concentration, including α-keto-glutarate (Ibacache, 1990). It is possible that the elevated ammonium had maximum toxicity in clusters prior to anthesis, when tissues were most sensitive and the plants had lowest dry weight

(Fig. 5-1). Plant dry weight decreased from bud break and then increased soon after anthesis when enough photosynthates from developed leaves were available for growth. Carbohydrate reserves in the vines are depleted to the lowest levels of the season at bloom (Winkler *et al.*, 1974). Consequently, at bloom the vine is dependent on currently assimilated carbon substrate rather than remobilized carbon reserves. Shading at this stage is likely to have a maximum effect on carbon substrate supply for ammonium assimilation, and therefore on the development of IN.

Shading increased tissue nitrate during and after anthesis and positive correlations existed between IN and nitrate concentrations as well. Most plants tolerate high levels of nitrate without any physiological disorder (Barker and Mills, 1980). We have demonstrated that incubation of clusters with ammonium induces a high incidence of IN while nitrate does not (Gu *et al.*, 1991). In fact, nitrate showed protective effects against IN from ammonium incubation at a late stage (Gu *et al.*, 1991) and it has alleviated ammonium toxicity in other species as well (Goyal *et al.*, 1982a, b). It is reasonable to believe that nitrate is not the direct causal agent of IN. The significant correlation between IN and tissue nitrate concentrations may be due to the correlation between nitrate and ammonium levels, since nitrate may have been a source of elevated tissue ammonium. More nitrate available for the reduction in shaded vines could increase tissue ammonium and cause IN.

Tissue Ammonium Concentrations: Tissue ammonium levels are dynamic pools influenced by input and export components. Ammonium input to a given tissue can occur directly by xylem translocation and penetration of the plasmalemma. Also, many catabolic processes can generate ammonium within the cells (Durzan and Stewart, 1983). The increase of tissue ammonium had little to do with root uptake of nitrogen, and ammonium concentration was generally unaffected by nitrogen source in the root medium (Chapter 6). Nitrate concentration in old roots was not influenced by shading, although the tissue nitrate concentration of new roots was considerably higher in shaded plants. In underground tissues -- stems, old roots, and new roots -- the ammonium concentration was not influenced by shading except at two weeks post-anthesis. Conradie (1986) found that in early season the demand for nitrogen in potted vines was greater than could be supplied by roots. The increase of ammonium induced by shading could be partially due to demobilized reserve nitrogen and the

release of ammonium. Nitrate reduction might elevate ammonium in above-ground tissues, especially at post-anthesis when tissue ammonium was significantly related to tissue nitrate concentration. Under shaded conditions, with decreased carbon substrate, energy, and reduction potential, ammonium assimilation was reduced and this would result in ammonium accumulation in the vines.

Elevated ammonium concentrations in shaded vines after anthesis was explained as a reflection of delayed vine development by Jordan (1989). This may not be the case near anthesis. Shaded and control vines bloomed at about the same time in studies with both one- and two-year-old vines, indicating that shading did not delay development in these greenhouse-grown young vines.

Tissue Nitrate Concentrations: Greater nitrate concentrations were found in various tissues of shaded than unshaded vines. It has been reported that nitrate accumulates in plants under shade (Hageman and Flesher, 1960). Shading increased nitrate concentrations considerably in new roots but did not affect old root nitrate concentration, suggesting that the nitrate transfer through old roots for shaded vines was similar to that for unshaded vines. However, unshaded plants could transfer more nitrate since they probably have a greater transpiration rate. Increased nitrate in above-ground tissues of shaded vines may reflect the decrease in nitrate reduction capacity due to reduced energy and the reduction potential needed by nitrate reduction. Increased nitrate could be from lower nitrate reductase activity because of its light dependency (Beevers *et al.*, 1965; Candela *et al.*, 1957; Hageman and Flesher, 1960; Sanderson and Cocking, 1964) and the inhibitory effect on enzyme induction by high ammonium concentration (Joy, 1969; Orebamjo and Stewart, 1975; Smith and Thompson, 1971). They reported that ammonium inhibits the induction of nitrate reductase in various species.

Summary

Shading reduced vine growth in one- and two-year-old 'Pinot noir' grapevines. There was 26.6% IN on shaded vines compared to only 0.6% on unshaded ones. Tissue ammonium and nitrate decreased during the season and were increased by shading in most tissues. Tissue ammonium correlated with nitrate in tissues at late stages.

Ammonium is one of the most toxic forms of inorganic nitrogen to many plants. Lack of sufficient carbohydrates from shading could lead to ammonium accumulation and cause IN. Significant correlations between IN and tissue ammonium concentrations and early occurrence of higher tissue ammonium in shaded vines suggest that elevated ammonium may be the causal agent for IN in 'Pinot noir' grapevines.

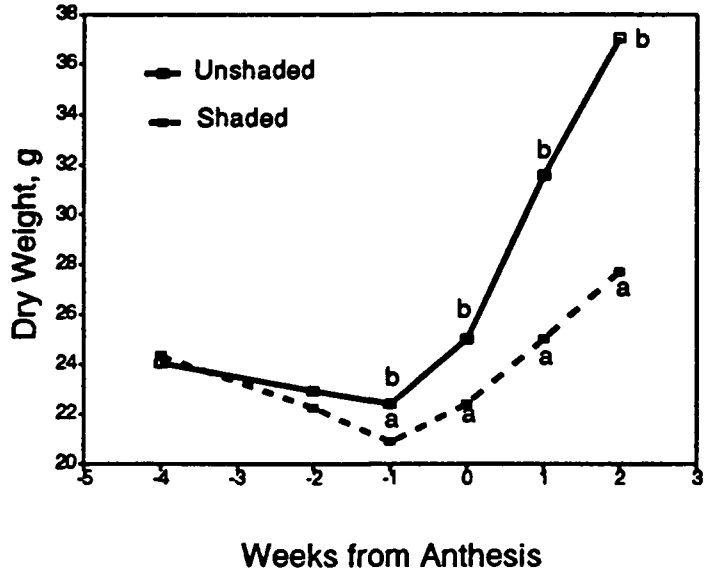

Fig. 5-1. Dry weight of 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

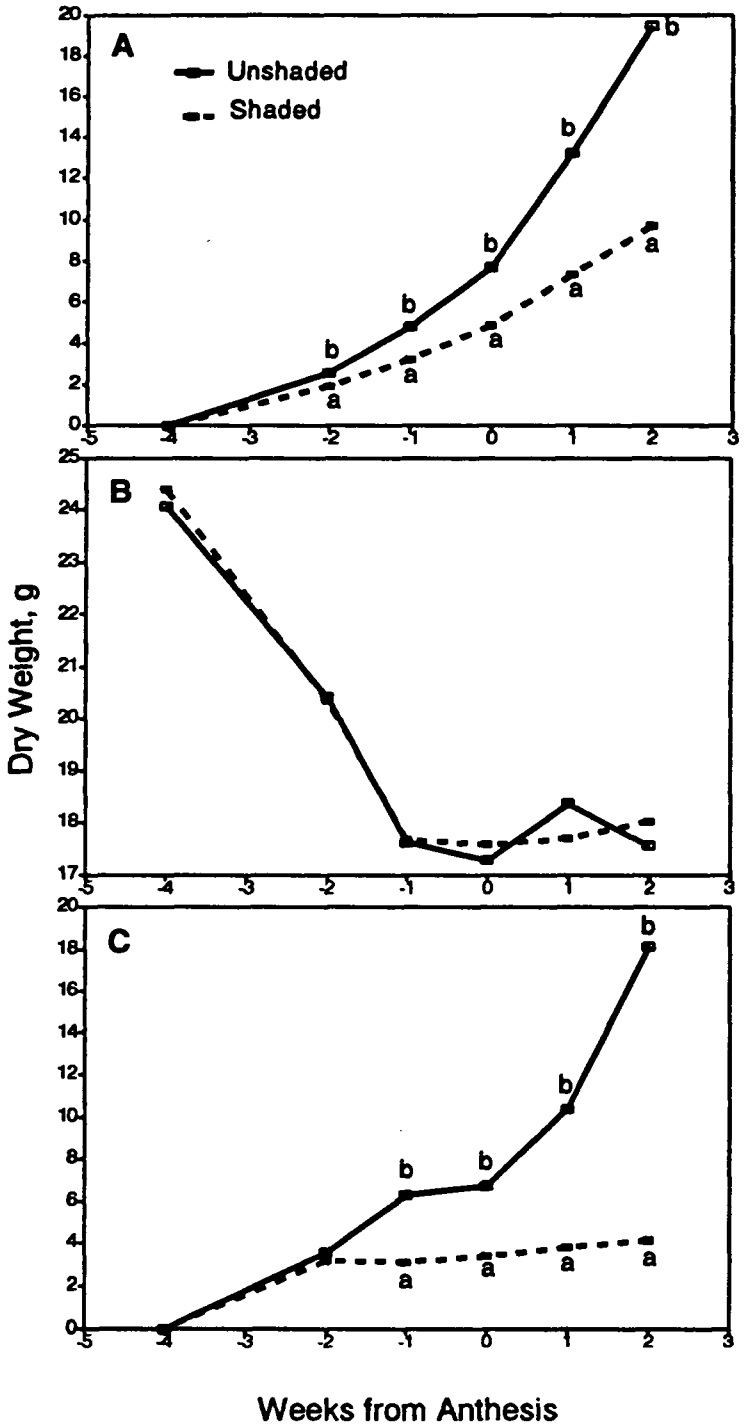

Fig. 5-2. Dry weight of top (A), stem + old roots (B), and new roots (C) of 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P = 0.05 level.

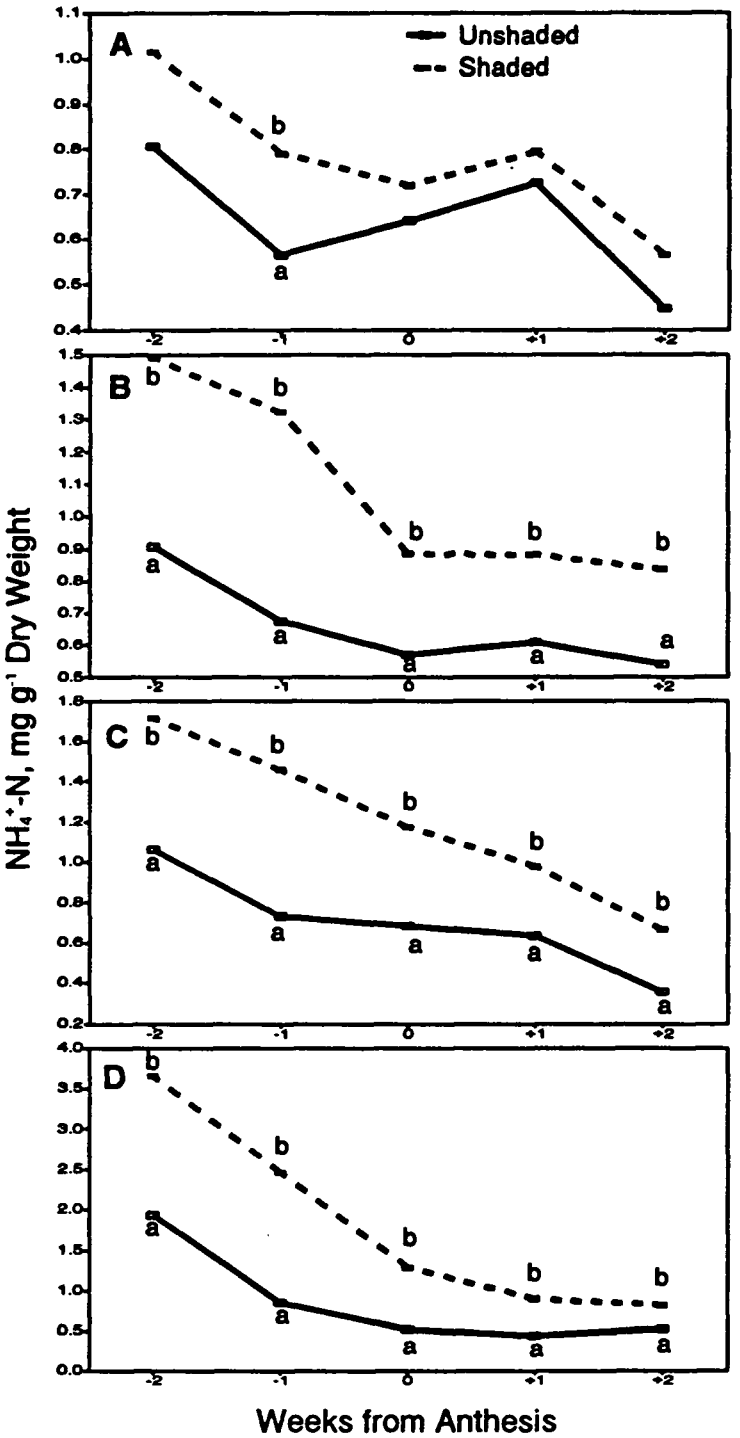

Fig. 5-3. Tissue ammonium of young laminas (A), young petioles (B), mature laminas (C), and mature petioles (D) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

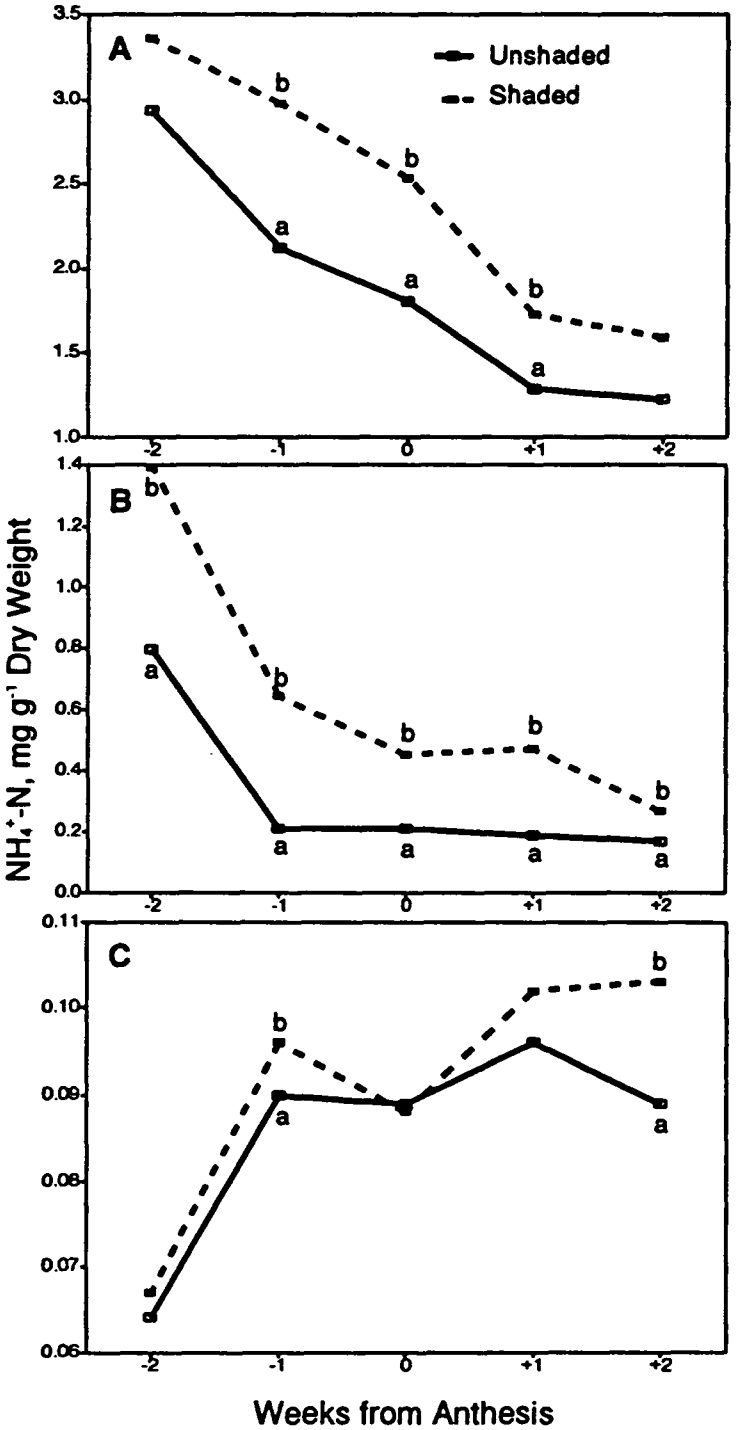

Fig. 5-4. Tissue ammonium of tendrils (A), shoots (B), and stem (C) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

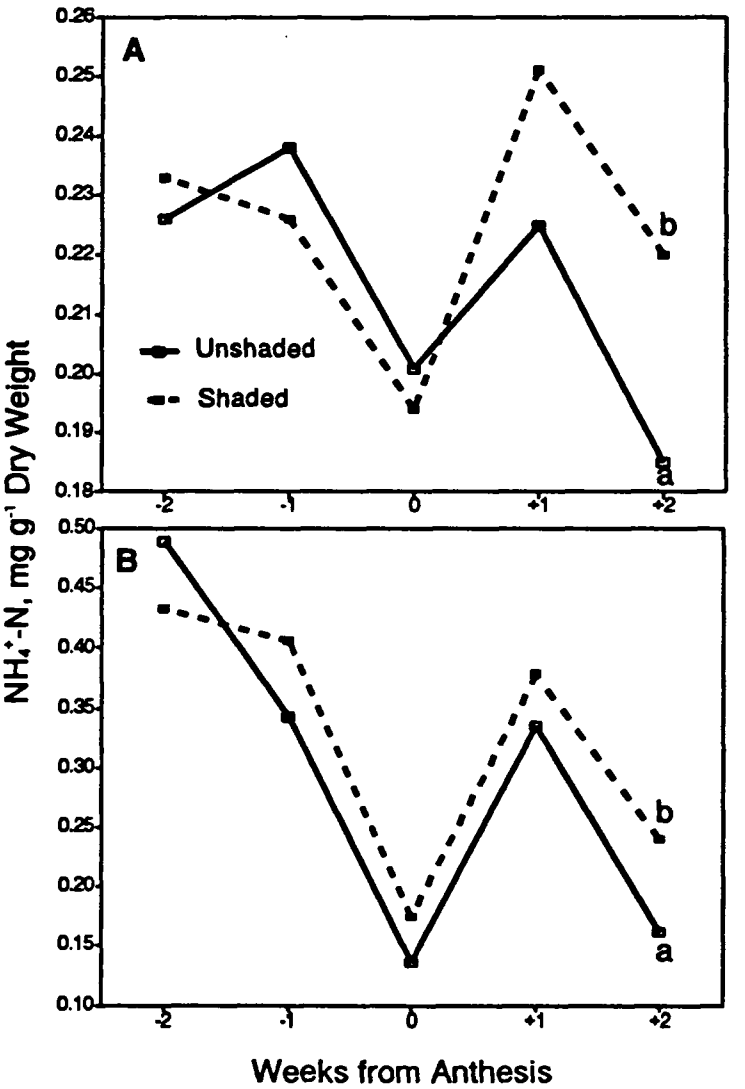

Fig. 5-5. Tissue ammonium of old roots (A) and new roots (B) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

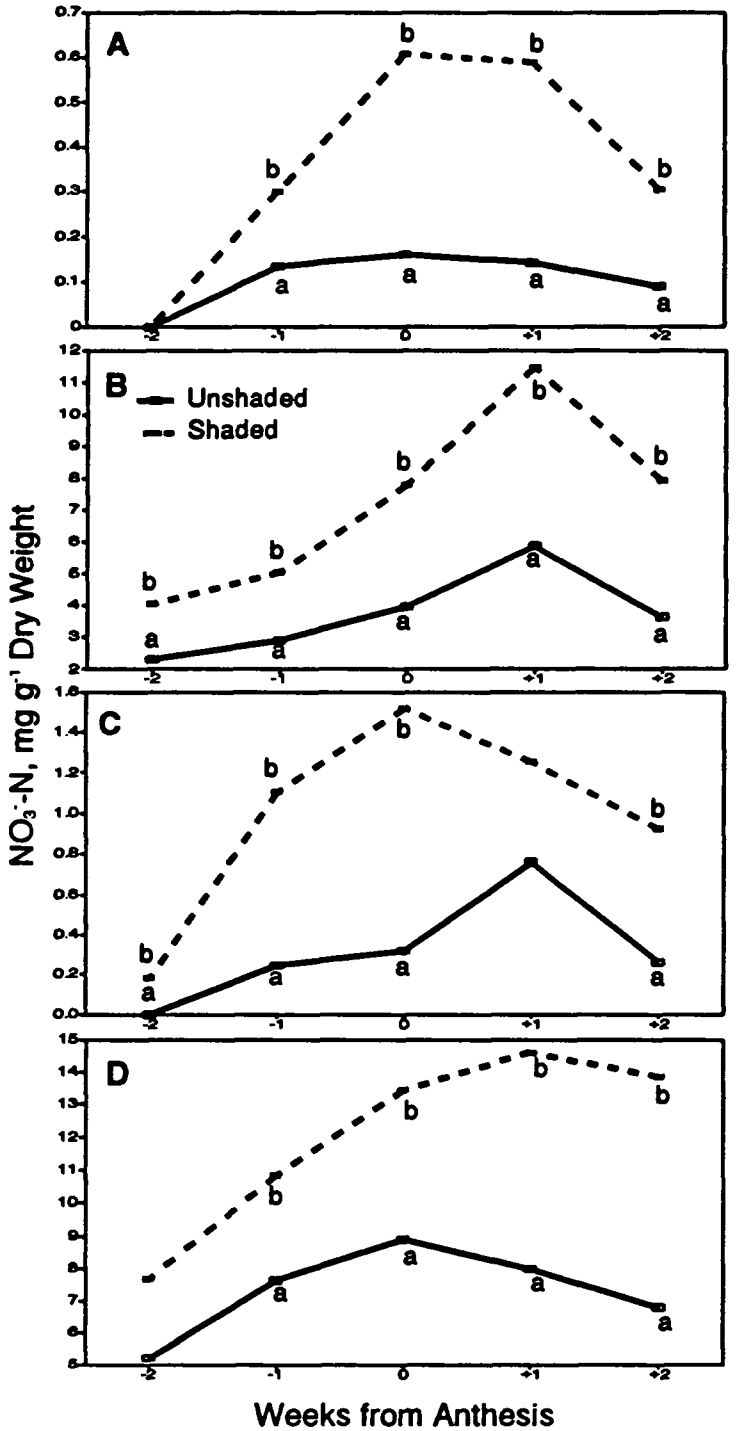

Fig. 5-6. Tissue nitrate of young laminas (A), young petioles (B), mature laminas (C), and mature petioles (D) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

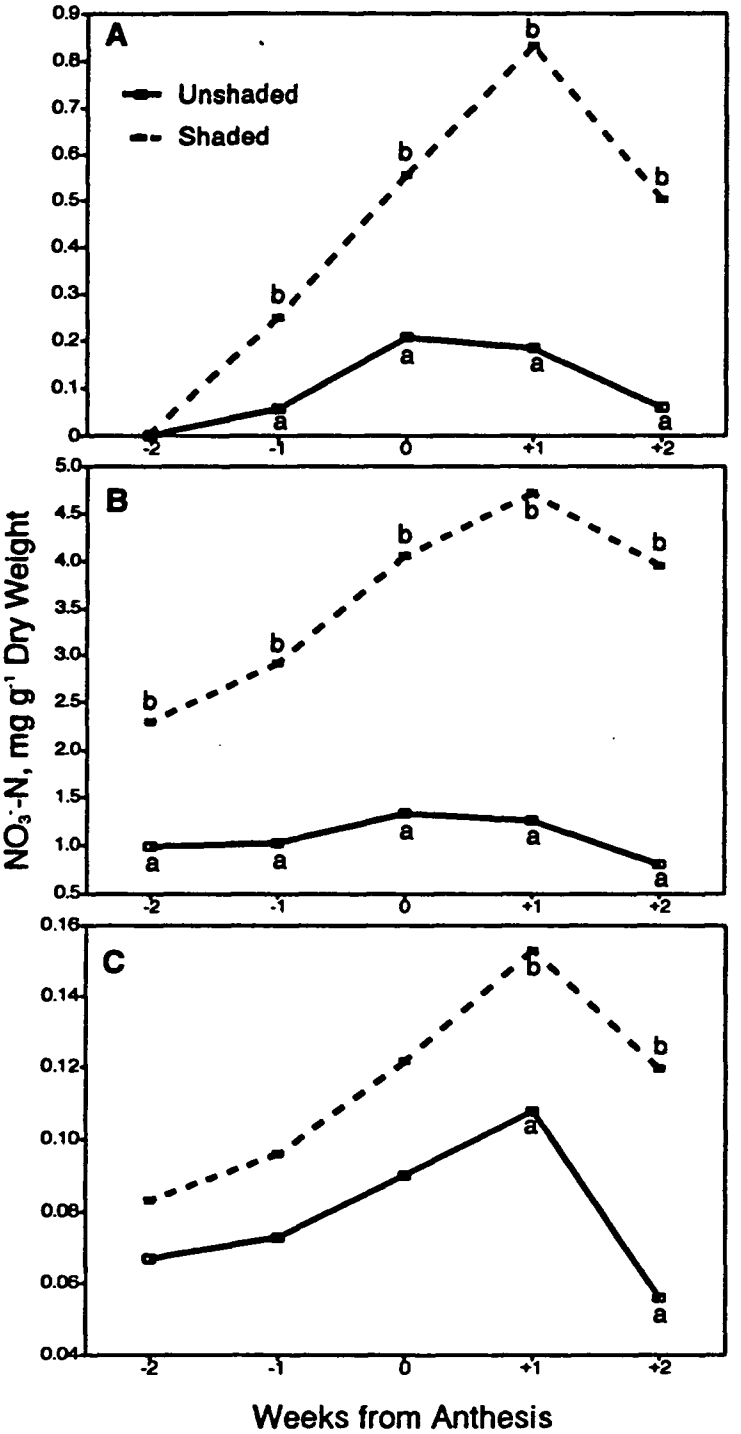

Fig. 5-7. Tissue nitrate of tendril (A), shoots (B), and stem (C) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

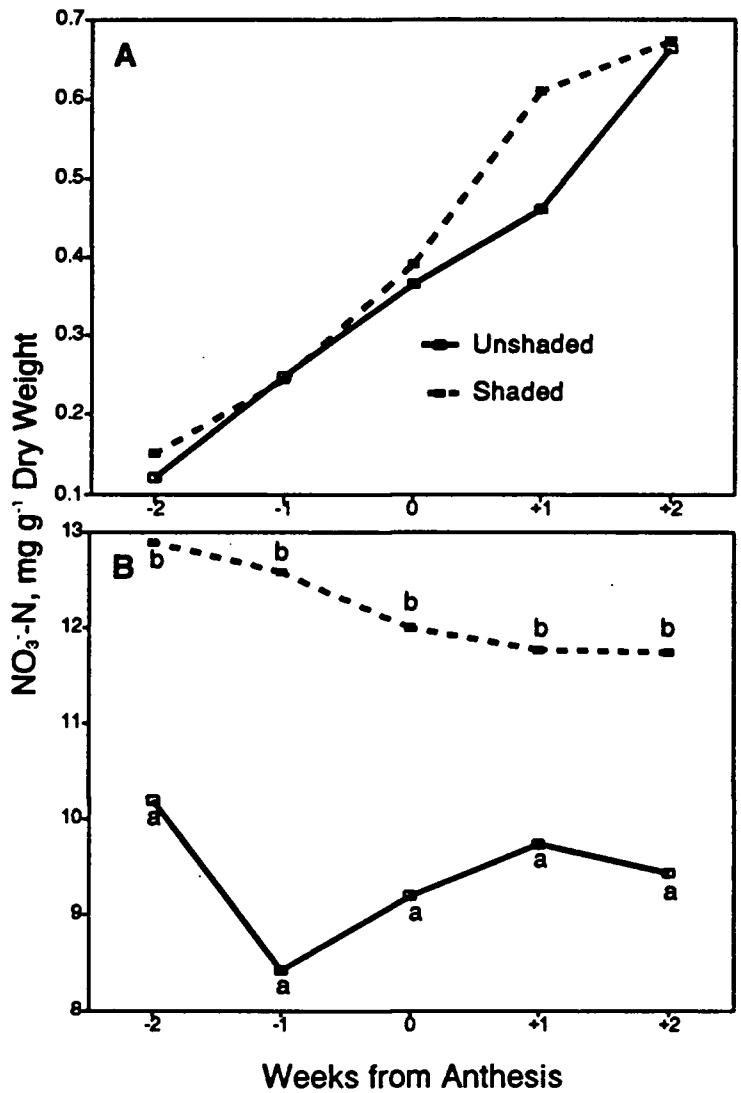

Fig. 5-8. Tissue nitrate of old roots (A) and new roots (B) in 60% shaded and unshaded one-year-old 'Pinot noir' grapevines (Expt. 1, 1989). Different letters for each sampling indicate significant difference at P=0.05 level.

Table 5-1. Effect of shading on growth and inflorescence necrosis in two-year-old 'Pinot noir' grapevines (two weeks post-anthesis, Expt. 2, 1990)

Treatment	Fresh Weight (g)		Number		Necrosis (%)
	Top	Cluster	Fruit	Cluster	
Unshaded	82.7 b[z]	12.1 b	236.7 b	2.3	0.6 a
Shaded	59.7 a	4.0 a	97.5 a	2.3	26.6 b

[z] Different letters indicate significant difference at P=0.05 level.

Table 5-2. Effect of shading on tissue ammonium and nitrate concentrations in two-year-old 'Pinot noir' grapevines (two weeks post-anthesis, Expt. 2, 1990)

Treatment	Rachis	Fruit	Lamina	Petiole	Tendril
		NH_4^+-N, mg g^{-1} Dry Weight			
Unshaded	0.19 a[z]	1.61	0.17 a	0.56 a	2.35
Shaded	2.29 b	1.81	0.79 b	1.15 b	2.41
		NO_3^--N, mg g^{-1} Dry Weight			
Unshaded	0.08 a	0.00	0.39 a	6.70 a	0.12
Shaded	2.76 b	0.06	2.65 b	16.60 b	0.63

[z] Different letters indicate significant difference at P = 0.05 level.

Table 5-3. Correlations between inflorescence necrosis (IN), tissue ammonium, and nitrate concentrations in two-year-old 'Pinot noir' grapevines (two weeks post-anthesis, Expt. 2, 1990)

Correlation	Lamina	Petiole	Fruit	Rachis	Tendril
IN with NH_4^+	.6853***[z]	.7374***	NS	.7751***	NS
IN with NO_3^-	.6106**	.6429**	NS	.7312***	.5493*
NH_4^+ with NO_3^-	.9502***	.7902***	.5421*	.9240***	NS

[z] NS, *, **, and *** indicates non-significant, significant at P=0.05, 0.01, and 0.001 levels, respectively.

Table 5-4. Correlations between tissue ammonium and nitrate concentrations in one-year-old 'Pinot noir' grapevines (Expt. 1, 1989)

	Weeks from Anthesis				
Tissue	-2	-1	0	+1	+2
Tendril	NS[z]	.7266**	NS	.8124**	NS
Young lamina	NS	.4914*	NS	NS	.4712*
Mature lamina	NS	.8079**	NS	.8891***	.8144***
Young petiole	NS	NS	NS	.6467**	.8222***
Mature petiole	NS	NS	NS	.6906**	.5963**
New shoot	NS	NS	NS	.8521***	.7395***
Stock stem	NS	NS	NS	NS	NS
Old root	NS	NS	NS	NS	NS
New root	.5227*	NS	NS	NS	.7294***

[z] NS, *, **, and *** indicates non-significant, significant at P=0.05, 0.01, and 0.001 levels, respectively.

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Chapter 6

Effects of Nitrogen Source on Growth, Tissue Ammonium and Nitrate Status, and Inflorescence Necrosis in 'Pinot noir' Grapevines

Abstract

Rooted cuttings and two-year-old 'Pinot noir' grapevines (*Vitis vinifera* L.) were potted with vermiculite and irrigated with modified Hoagland's nutrient solutions containing one of the following nitrogen sources: 16 mM NO_3^-, 8 mM NO_3^- + 8 mM NH_4^+, or 16 mM NH_4^+. No nitrogen served as control for two-year-old vines. Growth and inflorescence necrosis (IN) were monitored throughout the experiment. In rooted cuttings, ammonium concentration was higher in laminas, petioles, shoots, and new roots but less in tendrils of NH_4^+-fed vines compared to those received NO_3^-. NO_3^--fed vines had higher tissue nitrate in petioles, shoots, new roots, and tendrils, but less in laminas. Two-year-old vines fed either NH_4^+ or NO_3^- had higher rachis and fruit ammonium only at post-anthesis. NH_4^+-fed vines had higher ammonium levels than that of NO_3^--fed vines only in shoots (post-anthesis) and new roots (all three samplings). Tissue nitrate was proportional to the amount of NO_3^- fed to the vines in laminas, petioles, shoots, and new roots. A decrease in growth was observed with NH_4^+-fed vines. No IN was induced by any treatments. Nitrogen feeding is not a source of elevated ammonium for induction of IN.

Introduction

Inflorescence necrosis (IN), or early bunchstem necrosis of grapes (Ibacache *et al.*, 1991; Jackson and Coombe, 1988; Jordan *et al.*, 1991), has been described as a partial or complete breakdown of the rachis or pedicels near bloom. The cause of IN is unknown. No pathogens have been found on the affected tissue and the disorder appears to have a physiological cause (Jackson and Coombe, 1988). It is believed that IN is associated with inorganic nitrogen. Stresses such as shading and drought can increase its incidence (Ibacache *et al.*, 1991; Jackson, 1991; Jackson and Coombe, 1988). Elevated ammonium levels in rachis tissue are associated with IN (Ibacache *et al.*, 1991; Jordan *et al.*, 1991). Also, diammonium phosphate applied to inflorescences one week before flowering caused typical early bunchstem necrosis symptoms (Jackson and Coombe, 1988).

Tissue ammonium levels are dynamic pools influenced by input and export components. Ammonium input to a given tissue can occur directly by xylem translocation. Chang and Kliewer (1991) reported that NH_4^+-fed vines accumulated more free ammonium in petioles at veraison and in petioles and rachis at harvest, compared to those fed NO_3^-. However, there is no information about the effect of nitrogen source on tissue ammonium and nitrate status near anthesis, the critical time for development of IN.

The objective of this experiment was to determine the effect of nitrogen feeding and its source on growth, ammonium and nitrate status, and IN in 'Pinot noir' grapevines.

Materials and Methods

Rooted cuttings and two-year-old 'Pinot noir' grapevines were potted with vermiculite in a greenhouse at 25°/21°C (day/night) and irrigated with modified Hoagland's nutrient solutions containing one of the following nitrogen sources: 16 mM NO_3^-, 8 mM NO_3^- + 8 mM NH_4^+, or 16 mM NH_4^+. Control two-year-old vines received no nitrogen. IN severity was monitored throughout the experiment. Rooted cuttings were harvested two months after budbreak. Mature laminas, petioles, shoots, new roots, and tendrils were sampled for ammonium and nitrate analysis. Two-year-old vines were harvested one week pre-anthesis, anthesis, and one week post-anthesis. Top, stem, and new root fresh weight were taken for the growth responses. Mature laminas, mature petioles, shoots, tendrils, new roots, rachis, and flowers/fruit were sampled for ammonium and nitrate analysis.

Tissue Ammonium and Nitrate Analysis: Tissue samples were dried at 55°C for 48 hours and ground through a 20 mesh screen. A sample of 100 g dried tissue was extracted in 10 ml 2% (v:v) acetic acid solution for 1 hour on a shaker and then let stand for 30 minutes at room temperature. Extracts were filtered through an in-tube Plasma/Serum separator (Karlan Chem. Corp., Torrance, California). Ammonium concentration was determined on a Wescan Model 360 ammonia analyzer (Alltech Assoc., Inc./Wescan Instruments, San Jose, California). Nitrate was reduced to ammonium with a continuous flow reduction method developed by Carlson (1986) and analyzed in the same way as ammonium. Ammonium and nitrate concentration was expressed as mg N g^{-1} dry weight.

Experimental Design and Data Analysis: A completely randomized experimental design with 5 replications was used in this study. Means were separated with Duncan's multiple range test at $P=0.05$ level.

Results

Rooted Cuttings: Ammonium concentration increased with NH_4^+ feeding in laminas, shoots, and new roots (Table 6-1). But ammonium was highest in tendrils of NO_3^--fed vines. Tissue nitrate was increased by NO_3^- feeding in petioles, shoots, new roots, and tendrils. Lamina nitrate level was not influenced by nitrogen source. No IN data were available for rooted cuttings since no clusters developed on these plants.

Two-Year-Old Plants: Nitrogen source had significant influence on growth one week post-anthesis when top fresh weight was increased on NO_3^--fed vines, whereas growth of NH_4^+-fed vines was similar to that of the control (Fig. 6-1). Growth of clusters and new roots was not influenced by nitrogen source. No IN was induced by either the source or the feeding of nitrogen.

Tissue ammonium peaked at anthesis in new roots and rachis, but decreased in other tissues from pre-anthesis to post-anthesis (Figs. 6-2 to 6-4). Rachis and new root ammonium levels were higher than other tissues. Tissue ammonium was affected by nitrogen source. Compared to the control, vines fed any nitrogen had higher tissue ammonium levels in all sampled tissues one week post-anthesis (Figs. 6-2 to 6-4), in laminas, tendrils, and new roots at anthesis (Fig. 6-2A, Fig. 6-3A, C), and in new roots one week pre-anthesis (Fig. 6-3C). Tissue ammonium in laminas, new roots, and fruit was higher in NH_4^+-fed vines than NO_3^--fed vines.

Tissue nitrate levels increased from pre-anthesis to post-anthesis except in new roots and flowers/fruit (Figs. 6-5 and 6-6). Nitrate was higher in petioles and new roots than other tissues. Tissue nitrate increased in laminas, petioles, shoots and new roots at all three samplings with NO_3^- feeding (Fig. 6-5). The increase of tissue nitrate was nearly proportional to the NO_3^- concentration in the nutrient solutions. Highest tissue nitrate was found in the vines fed with 16 mM NO_3^-, followed by vines fed with 8 mM NO_3^- + 8 mM NH_4^+, and lowest in vines fed with 16 mM NH_4^+ and no nitrogen control. Tissue nitrate levels were not influenced by either NO_3^- or NH_4^+ feeding in tendrils, rachis, flowers, and fruit (Fig. 6-6). NH_4^+ feeding did not increase tissue nitrate over the control vines in any tissue.

Discussion

Growth: Nitrogen source had a significant influence on top fresh weight, and the effect seemed to be accumulative. NO_3^- feeding was superior over NH_4^+ and no nitrogen for vine growth, suggesting the preference of 'Pinot noir' grapevines for NO_3^-. This is consistent with that reported by Chang and Kliewer (1991). Lack of growth responses to nitrogen source before anthesis may be have been due to the dependence of the vine growth on reserve nitrogen in the permanent parts of the vines (Conradie, 1986, 1991). The reduction of growth on NH_4^+-fed vines may be due to the uncoupled photophosphorylation induced by ammonium toxicity (Good, 1977; Hageman, 1984; Sauer *et al.*, 1987), and higher demand of carbohydrate channeled to ammonium assimilation (Allen *et al.*, 1985) and detoxification (Givan, 1979).

Tissue Ammonium and Nitrate Concentrations: Ammonium concentrations in various tissues were influenced either by amount of nitrogen supplied or by nitrogen source in the later stages. Tissue ammonium responded to NH_4^+ and/or NO_3^- feeding only until anthesis (lamina and tendrils), or even one week post-anthesis (petioles, shoots, flowers/fruit, and rachis), but ammonium was increased in new roots at all samplings from NH_4^+-fed vines. This suggests the dependence of tissue ammonium on reserve nitrogen (Conradie, 1986, 1991). NH_4^+ feeding increased tissue ammonium concentration only in new roots (all three samplings) and shoots (post-anthesis), compared to NO_3^- feeding. But supplementary nitrogen feeding increased ammonium concentrations over no nitrogen control in other tissues. However, ammonium was influenced by nitrogen source in all sampled tissues of rooted cuttings. These results suggest that grapevines take up ammonium from the culture media and transfer it to above-ground tissues. The response of tissue ammonium to nitrogen source was less than tissue nitrate, indicating supplied ammonium is at least no more efficient than nitrate in elevating ammonium or nitrate concentration in aerial tissues especially clusters. Therefore, the differences between rooted cuttings and two-year-old vines may reflect the effect of established root system.

New root, shoot and petiole nitrate levels were related proportionally to the amount of NO_3^- supplied, in contrast to that of tendrils and cluster tissues. The proportional relationship between tissue nitrate levels and nitrate concentration in

nutrient solution may suggest that the absorption and translocation of nitrate is driven by transpiration flow.

IN and its Relationship to Tissue Ammonium and Nitrate Concentration: IN begins just before anthesis and is associated with tissue ammonium (Ibacache *et al.*, 1991; Jordan *et al.*, 1991; Gu *et al.*, 1991). So the concentration of ammnium in cluster tissues before anthesis may play an important role in induction of IN. The results indicate that rachis and flower ammonium concentration is not increased by either NO_3^- or NH_4^+ feeding until post-anthesis and no IN developed in reponse to either nitrogen source. Therefore, the NH_4^+ or NO_3^- supplied in culture media is not the source for elevated tissue ammonium levels responsible for the development of IN.

Summary

In rooted cuttings, tissue ammonium concentration was higher in laminas, petioles, shoots, and new roots of NH_4^+-fed vines. NO_3^--fed vines had higher tissue nitrate concentrations in petioles, shoots, new roots, and tendrils. Two-year-old vines fed either NH_4^+ or NO_3^- had higher ammonium concentrations in rachis and fruit only at post-anthesis. NH_4^+-fed vines had higher ammonium levels than those fed NO_3^- only in shoots (post-anthesis) and new roots (all three samplings). Tissue nitrate concentration was higher in laminas, petioles, shoots, and new roots of NO_3^--fed vines. IN was not induced by either nitrogen source. Supplementary nitrogen feeding is not the major source for elevated ammonium responsible for the development of IN.

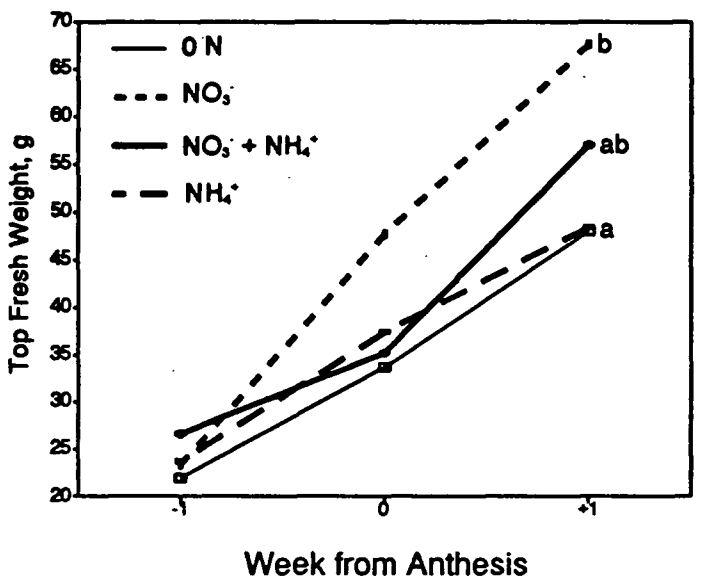

Fig. 6-1. Top growth of two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^- + 8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P = 0.05 level.

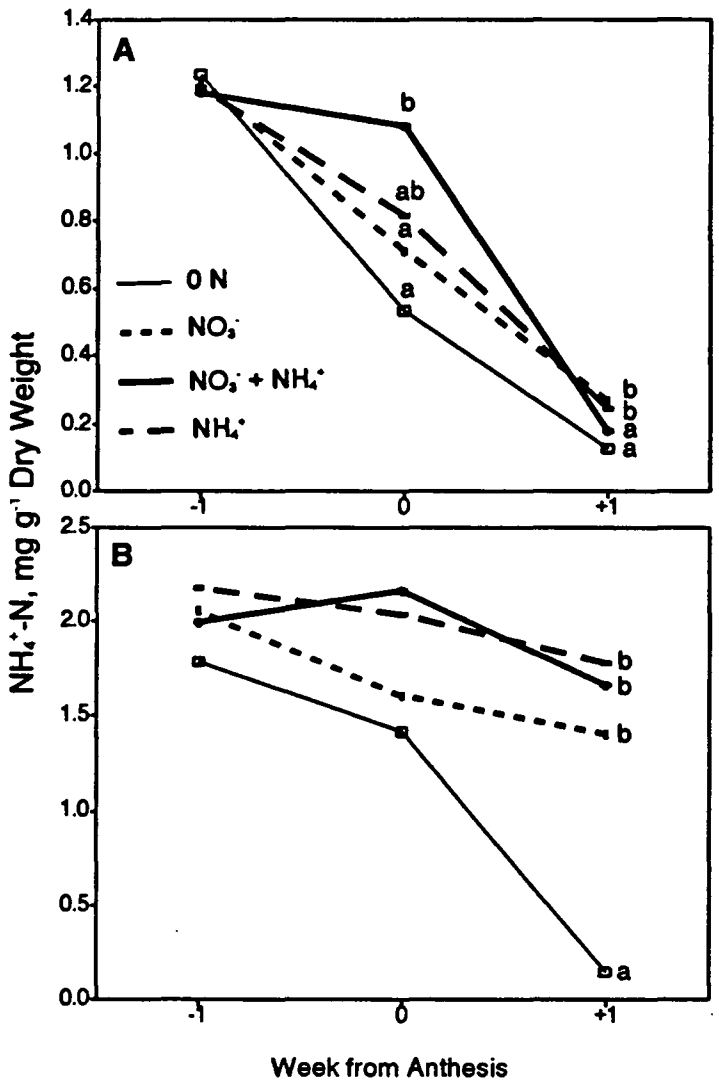

Fig. 6-2. Tissue ammonium concentrations of laminas (A) and petioles (B) in two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^- + 8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P = 0.05 level.

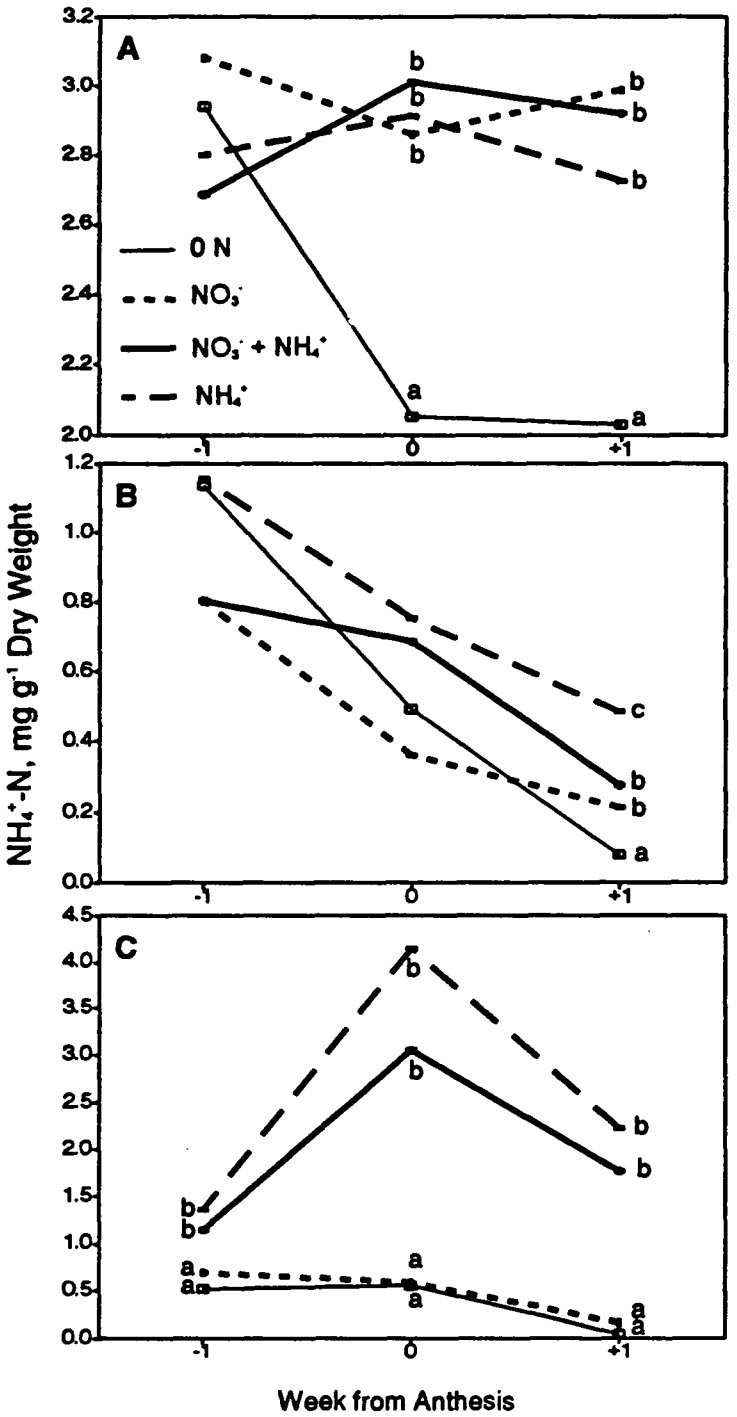

Fig. 6-3. Tissue ammonium concentrations of tendril (A), shoots (B), and new roots (C) in two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^- + 8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P = 0.05 level.

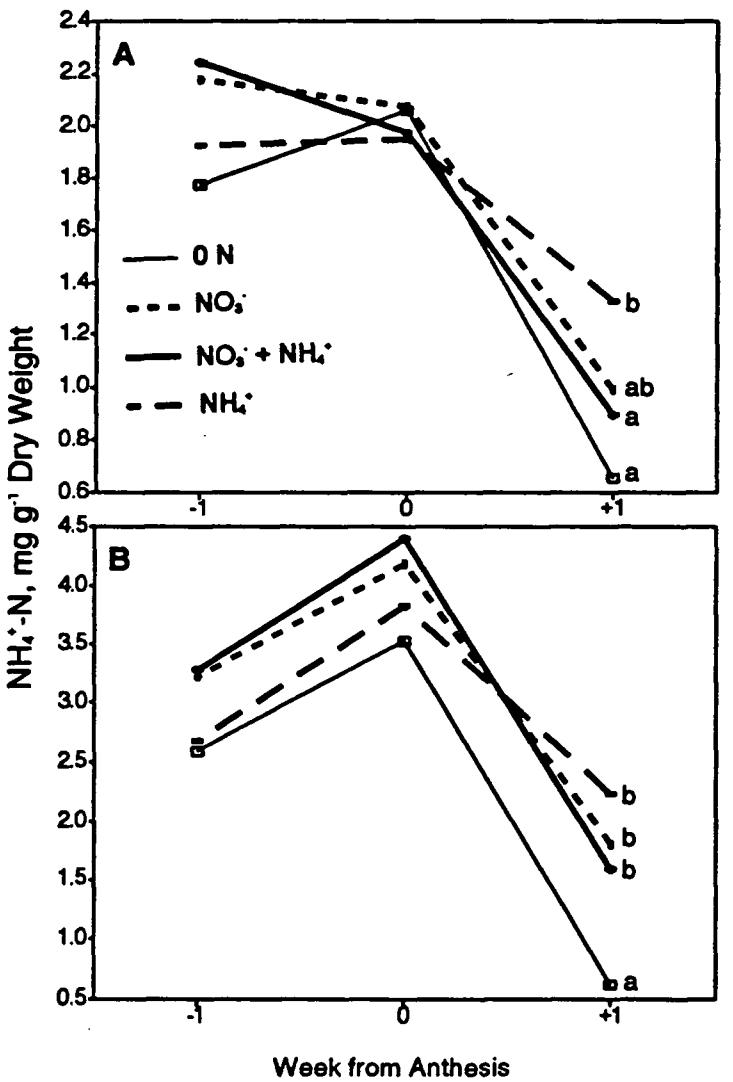

Fig. 6-4. Tissue ammonium concentrations of flowers/fruit (A) and rachis (B) in two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^-+8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P=0.05 level.

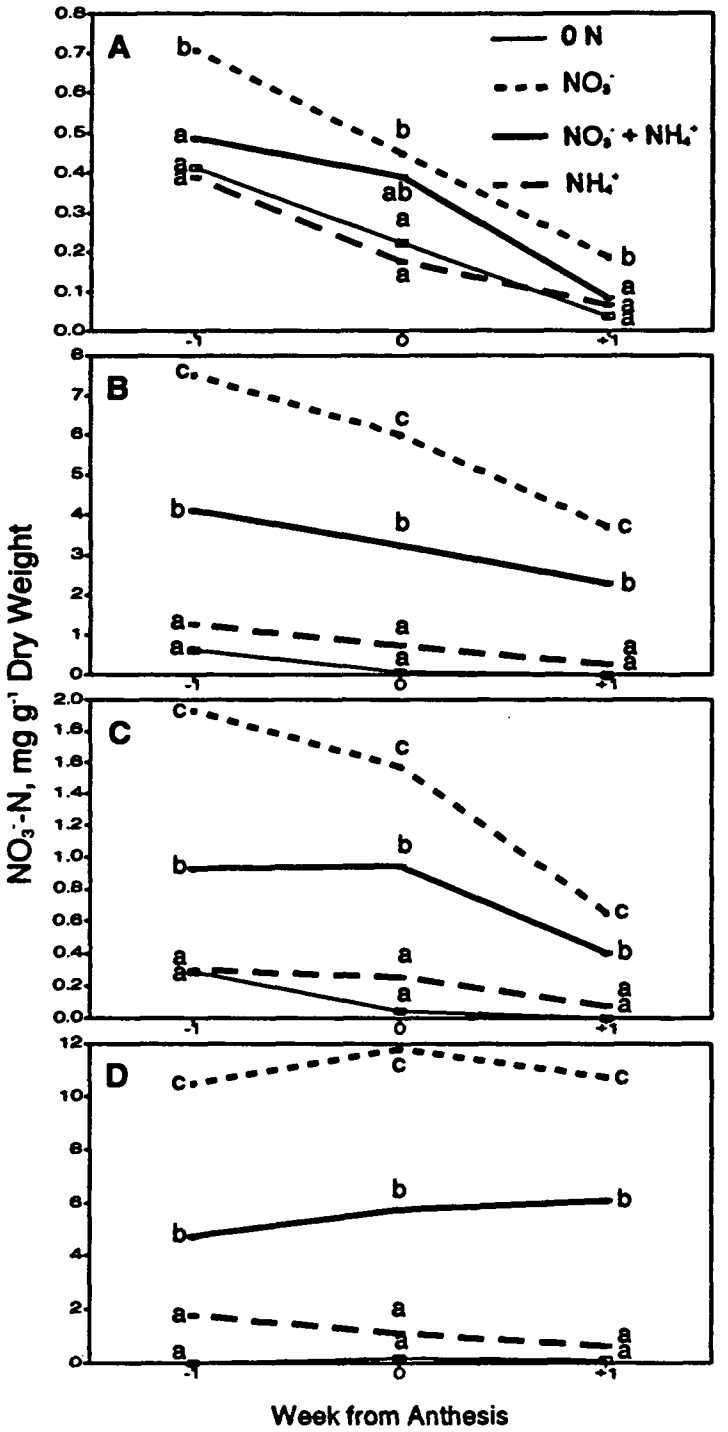

Fig. 6-5. Tissue nitrate concentrations of laminas (A), petioles (B), shoots (C), and new roots (D) in two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^-+8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P=0.05 level.

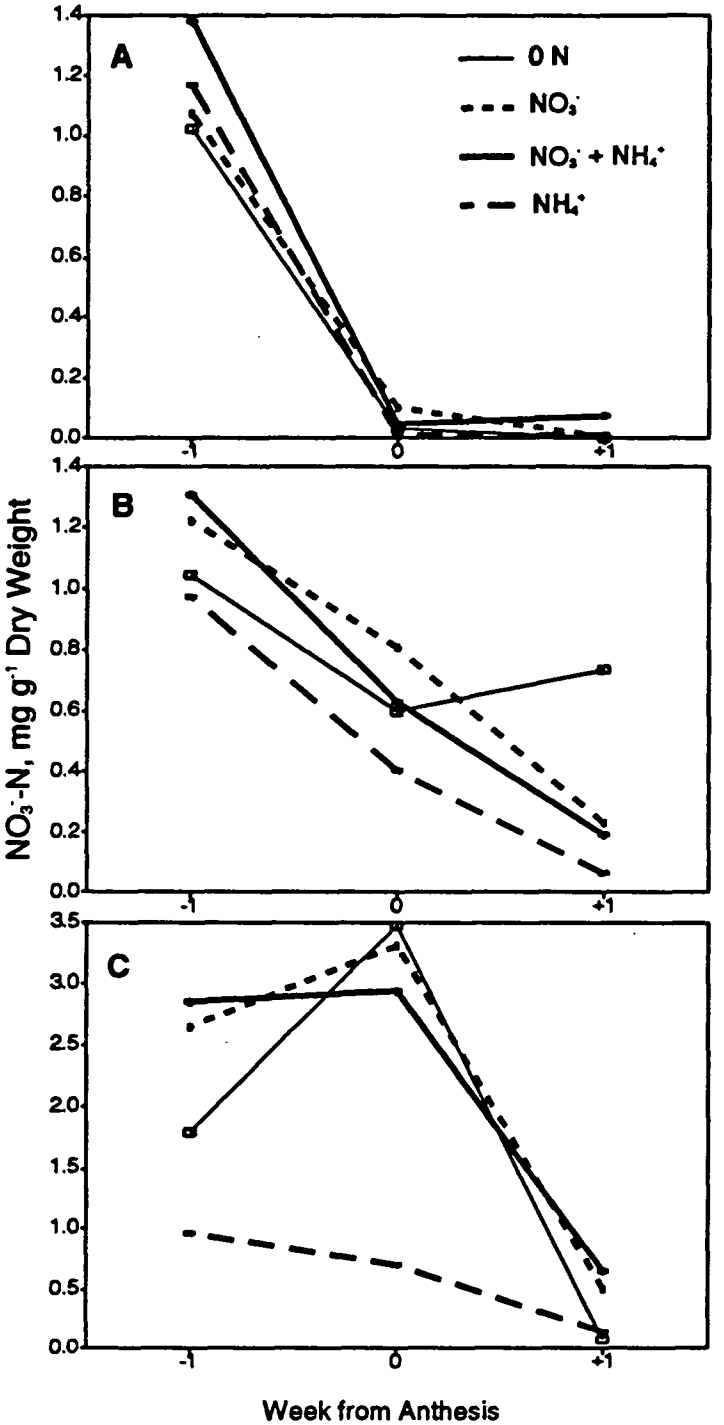

Fig. 6-6. Tissue nitrate concentrations of tendrils (A), flowers/fruit (B), and rachis (C) in two-year-old 'Pinot noir' grapevines fed with no N, 16 mM NO_3^-, 8 mM NO_3^-+8 mM NH_4^+, and 16 mM NH_4^+. Mean separation for each sampling by Duncan's multiple range test at P=0.05 level.

Table 6-1. Effect of nitrogen source on tissue ammonium and nitrate concentrations in rooted cuttings of 'Pinot noir' grapevines

N Feeding	Lamina	Petiole	Shoot	Root	Tendril
		NH_4^+-N, mg g^{-1} Dry Weight			
16 mM NO_3^-	0.34 a[z]	0.69 a	0.21 a	0.19 a	2.42 b
8 mM (NO_3^- + NH_4^+)	0.40 ab	1.38 b	0.27 a	0.68 b	1.97 a
16 mM NH_4^+	0.53 b	2.22 c	0.40 b	1.54 c	2.03 a
		NO_3^--N, mg g^{-1} Dry Weight			
16 mM NO_3^-	1.05	9.19 b	2.52 c	11.92 c	3.12 b
8 mM (NO_3^- + NH_4^+)	0.80	9.59 b	2.02 b	9.72 b	2.53 a
16 mM NH_4^+	0.84	5.92 a	1.21 a	3.03 a	2.59 a

[z] Mean separation in columns for ammonium or nitrate by Duncan's multiple range test at P = 0.05 level.

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