Regulation of growth, water use efficiency and δ^{13}C by the nitrogen source in *Casuarina equisetifolia* Forst. & Forst.

R. Martínez-Carrasco*[1], P. Pérez[1], L. L. Handley[2], C.M. Scrimgeour[2], M. Igual[1], I. Martín del Molino[1] and L. Sánchez de la Puente[1]

[1]Instituto de Recursos Naturales y Agrobiología de Salamanca, CSIC. Apartado 257, 37071 Salamanca, Spain.

[2]Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

*Correspondence to: Rafael Martínez-Carasco, Instituto de Recursos Naturales y Agrobiología de Salamanca, CSIC. Apartado 257, 37071 Salamanca, Spain. E-mail: rafaelmc@gugu.usal.es; FAX: 34 23219609; Telephone 34 23219606

Abstract

Carbon isotope composition (δ^{13}C) was measured in a glasshouse experiment with N$_2$-fixing and NO$_3^-$- or NH$_4^+$-fed *Casuarina equisetifolia* Forst. & Forst plants, both under well-watered and drought conditions. The abundance of ^{13}C was higher (more positive δ^{13}C) for NH$_4^+$ than for NO$_3^-$-grown plants and was lowest for N$_2$-fixing plants. NH$_4^+$-fed plants had more leaf area and dry weight and higher water use efficiency (on a biomass basis) than N$_2$- and NO$_3^-$-grown plants and had lower water consumption than plants supplied with NO$_3^-$, either with high or low water supply. Specific leaf areas and leaf area ratios were higher with NH$_4^+$ than with NO$_3^-$ or N$_2$ as the N source. The difference observed in δ^{13}C between plants grown with different N sources was higher than that predicted by theory and was not in the right direction (NH$_4^+$-grown plants with a more negative δ^{13}C) to be explained by differences in plant composition and engagement of the various carboxylation reactions. The more positive δ^{13}C in NH$_4^+$-than in NO$_3^-$-grown plants is probably due to a decreased ratio of stomatal to carboxylation conductances, which accounts for the lower water cost of C assimilation in NH$_4^+$-grown plants.

Key-words: *Casuarina equisetifolia* Forst. & Forst; actinorhizal plants; ammonium; carbon isotope composition; ; conductance; $\delta^{13}C$; growth; nitrate; nitrogen; nitrogen fixation; water use efficiency.

Introduction

NH$_4^+$ and NO$_3^-$ are the main sources of inorganic nitrogen for higher plants. Actinorhizal plants and legumes in symbiotic association with *Frankia* and *Rhizobium*, respectively, can also fix N$_2$ from the atmosphere. While more energy is required for the uptake and assimilation of N$_2$ and NO$_3^-$ than of NH$_4^+$ (Ryle, Powell & Gordon 1979; Raven 1985), plants frequently exhibit faster growth with NO$_3^-$ than with NH$_4^+$ (Salsac et al. 1987; Raab & Terry 1994). We have found no information on the growth differences caused by N sources for *Casuarina*. However, higher dry matter yields have been reported for other actinorhizal plants (*Alnus incana* and *Myrica gale*) in the presence of NH$_4^+$ (Troelstra, Van Dijk & Blacquière 1985; Troelstra, Wagenaar & Smant 1992).

N and C metabolism are closely associated and C assimilation is, in turn, inevitably linked to water loss through the stomata. The interaction of N metabolism, photosynthesis and C metabolism with water use has been shown in studies of transgenic *Nicotiana* plants with decreased Rubisco expression where low Rubisco levels increased the water costs of photosynthetic CO$_2$ fixation at the expense of a lower N cost (Stitt & Schulze 1994). At leaf level, water use efficiency (the ratio of dry matter production to transpiration) depends on the ratio of CO$_2$ assimilation rate to transpiration rate; the latter, in turn, depends on the diffusive conductance to water vapour. Farquhar, O'Leary and Berry (1982) showed that C$_3$ plants should exhibit a negative association between the ratio of assimilation to conductance and discrimination against ^{13}C. It has also been shown that differences in the N source for growth and N content can alter the contribution of non-Rubisco carboxylases to net C acquisition, thereby altering the δ^{13}C signature of plants (Farquhar, Ehleringer & Hubick 1989; Raven & Farquhar 1990). Raven (1985) and Raven and Sprent (1993) predicted that at given ratios of assimilation to conductance and water vapour pressure gradients between the intercellular spaces and the bulk air, the water cost of C assimilation is greater with N$_2$, and similar or greater with NO$_3^-$ than with NH$_4^+$ as the N

source. The experimental data show, however, that the water cost of C accumulation with NO_3^- is less than with NH_4^+ in several plant species (Raven & Sprent 1993).

The aim of this work was to investigate (1) the relative effects of the source of N on C assimilation and conductance and (2) whether water use efficiency with different N sources for growth can be accounted for by the predicted water costs of C assimilation, or rather by the ratio of C assimilation to gas-phase conductance. We analysed $\delta^{13}C$, the expansion of the photosynthetic surface, dry matter accumulation and water consumption of *Casuarina equisetifolia* plants relying on symbiotic N_2 fixation or grown with NH_4^+ or NO_3^-, under both well-watered and drought conditions.

Materials and methods

Seeds of *Casuarina equisetifolia* Forst. & Forst. were germinated in trays containing perlite under 200 µmol m^{-2} s^{-1} illumination at 25 ^{o}C. Thirty-nine days later the seedlings were transferred to hydroponic culture containing 1/4-strength Crone's medium (Hewitt 1966) without N. After 21 days, Crone's medium was increased in concentration to 1/2-strength without N for N$_2$-fixing plants, with 3.71 mM KNO$_3$ for NO$_3^-$-fed plants or NH$_4$Cl for NH$_4^+$-fed plants. The N$_2$-fixing treatments were inoculated with a suspension of crushed nodules of *Casuarina equisetifolia* (Cañizo & Rodriguez-Barrueco 1976) 2 months after sowing. At 166 days after sowing, seedlings were planted in 2-L pots containing perlite, which were placed in a glasshouse with 30/13°C maximum/minimum temperatures and 1000 µmol m^{-2} s^{-1} maximum photon flux density. The nutrient solution used subsequently contained 4 mM CaCl$_2$, 1.5 mM MgSO$_4$, 0.4 mM HNa$_2$PO$_4$, 0.94 mM H$_2$NaPO$_4$, 0.1 mM iron citrate and micronutrients (Hewitt 1966). In addition, the solution for N$_2$-fixing plants contained 10.1 mM KCl, that for NO$_3^-$-fed plants 10.1 mM KNO$_3$ and that for NH$_4^+$-fed plants 10.1 mM NH$_4$Cl and 10.1 mM KCl.

The experiment consisted of all of the factorial combinations of the N sources with two levels of water supply, replicated three times. The two watering levels were achieved by watering the pots to field capacity once a week and once every two weeks. The pots were initially flushed with a large, known amount of water to wash out the rooting medium. Then, the nutrient solution was added and the total drainage measured. Water use by evapotranspiration was estimated as the difference between added and drained water.

At 231 days after sowing, one plant per factorial combination and replicate was harvested and the green area was measured with an electronic planimeter (LI-3000, LiCor, USA). Plants were separated into green, non-green (including the stem and senescent cladodes) and root fractions and then oven dried at 60°C for 16 h and weighed. Plant C and N contents and δ^{13}C were analysed using an elemental analyzer coupled to a mass spectrometer (Europa Scientific Tracermass with Roboprep-CN

sample converter, Europa Scientific Ltd., Crewe, UK; Handley et al. 1993). Carbon isotope composition ($\delta^{13}C$) was defined as $[(^{13/12}\text{Ratio}_{\text{sample}} - {}^{13/12}\text{Ratio}_{\text{standard}})/^{13/12}\text{Ratio}_{\text{standard}}] \times 10^3$. Analyses of variance and orthogonal contrasts between treatments were performed as in a fully randomized factorial experiment.

Results

Plant growth and water use

At 231 days after sowing, NH_4^+-fed plants had greater dry weight and green area than those supplied with NO_3^-, which in turn showed higher values than N_2-fixing plants. These effects were found for all plant fractions, and only the total dry weight and green area are shown in Table 1. The plants supplied with NH_4^+ showed higher specific leaf areas (area/dry weight ratios for green cladodes) and leaf area ratios (green area/total plant dry weight ratios) than NO_3^-- and N_2-grown plants. The shoot:root ratio was not significantly affected by the N source. The described effects of the N source on dry matter production and intra-plant distributions were observed under both drought and well-watered conditions. Drought decreased both the dry weights and amounts of green area, although it had little effect on N_2-supplied plants. Drought had no significant effect on specific leaf area, the leaf area ratio or the shoot:root ratio (Table 1).

Total water use was greater for NO_3^-- than for NH_4^+- and N_2-grown plants, both under well-watered and drought conditions (Table 1), although total water use was decreased by drought. The ratio of total dry weight to water consumed (water use efficiency, WUE) was highest in NH_4^+-grown plants and lowest in N_2-grown plants (Table 1). We found that evaporative losses from pots without plants irrigated following the well-watered treatments were similar to those of well-watered N_2-grown plants (data not shown). This indicates that relatively more water was evaporated from pots with plants consuming less of the available water, leading to overestimations of the water used, and thus to underestimations of WUE of N_2- as compared to NH_4^+- and NO_3^--grown plants. Differences in transpiration and WUE between well-watered and droughted plants would thus be smaller and greater, respectively, than estimated. On the other hand, the observed differences between NH_4^+- and the higher water-consuming NO_3^--grown plants are unlikely to be reversed.

Carbon isotope composition

N_2-fixing plants had more negative $\delta^{13}C$ values in the green cladodes than did NO_3^--

fed plants, which in turn had lower $\delta^{13}C$ values than NH_4^+-grown plants (Table 2). This effect of the N source on $\delta^{13}C$ was observed both in well-watered and drought-stressed plants. The carbon isotope composition in other plant fractions showed similar responses to the N source (data not shown). Drought had no significant effect on $\delta^{13}C$.

The carbon isotope composition of the green cladodes showed a significantly negative relationship with whole plant water use efficiency (Fig. 1). The low values for water use efficiency and the high extrapolated value for $\delta^{13}C$ at zero water use efficiency in Fig. 1 are due to the use of evapotranspiration rather than transpiration in the computations.

Carbon and nitrogen contents

There were few differences in C content (per cent dry weight) of the green cladodes among plants with different N sources (Table 2). The N content (per cent dry weight) of the green cladodes was higher with NH_4^+ than with NO_3^- and N_2 as N sources; N_2-grown plants showed the lowest values. The C and N contents of other plant fractions had similar responses to the N source (data not shown). The whole-plant N contents were very low in N_2- as compared to NH_4^+- and NO_3^--grown plants (Table 2). The C/N ratio was lowest in NH_4^+-fed plants and highest in N_2-fixing plants (Table 2). Drought decreased the N content and increased the C/N ratio in NO_3^--grown plants. The water cost of carbon assimilation (the molar ratio of water consumed by evapotranspiration to whole-plant C content) was lower for NH_4^+- than for NO_3^--grown plants (Table 2); the very high water cost with N_2 as the N source resulted from the relatively high evaporation from perlite in pots with low transpiration. Drought had no significant effect on the water cost of carbon assimilation.

Discussion

NH$_4^+$-grown *Casuarina equisetifolia* plants had a less negative δ^{13}C than plants with other N sources, which according to Farquhar et al. (1982) indicates they had a higher ratio of C assimilation to diffusive conductance. The higher rate of C assimilation found with NH$_4^+$ than with other N sources is attributable to a change in dry matter allocation within the plant, with greater generation of green area per unit of green and total plant biomass in NH$_4^+$-grown plants, as indicated by the specific leaf area and the leaf area ratio (Table 1). Work is currently in progress to examine whether C fixation per unit leaf area is also affected by the source of N. The lower water use in NH$_4^+$- than in NO$_3^-$-grown plants, in spite of the larger leaf area of the former, suggests that NH$_4^+$ decreased diffusive conductance. Thus, the increased ratio of C assimilation to diffusive conductance and hence the higher WUE of NH$_4^+$-grown plants were the result of both increased dry matter production and decreased conductance.

Theory (Raven 1985; Raven & Sprent 1993) predicts that the water cost of carbon assimilation is greater with N$_2$, and similar or greater with NO$_3^-$ than with NH$_4^+$ as the N source. Contrary to the results of other authors (Raven & Sprent 1993 and references therein), our results with *Casuarina equisetifolia* are in agreement with the theory. This occurred despite the higher C/N ratio (Table 2) for NO$_3^-$-grown and N$_2$-grown than for NH$_4^+$-grown plants, which should reduce the predicted increment of water cost of carbon assimilation caused by NO$_3^-$ and N$_2$ rather than NH$_4^+$ as the N source (Raven & Sprent 1993). However, our estimates of both the water cost of C assimilation and the differences in water cost associated with the source of N are higher than the theoretical prediction (Raven & Sprent 1993). Evapotranspiration rather than transpiration was used for computations, and this accounts for the very high water cost with N$_2$ as the N source, but not for the higher than predicted difference in water cost of C assimilation between NO$_3^-$- and NH$_4^+$-grown plants. On the basis of differences in composition and the occurrence and location of carboxylation reactions, a difference in δ^{13}C of 0.11 $^o/_{oo}$ between NO$_3^-$- and NH$_4^+$-grown *Ricinus communis* plants is predicted, NO$_3^-$-grown

plants being more positive (Raven & Farquhar 1990). The observed differences between *Casuarina* plants with these N sources is 1.12 $^o/_{oo}$ and 2.19 $^o/_{oo}$ for droughted and well-watered plants, respectively, NH_4^+-grown plants being more positive. This departure from the predictions suggests (Raven & Farquhar 1990) that rather than plant composition and involvement of the various carboxylases, a lower ratio of intercellular (p_i) to bulk air (p_a) partial pressures of CO_2 and hence a decreased ratio of stomatal to carboxylation conductances, which is associated with a higher (less negative) $\delta^{13}C$ (Farquhar, O'Leary & Berry 1982), would be the cause of the large difference in water cost of carbon acquisition between NH_4^+-grown plants and those supplied with NO_3^-.

The basis for the decreased p_i/p_a ratio of the NH_4^+-grown plants is unclear. Increased photosynthesis in sugar beet plants supplied with NH_4^+ reduced the intercellular CO_2 concentration and had no effect on stomatal conductance (Raab & Terry 1994) while NH_4^+ decreased conductance in *Casuarina equisetifolia*. Other experiments in our laboratory (Sánchez-Rodriguez, Martínez-Carrasco & Pérez, unpublished) indicate that NH_4^+-grown plants have lower water potentials than those with N_2 and NO_3^- as the chief N source. We speculate that this may have decreased the stomatal conductance and thus the p_i/p_a ratio of plants supplied with NH_4^+. Work is under way to test this hypothesis.

Acknowledgments

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Table 1. Total dry weight (g plant^{-1}), green area (cm^2 plant^{-1}), specific leaf area (SLA, cm^2 g^{-1}), leaf area ratio (LAR, cm^2 g^{-1}), ratio of shoot to root dry weights, cummulative water use (dm^3 plant^{-1}) and water use efficiency (WUE, g l^{-1}) of droughted and well-watered *Casuarina equisetifolia* plants grown with N$_2$, NO$_3^-$ or NH$_4^+$ as N source at 231 days after sowing. s.e.d, standard error of the difference between two means. Each value is the mean of 3 replicates.

Plant variables	N$_2$		NO$_3^-$		NH$_4^+$		
	drought	control	drought	control	drought	control	s.e.d
D. Weight	0.24	0.32	1.91	3.56	2.68	4.81	0.484
Area	2.50	3.80	23.5	50.1	45.8	82.0	10.26
SLA	18.7	22.5	20.4	23.0	28.8	27.1	2.58
LAR	10.5	13.3	12.1	14.5	16.8	17.0	1.99
Shoot/ root	2.48	2.47	2.65	2.66	2.74	2.99	0.377
Water use	2.14	3.38	2.67	3.88	2.48	3.52	0.145
WUE	0.11	0.09	0.72	0.93	1.08	1.37	0.115

Table 2. δ^{13}C, C and N content (% dry weight) and molar C/N ratios of green cladodes, total N content (mg plant^{-1}) and water cost of carbon assimilation (mol H$_2$O consumed by evapotranspiration/ mol C) of droughted and well-watered *Casuarina equisetifolia* plants grown with N$_2$, NO$_3^-$ or NH$_4^+$ as N source at 231 days after sowing. s.e.d, standard error of the difference between two means. *, s.e.d. for NO$_3^-$- and NH$_4^+$-grown plants. Each value is the mean of 3 replicates.

	N$_2$		NO$_3^-$		NH$_4^+$		
	drought	control	drought	control	drought	control	s.e.d.
δ^{13}C	-28.7	-28.6	-27.2	-27.7	-26.0	-25.5	0.29
C%	43.4	43.7	44.5	45.6	44.5	44.7	2.96
N%	1.72	1.76	2.02	2.45	2.80	2.67	0.110
total N	3.14	4.40	33.1	69.4	63.3	109.9	8.63
C/N	29.6	29.0	25.8	21.8	18.6	19.5	1.37
Water cost	14100	19437	2151	1870	1436	1101	*247

Legends to figures

Fig. 1. Relationship between carbon isotope ratio ($\delta^{13}C$) of green cladodes and water use efficiency (total dry weight/water consumption) of droughted and watered *Casuarina equisetifolia* plants grown with N_2 (circles) NO_3^- (squares) or NH_4^+ (triangles) as N source at 231 days after planting. The regression line is:
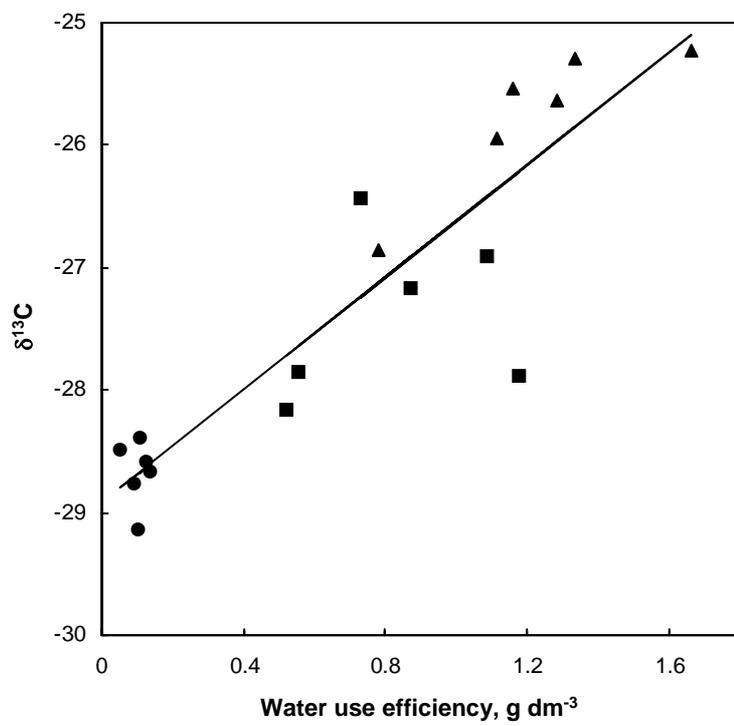 $y= -28.916(\pm0.240)+2.294(\pm0.273)x$, $R^2=0.82$.

Figure 1