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Apoplastic pH and Ammonium Concentration in Leaves of *Brassica napus* L.[1]

Søren Husted* and Jan Kofod Schjoerring

Plant Nutrition Laboratory, Department of Agricultural Sciences, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark

A vacuum infiltration technique was developed that enabled the extraction of apoplastic solution with very little cytoplasmic contamination as evident from a malate dehydrogenase activity of less than 1% in the apoplastic solution relative to that in bulk leaf extracts. The volume of apoplastic water, a prerequisite for determination of the concentration of apoplastic solutes, was determined by vacuum infiltration of indigo carmine with subsequent analysis of the dilution of the dye in apoplastic extracts. Indigo carmine was neither transported across the cell membrane nor significantly adsorbed to the cell walls, ensuring reproducible (SE < 2%) and precise determination of apoplastic water. Analysis of leaves from four different positions on senescing *Brassica napus* plants showed a similar apoplastic pH of 5.8, while apoplastic NH_4^+ increased from 1.1 mM in lower leaves to 1.3 mM in upper leaves. Inhibition of glutamine synthetase in young *B. napus* plants resulted in increasing apoplastic pH from 6.0 to 6.8 and increasing apoplastic NH_4^+ concentration from 1.0 to 25.6 mM, followed by a marked increase in NH_3 emission. Calculating NH_3 compensation points for *B. napus* plants on the basis of measured apoplastic H^+ and NH_4^+ concentrations gave values ranging from 4.3 to 5.9 nmol NH_3 mol^{-1} air, consistent with an estimate of 5.3 ± 3.6 nmol NH_3 mol^{-1} air obtained by NH_3 exchange experiments in growth chambers. A strong linear relationship was found between calculated NH_3 compensation points and measured NH_3 emission rates in glutamine synthetase-inhibited plants.

NH_3 is a critical air pollutant with major impacts on atmospheric chemistry and ecosystem stability and biodiversity (Heil and Diemont, 1983; Bobbink et al., 1992; Sutton et al., 1993). There is increasing evidence showing that plants may both absorb NH_3 from the atmosphere and emit NH_3 to the atmosphere (Farquhar et al., 1980; Langford and Fehsenfeld 1992; Schjoerring et al., 1993; Sutton et al., 1995). Fertilized agricultural vegetation, including *Brassica napus*, seems in most cases, particularly during senescence, to be a net source of atmospheric NH_3 (Sutton et al., 1994).

The χ_{NH3} is equal to the mole fraction of gaseous NH_3 above the water film (apoplast) in the cell walls of the mesophyll cells. NH_3 emission occurs if the atmospheric mole fraction of NH_3 is below χ_{NH3}. In contrast, NH_3 is absorbed if the atmospheric mole fraction is higher than χ_{NH3}. Since χ_{NH3} depends on apoplastic H^+ and NH_4^+ concentrations, information about these parameters is essential to obtain a better understanding of the regulation of plant-atmosphere NH_3 exchange.

Several attempts have been made to measure apoplastic pH because of its importance in cell-wall extension and cell-wall synthesis (Jacobs and Ray, 1976) and in the regulation of carbohydrate uptake and phloem loading (Giaquinta, 1977; Tetlow and Farrar, 1993). A commonly used technique is to measure pH directly in exudates obtained by leaf pressurization. However, the application of high pressure may lead to leakage of cytoplasm into the apoplast and to overestimation of pH (Hartung et al., 1988). Pfanz and Dietz (1987) used fluorescence emission spectroscopy to measure pH by infiltrating the apoplast with the fluorescent dye 6-glucoxy-7-hydroxycoumarin. This technique can lead to an underestimation of pH because the dye may penetrate into the mesophyll cells. Furthermore, quenching of the fluorescent dye by cell walls has been observed to lead to a severe underestimation of the pH values (Tetlow and Farrar, 1993). Hoffmann et al. (1992) modified the fluorescent technique by coupling fluorochrome fluorescein isothiocyanate to dextran, thereby preventing the dye from penetrating into the cytoplasm. Starrach and Mayer (1989) measured apoplastic pH in *Phaseolus pulvinus* by direct insertion of a microelectrode using a micomanipulator and measured pH values of 5.9 and 6.7 in light and darkness, respectively. This technique may allow measurements of pH gradients in the cell-wall system but is very time consuming and cannot be used as a routine method to determine apoplastic pH in a large number of samples.

A vacuum infiltration technique was used by Cosgrove and Cleland (1983) and Speer and Kaiser (1991) to measure several organic and inorganic solutes in the apoplast of *Pisum sativum* and *Spinacia oleracea*. In these studies, leaves were infiltrated with an aqueous solution under vacuum; a centrifugation step followed in which the solution was collected. Apoplastic air volume and apoplastic water volume were determined by use of high-viscosity silicon oil and [^{14}C]sorbitol or indigo carmine, enabling correction for dilution of solutes in the apoplastic water film during

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* Corresponding author; e-mail soeren.husted@agsci.kvl.dk.; fax 4535283460.

Abbreviations: χ_{NH3}, NH_3 compensation point; GS, Gln synthetase (EC 6.3.1.2); MDH, malate dehydrogenase; MSO, Met sulfoximine (DL-methionine-DL-sulfoximine); PE, polyethylene; r_{NH3}, NH_3 leaf resistance.

infiltration. However, the vacuum infiltration technique has so far not been used to determine apoplastic pH and NH_4^+. Moreover, the technique has never been tested for artifacts caused by cytoplasmic leakage during infiltration and centrifugation. Since even small errors in the determination of apoplastic pH and NH_4^+ may lead to serious errors in the calculation of χ_{NH3}, it is crucial to optimize the analytical procedures involved in the vacuum infiltration technique.

The objective of the present work was to develop a precise and rapid method for analyzing apoplastic pH and NH_4^+ in *B. napus* L. and to verify that the results obtained by the method were reliable, i.e. not biased by contamination with cytoplasmic material. In addition, the hypothesis that plant-atmosphere NH_3 exchange is strongly related to apoplastic NH_4^+ and H^+ concentrations was tested.

MATERIALS AND METHODS

Plant Material

Seeds of rape (*Brassica napus* L. cv Global) were soaked overnight in aerated tap water and germinated in the dark on filter paper for 4 d at 25°C before planting in 0.01-m^3 self-watering pots filled with a 1:1 mixture of soil and sand containing (in g nutrient kg^{-1} soil mixture) 0.4 N, 0.15 P, 0.43 K, 0.07 Mg, 0.1 S, 0.005 Cu, and 2.5 $CaCO_3$. Plants were grown in a greenhouse at 20 ± 2°C under a 16-h photoperiod provided by natural light and supplemented with high-pressure sodium lamps, ensuring photon flux densities greater than 400 μmol m^{-2} s^{-1}. Unless otherwise stated, only plant material from the early vegetative growth period (6–8 weeks old) was used in the experiments.

Apoplastic Air Volume

Leaf segments of 4 cm^2 were weighed, infiltrated with high-viscosity silicone fluid (polydimethylsiloxane; viscosity 5 cs; δ 0.904 g cm^{-3}; Dow Corning, Poole, UK), which is impermeable to the plasma membrane, under vacuum in a 50-mL PE syringe, and reweighed. The leaf surface was blotted dry with thin tissues (Kleenex; Kimberley Clark GmbH, Koblenz, Germany), and the weight increase (corrected for the density of silicone oil) was used for calculation of the apoplastic air (V_{air}, cm^3 air cm^{-3} tissue). The amount of silicone oil adsorbed to the cuticle made an insignificant contribution to the increase in weight.

Apoplastic Water Volume

Leaf segments cut from washed and blotted dry leaves were weighed and infiltrated with 50 μM indigo carmine (indigo-5,5′-disulfonic acid) or 6 μM bromocresol blue (3′,3″,5′,5″-tetrabromo-*m*-cresolsulfonphtalein) dissolved in 50 mM phosphate buffer at pH 6.2. The infiltration was carried out using a 50-cm^3 syringe under pulsed periods of 30 s with vacuum and pressure, respectively. Immediately after infiltration, leaves were reweighed, put into a small, zippered PE bag to eliminate water vaporization, and stored for a maximum of 15 min at 4°C before extraction. Assuming a leaf density of 1 g cm^{-3} the difference in weight before and after infiltration was used for calculation of the infiltration volume (V_i). The leaf segments were then centrifuged at 2000*g* for 15 min at 10°C, and the dilution of dyes (D_{dye}) was measured spectrophotometrically in a 70-μL sample at 610 and 616 nm for indigo carmine and bromocresol blue, respectively. The apoplastic water volume (V_{apo}, cm^3 H_2O cm^{-3} tissue) was calculated using the equation:

$$V_{apo} = \frac{D_{dye} \times V_i}{1 - D_{dye}} \quad (1)$$

where $0 \leq D_{dye} \leq 1$.

To compare results obtained by dye infiltration, a similar procedure was applied using [^{14}C]sorbitol with which the dilution was measured by liquid scintillation. Leaves were infiltrated with 0.01 μCi [^{14}C]sorbitol mL^{-1} (Amersham Life Science, Birkerød, Denmark).

Extraction of Apoplastic H^+ and NH_4^+

Leaves were infiltrated with a 350 mOsmol isotonic sorbitol solution (0.28 M) and extracted as described above. Infiltrated leaves were stored at 4°C for ≤15 min before extraction, and pH was measured in a 50-μL sample by a microglass electrode (Metrohm, Herisan, Switzerland). NH_4^+ was measured spectrophotometrically using the indophenol method of Krom (1980) or by flow injection analysis (Tecator FIA Star 5020, Höganäs, Sweden). The concentration of apoplastic H^+ and NH_4^+ was corrected for dilution with the infiltration medium by multiplication with the dilution factor (F_{dil}):

$$F_{dil} = \frac{V_{apo} + V_{air}}{V_{apo}} \quad (2)$$

Cytoplasmic Contamination

Apoplastic extracts were screened for cytoplasmic contamination by measuring their activity of MDH relative to bulk leaf extracts. Randomly selected leaf sections were homogenized at 4°C by using an Ultra Turrax T25 (Janke and Kunkel Labortechnik, Staufen, Germany). The ratio between plant material and extraction buffer was 1:5 (w/w). The extraction buffer solution (0.1 M Tes, 0.2 mM EDTA, 2 mM DTT, pH = 7.5) was centrifuged at 10,000*g* (4°C, 10 min), and the enzyme activity was thereafter assayed spectrophotometrically (340 nm) at 25°C in a medium consisting of 0.094 mM NADH, 0.17 mM oxaloacetate, and 0.05 M Tes by adding 100 μL extract to 3 mL of assay medium.

A further check of cytoplasmic contamination was carried out by comparing the osmotic potential in the apoplast with that in bulk leaf extracts. Both were determined in 15-μL samples on a cryoscopic osmometer (Osmomat 030; Gonotec, Germany).

Adsorption of Dyes to Cell Wall

Solid-phase extraction columns were used for screening the dyes for their ability to adsorb to negative charges,

since this property would indicate a possible binding of the dyes to cell walls in the apoplast. A strong cation-exchange column (benzenesulphonic acid, Isolute SCX; International Sorbent Technology, Hengoed, UK) was equilibrated with 1 M NaCl and 100 mM buffers (Mes, pH 6.0; TES, pH 7.0; or Tris, pH 8.0). The dyes were mixed with the buffers and continuously recirculated through the columns for 5 min, after which the change in A was measured as previously described.

Cell-Wall Isolation

Isolation of cell walls was accomplished by a modified procedure adapted from Sentenac and Grignon (1981) and Bush and McColl (1987). Leaves were cut into 2-cm^2 pieces and plasmolyzed under vacuum in 25 mL of a solution containing 0.4 M mannitol, 25 mM Tris (pH 8.0), and 0.01% Triton X-100 for 30 min. Leaves were homogenized in a blender containing 0.25 M Suc, 10 mM DTT, and 25 mM Tris (pH 8.0) and subsequently in a homogenizer (Ultra Turrax T25, Janke and Kunkel Labortechnik) at 11,000 rpm. The sample was centrifuged four times at 1000g for 10 min at 4°C in a solution containing 10 mM DTT, 0.2 M KCl, and 50 mM Tris (pH 8.0). The pellet was washed and filtered on a 30-μm PE sheet under vacuum with 0.01% Triton X-100, 1 M NaCl, and 0.5 M $NaHCO_3$ and finally with acetone to remove Chl. All procedures were performed at 4°C except plasmolysis and initial fragmentation. Cell walls were dried in a desiccator for at least 24 h before being used for the experiments. Isolated cell walls (10 mg) were mixed with 6 μM indigo carmine and 50 μM Mes buffer adjusted to pH 6.2 in a 2.0-mL microcentrifuge vial and shaken for 2 h at 20°C, whereafter the change in A was measured.

GS Activity

Plant material (200 mg) was frozen in liquid N_2 and homogenized at 4°C in 2 mL of an extraction buffer containing 75 mM Tris (pH 7.2), 1 mM EDTA, 2 mM DTT, 10 mM $MgSO_4$, 5 mM glutamate, and 10% 1,2-ethanediol, followed by centrifugation at 25,000g (5°C) for 5 min. GS was assayed at 30°C by addition of 800 μL of supernatant to 2 mL of incubation solution containing 75 mM Tris (pH 7.2), 90 mM glutamate, 5 mM hydroxylamine, 50 mM $MgSO_4$, and 16 mM ATP. After 20 min the reaction was terminated by addition of 2.8 mL of solution containing 2.5% $FeCl_3$ and 20% TCA in 1.5 M HCl, followed by centrifugation at 10,000g (5°C) for 5 min. The GS activity was quantified spectrophotometrically at 540 nm, by measuring the amount of γ-glutamylhydroxamate formed.

NH_3 Emission from MSO-Treated Plants

NH_3 emission was determined from young *B. napus* plants in early vegetative growth stages. Plants were grown hydroponically in a nutrient solution with the composition described by Mattsson et al. (1992) and supplied with 3 mM NO_3^-. Inhibition of GS activity in the plants was obtained by adding 0.75 mM MSO (Sigma) to the nutrient solution. Plants were positioned in gas-tight polycarbonate cuvettes of 8 L and flushed with NH_3-free air cleaned on a filter containing Chemviron Carbon F22 (Chemviron Carbon, Neu-Irsenburg, Germany) and Purafil (Purafil, Inc., Atlanta, GA) at a flow rate of 30 L min^{-1}. The air was conditioned to 25°C and 60% RH, and the plants were exposed to a photon flux density of 400 μmol m^{-2} s^{-1} provided by high-pressure sodium lamps (Power Star; Osram, Germany). Leaf temperatures were measured by small thermocouples. The air was distributed through a nozzle in the bottom of the cuvette and led to the NH_3 monitor (NH_3 continuous flow wet denuder, model 1263 B; ECN Real Time Systems, Petten, The Netherlands) through an opening in the top of the cuvette. Transpiration and photosynthesis were measured on a CIRAS-1 monitor (PP-Systems, Hertz, UK) and data were logged on a computer every 60 s. Leaf resistances to NH_3 transport were derived by applying Fick's first law to the relationship between NH_3 emission and NH_3 compensation point:

$$r_{NH3} = \frac{\chi_{NH3} + \chi_{atm}}{F} \quad (3)$$

where r_{NH3} is the resistance against NH_3 diffusion in m^2 s mol^{-1}, χ_{NH3} is the compensation point in nmol NH_3 mol^{-1} air, χ_{atm} is the atmospheric mole fraction of NH_3 in nmol NH_3 mol^{-1} air, and F is the NH_3 flux in nmol m^{-2} s^{-1}.

Determination of NH_3 Compensation Points

NH_3 compensation points were determined by fumigating plants at five different NH_3 mole fractions ranging from 0 to 30 nmol NH_3 mol^{-1} air in a cuvette system (Husted and Schjoerring, 1995). The plants were grown as described above. To prevent gas exchange between soil and the cuvette atmosphere, the aerial plant parts were sealed from the soil with neoprene plugs inserted into plastic discs in which 10-mm (i.d.) holes were drilled for the plants. Gas samples were automatically drawn from the inlet and outlet air streams of five plant cuvettes exposed to 0, 5, 15, 20, and 30 nmol NH_3 mol^{-1} air by time-programmed valves and led to a modified chemiluminescent NO_x/NH_3 monitor (model 14; Thermo Environmental Instruments, Franklin, MA) and CO_2/H_2O monitor (model 6262; Li-Cor, Lincoln, NE). Data were automatically logged to a computer every 3 min.

RESULTS

Effect of Infiltration and Extraction on Contamination with Cytoplasmic Solutes

MDH activities in apoplastic extracts were independent of the centrifugal force in the range from 1,000 to 12,000g and always remained below 1% of that in bulk leaf extracts, showing very little damage to the cells during extraction (Fig. 1, top). Cutting a small leaf vein with a scalpel prior to centrifugation resulted in a 6-fold increase in MDH activity (data not shown). The osmolality of the apoplastic extract changed from 56 mOsmol kg^{-1} at 2,000g to only 61 mOsmol kg^{-1} at 12,000g (Fig. 1, top). In leaf homogenates the osmolality ranged between 350 and 450 mOsmol kg^{-1}. Similarly, neither proton activities (pH) nor NH_4^+ concen-

trations were affected by the centrifugal force applied to the leaf discs (Fig. 1, bottom). All future extractions were conducted at 2,000*g*, yielding approximately 100 μL of extract from leaf sections of 4 cm^2 (600 mg), which was sufficient for analysis of pH and NH_4^+ and for the other analyses described.

Apoplastic Water Volume

Apoplastic water constituted 10 to 13% of the leaf volume in *B. napus* plants in early vegetative growth whether determined by indigo carmine, bromocresol blue, or ^{14}C-labeled sorbitol ($P < 0.001$; Fig. 2). In leaves from plants in late vegetative growth stages, apoplastic water volumes increased from 8.3% in upper leaves to 14.4% in lower leaves (Table I). No detectable quantities of [^{14}C]sorbitol or indigo carmine were transported across the plasmalemma of the mesophyll cells during incubation of infiltrated leaves at 5°C for 15 min. However, both compounds may be transported across the plasmalemma when stored for

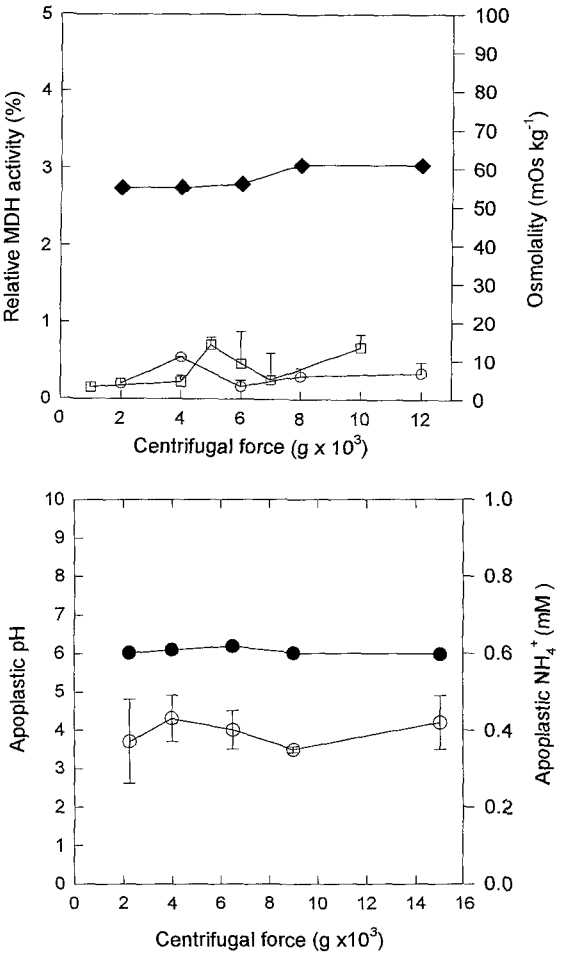

Figure 1. Top, MDH activity and osmolality of the apoplastic solution extracted at increasing centrifugal force applied to leaf cut sections of *B. napus*. ♦, Osmolality; ○, MDH upper leaves; □, MDH lower leaves. Bottom, NH_4^+ concentration and pH in the apoplast at increasing centrifugal force applied to leaf cut sections of *B. napus*. ●, pH; ○, NH_4^+.

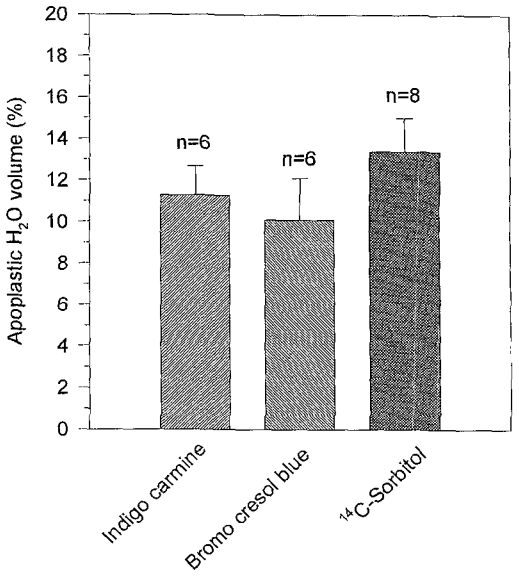

Figure 2. Apoplastic water volume in *B. napus* leaves obtained by measuring the dilution of indigo carmine, bromocresol blue, and [^{14}C]sorbitol.

longer periods and careful adjustment of the osmolality in the infiltration solution and the storage time is therefore crucial (data not shown). Less than 1.8% indigo carmine and 0.8% bromocresol blue were adsorbed to negative charges on a strong cation-exchange column in the pH interval 6.0 to 8.0 (data not shown). Similarly, only 2.1% of indigo carmine was adsorbed to purified, Na^+-saturated cell walls at pH 6.0 during a 2-h period (data not shown). Bromocresol blue had the disadvantage of a pH-sensitive light absorption spectrum at pH values of less than 5.8, whereas that of indigo carmine was uniform below pH 11.0 (data not shown). Moreover, bromocresol blue showed severe interference with Ca^{2+} in some instances and was consequently excluded from further experiments.

Apoplastic pH and NH_4^+

Neither apoplastic H^+ nor NH_4^+ concentrations changed significantly with the time, ranging from 2 to 32 min, between infiltration and extraction at 5°C ($P < 0.01$; data not shown). Similarly, both parameters were unaffected by the NH_4^+ concentration in the infiltration medium throughout a 15-min period (Fig. 3). Thus, NH_4^+ was rapidly transported from the apoplast to the cytoplasm across the plasmalemma of the mesophyll cells, thereby maintaining a constant apoplastic NH_4^+ concentration (Fig. 3, bottom).

Infiltrating the leaf apoplast with increasing K^+ concentrations ranging from 0 to 100 mM did not change the apoplastic NH_4^+ concentration (data not shown). However, apoplastic pH decreased from 6.1 to 5.4 when the K^+ was increased from 0 to 100 mM. This indicates that only a small amount of NH_4^+ was adsorbed to the cell-wall surface and that a significant amount of H^+ could be desorbed from the cell walls by ion exchange or that K^+ influx was associated with H^+ efflux.

Table I. *Apoplastic pH, NH_4^+, and calculated NH_3 compensation points in four different leaf positions for B. napus (n = 4–6, mean ± SE)*

Leaf Position	Air Volume	Water Volume	NH_4^+	pH	χ_{NH3}	χ_{NH3}
	%	%	mM		*I = 0 mM* nmol mol^{-1}	*I = 25 mM* nmol mol^{-1}
Acropetal direction						
6–9	27.2	14.4 ± 1.1	1.10 ± 0.24	5.77 ± 0.19	4.9	4.2
8–10	24.5	11.3 ± 0.5	1.06 ± 0.16	5.73 ± 0.16	4.3	3.7
12–16	18.3	12.2 ± 1.8	1.03 ± 0.14	5.83 ± 0.08	5.3	4.5
15–21	15.0	8.3 ± 1.0	1.30 ± 0.08	5.78 ± 0.07	5.9	5.1

Apoplastic pH in *B. napus* plants in late vegetative growth stages was on average 5.8 ± 0.2 and was not affected by leaf age (Table I). Young leaves had an apoplastic NH_4^+ of 1.3 mm, which was significantly ($P < 0.05$) higher than that in older leaves, in which the average apoplastic NH_4^+ was 1.06 mM (Table I). Bulk leaf NH_4^+ on a tissue water basis was 10.1 ± 0.5 mM (data not shown).

NH_3 Compensation Points in Relation to Apoplastic pH and NH_4^+

The χ_{NH3} was 5.3 ± 3.6 nmol NH_3 mol^{-1} air for *B. napus* in late vegetative growth stages (Fig. 4). As the atmospheric NH_3 mole fraction approached zero, NH_3 emission fluxes increased to 3.8 nmol NH_3 m^{-2} s^{-1}. Above the compensation point NH_3 absorption increased linearly with increasing external NH_3 mole fractions, reaching an uptake flux of approximately 7 nmol NH_3 m^{-2} s^{-1} at an atmospheric NH_3 concentration of 29 nmol mol^{-1} air.

Based on the data for apoplastic pH and NH_4^+ in Table I, calculations of the NH_3 compensation point resulted in values ranging from 4.3 to 5.9 nmol NH_3 mol^{-1} air (Table I). Assuming an ionic strength of 25 mm (Speer and Kaiser, 1991) instead of 0 mM and using the extended Debye-Hückel equation (Atkins, 1990) decreased the estimated χ_{NH3} to values between 3.7 and 5.1 nmol NH_3 mol^{-1} air (Table I).

Inhibition of GS Activity by MSO

GS was effectively inhibited by MSO (Fig. 5). Addition of 0.75 mm MSO to *B. napus* petioles resulted in a 70% decrease in GS activity after 4 h of incubation, after which the enzyme activity remained fairly stable. The inhibition of GS was followed by a marked increase in both symplastic and apoplastic NH_4^+ concentrations. However, the symplastic NH_4^+ concentration continued to increase after 4 h of incubation, whereas the apoplastic NH_4^+ concentration leveled off together with the reduction in GS activity. Apoplastic pH increased from 5.9 to 6.5 during the period of incubation (data not shown).

Addition of MSO to young vegetative plants after 2 h was followed by a marked increase in apoplastic NH_4^+ in upper leaves (leaves 4–6 in acropetal direction), whereas the NH_4^+ concentrations in lower leaves (leaves 1–3) showed only a relatively small increase (Fig. 6, top). In upper leaves the NH_4^+ concentration peaked at 25.6 mM after 7.5 h, whereas that in lower leaves increased to only 5.6 mM NH_4^+. Apoplastic pH increased from approximately 6.0 to 6.7 during the experiment, and no significant differences in the rate of pH increase was observed between upper and lower leaves (Fig. 6, middle). Leaf CO_2 uptake rates started to decline 2 h after MSO addition, whereas transpiration remained fairly stable throughout the experimental period (data not shown).

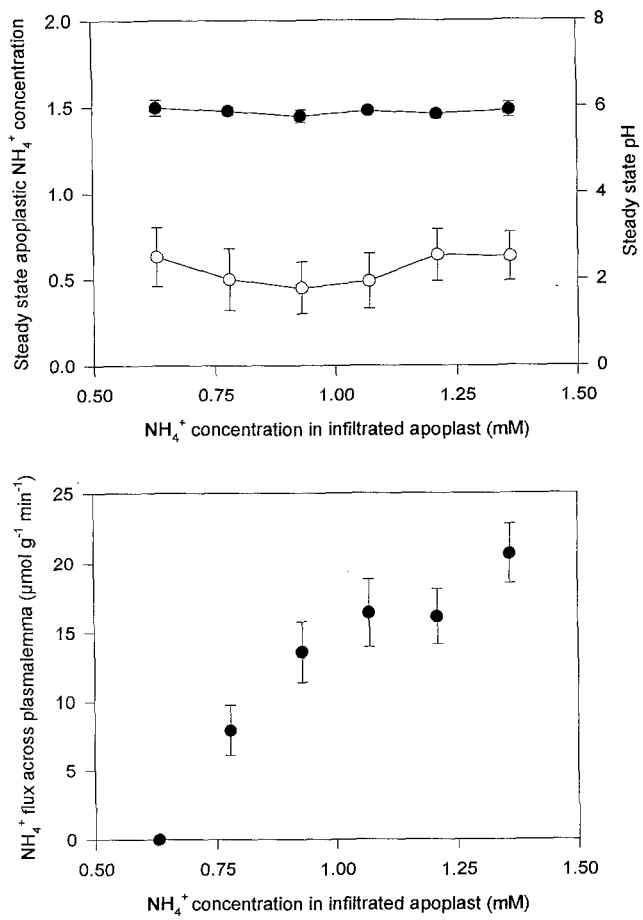

Figure 3. Top, NH_4^+ concentration and pH in the apoplast of *B. napus* leaves at start of the experiment in relation to steady-state values in the apoplast after incubation at 5°C for 15 min ($n = 3$) The apoplast was infiltrated with an isotonic sorbitol solution containing 0 to 1.25 mM NH_4^+ in $(NH_4)_2SO_4$ and adjusted to pH 6.2 with 50 mm Na_2HPO_4. ○, NH_4^+; ●, pH. Bottom, The NH_4^+ flux across the mesophyll plasmalemma during the incubation period.

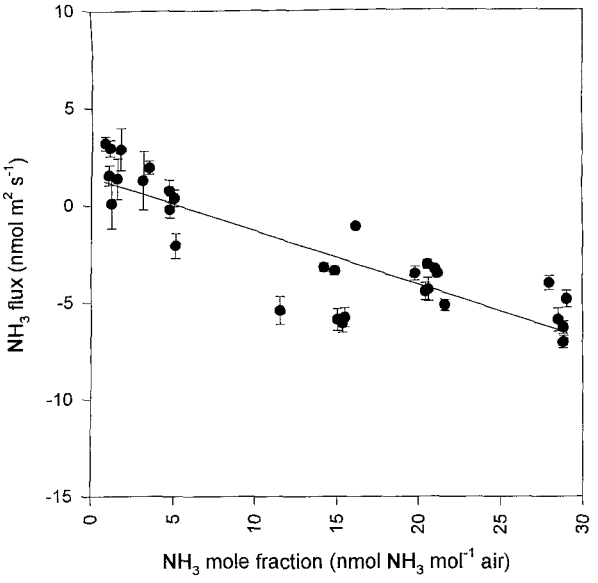

Figure 4. Determination of the NH_3 flux between *B. napus* in early vegetative growth and the atmosphere fumigated with different NH_3 mole fractions at realistic ambient levels. Positive and negative numerals denote emission and adsorption, respectively.

NH_3 compensation points calculated on the basis of measured apoplastic H^+ and NH_4^+ concentrations in MSO-treated plants correlated very well with actually measured NH_3 emissions (Fig. 6, bottom).

During the first 5 h after MSO addition the accumulation rate of NH_3 in the leaf apoplast was 16.9 nmol NH_3 m^{-2} leaf s^{-1} (calculated on the basis of data in Fig. 6, top), compared to measured NH_3 emission rates ranging from 0.05 to 1.5 nmol m^{-2} s^{-1} (Fig. 6, bottom). Thus, NH_3 emission was too slow to remove all NH_3 produced after inhibition of GS, leading to excessive accumulation of NH_3 and a high leaf resistance to NH_3 diffusion (r_{NH3} 66.7 m^2 s mol^{-1}; calculated using Eq. 3 and data in Fig. 6, bottom) as compared to the leaf resistance obtained in fumigation experiments performed under steady-state conditions in growth chambers (r_{NH3} 3.6 m^2 s mol^{-1}; calculated using Eq. 3 and data from Fig. 4).

DISCUSSION

Even relatively small errors in the determination of apoplastic pH and NH_4^+ would lead to severe errors in the calculation of χ_{NH3}. Changing pH from 5.8 to 6.0 with an apoplastic NH_4^+ of 1.10 mM would increase χ_{NH3} from 5.2 to 8.2 nmol NH_3 mol^{-1} air. Thus, because cytoplasmic NH_4^+ and pH levels usually are higher than apoplastic levels (Grignon and Sentenac, 1991; Wang et al., 1993a), it is important to extract uncontaminated apoplastic solutions. The low MDH activities and osmolalities (Fig. 1) of the apoplastic solution in the present work show that, together with constant NH_4^+ and pH values at increasing centrifugal force up to 15,000*g*, the vacuum infiltration technique allows extraction of apoplastic H^+ and NH_4^+ in *B. napus*, essentially free from cytoplasmic contamination. Using a similar vacuum technique to extract apoplastic sugars from *Hordeum vulgare*, Tetlow and Farrar (1993) found less than 0.01% contamination when measuring MDH activities. Terry and Bonner (1980) also used MDH as a cytoplasmic marker enzyme in extraction of apoplastic solutes from *Pisum sativum* stems by centrifugation and found a marked increase in MDH activity as the centrifugal force exceeded 5,000*g*. The fact that *B. napus* cells did not burst even at a centrifugal force of 15,000*g* (Fig. 1) indicates that they have relatively strong cell walls.

Less than 2% of indigo carmine and bromocresol blue was adsorbed to negative charges on a strong cation-exchange resin and on isolated cell walls. An adsorption of this magnitude would increase the dye dilution coefficient (D_{dye}; Eq. 1) from typically 0.34 to 0.35, which would lead to a relative error smaller than 5% on the apoplastic water volume. Cosgrove and Cleland (1983) used indigo carmine and [^{14}C]sorbitol for analyzing the free space water content of stems from different plant species and found that both compounds were completely extracted from *P. sativum* and *Glycine max*, whereas indigo carmine was retained in some way by *Cucumis sativus*.

Infiltration of leaves with isotonic sorbitol solutions at increasing NH_4^+ concentrations did not change the apoplastic H^+ and NH_4^+ concentrations after an incubation period of 15 min, showing that a rapid NH_4^+ net influx across the plasmalemma buffered the apoplast against changes in external NH_4^+ concentration (Fig. 3). The NH_4^+ net influx increased almost linearly with NH_4^+ in the infiltration solution and an increase of 1 mM NH_4^+ in the infiltration solution increased NH_4^+ net influx with approximately 26 μmol NH_4^+ + $NH_{3(aq)}$ g^{-1} min^{-1}. Data concerning NH_4^+ transport across the mesophyll plasmalemma are very scarce in higher plants, but Raven and

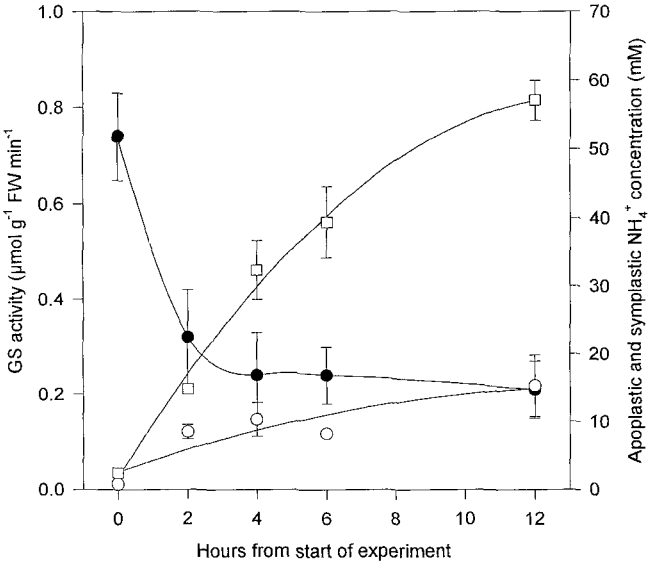

Figure 5. Effectiveness of the inhibition of GS in *B. napus* leaves with 0.75 mM MSO. ●, GS; ○, apoplast; □, symplast. FW, Fresh weight.

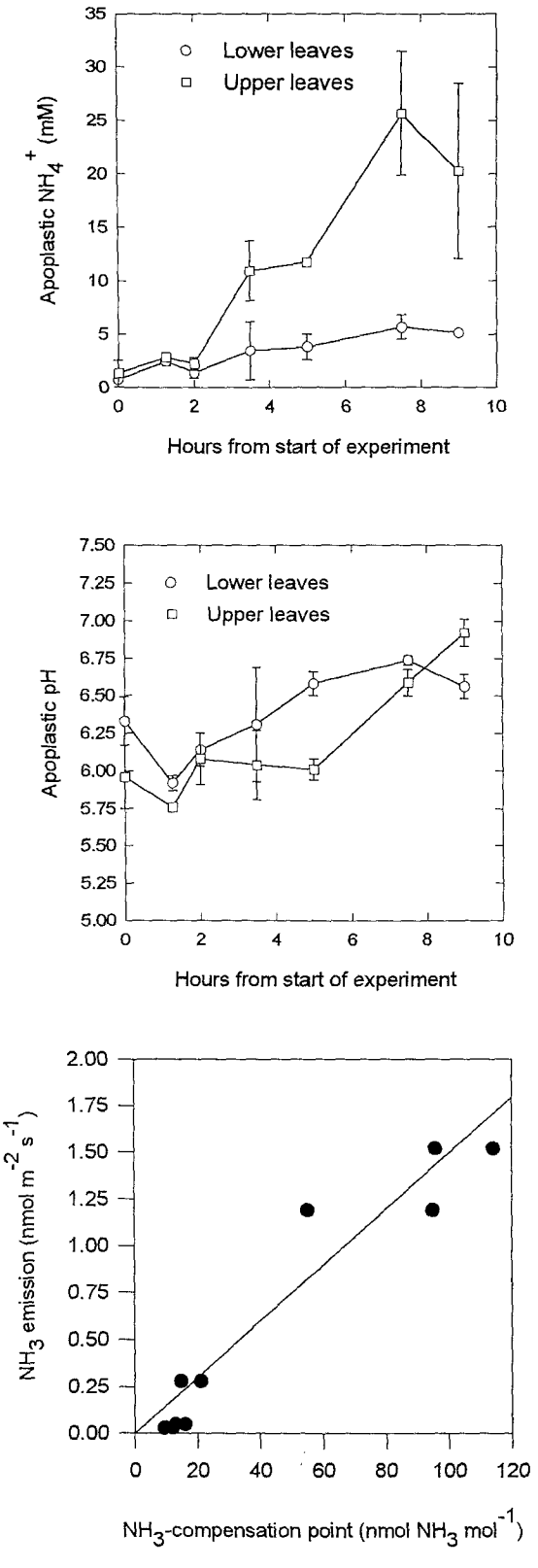

Figure 6. Top, Time series of NH_4^+ concentrations in the apoplast for upper and lower leaves of *B. napus* after inhibition of GS with MSO. Middle, Time series of apoplastic pH after GS inhibition. Bottom, Calculated NH_3 compensation points in relation to experimentally determined NH_3 emissions.

Farquhar (1981) measured the influx of $^{14}CH_3NH_3^+$ from a 0.1 mM bathing solution to leaf sections of *Phaseolus vulgaris* and determined an influx of 25 μmol NH_4^+ + $NH_{3(aq)}$ g^{-1} min^{-1}. $^{13}NH_4^+$ influx in roots of *Oryza sativa*, exposed to external NH_4^+ concentrations ranging from 0 to 40 mM, was 0.2 μmol g^{-1} min^{-1} for plants exposed to an external NH_4^+ concentration of 1 mM (Wang et al., 1993b). Transport of NH_4^+ across plasmalemma of mesophyll cells thus seems to be rapid relative to that across plasmalemma of root cells. Raven and Farquhar (1981) and Karasawa et al. (1994) suggested that NH_4^+ was transported across the leaf plasmalemma by an uniport, actively controlling the NH_4^+ and $NH_{3(aq)}$ concentration in the apoplast via induction/repression and feedback inhibition.

The NH_4^+ concentration in leaves of *B. napus* ranged from 0.4 to 1.3 mM in the present experiments. To our knowledge no experiments have been undertaken to measure apoplastic NH_4^+ in leaves, making a direct comparison with other studies impossible. However, Cramer and Lewis (1993) analyzed the NH_4^+ concentration in xylem sap from *Triticum aestivum* and *Zea mays* and found values ranging from 0.3 to 2.6 mM for plants grown in 4 mM NO_3^- or NH_4^+. By compartmentation analysis, Wang et al. (1993a) measured cell-wall NH_4^+ in rice roots grown in 2 μM to 1 mM $^{13}NH_4^+$ and found concentrations ranging from 0.6 to 14.4 mM.

The fact that neither NH_4^+ nor H^+ concentrations changed with increasing time between infiltration and extraction shows that infiltrating a leaf prior to extraction, and thereby diluting the apoplastic solution, did not induce an efflux of these ions. No efflux of NH_4^+ from the cell interior to the apoplast is in accordance with a lower electrochemical potential of NH_4^+ in the cytoplasm than in the apoplast (Wang et al., 1993a). However, for dissolved $NH_{3(aq)}$ the situation is quite different because symplastic pH (Roberts et al., 1982) and NH_4^+ (Wang et al., 1993a) are higher than apoplastic values, thus supporting a concentration gradient directed outward. Efflux of dissolved NH_3 would in addition be favored by its high plasmalemma permeability (Kleiner, 1981). The increase in both apoplastic NH_4^+ and pH after inhibition of GS also indicates that NH_3 efflux actually occurred (Fig. 6). However, in this case the NH_3 efflux must have been accompanied to some extent by H^+ efflux via a proton pump, because the apoplastic pH would otherwise have increased to approximately 10.5, assuming a negligible buffering capacity by cell walls and dissolved buffer species.

NH_3 compensation points calculated on the basis of the measured values for NH_4^+ and H^+ concentrations in the apoplast ranged from 4.3 to 5.9 nmol NH_3 mol^{-1} air and agreed well with experimentally determined compensation points (Figs. 4 and 6, bottom). Thus, apoplastic H^+ and NH_4^+ concentrations seem to be a very valuable tool for predicting NH_3 compensation points. However, correction for ionic strength of the apoplastic solution may be important (Table I), in particular if the ionic strength is as high as observed under drought stress and in plants exposed to salt stress, in which it may exceed 100 mM (Speer and Kaiser, 1991; Speer et al., 1994).

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