Inflorescence Necrosis Induced from Ammonium Incubation and Deterred by α-Keto-Glutarate and Ammonium Assimilation in Pinot noir Grapevines

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Inflorescence necrosis (IN) and increased tissue NH_4^+ occurred after incubating single-node, field-grown, Pinot noir grape (*Vitis vinifera* L.) cuttings with one leaf and cluster in 120 m*M* or higher NH_4^+ solutions of NH_4NO_3 or $(NH_4)_2SO_4$. Incubation with solutions of NO_3^-, $SO_4^=$, and K^+ at the same concentrations did not induce IN. The addition of 80 m*M* KG, a substrate for ammonium assimilation, to an incubation solution containing 160 m*M* NH_4^+ deterred IN and lowered flower or fruit NH_4^+ to near control levels. The presence of glutamine synthetase/glutamate synthase (GS/GOGAT) pathway of ammonium assimilation was detected in flowers, fruit, and pedicels, but not in rachis of cuttings treated with GS/GOGAT inhibitors: *i.e.*, 10 m*M* MSX (a GS inhibitor) or 5 m*M* AS (a GOGAT inhibitor). Glutamate dehydrogenase (GDH) is not involved in ammonium assimilation and detoxification in grape inflorescence. Chemical names used: α-keto-glutarate (KG), methionine sulfoximine (MSX), azaserine (AS).

KEY WORDS: ammonium, inflorescence necrosis, glutamine synthetase/glutamate synthase, glutamate dehydrogenase, *Vitis vinifera*

Nitrogen metabolism in grapes and high levels of tissue NH_4^+ have recently been associated with two disorders: inflorescence necrosis (IN), or early bunchstem necrosis (12,13,14), and bunchstem necrosis, or waterberry (5,14). Both disorders can cause economic crop losses (14).

IN is described as a partial or complete breakdown of the rachis or pedicel near bloom (12). The cause of IN is unknown. No pathogens have been associated with the affected tissue, and the disorder appears to have a physiological cause (13). Stresses such as shading and drought can increase its incidence (12,13). Elevated NH_4^+ level in rachis tissue was linked with a higher incidence of IN (12,14), and diammonium phosphate applied to inflorescences one week before flowering caused typical symptoms of early bunchstem necrosis (13). 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 the low availability of carbon substrates essential for ammonium assimilation (3,9). Assimilation of ammonium into amino acids and proteins is the main defensive mechanism for plants to prevent NH_4^+ accumulation and toxicity (9). Jordan (14) reduced NH_4^+ concentration and necrosis with a carbon substrate, α-keto-glutarate (KG), after NH_4^+ incubation in grape tendrils. To understand the factors which affect the buildup of NH_4^+ in grape tissues, it is necessary to understand the enzyme systems for ammonium assimilation and detoxification. The activity of ammonium assimilation pathways in grape, especially in pedicel and rachis tissues, may be critical to the development of these two disorders.

The glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is the primary route for ammonium assimilation in leaves of higher plants (15,24). Several researchers have suggested an assimilatory role for glutamate dehydragenase (GDH) in some plant tissues (17,23,25). GS and GDH activities have been detected in grape berries, roots, and leaves (8,20,21). However, Roubelakis-Angelakis and Kliewer (21) were unable to detect GOGAT activity.

Inhibitors of the GS/GOGAT pathway, such as methionine sulfoximine (MSX) and azaserine (AS), have been widely used in studies of plant carbon-nitrogen metabolic pathways. Treatment of photosynthesizing plants with MSX, a specific GS inhibitor, leads to a large-scale NH_4^+ accumulation in various species (1,2,7,18,22), while the CO_2 fixation process (19), nitrate reductase (7), or GDH activity (2,4) were not affected. AS, an analog of glutamine, inhibits all glutamine amide transfer reactions, including GOGAT, but it has no effect on GDH activity (6,15).

Our objectives were to investigate NH_4^+ as the possible cause of IN, to determine the effect of KG on IN and NH_4^+ accumulation, and to verify the presence of the GS/GOGAT and GDH pathways in cluster tissues of Pinot noir grapevines.

Materials and Methods

Plant material from mature Pinot noir grapevines (*Vitis vinifera* L.) grown at Beaver Creek Vineyard near Corvallis, Oregon, were used in the incubation experiments. The vineyard is excessively vigorous and has a history of severe IN.

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Incubation experiments were conducted one week pre-anthesis, anthesis, and one week post-anthesis in 1990 and 1991. Plant material for all incubations was collected as follows. 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. To incubate, the basal ends of the cuttings were immersed in the treatment solutions in 20-mL plastic vials. Additional solution was added as necessary. Incubation was continued for 48 hours in a greenhouse at temperatures of 25°C/21°C (day/night) in Experiment 1 or six hours at 25°C in Experiments 2 and 3.

Induction and prevention of IN (Expt. 1, 1990 and 1991): The treatment solutions for incubating the cuttings were: 160 m*M* NH_4NO_3 (160 m*M* NH_4^+ and 160 m*M* NO_3^-); 80 m*M* $(NH_4)_2SO_4$ (160 m*M* NH_4^+); 80 m*M* $(NH_4)_2SO_4$ plus 80 m*M* KG; and distilled water as a control. All solutions except the distilled water were adjusted to pH 6.5 with KOH. Solution of KNO_3 and K_2SO_4 with K^+ and $SO_4^=$ at 160 m*M* were used to test the effect of these ions on IN. Cuttings were also incubated in 0, 60, 80, 120, 160, and 200 m*M* NH_4^+ solutions of $(NH_4)_2SO_4$ to establish the NH_4^+ threshold for IN development and the relationship between IN and tissue NH_4^+ concentration.

GS/GOGAT inhibition (Expt. 2, 1990 and 1991): Cuttings were incubated in 10 m*M* KNO_3 solutions with or without the GS/GOGAT inhibitors, MSX (at 10 m*M*), or AS (at 5 m*M*) to detect the presence of GS/GOGAT pathway of ammonium assimilation. The AS treatments were applied only one week post-anthesis in 1990.

GDH pathway (Expt. 3, 1991): Cuttings were incubated in 40 m*M* NH_4^+ solutions of $(NH_4)_2SO_4$ with or without 10 m*M* MSX and/or 20 m*M* KG to test for the presence of the GDH pathway.

Necrosis was determined as a percentage of necrotic tissue area on the rachis and pedicel.

Flowers at one week pre-anthesis and at anthesis, or fruit at one week post-anthesis, with pedicels (Expt. 1) or without pedicels (Expts. 2 and 3), pedicels (Expts. 2 and 3), and the rachis were sampled after 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 one hour on a shaker and then left to stand for 30 minutes at room temperature. Extracts were filtered through an in-tube Plasma/Serum separator (Karlan Chem. Corp., Torrance, CA). Ammonium concentration was determined on Wescan Model 360 ammonia analyzer (Alltech Assoc., Inc./Wescan Instruments, San Jose, CA) and expressed as NH_4^+-N mg/g dry weight.

A completely randomized experimental design with five replications was used for all experiments. Data were analyzed by ANOVA or correlation/regression analysis as appropriate. Mean separation was by Fisher's LSD test.

Results

Induction and prevention of IN (Expt. 1): IN symptoms (both rachis and pedicel necrosis) occurred only when cuttings were incubated in NH_4NO_3 and $(NH_4)_2SO_4$ (Fig. 1A, B). More severe damage occurred on pedicels than rachis at all stages in both years. There

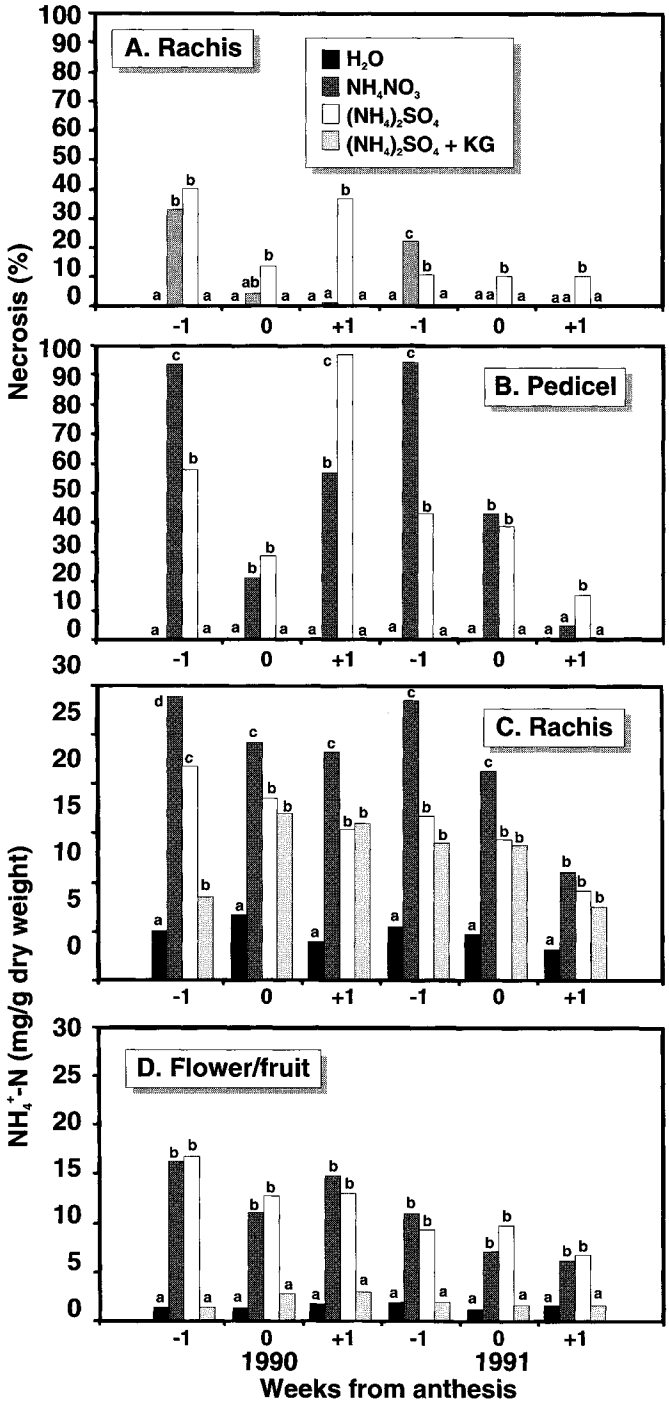

Fig. 1. Effect of incubating single-node Pinot noir grape cuttings with NH_4NO_3, $(NH_4)_2SO_4$, and α-keto-glutarate (KG) solutions on necrosis of rachis (A) and pedicel (B) and tissue NH_4^+ of rachis (C) and flowers/fruit (D) (Expt. 1, 1990 and 1991). All solutions were adjusted to a pH of 6.5 with KOH except H_2O. Mean separation for each sampling by Fisher's LSD test at $p = 0.05$ level.

was no IN in the $(NH_4)_2SO_4$ solution with added KG. No IN was found on clusters incubated with KNO_3 and K_2SO_4, but leaf injury occurred with 160 m*M* solution of K_2SO_4 (data not shown). The incubation with $(NH_4)_2SO_4$ induced similar rachis necrosis pre-anthesis in 1990, but greater rachis necrosis pre-anthesis in 1991 compared to that with with NH_4NO_3. Also, $(NH_4)_2SO_4$ gave higher rachis necrosis at anthesis and post-anthesis in both years. NH_4NO_3 pre-anthesis induced greater pedicel necrosis, whereas $(NH_4)_2SO_4$ post-anthesis gave greater rachis necrosis (Fig. 1A, B).

Table 1. Effect of incubating single-node cuttings with $(NH_4)_2SO_4$ on inflorescence necrosis severity and NH_4^+ concentration of rachis and flowers/fruit in Pinot noir grapevines (Expt. 1, 1991).

NH_4^+-N concentration (m*M*)	Necrosis severity (%)		NH_4^+-N (mg/g dry wt)	
	Pedicel	Rachis	Rachis	Flower/fruit
		Pre-anthesis		
0	0	0	5.43	1.97
60	0	0	11.64	2.80
80	0	0	13.20	3.85
120	8	2	16.53	6.24
160	43	10	16.65	9.33
200	90	56	19.42	14.03
Significance[z]	**L **Q	*L	***L	***L ***Q
		Anthesis		
0	0	0	4.56	1.00
60	0	0	8.34	1.67
80	0	0	10.84	2.53
120	35	0	10.58	6.50
160	38	11	14.40	9.91
200	72	52	12.97	13.44
Significance	**L	*L*Q	*L	***L*Q
		Post-anthesis		
0	0	0	2.81	1.42
60	0	0	3.55	1.87
80	0	0	5.35	4.44
120	11	13	7.96	5.24
160	15	10	9.06	6.56
200	42	24	8.90	7.56
Significance	**L *Q	*L	*L	**L

z *, ** significant at $p = 0.05$ and 0.01 levels, respectively.
L, linear; Q, quadratic.

Table 2. Correlation coefficients between inflorescence necrosis severity and tissue NH_4^+ concentrations in single-node Pinot noir grape cuttings incubated with 0 to 200 m*M* NH_4^+ (Expt. 1, 1991).

Tissue NH_4^+-N	Necrosis severity	
	Pedicel	Rachis
	Pre-anthesis	
Rachis	0.638**z	0.504**
Flower	0.883**	0.803**
	Anthesis	
Rachis	ns	ns
Flower	0.757**	0.689**
	Post-anthesis	
Rachis	ns	ns
Fruit	0.459*	ns

[z]ns, *, ** non-significant, significant at $p = 0.05$ and $p = 0.01$ levels, respectively.

Tissue NH_4^+ concentrations increased several-fold with both NH_4NO_3 and $(NH_4)_2SO_4$ incubation (Fig. 1C, D). Flowers and fruit had lower NH_4^+ concentrations than the rachis. Rachis NH_4^+ was generally higher when cuttings were incubated in NH_4NO_3 than $(NH_4)_2SO_4$. Flower and fruit NH_4^+ varied but was similar for NH_4NO_3 and $(NH_4)_2SO_4$ incubations. The addition of KG reduced flower and fruit NH_4^+ to the control level at all three stages in both years, while rachis NH_4^+ concentration was reduced only pre-anthesis in 1990.

Tissue NH_4^+ concentration and necrosis exhibited either a linear or quadratic correlation to the NH_4^+ concentration of incubation solutions (Table 1). Necrosis was associated with solution NH_4^+ levels above 120 m*M*. The strongest correlation between IN severity and tissue NH_4^+ was found pre-anthesis (Table 2). Flower NH_4^+ had a better correlation with necrosis severity than did rachis NH_4^+. Correlation between flower NH_4^+ concentration and pedicel or rachis necrosis pre-anthesis is presented in Figure 2.

GS/GOGAT inhibition (Expt. 2): The GS inhibitor MSX increased tissue NH_4^+ concentration in flowers, fruit, and pedicels in both years (Fig. 3). MSX increased NH_4^+ concentration in flowers by 17% and in fruit by 80% in 1990 and in flowers by 69% one week pre-anthesis in 1991 (Fig. 3A). MSX also increased NH_4^+ in pedicels by nearly double post-anthesis in 1990 and pre-anthesis in 1991 (Fig. 3B). MSX did not raise the rachis NH_4^+ concentration (Fig. 3C).

The effect of AS on NH_4^+ accumulation was similar to that of MSX when applied at 5 m*M*, but only post-

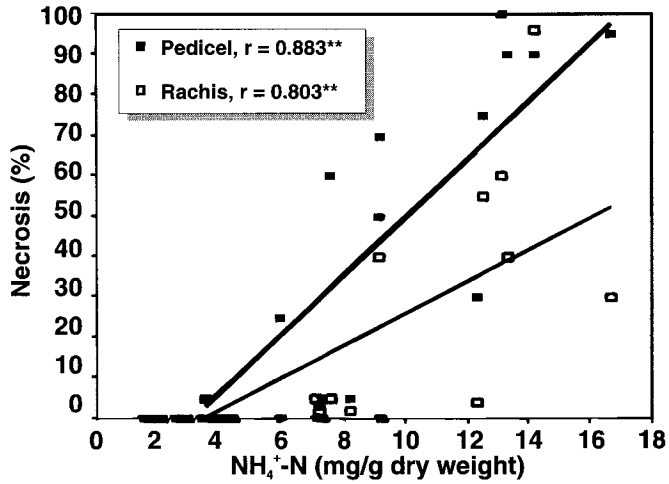

Fig. 2. Relationship between flower NH_4^+ concentration and rachis or pedicel necrosis of single-node Pinot noir grape cuttings incubated with $(NH_4)_2SO_4$ (0 to 200 m*M* NH_4^+) solutions one week pre-anthesis (Expt. 1, 1991). Correlation coefficients significant at $p = 0.05$ (**) level.

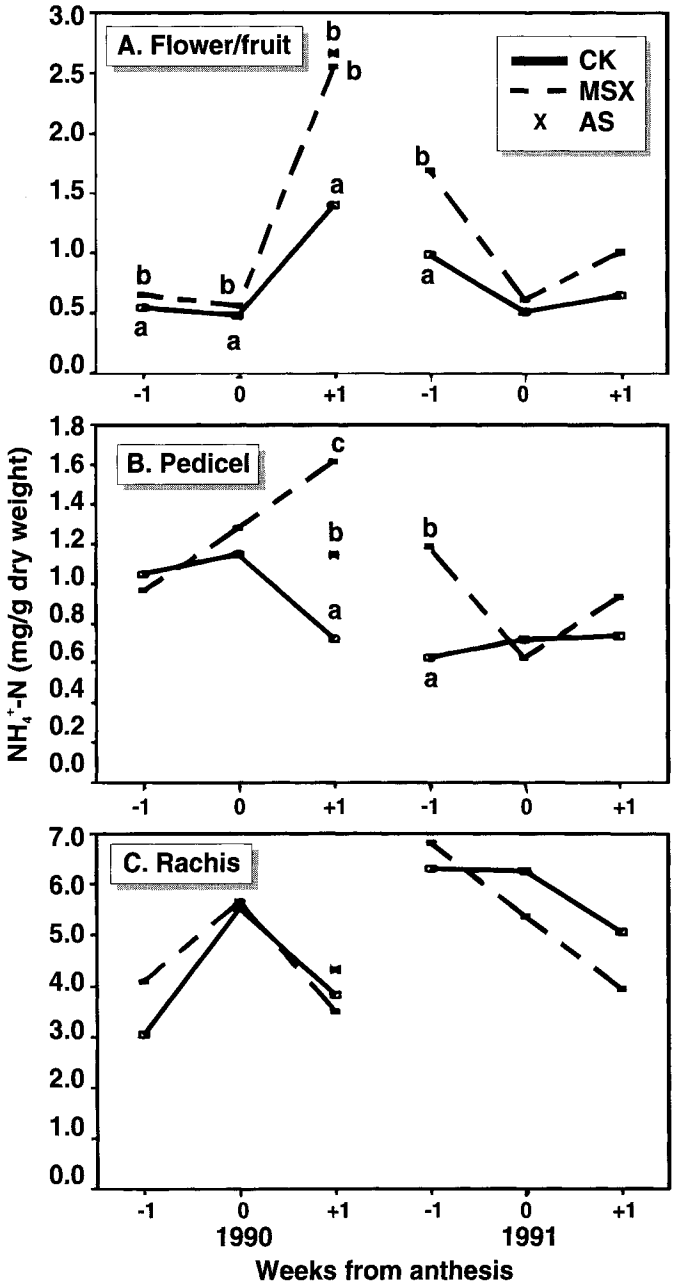

Fig. 3. Effect of methionine sulfoximine (MSX) and azaserine (AS), compared to control (CK), on NH_4^+ accumulation of flowers/fruit (A), pedicels (B), and rachis (C) in single-node Pinot noir grape cuttings (Expt. 2, 1990 and 1991). Mean separation among treatments for each sampling by Fisher's LSD test at $p = 0.05$ level.

anthesis in 1990. There was no difference in NH_4^+ accumulation in response to AS and MSX treatments in all sampled tissues except the pedicels which accumulated less NH_4^+ when treated with AS (Fig. 3).

GDH pathway (Expt. 3): MSX increased flower and pedicel NH_4^+ at the first two samplings and pedicel NH_4^+ post-anthesis, with or without the addition of KG (Table 3). Rachis NH_4^+ was affected by neither KG nor MSX. No interactions were found between KG and MSX treatments in any tissue tested at any time.

Table 3. Effect of incubating cuttings with 40 m*M* NH_4^+ and 20 m*M* α-keto-glutarate (KG) or 10 m*M* methionine sulfoximine (MSX) on NH_4^+ accumulation of cluster tissues in Pinot noir grapevines (Expt. 3, 1991).

Treatment	NH_4^+-N (mg/g dry weight)		
	Flower/fruit	Pedicel	Rachis
		Pre-anthesis	
NH_4^+	1.00 a	0.73 a	7.12
NH_4^+ + MSX	1.71 b	2.07 b	7.72
NH_4^+ + KG	1.02 a	0.55 a	6.35
NH_4^+ + KG + MSX	1.60 b	1.69 b	7.58
Significance			
KG	ns	ns	ns
MSX	**	**	ns
MSX X KG	ns	ns	ns
		Anthesis	
NH_4^+	0.46 a	0.63 ab	7.21
NH_4^+ + MSX	0.80 ab	0.78 bc	6.59
NH_4^+ + KG	0.51 ab	0.36 a	5.93
NH_4^+ + KG + MSX	0.85 b	1.04 c	6.69
Significance			
KG	ns	ns	ns
MSX	**	**	ns
MSX X KG	ns	ns	ns
		Post-anthesis	
NH_4^+	1.09	0.69	5.52
NH_4^+ + MSX	0.77	0.98	5.44
NH_4^+ + KG	1.27	0.71	5.53
NH_4^+ + KG + MSX	0.89	1.23	6.12
Significance			
KG	ns	ns	ns
MSX	ns	*	ns
MSX X KG	ns	ns	ns

[z]Mean separation in column for each sampling by Fisher's LSD test at $p = 0.05$ level.

[y]ns, *, ** non-significant, significant at $p = 0.05$ and $p = 0.01$ levels, respectively.

Discussion

Induction and prevention of IN: No evidence was found that NO_3^-, $SO_4^=$, or K^+ ions are associated with IN, since incubation of single-node cuttings with solutions of 160 m*M* KNO_3, or K_2SO_4 did not induce IN. Only NH_4^+ from NH_4NO_3 or $(NH_4)_2SO_4$ incubation was able to induce IN, indicating that NH_4^+ is involved in the development of IN. The involvement is also evidenced by the high correlation between necrosis severity and tissue NH_4^+. However, NH_4^+ levels in flowers or fruit were better indicators for necrosis than rachis NH_4^+ (Table 2).

Pedicels appeared to be more sensitive to NH_4^+ since they had lower NH_4^+ levels but greater necrosis. Carbon substrates and activities of assimilation enzymes are critical for ammonium assimilation and detoxification. Flowers and fruit appeared to have higher enzyme activity (Fig. 3), which could explain why they are affected less by necrosis. The variation in tissue re-

sponse to NH_4^+ could be due to differences in the rate of ammonium assimilation, the availability of substrates, and the structure of these tissues.

Clusters responded differently to NH_4^+ and NO_3^- incubation from pre- to post-anthesis during the development of fruit from flowers. Nitrate incubation had reduced the susceptibility of cluster tissues to IN at later stages as evidenced by the lower IN severity when incubated with NH_4NO_3, compared with $(NH_4)_2SO_4$ (Fig. 1A, B). The protective effect of NO_3^- on ammonium toxicity has been reported for various species (10,11,16).

Ammonium assimilation pathways: Increases in tissue NH_4^+ accumulation as a result of MSX and AS incubation suggest that ammonium assimilation by the GS/GOGAT cycle was blocked. The elevated NH_4^+ concentrations in flowers, fruit, and pedicels treated with the inhibitors indicates the importance of the GS/GOGAT pathway in nitrogen assimilation of grape cluster tissues, and is consistent with previous reports for other species (1,2,7,18,22). In all sampled tissues, AS showed an effect similar to MSX on NH_4^+ accumulation, indicating that GOGAT is tightly coupled with GS. Since these inhibitors had no effect on NH_4^+ accumulation in rachis, we assume that rachis has none or very low levels of GS/GOGAT activity.

An ammonium assimilatory role for GDH was recently suggested for some plant tissues (17,23,25). Jordan (14) found that the addition of 10 m*M* MSX did not prevent a reduction in NH_4^+ concentration of tendrils incubated with KG and NH_4^+. This suggests that the assimilation stimulated by KG did not involve GS, but that another assimilation pathway, most likely catalyzed by GDH, may have operated. However, in this study, when the cuttings were incubated with MSX, the addition of KG did not reduce tissue NH_4^+ concentration (Table 3). This indicates the absence or very low level of GDH activities in Pinot noir grape cluster tissues.

The response to the MSX, AS, and KG treatment varied among flower, fruit, pedicel, and rachis. NH_4^+ concentration in flowers, fruit, and pedicels increased after treatment with the inhibitors, whereas only slight differences were noted in rachis (Fig. 3). Also, addition of KG reduced NH_4^+ levels in flowers, fruit, and pedicels but not in rachis (Fig. 1). It is reasonable to expect variation in enzyme systems, enzyme activities, and/or their substrates among these tissues. Flower, fruit, and pedicels showed high GS/GOGAT activities and no or very low GDH activity, whereas no or very low GS/GOGAT and GDH activities were found in rachis. This may explain, at least in part, why the NH_4^+ concentration in rachis was several-fold higher than that in flowers, fruit, and pedicels. Differences in tissue NH_4^+ accumulation with or without inhibitors also varied with developmental stages of the cluster. Thesr differences may be associated with the development of fruit from flowers.

Plants can detoxify NH_4^+ by converting it into amino acids. Givan (9) suggested three assimilation pathways for removal of free NH_4^+. GS/GOGAT is believed to be the usual pathway for the assimilation of NH_4^+ at low (*i.e.*, normal) intercellular concentration, while GDH and asparagine synthetase reactions probably assimilate NH_4^+ only when the intercellular NH_4^+ concentration is unusually high. We could determine only the presence or absence of GS/GOGAT and GDH pathways in various cluster tissues of Pinot noir grapevines. If excessive NH_4^+ is the causal agent of IN, our data indicate that the rachis may serve as a strong NH_4^+ pool for the development of this disorder.

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