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Whole-Nodule Carbon Metabolites Are Not Involved in the Regulation of the Oxygen Permeability and Nitrogenase Activity in White Clover Nodules[1]

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To test the hypothesis of an indirect or direct involvement of carbon metabolites in the short-term regulation of nitrogenase activity, nodule O_2 permeability was manipulated either by defoliation or by varying rhizosphere O_2 partial pressure. In contrast to defoliation, a 50% reduction of the nodule O_2 permeability, due to adapting nodules to 40 kPa O_2, had no effect on nodule sucrose concentration. Likewise, total concentrations of other carbon metabolites such as fructose, starch, L-malate, and succinate tended to be differentially affected by the two treatments. Upon defoliation, carbon metabolites in roots responded in a manner similar to those in nodules. Sucrose concentration in nodules decreased significantly after the removal of 40% of the leaf area, which is known to have no effect on nitrogenase activity and O_2 permeability. During regrowth after a 100% defoliation, nitrogenase activity could be increased at any time by elevating rhizospheric O_2 partial pressure. Thus, during the entire growing cycle nitrogenase activity seems primarily oxygen limited. Changes in whole nodule sucrose pools after defoliation have to be viewed as secondary effects not necessarily linked to nodule activity. Whole-nodule carbon metabolites appear not to be determinants of nodule activity, either through direct metabolic involvement or through indirect effects such as triggering O_2 permeability.

Several experiments have demonstrated that nitrogenase activity is very sensitive to various forms of stress, such as stem girdling, drought, defoliation, and extended darkening (Durand et al., 1987; Hartwig et al., 1987, 1990; Vessey et al., 1988). Initially, the decline in nitrogenase activity after defoliation was assumed to be directly induced by an interruption in the supply of photosynthates due to the removal of photosynthetically active tissue. Further investigations gave clear evidence that the decrease in nitrogenase activity after defoliation cannot be explained by a shortage of photosynthates from reserves or from current photosynthesis (Hartwig et al., 1990, 1994). Nitrogenase activity, however, appears to be regulated through the availability of oxygen in the infected cell zone of nodules (Hartwig et al., 1987; Denison and Layzell, 1991; Sung et al., 1991; Denişon et al., 1992; Diaz del Castillo et al., 1992). It has been proposed that a variable oxygen permeability regulates the oxygen supply that supports nitrogenase activity (Witty et al., 1984, 1987; Hunt et al., 1987; Weisz and Sinclair, 1987). Mechanisms involved in the short-term regulation of O_2 permeability in nodules are poorly understood. Previous studies have shown that the nodule oxygen permeability may be related to osmotically induced changes in the size or shape of cells or intercellular spaces in the cortex and in the infected zone (van Cauwenberghe et al., 1993; Serraj et al., 1995). Such a concept seems plausible, since nodule Suc concentration was reported to decrease after defoliation (Gordon et al., 1986; Streeter and Salminen, 1993) and after nitrate fertilization (Minchin et al., 1986), treatments that result in changes in nodule O_2 diffusion. Other carbon metabolites that could also be envisaged as osmotically active molecules, such as L-malate or succinate, were not investigated in these studies. Since Steeter and Salminen (1993) found that a defoliation-induced decline in whole-nodule Suc concentration is representative also for the nodule cortex, this study was performed on the basis of whole nodules.

After the nearly instantaneous decrease in nitrogenase activity upon defoliation, nitrogenase activity recovers as the plant regrows. During this period, the regrowing shoot is a strong sink for carbon metabolites, and thus nodule activity may suffer from carbon shortage. It is not known whether the import of carbon metabolites to nodules is sufficient during this period and hence if nitrogenase activity would still be oxygen limited.

To test the hypothesis that Suc or other osmotically active carbon metabolites could be involved in regulating the O_2 permeability in white clover (*Trifolium repens* L.) nodules, nodule O_2 permeability was manipulated either by defoliation or by adapting the nodulated root system to various rhizosphere pO_2 values. Moreover, concentrations of carbon metabolites were monitored after a 40% removal of leaves, a treatment that does not affect nitrogenase activity. Finally, the possibility of a carbon limitation of nitrogenase activity during several days of regrowth was monitored.

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Abbreviations: ANA, apparent nitrogenase activity (H_2 evolution in N_2:O_2 = 80:20); PNA, potential nitrogenase activity (highest H_2 evolution in Ar:O_2 at elevated pO_2); pO_c, rhizospheric O_2 partial pressure; pO_2, oxygen partial pressure; TNA, total nitrogenase activity (peak H_2 evolution in Ar:O_2 = 80:20).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Defoliation Treatments

White clover (*Trifolium repens* L. cv Milkanova) from a single clone was grown in growth chambers (PGR-15, Conviron Instruments Co., Winnipeg, Manitoba, Canada) at 18/13°C day/night temperatures and 90% RH, in a 16-h photoperiod and a photon flux density of 500 μmol quanta PAR m^{-2} s^{-1} (fluorescent [Cool White, 160 W] and incandescent [138 L 100 W], Sylvania GTE, Geneva, Switzerland). For all experiments, plants were established by transplanting stolon tips, including the first five to six internodes, into gas-tight, sealable pots with a volume of 250 mL, filled with silica sand (0.8–1.2 mm). Plants were watered for 8 d with nutrient solution similar to that of Hammer et al. (1978), supplemented with 7.5 mM nitrate. From d 9 to 14 the nitrate concentration was 1 mM, and an N-free nutrient solution was given during the rest of the experiment. We inoculated with *Rhizobium trifolii* strain RBL 5020 (generously provided by Dr. H.P. Spaink, Leiden University, Leiden, The Netherlands) at d 14 and 19 after planting. Experiments were performed 6 to 8 weeks after planting.

Plants were either completely (100%) defoliated or only 40% of the leaf area was removed as described by Hartwig et al. (1994). A 40% defoliation has been reported to have no effect on nitrogenase activity or nodule oxygen permeability (Hartwig et al., 1994).

Measurement of TNA, PNA, and ANA and Determination of O_2 Limitation of Nitrogenase Activity

The gas-exchange system used to measure nitrogenase activity in all experiments was similar to that described by Minchin et al. (1983); all details are described by Heim et al. (1993). To assess O_2 permeability, oxygen limitation of nitrogenase activity was measured as the pO_2 required for highest nitrogenase activity; all details are described by Heim et al. (1993). TNA, PNA, and ANA were measured in a manner similar to that of Diaz del Castillo et al. (1992). TNA represents peak H_2 evolution under Ar:O_2 (80:20). For measuring PNA, the initial external oxygen partial pressure of 20 kPa in Ar was increased at a rate of 2 kPa min^{-1} until nitrogenase activity showed no further increase. ANA represents H_2 evolution under N_2:O_2 (80:20).

Adaptation of Nodules to Different pO_2 Values

In these experiments, the same gas-exchange system described above was used. For the process of adaptation, plants were exposed to a gas stream of synthetic air (N_2:O_2 = 80:20). When a steady state of ANA was reached, pO_2 was changed at a rate of 1 kPa min^{-1} to 10 or 40 kPa O_2. Treatments lasted until ANA reached a new steady state (see also "The Short-Term Response of Carbon Metabolites to Manipulations of O_2 Permeability by Varying pO_e" in "Results"). Upon this treatment, nodule O_2 permeability was either decreased (40 kPa O_2) or increased (10 kPa O_2) according to Fick's law (Weisz and Sinclair, 1987; Davey and Simpson, 1989). To assure that the 40-kPa O_2 treatment did not damage nitrogenase, pO_2 was readjusted to 20 kPa O_2 and ANA was checked again.

Preparation of Plant Extracts for All Analyses

Plants used for analysis were rapidly removed from their pots, washed in cold water, frozen in liquid N_2, and stored until freeze-drying at −80°C. Freeze-dried material was separated into roots and nodules. Aliquots of ground material (about 100 mg) were extracted at room temperature in 1 mL of ethanol using a mortar and pestle. To remove all water-soluble substances, the pellet from a 15-min centrifugation at 3000*g* was twice resuspended in 1 mL of 65% ethanol and centrifuged again. The pellet was used for starch analysis, and the collected supernatants were dried in vacuo and resuspended in 0.5 mL of water for analysis of soluble sugars and organic acids.

Analysis of Sugars (Suc, Glc, Fru), Cyclitols (D-Pinitol, *myo*-Inositol), Starch, and Organic Acids

For analysis of sugars and cyclitols, extracts were divided into neutral and ionic fractions using modified ion-exchange columns (Bachmann et al., 1994). Columns were filled with 100 μL of Dowex 1-*8 (HCO_3^-), 200 to 400 mesh, and Dowex 50-*8 (H^+), 200 to 400 mesh, omitting the PVP layer. The filled columns were washed with 1 mL of water and centrifuged for 20 min at 3000*g* before use. Fifty microliters of the extract were filtered through the column and rinsed with 50 μL of water. The wash was filtered through a new column in the same manner. The ionic fraction was discarded, and sugars and cyclitols from the neutral fraction were separated by HPLC (RI-Detector, ERC-7512, Erma, Tokyo, Japan) using a SS-100, Pb^{2+} carbohydrate column (7.8 × 300 mm, Sierra Separations, Reno, NV) in an isocratic solvent system with twice-distilled water as the mobile phase. The column temperature was 80°C. Software from Pyramid (Axxiom Chromatography, Moorpark, CA) was used for recording and integration of chromatograms.

The amount of starch was determined in the pellet, which was dissolved in 0.6 mL of water and heated to 90°C before the addition of 150 μL of a solution containing Termamyl (0.5 mL of Termamyl 120L, a heat-resistant α-amylase from *Bacillus licheniformis*, in 10 mL of H_2O; Novo Nordisk Fermant Ltd., Dittingen, Switzerland). The mixture was boiled for 15 min in a heating block and immediately chilled on ice. After addition of 0.5 mL of sodium-acetate buffer (70 mM, pH 4.3) and 120 μL of a solution of amyloglucosidase (10 mg of amyloglucosidase 60U [Boehringer, Mannheim]; dissolved in 1 mL of sodium-acetate buffer, pH 4.6), the reaction mixture was incubated at a temperature of 60°C for 30 min and then cooled. All samples were filtered (LS 14, Schleicher & Schuell) and diluted with water to 10 mL. For Glc determination, 0.05-mL aliquots were added to a mixture of 0.6 mL of triethanolamine-HCl buffer (750 mM, pH 7.6) with 900 mM ATP and 13 mM NADP, 0.98 mL of water, and 0.01 mL of hexokinase (450 units mg^{-1}) according to the manufactur-

er's instruction (Boehringer Mannheim) and analyzed immediately. The amount of D-Glc from starch breakdown was determined spectrophotometrically at 340 nm (LKB Bilchrom, Ultraspec 4050, Turku, Finland) based on the conversion of NADP to NADPH.

For detection of organic acids by HPLC, an Aminex HPX-87H column (7.8 × 300 mm, Bio-Rad) was used in an isocratic solvent system with 6 mM $H_2SO_4^{2-}$ as the mobile phase at a flow rate of 500 μL min^{-1}. Column temperature was 15°C. Prior to injection, all samples were filtered through a microfilter ACRO LC 3A, 0.45 μm (Disposable Filter Assembly, Gelman Sciences, Ann Arbor, MI). Chromatograms were recorded and integrated using software from Waters (series 990 PDA, Millipore). Integration of peaks was performed at a wavelength of 210 nm using the Waters photodiode array detector system 991. All standards used for calibration were purchased from Fluka with the exception of D-pinitol, which was a generous gift from Dr. S. Baumgartner (Institute of Food Technology, Swiss Federal Institute of Technology, Zurich, Switzerland).

The concentrations of carbohydrates in nodules from undefoliated plants are comparable to those described by Gordon et al. (1986) and Minchin et al. (1986); the present data on organic acids are substantially higher (more than a factor of 10) compared to those described by Davis and Nordin (1983). Different growing conditions, plant age, nutrient status, plant and/or bacteria cultivars, or methodological reasons could explain such differences.

Statistical Analysis

Data were subjected to analysis of variances using SAS (Statistical Analyses System Institute, Cary, NC).

RESULTS

The Short-Term Response of Carbon Metabolites to Manipulations of Nitrogenase Activity and O_2 Permeability by a 100% Defoliation

Similar to the reduction in nitrogenase activity and a decrease in O_2 permeability due to a 100% defoliation (data not shown), Suc concentration in roots and nodules declined by 50% (Fig. 1) within the 1st h of defoliation. Fru, Glc, D-pinitol, and starch concentrations in roots and nodules were not significantly affected over the 4-h period immediately following defoliation (Fig. 1). Although D-pinitol and Fru concentrations were always about 5-fold lower in roots than in nodules, Glc concentration in roots was much higher than in nodules, where it was near the limit of detection. No *myo*-inositol could be found in roots or in nodules. L-Malate and succinate contributed 70% of the organic acid fraction found in nodules; no significant response to defoliation was observed (Fig. 1). Concentrations of the measured organic acids were substantially higher in nodules than in roots (data not shown; Fig. 1).

The Short-Term Response of Carbon Metabolites to Manipulations of O_2 Permeability by Varying pO_e

To manipulate the nodule O_2 permeability without interrupting the phloem sap supply, the pO_e was modified

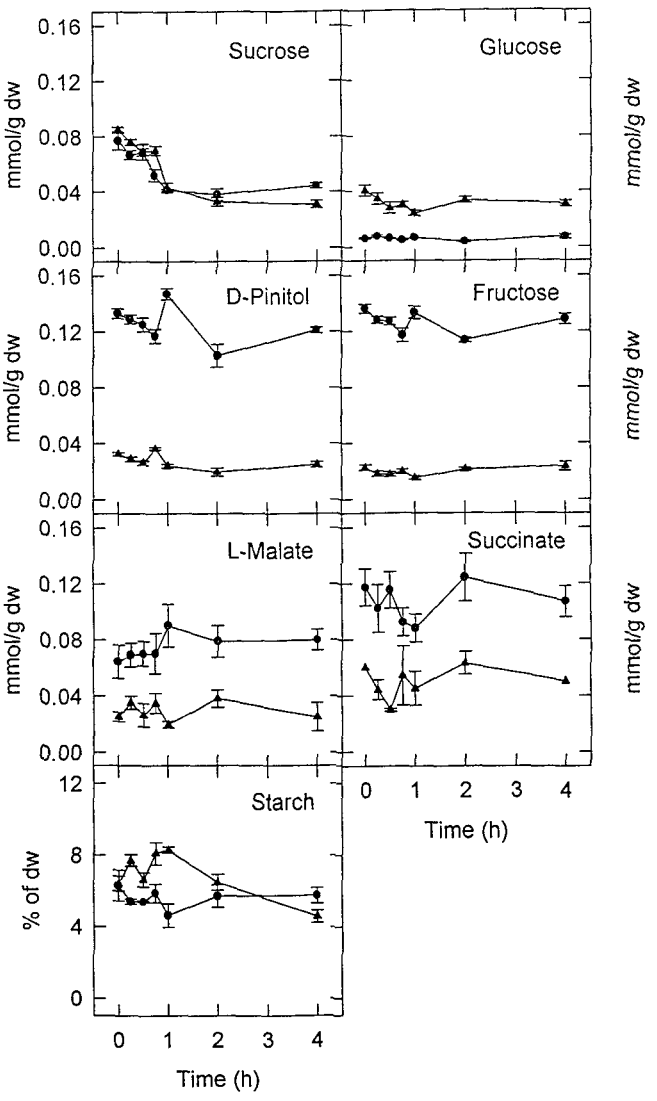

Figure 1. Concentrations of Suc, Glc, D-pinitol, Fru, L-malate, succinate, and starch in roots (▲) and nodules (●) over a 4-h period after complete defoliation (at time 0) of white clover. Means ± SE of seven replicates are shown. dw, Dry weight.

(Figs. 2 and 3). In response to an exposure of the root system to a pO_e of 40 kPa, nitrogenase activity increased initially and was reestablished within 1 h at a level slightly higher than that observed under 20 kPa O_2 (Fig. 2). According to Fick's law, the O_2 permeability must have been decreased to 50% of its initial value. To ensure that nitrogenase was not damaged by this treatment, nodules were re-exposed to 20 kPa O_2 after the experiment. Since nitrogenase activity recovered completely to its initial value, it is evident that no nitrogenase damage had occurred. A decrease of pO_e from 20 to 10 kPa caused an initial decline in nitrogenase activity, which was partially readjusted after 1 h (Fig. 3). Since pO_e was decreased by 50% and nitrogenase activity showed 80% of its initial value, the O_2 permeability must have been increased to 160% of its initial level (Fick's law).

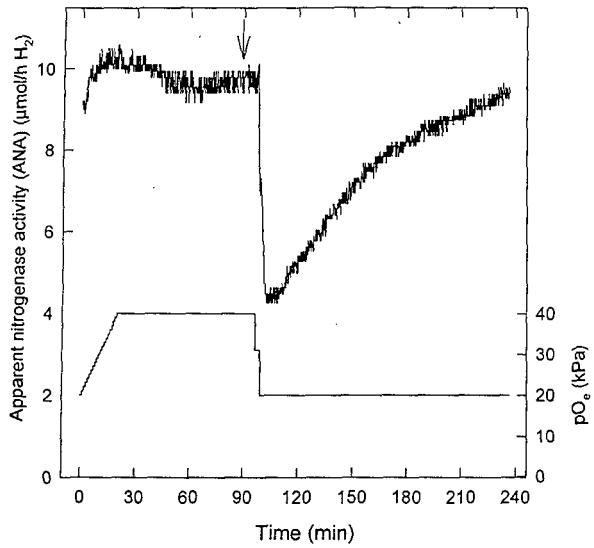

Figure 2. Response of ANA to an increase of external oxygen pressure (pO_e) from 20 to 40 kPa. Samples were taken when ANA reached steady-state values (arrow). To assure that nitrogenase was not damaged by the treatment, pO_e was readjusted to 20 kPa O_2 and ANA was checked again.

Under conditions of undisturbed phloem sap supply but decreased O_2 permeability at a pO_e of 40 kPa, Suc concentration in nodules showed no response (Table I). However, with increased O_2 permeability and reduced nitrogenase activity at a pO_e of 10 kPa, Suc concentration was 30% higher than under 20 kPa O_2 (Table I). Changes in Fru concentrations in response to altered pO_e were similar to those for Suc, whereas D-pinitol and starch concentrations did not change. Glc concentration was below the limit of detection in this experiment. Both L-malate and succinate were unaffected by 10 kPa O_2 but tended to decrease under 40 kPa O_2.

Table I. *Concentrations of carbon metabolites in white clover nodules after adaptation of nodules to various pO_e*

Means of at least five replicates are shown.

Carbon Metabolite	pO_e		
	10 kPa	20 kPa	40 kPa
Starch[a]	55.4 ± 13.2[c]	63.4 ± 6.6[c]	39.4 ± 4.0[c]
Suc[b]	0.163 ± 0.006[d]	0.108 ± 0.004[c]	0.108 ± 0.001[c]
Fru[b]	0.134 ± 0.013[d]	0.093 ± 0.007[c]	0.100 ± 0.016[c]
D-Pinitol[b]	0.075 ± 0.003[c]	0.066 ± 0.004[c]	0.061 ± 0.008[c]
L-Malate[b]	0.064 ± 0.009[c]	0.070 ± 0.006[c]	0.047 ± 0.008[c]
Succinate[b]	0.286 ± 0.022[c]	0.230 ± 0.002[c]	0.165 ± 0.017[c]

[a] mg g^{-1} nodule dry weight. [b] mmol g^{-1} nodule dry weight. [c,d] Values with dissimilar letters differ significantly from each other ($P < 0.05$).

The Short-Term Response of Suc to a Degree of Defoliation Not Affecting Nitrogenase Activity and Nodule O_2 Permeability

To detect possible links between nodule carbohydrate content, nitrogenase activity, and nodule O_2 permeability, Suc concentrations in nodules of plants defoliated by 0, 40, and 100% were compared. Although nitrogenase activity does not respond within 120 min to a 40% defoliation (Hartwig et al., 1994), the nodular Suc concentration dropped significantly, by about one-third, within only 15 min (Fig. 4). In 100% defoliated plants, the decrease in nodule Suc concentration was initially slower. Yet by 120 min after 100% defoliation, the Suc concentration was reduced by two-thirds. Both Glc and Fru concentrations were unaffected by either of the defoliation treatments (data not shown).

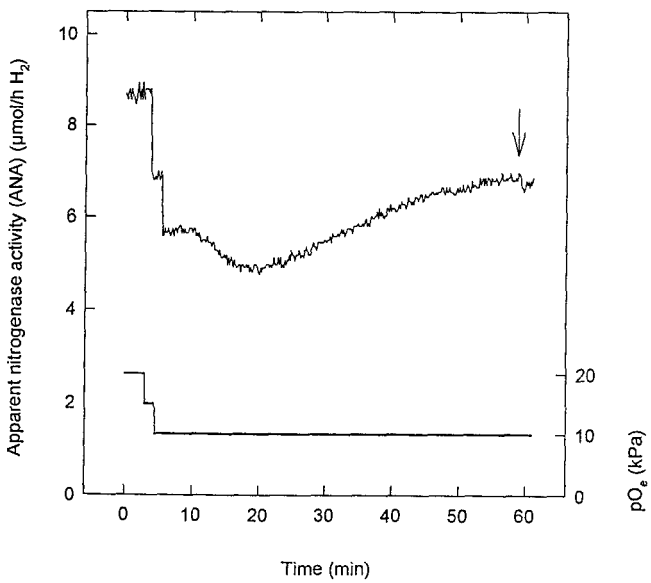

Figure 3. Response of ANA to a decrease of external oxygen pressure from 20 to 10 kPa. Samples were taken when ANA reached steady-state values (arrow).

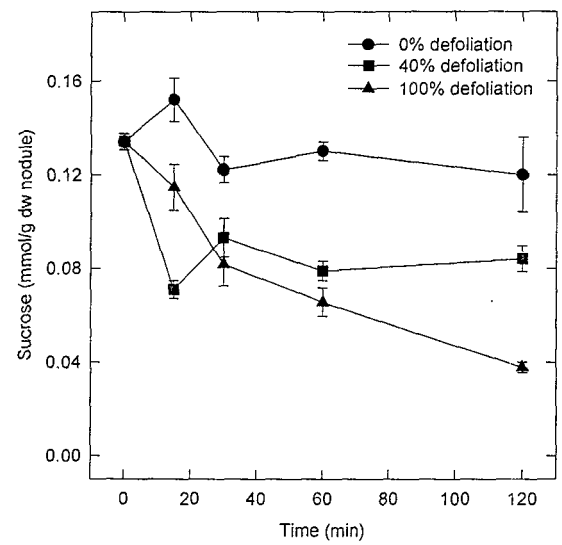

Figure 4. Suc concentrations of white clover nodules after partial (40% of leaf area removed) and complete defoliation (at time 0). Means ± SE of five replicates are shown. dw, Dry weight.

The Time Course of TNA and PNA during Regrowth after the Removal of 100% of the Leaf Area

To examine whether the carbon pools in nodules can represent a limiting factor for nitrogenase activity after defoliation and during regrowth, PNA was monitored (Fig. 5). Nitrogenase activity could always be significantly increased by elevating pO_e in the rhizosphere.

DISCUSSION

Correlation between Nodule Carbon Metabolites and O_2 Permeability

In the first experiment, oxygen permeability was manipulated by defoliation, a treatment known to cause a decline in nitrogenase activity and a concurrent decrease in nodule O_2 permeability (Hartwig et al., 1987; Denison and Layzell, 1991; Sung et al., 1991; Denison et al., 1992; Diaz del Castillo et al., 1992). Within the 1st h after 100% defoliation, Suc concentrations declined to 50% of their initial values in both roots and nodules (Fig. 1). This finding is consistent with the results of Gordon et al. (1986). Unexpectedly, the concentration of other metabolites in roots and nodules, especially those of Fru and Glc, were not affected by removal of 100% of the leaf area (Fig. 1). Nodule Glc concentration was very low and near the limit of detection, as was also found by Davis and Nordin (1983). Since Gordon et al. (1990) provided evidence for the presence of Suc synthase in white clover nodules, this result may indicate that Suc in white clover nodules is cleaved mainly by Suc synthase, because UDP-Glc, the product resulting from this cleavage, cannot be detected by the method used in this study. The substantial level of Glc found in soybean nodules by Streeter and Salminen (1993) may suggest that alkaline invertase plays a more significant role in Suc cleavage in soybean compared to white clover nodules.

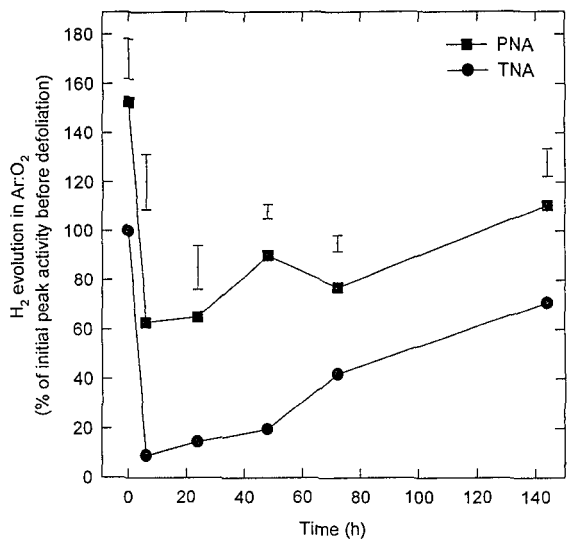

Figure 5. Changes in TNA and PNA after complete defoliation (at time 0) of white clover. Means ± SE of at least three replicates are shown. PNA differs significantly from TNA ($P \approx 0.0067$).

Of all organic acids analyzed, only results for L-malate and succinate are shown because these two metabolites are recognized as the main organic acids both in quantity and in support of bacteroid function (Day and Copeland, 1991). Their concentrations were found to be significantly higher in effective nodules than in ineffective ones (Rosendahl et al., 1990; Vance and Gant, 1992). Furthermore, L-malate and succinate concentrations found in nodules may develop a high osmotic activity and could therefore be involved in the regulation of the nodule O_2 permeability. However, neither L-malate nor succinate concentrations in nodules or roots were significantly affected by defoliation (Fig. 1), indicating the lack of a relationship on a whole-nodule basis between their concentrations and nodule O_2 permeability. Thus, Suc is the only whole-nodule metabolite analyzed that potentially could influence oxygen permeability.

In a second experiment, O_2 permeability was manipulated without interrupting phloem sap supply by altering pO_e. For this purpose, nodulated roots were adapted to either 40 or 10 kPa O_2 (Figs. 2 and 3). In contrast to a defoliation-induced decrease in nodule O_2 permeability, the reduction in O_2 permeability induced by 40 kPa pO_e had no effect on the concentrations of Suc and other carbohydrates (Table I). The decreased concentrations of L-malate and succinate in response to 40 kPa O_2, although not statistically significant (Table I), could indicate a depletion of these two metabolites due to slightly increased nitrogenase activity under 40 kPa O_2 (Fig. 2).

These findings do not support the hypothesis that total overall concentrations of Suc or other carbon metabolites are involved in triggering nodule O_2 permeability. This would be consistent with the fact that Suc concentration in roots and nodules drops in a similar manner after defoliation (Fig. 1), which makes it unlikely that nodule Suc concentrations are related specifically to nodule gas permeability. This holds true on the basis that possible localized changes in carbon metabolite concentrations are not masked by large amounts from the whole nodules.

Indication That the Import of Suc into Nodules Is Not Controlled by Nodule Sink Activity

Complete defoliation resulted in similar responses in root and nodule carbon metabolites (Fig. 1). This suggests that export of Suc from shoot to root is governed by the shoot's supply (source) and would imply that under the conditions tested in the present study, variations in the Suc supply to nodules are not determinants for nodule activity. To test this hypothesis, the source of Suc was manipulated by the degree of defoliation (40% of leaf area removed) known to have no effect on nodule function (Hartwig et al., 1994). Although nitrogenase activity and O_2 permeability were not affected by 40% defoliation, Suc concentrations declined substantially (Fig. 4). Obviously, full nodule function continued even under a significantly reduced Suc availability. The measured reduced Suc concentrations under this treatment may indicate a reduced supply from the shoot. Further evidence for the hypothesis that nodule activity is independent of a reduced supply of Suc is the fact that Suc concentration increased rather than declined after ANA was reduced to 70%

under a pO_e of 10 kPa (Table I; Fig. 3). Under this treatment, import of Suc into nodules may have continued at high rates in relation to the reduced nodule activity, and hence this sugar accumulated. These data strongly suggest that Suc supply to the nodules is not governed by the sink activity.

It is noteworthy that a new steady-state level of nodule Suc concentration was reached within 15 min after 40% defoliation, whereas it took at least 2 h after the 100% defoliation (Fig. 4). Under 40% defoliation, nitrogenase activity is unaffected (Hartwig et al., 1994) and thus carbon consumption remains high. Assuming that the supply of Suc decreased, a rapid drop in Suc concentration would be the obvious result. However, a 100% defoliation resulted in an immediate and severe decrease in nitrogenase activity and thus an immediately reduced carbon consumption. Since some Suc may still be supplied to the nodules from the large carbon storage in the stolon, the decrease in nodule Suc concentration was less abrupt. This is consistent with the idea that mechanisms other than carbon availability are governing nitrogenase activity (e.g. the proposed N-feedback mechanism; see Hartwig and Nösberger, 1994).

Results from these experiments led us to question whether nodule function is ever limited by carbon supply, even during periods of severe stress such as a complete defoliation. This, indeed, seems not to be the case, since the level of nitrogenase activity could always be significantly increased by increasing the pO_e (Fig. 5). This observation is consistent with those of Denison et al. (1992), who reported that after defoliation of alfalfa the decrease in nodule oxygen permeability preceded the decrease in the oxygen-saturated nodule respiration, which may depend mainly on nodule carbohydrate availability. It is possible that carbon resources for nodule activity become depleted and hence limiting if excised nodules are studied, as reported by Sung et al. (1991). It is also feasible that plants severely depleted of carbohydrates before defoliation or with a very limited capacity to store carbon metabolites cannot support adequate nodule activity during periods of stress such as defoliation. However, white clover, grown under favorable conditions with stolons that are not affected by defoliation and in which large amounts of starch can be stored, seems to supply nodules with Suc in excess. In fact, N_2 fixation in white clover after defoliation was proven to be adequate to supply sufficient nitrogen for regrowth (Hartwig et al., 1994).

Although a regulatory function for carbon metabolites in the short-term regulation of the nodule O_2 permeability and thus of the nitrogenase activity cannot be supported based on the present data, the presence of a variable O_2 permeability is unquestioned. Alternative mechanisms such as the rapid and reversible depolarization of membranes in nodules accompanying decreased O_2 permeability (Denison and Kinraide, 1995) and/or the involvement of glycoproteins in the regulation of a nodule O_2 barrier (de Lorenzo et al., 1993; Iannetta et al., 1993) may help to explain rapid changes in oxygen permeability.

CONCLUSIONS

This study provides evidence that after defoliation and during regrowth, carbon metabolites at the whole-nodule level appear not to be determinants of nodule activity, either through a direct metabolic involvement or through indirect effects like triggering the oxygen permeability. Even under the severe stress of a complete defoliation, nitrogenase activity seems primarily oxygen limited; changes in the whole-nodule Suc pool after defoliation have to be viewed as secondary effects not necessarily linked to nodule activity.

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