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ORIGINAL PAPER

Nitrate reductase activity and nitrogen compounds in xylem exudate of *Juglans nigra* seedlings: relation to nitrogen source and supply

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Abstract Nitrogen (N) limits plant productivity and its uptake and assimilation may be regulated by N source, N availability, and nitrate reductase activity (NRA). Knowledge of how these factors interact to affect N uptake and assimilation processes in woody angiosperms is limited. We fertilized 1-year-old, half-sib black walnut (*Juglans nigra* L.) seedlings with ammonium (NH_4^+) [as $(NH_4)_2SO_4$], nitrate (NO_3^-) (as $NaNO_3$), or a mixed N source (NH_4NO_3) at 0, 800, or 1,600 mg N plant^{-1} season^{-1}. Two months following final fertilization, growth, in vivo NRA, plant N status, and xylem exudate N composition were assessed. Specific leaf NRA was higher in NO_3^--fed and NH_4NO_3-fed plants compared to observed responses in NH_4^+-fed seedlings. Regardless of N source, N addition increased the proportion of amino acids (AA) in xylem exudate, inferring greater NRA in roots, which suggests higher energy cost to plants. Root total NRA was 37% higher in NO_3^--fed than in NH_4^+-fed plants. Exogenous NO_3^- was assimilated in roots or stored, so no difference was observed in NO_3^- levels transported in xylem. Black walnut seedling growth and physiology were generally favored by the mixed N source over NO_3^- or NH_4^+ alone, suggesting NH_4NO_3 is required to maximize productivity in black walnut. Our findings indicate that black walnut seedling responses to N source and level contrast markedly with results noted for woody gymnosperms or herbaceous angiosperms.

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Introduction

Black walnut (*Juglans nigra* L.) is one of the most valuable species of the Central Hardwood Forest Region of the United States. The species is used for high quality lumber and veneer, has value for nut production and serves as a food source for wildlife (Williams 1990). Considering the value and increasing significance of black walnut (Jacobs et al. 2004), optimizing growth and physiology of juvenile seedlings is important to enhance plantation productivity. Nitrogen (N) supply is a key factor limiting plant growth and appropriate N fertilization provides an opportunity to promote productivity of tree crops (Allen 1987), especially for young plantations.

Plants may assimilate organic N (Näsholm et al. 1998; Raab et al. 1999; Persson et al. 2006). However, the major source of N for plant uptake and assimilation is inorganic N as nitrate (NO_3^-) or ammonium (NH_4^+) (Hageman 1980; Walecka-Hutchison and Walworth 2007). Absorbed NH_4^+ is quickly assimilated into amino acids (AA) and proteins for storage in roots because low levels can cause NH_4^+ toxicity, such as ammonium-induced element deficiency associated with impaired uptake of other cations and rhizosphere acidification (Haynes 1986). By contrast, absorbed NO_3^- may be stored or assimilated into organic forms for storage in roots or transported in xylem exudate along with AA into leaves where it is reduced. These processes may be regulated by nitrate reductase activity (NRA), N source supplied to plants, and N application levels.

Studies have shown that preferred N source is largely species-specific. For example, woody gymnosperms

generally grow on acidic soils and have adapted mechanisms to use NH_4^+ preferentially, such as storing NH_4^+ in acidic vacuoles (Aarnes et al. 2007). By contrast, herbaceous angiosperms grow on less acidic soils and have not adapted such mechanisms for using NH_4^+, so they preferentially use NO_3^- (Hageman 1980). It is unclear as to whether N form preference of woody angiosperms would more closely resemble that of woody gymnosperms or herbaceous angiosperms, though woody angiosperms may be better adapted to take up NO_3^- than gymnosperms (Min et al. 1999).

Nitrate reductase activity is a measure of capacity for plants to reduce NO_3^- into usable forms for assimilation, and location of NO_3^- reduction has a critical influence on plant energy costs. Reduction of NO_3^- in the roots is associated with increased root respiration because greater energy is required to assimilate NO_3^- (Zogg et al. 1996). Conversely, assimilation of NO_3^- in leaves occurs at less energy cost to plants because reduction in leaves is powered mostly from direct products of photosynthesis (Schrader 1984). Nitrogen source may influence NRA. It has been suggested that NH_4^+ may decrease NRA because of feedback inhibition from end products of NH_4^+ assimilation (Orebamjo and Stewart 1975). By contrast, other studies contend that NH_4^+ inhibition rarely occurs and NH_4^+ is often required for optimal NRA (Mohanty and Fletcher 1976; Beevers and Hageman 1980). Furthermore, NO_3^- is known to induce NRA (Wang et al. 2003) and a mixed N source may optimize NRA (Hageman 1980). Location of NRA can be estimated by xylem exudate composition (Pate 1973; Andrews 1986) because the proportion of NO_3^- to total N in xylem exudates reflects partitioning of NRA in roots and shoots. This may be controversial because N compounds can also be remobilized from storage or transferred in phloem to skew the correlations (Malaguti et al. 2001; Guak et al. 2003). Low amounts of NO_3^- in xylem exudate suggest most NO_3^- is assimilated in roots (Radin 1978), while greater amounts suggest that NO_3^- is assimilated more in leaves. Like NRA, xylem exudate composition is species-specific and affected by different N sources.

However, few studies have examined N source effects on xylem exudate composition and on NRA in woody angiosperms. For instance, NRA in red ash (*Fraxinus pennsylvanica* Marsh.) seedlings was not changed with increasing NH_4^+, but was increased with increasing NO_3^- (Truax et al. 1994). In the same study, there was no change in root NRA with increasing NH_4^+, but an increase was noted with NO_3^-. In red maple (*Acer rubrum* L.), NRA fluctuated (up and down) as N was increased with NO_3^- or NH_4^+ addition (Downs et al. 1993). These divergent results indicate that it is unclear how NRA of woody angiosperms in general is affected by N source. We are not aware of any published research that has quantified xylem exudate N composition or measured NRA to explore how NRA might influence N assimilation processes in black walnut. A few studies exist for other *Juglans* species. For example, little of the total N in xylem exudate consisted of AA in 6-year-old English walnut (*Juglans regia* L.), suggesting that most NO_3^- was transported to leaves for assimilation (Ding and Xi 1993). By contrast, the majority of xylem exudate N consisted of AA in 5-year-old English walnut, signifying more NO_3^- was assimilated in roots (Prima-Putra and Botton 1998).

Although NRA may increase in walnut when supplied with NH_4NO_3 than with NO_3^- alone (Nicodemus et al. 2007), the effects of increased fertility and varied N source on xylem exudate N composition and location of NO_3^- assimilation have yet to be quantified and elucidated for this species. In the current study, we examined growth and physiological responses of black walnut to varied N source and supply, and quantified NRA to assess location of NO_3^- assimilation and implications on plant energy cost. This knowledge is important to help select desired N species and fertilizer regimes that will result in lower plant energy cost and improved productivity. We expected (1) black walnut seedlings to prefer NH_4NO_3 rather than NH_4^+- or NO_3^--based fertilizers (Nicodemus et al. 2007), (2) greater growth, N uptake, and NRA associated with preferred N source, and (3) higher percentage of NO_3^- and AA in the xylem exudate with increased N supply, which would be associated with greater NRA in leaves and overall less energy cost to plants.

Materials and methods

Plant material and treatments

Half-sib (from expired patent Purdue #1 mother tree) black walnut seedlings were grown under operational bareroot production systems for one season in the Indiana State Nursery near Vallonia, IN (38°85′N, 86°10′W). Seedling production protocols are detailed in Jacobs (2003). Use of half-sib seed helped minimize genetic variation in responses, because NRA and xylem exudate composition are sensitive to genotype (Hirel et al. 2001; Majerowicz and Kerbauy 2002) as well as physiological responses to fertilization (Zornoza and Gonzalez 1998). In October 2004, seedlings were lifted and overwintered (3–5°C) in a cooler at the Purdue University Forestry and Natural Resources Farm. In April 2005, seedlings were removed from cold storage and potted into 10.7 l Treepot™ containers (Steuwe and Sons, Corvallis, OR, USA) at the Purdue University Horticulture and Landscape Architecture Plant Growth Facility (40°4′N, 86°30′W). The planting medium was

Berger BM-6^{TM} (Berger Peat Moss, St Modeste, QC, Canada), which consists of 4:1 peat:perlite (by volume). Plants were watered equally twice a week as needed ($\sim$1 l plant^{-1} week^{-1}) based on gravimetric techniques (Timmer and Armstrong 1989) with pH adjusted to $\sim$6.0, which is known to be within the ideal range for growing walnut seedlings (Williams 1990). Greenhouse air temperature ranged from 20 to 24°C and supplemental light (350–400 μmol m^{-2} s^{-1}) was used to provide 16 h of daylight.

Plants were fertilized with N as NH_4NO_3, $NaNO_3$, or $(NH_4)_2SO_4$ at 0, 800, or 1,600 mg N plant^{-1} season^{-1}. These rates were chosen within the general fertilization rates (range 160–400 kg N ha^{-1}) recommended for black walnut (Villarrubia 1980; Ponder 1996, 1998). Ammonium nitrate and $NaNO_3$ were selected because they have been shown to positively influence black walnut (Gray and Garrett 1998; Nicodemus et al. 2007), and $(NH_4)_2SO_4$ is a common source for NH_4^+. Thus, the experimental design was a 3 × 3 (N source × N level) completely randomized factorial design with eight replications of each source × level treatment. Initial N content (N_s = 612 mg N plant^{-1}) was determined from five plants, which was used to compute exponential fertilization regimes as detailed elsewhere (Timmer 1997). The total seasonal fertilizer dose (N_T) was supplied over seven applications, with the initial fertilization immediately after all seedlings broke bud and following every 2 weeks. Exponential fertilization was used to help maximize plant N uptake and physiological response to treatments. Exponential fertilization has been successfully applied to 1-year-old tree seedlings (Dumroese 2003; Nicodemus et al. 2008) and for seedling culture of other hardwood species (Close et al. 2005; Salifu and Jacobs 2006). The approach has demonstrated merit for optimizing N uptake and minimizing potential leaching losses (Timmer 1997; Dumroese et al. 2005). Two months following final N application, physiological measurements were conducted and seedlings were processed to determine dry weight of components and N concentration. Component N content was computed as concentration multiplied by plant dry mass.

Nitrate reductase activity

Nitrate reductase activity was assessed using an in vivo assay 2 months after final fertilization (Truax et al. 1994). Measurements were initiated between 1,000 and 1,400 h solar time. Roots and leaves were rinsed and then dried of surface water before sampling to minimize contamination and ensure weights were not skewed by water on samples. Roots <1 mm diameter and alive (white or light brown in color) were cut into <1 cm sections using a knife. Leaf disks were cut using a hole punch and taken from the second leaflet from the terminus of the youngest fully expanded leaf. A 0.2 g fresh tissue sample was placed in a test tube containing 2 ml incubating solution [100 mM phosphate buffer (pH 7.5), 40 mM KNO_3, and 1.2% 1-propanol], which was sealed and placed in the dark for 1 h at room temperature (24°C). The enzymatic reaction was stopped by removing plant tissue. A 1 ml aliquot was extracted from the tube with a pipette, mixed with 1 ml naphthyl ethylenediamine (NED) (0.02%) and 1 ml sulfanilic acid, and the initial absorbance at 540 nm was read on a Perkin-Elmer LC-95 UV/visible spectrophotometer (Perkin-Elmer Inc., Norwalk, CT, USA). After 30 min, samples were again measured for absorbance at 540 nm (Truax et al. 1994).

Xylem exudate analysis

Xylem exudate was extracted according to procedures described for hardwood species (Siebrecht and Tischner 1999). Stems were severed with clippers at the root collar. Severed stem with intact leaves was fitted into a Scholander chamber. Bark was removed from the section of stem that was exposed to the outside of the chamber and the stem was rinsed with distilled water to prevent contamination with phloem exudate. The initial exuded sap was discarded to minimize contamination by damaged tissues. A section of Tygon tubing was attached to the exposed end of the stem and directed to a vial to collect the exudate. Pressure was increased in the chamber until sap flowed from the exposed stem. The exudate was collected in the vial and immediately frozen at −20°C until analyzed. Samples were all taken within 3 days and collected between 1,000 and 1,400 h solar time (Siebrecht and Tischner 1999).

At time of analysis, exudate samples were allowed to thaw at room temperature. Nitrate and NH_4^+ were measured on 100 μl of exudate (made up to 1 ml with distilled water) using a Jenco 6251 ion meter (Jenco Electronics, Inc., San Diego, CA, USA). Total AA concentration was measured using the ninhydrin assay (Pandey and Upadhyay 2006). One hundred microliters of xylem exudate was added to 900 μl of water to adjust to 1 ml of sample. To this was added 1.2 ml of ninhydrin reagent (0.575 g ninhydrin in 50 ml 2-methoxyethanol plus 0.02 g ascorbic acid in 2 ml water made up to 60 ml with 2-methoxyethanol) and 0.5 ml 0.8 M citrate buffer (168 g citric acid plus 64 g NaOH per liter water). This mixture was heated at 100°C on a hot plate for 30 min, with a marble on top of each test tube to prevent evaporation. Samples were removed and allowed to cool for at least 5 min. Three ml of 60% ethanol was added and the absorbance read at 570 nm on a Perkin-Elmer LC-95 UV/visible spectrophotometer (Perkin-Elmer Inc., Norwalk, CT, USA). The absorbance was compared to a reference curve made from known amounts of AA.

Plant sampling, chemical, and statistical analysis

Plants were separated into leaves, roots, new and old stems following physiological measurements. New stems were those taken from the stem between the location of initial terminus (at planting) and final terminus (at harvest). These samples were placed in an oven for 72 h at 68°C and component dry mass determined. Subsequently, samples were ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a 20 mesh sieve. For carbon (C) and N determination, 200 mg of sample was used. Carbon and N were determined by combustion using a LECO CNS-2000 analyzer (Leco Corporation, St Joseph, MI, USA).

Analysis of variance (ANOVA) was conducted on measured data with treatment effects tested at $P < 0.05$ and where significant, treatment means were ranked according to Tukey's highly significant difference (HSD) test at $\alpha = 0.05$ using SAS software (SAS Institute, Cary, NC, USA). To minimize possibility of Type II error for specific NRA for leaf and roots, ANOVA treatment effects were considered significant for these variables at $P < 0.10$ and where significant, treatment means were ranked according to Tukey's HSD test at $\alpha = 0.10$. Prior to ANOVA, data were tested and found to meet the ANOVA assumptions for normality (Shapiro–Wilks test) and homogeneity of variances (Bartlett test). The linear model for the ANOVA is given as:

$$Y_{ijk} = \mu + S_i + R_j + SR_{ij} + \varepsilon_{(ij)k}$$

where Y_{ijk} is plant component dry mass, or measured physiological response of the kth replicate ($k = 1, 2, 3,\ldots,8$), estimated from the jth N level ($j = 1, 2, 3$) from the ith N source ($i = 1, 2, 3$); μ = overall mean; S_i = fixed effect of the ith N source; R_j = fixed effect of the jth N level; followed by interaction effects and ε is error associated with measured seedling dry mass or physiological response from bulk replicates. Significant N source and level interaction effects were separated by orthogonal contrasts (Nogueira 2004), which examined N source effects within the 800 and 1,600 mg N plant^{-1} levels.

Results

Nitrogen level effects on growth and physiology

Black walnut seedling growth was significantly affected by N level for the whole plant as well as in plant components (Fig. 1a, Table 2). For example, plant dry mass at the 1,600 mg N level increased 27% compared with the control.

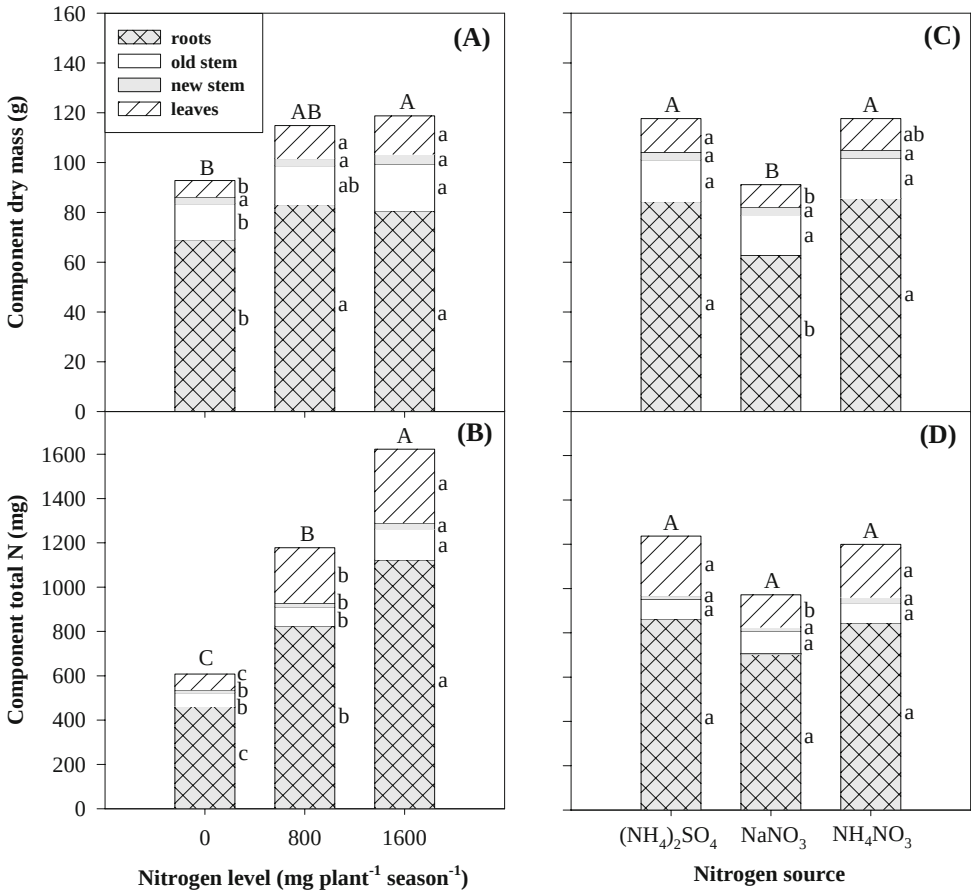

Fig. 1 One-year old black walnut seedling component dry mass as affected by N application level (*left*) or N source (*right*) grown under greenhouse conditions. *Lowercase letters* show significant differences (Tukey, $\alpha = 0.05$) within each component and *uppercase letters* show whole plant responses

Similarly, plant as well as plant component N contents differed by N level. Compared with unfertilized seedlings, plant N content was nearly double in the 800 mg N treatment and more than 2.5 times higher in the 1,600 mg N treatment (1,623 mg) (Fig. 1b). Plant component N concentration and C:N were highly affected by N level (Table 1). For example, leaf N concentration was twice as high for the 1,600 mg N level compared with unfertilized seedlings. However, leaf N concentration for plants fertilized at 1,600 and 800 mg N were similar (Table 1). By contrast, C:N for control was nearly twice that of the 1,600 mg N treatment. Root N concentration was 2 and 2.5 times higher in plants fertilized at 800 and 1,600 mg N, respectively, compared with controls and differed significantly (Table 1). Total NRA was significantly affected by N level in plant components (Fig. 2a, Table 2). Total NRA was much higher in roots than leaves (Fig. 2a), due to much higher root mass (Fig. 1a). The percentage of AA in xylem exudate increased with increasing fertilization, whereas NO_3^- and NH_4^+ decreased (Fig. 3a, Table 2).

Table 1 Carbon, N, and C:N by plant component for black walnut seedlings as affected by N level and source

Treatment	Measured parameter	Plant component			
		Leaf	Root	Old stem	New stem
N level (mg N)					
	C (g kg^{-1})				
0		467.2a	474.8b	481.8a	484.1a
800		468.3a	479.4ab	482.4a	483.1a
1,600		465.0a	485.3a	481.8a	484.2a
	P value	0.7579	*0.0411*	0.4199	0.9452
	N (g kg^{-1})				
0		10.0b	6.7c	4.4c	5.0b
800		18.6a	10.1b	5.8b	6.7ab
1,600		20.8a	14.9a	7.5a	8.7a
	P value	*<0.0001*	*<0.0001*	*<0.0001*	*0.0003*
	C:N				
0		53.99a	80.88a	128.44a	110.65a
800		26.96b	50.33b	88.03b	79.89b
1,600		23.85b	35.05c	68.64b	66.19b
	P value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
N source					
	C (g kg^{-1})				
$(NH_4)_2SO_4$		467.2a	475.3b	481.6a	480.5a
$NaNO_3$		468.3a	485.4a	486.4a	487.0a
NH_4NO_3		465.0a	478.7ab	482.1a	483.8a
	P value	0.1728	*0.0502*	0.2907	0.2365
	N (g kg^{-1})				
$(NH_4)_2SO_4$		16.9a	9.97a	5.3b	5.8b
$NaNO_3$		15.4a	11.7a	6.4a	8.0a
NH_4NO_3		17.0a	10.1a	5.9ab	6.5ab
	P value	0.3471	*0.0870*	*0.0662*	*0.0384*
	C:N				
$(NH_4)_2SO_4$		36.48a	53.58a	107.16a	96.67a
$NaNO_3$		34.18a	52.77a	85.30a	73.72b
NH_4NO_3		34.13a	59.91a	92.65a	86.34ab
	P value	0.8017	0.3897	0.1734	*0.0332*

Italic *P* values and *lowercase letters* indicate significant differences (Tukey, $\alpha = 0.10$)

Nitrogen source effects on growth and physiology

Plant and component dry mass was significantly lower in NO_3^--fed plants compared with those fertilized with NH_4^+ or NH_4NO_3 (Fig. 1c). However, plant component N content was similar across all treatments except for leaves (Fig. 1d). Leaf N concentration was not significantly affected by N source (Table 1), though N concentration for NO_3^--fed plants (15.4 g kg^{-1}) was lower than that measured in plants fed with NH_4^+ (16.9 g kg^{-1}) or NH_4NO_3 (17.0 g kg^{-1}). Nitrogen concentration was higher in old and new stems of NO_3^- than in NH_4^+-fed plants (Table 1). Similarly, new stem C:N differed by N source (Table 1). Total NRA was unaffected by N source for leaves or roots, but higher in roots than leaves (Fig. 2b). Amino acids and NO_3^- in the xylem exudate were not significantly affected by N source (Table 2, Fig. 3b). Ammonium was significantly affected by N source (Table 2) with NH_4^+-fed plants exhibiting higher NH_4^+ levels than the other sources (Fig. 3b).

Interaction effects (source × level) on growth and physiology

Treatment interaction effects of N source × level were significant for leaf C:N and leaf N content. Carbon to N ratio was not significantly affected by N source at the 800 mg N level, but at the 1,600 mg N level, leaf C:N was higher in plants fed with NO_3^- than NH_4^+ source (Fig. 4a). Leaf N was higher in plants fed with NH_4NO_3 than NO_3^- source at the 800 mg N level. But, at the 1,600 mg N level, leaf N was higher in NH_4^+ treated plants compared with those fed with NH_4NO_3 (Fig. 4b). Leaf N concentration was similar between N sources at the 800 mg N level, but significantly increased in NH_4^+ than in NO_3^--fed plants at the 1,600 mg N level (Fig. 5a). Root N concentration of NO_3^- treated plants was higher than the other sources at the 800 mg N level (Fig. 5b). Specific NRA also showed a significant source × level interaction effect in leaves and roots (Table 2). At the 800 mg N level, specific NRA in leaves of seedlings treated with NO_3^- or NH_4NO_3 was more than twice that of NH_4^+-fed plants. However, at the 1,600 mg N level, specific NRA in leaves of NH_4^+-treated plants increased while that of NO_3^--fed plants decreased

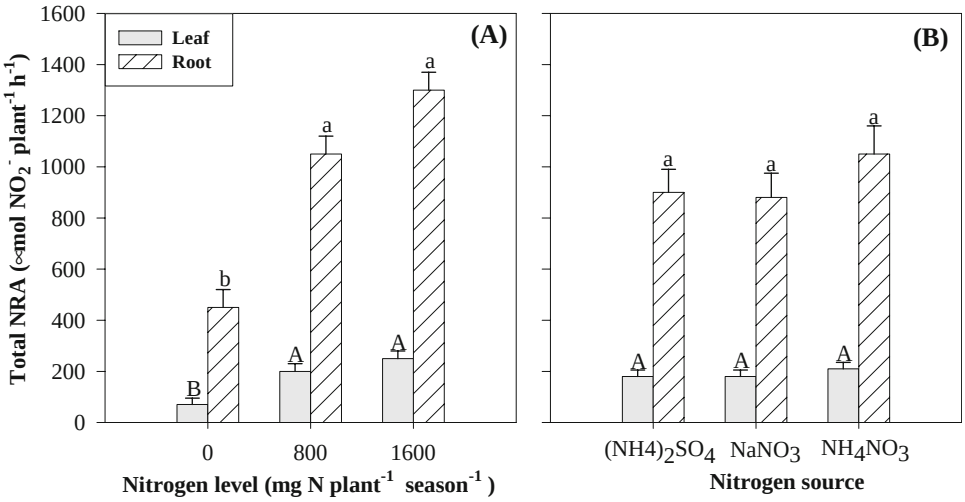

Fig. 2 Total nitrate reductase activity in greenhouse grown 1-year-old black walnut seedling roots and leaves as affected by N application level (*left*) or N source (*right*). *Letters above bars* indicate significant differences (Tukey, $\alpha = 0.05$). *Bars* represent standard error associated with mean estimates

Table 2 ANOVA table for component dry mass, xylem N, and nitrate reductase activity (NRA)

Source of variation	Dry mass						Xylem N%			Specific NRA		Total NRA		
	Leaf	Root	Old stem	New stem	Shoot total	Plant	Amino acids	NO_3^-	NH_4^+	Leaf	Root	Leaf	Root	Plant
N source	*0.0151*	0.2514	0.8857	0.8510	0.2435	*0.0191*	0.4351	0.4277	*0.0108*	*0.0157*	*0.0287*	0.1692	0.3639	0.1936
N level	*0.0001*	*0.0206*	*0.0292*	0.2389	*0.0001*	*0.0351*	*0.0555*	*0.0618*	*0.0032*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
Source × level	*0.0039*	0.3062	*0.0598*	0.7003	0.2855	0.2480	0.8553	0.8455	0.9266	*0.0703*	*0.0877*	0.1082	0.7644	0.5614

Italic numbers indicate significance at $P < 0.10$

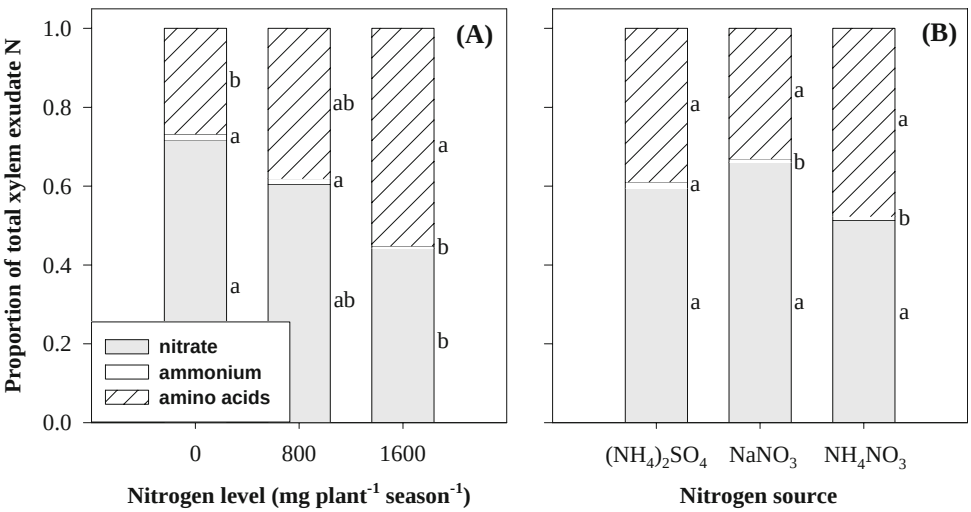

Fig. 3 Proportion of the total N in nitrate, ammonium, and amino acids in xylem exudate of greenhouse grown 1-year-old black walnut seedlings as affected by N application level (*left*) or N source (*right*). *Lowercase letters* indicate significant differences among components (Tukey, $\alpha = 0.05$)

(Fig. 6a). For roots, specific NRA was consistently higher in NO_3^--fed plants (Fig. 6b).

Discussion

Nitrogen level effects on growth and physiology

Foliar N concentration below 20.0 g kg^{-1} is considered deficient for black walnut seedling growth (Phares and Finn 1971; Ponder 2004). Foliar N concentration of unfertilized seedlings was 10.0 g kg^{-1}, which clearly demonstrates deficiency. Total N for unfertilized seedlings at the end of the study was nearly the same as the pre-season level (600–612 mg N $plant^{-1}$; Fig. 1b). Thus, this medium appears adequate for testing N fertilization and source effects on plant growth and metabolism.

The increase in NRA with fertilization could be associated with NO_3^- induction of NRA (Wang et al. 2003)

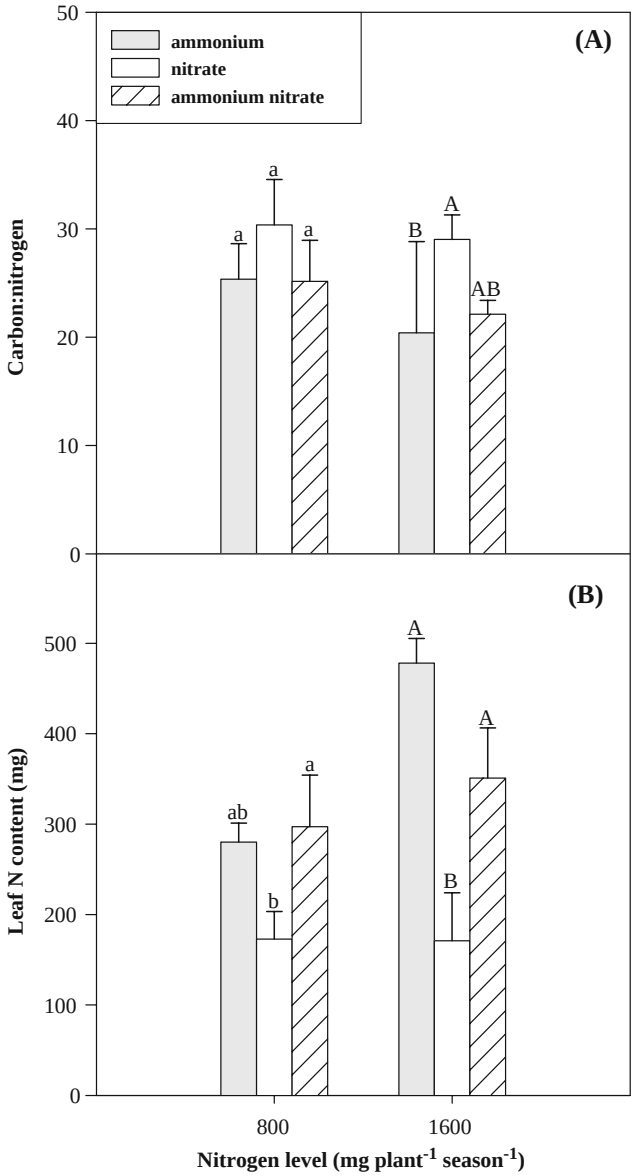

Fig. 4 Interaction effects of N application level by source on leaf carbon to N ratio (**a**) and leaf N content (**b**) in 1-year old greenhouse grown black walnut seedlings. *Letters above bars* indicate significant differences (Tukey, $\alpha = 0.10$). *Bars* represent standard error associated with mean estimates

and (or) optimal NRA noted with NH_4^+ supplementation (Beevers and Hageman 1980; Mohanty and Fletcher 1976). Greater total NRA in roots compared with leaves is important because of the extra energy cost to the plant associated with increased root respiration (Zogg et al. 1996). Supplied NO_3^- can be assimilated in roots, stored, or transported to leaves. Thus, it is likely that acquired NO_3^- was either stored or assimilated in roots, which partly explains observed greater total NRA in roots. The increase in total NRA with N addition (Fig. 2a) correlated with greater plant dry mass and N content at higher fertility (Fig. 1b). Some older studies have suggested that NRA could be a more sensitive indicator of leaf N status than N concentration because NRA is more related to leaf physiology (e.g., Bar-Akiva et al. 1970). This contention is supported by our data where leaf NRA was more responsive to leaf N content than concentration.

Nitrate and AA are the predominant forms of N transported in xylem. Decreased NO_3^- levels in xylem exudate with N addition observed in our study (Fig. 3a) are consistent with trends noted for spruce (*Picea abies* L. Karst) seedlings fertilized with nitrite (Muller et al. 1996). These trends contrast markedly with increased NO_3^- levels at higher fertility noted for cherry (*Prunus avium* L.) seedlings (Grassi et al. 2002). An increase in N availability apparently stimulates N assimilation in roots, which is implicated by more AA loading into xylem. Thus, increased fertilization promotes NRA, N assimilation, and N transport processes. Increase in AA in xylem exudate has similarly been reported for jack pine (*Pinus banksia* Lamb.) suggesting that AA may be more sensitive indicators of N status and response to fertilization (Kim et al. 1987). Generally, our study results suggest that increased N addition in black walnut seedlings favors N assimilation in roots as opposed to transport in xylem sap for subsequent reduction in leaves.

Nitrogen source effects on growth and physiology

Preferred N source for growth and metabolism varies with plant species (Hageman 1980). A mixed NH_4^+ and NO_3^- source is often best, but in some cases, one form is preferred over another. The reduced energy cost required to assimilate NH_4^+- compared with NO_3^-–N would be one mechanism to explain plant preference for the different N species. Additionally, this may be related to inability of the NO_3^--reducing system to supply the plant with maximum usable levels of reduced N (see citations in Haynes 1986, p. 347). Furthermore, NO_3^- uptake is coupled with H^+ uptake, whereas NH_4^+ is coupled with a change in membrane polarization caused by releasing H^+ into the external medium. Thus, a mixed N source should result in no net change in cellular or external pH, which benefits pH-sensitive plants.

The lower biomass and N content in NO_3^--fed plants than with NH_4^+ or NH_4NO_3 noted in this study suggests that NO_3^- may be less preferred to NH_4^+ or NH_4NO_3 for black walnut. Other studies have noted better growth with NH_4NO_3 than with NO_3^- or NH_4^+. For example, Douglas-fir [*Pseudotsuga menziesii* (Mirb) Franco] exhibited higher growth and net photosynthesis with NH_4NO_3 than with urea (which rapidly hydrolyses to NH_4^+ forms) (Brix 1981). For pecan [*Carya illinoisensis* (Wangenh.) K. Koch], a tree species in the same family as walnut

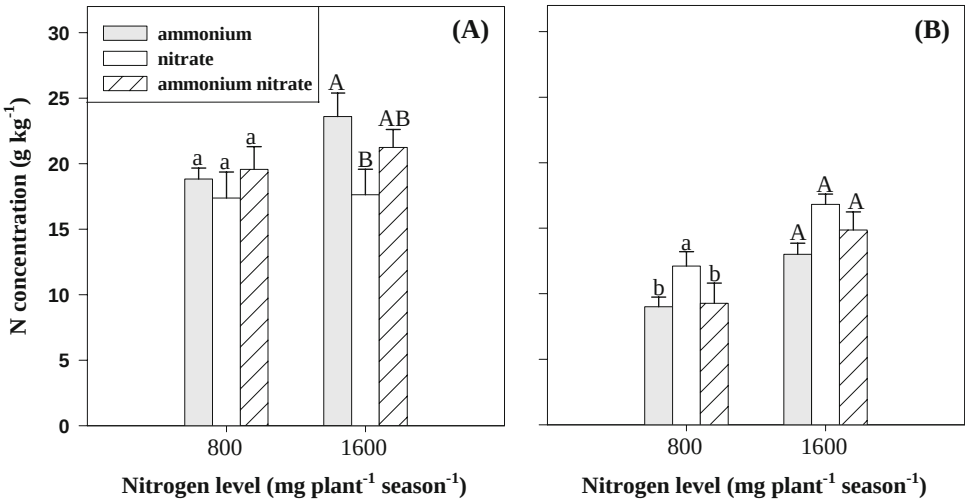

Fig. 5 Interaction effects of N application level by source on N concentration in leaves (**a**) and in roots (**b**) of greenhouse grown 1-year-old black walnut seedlings. *Letters above bars* indicate significant differences (Tukey, $\alpha = 0.10$). *Bars* represent standard error associated with mean estimates

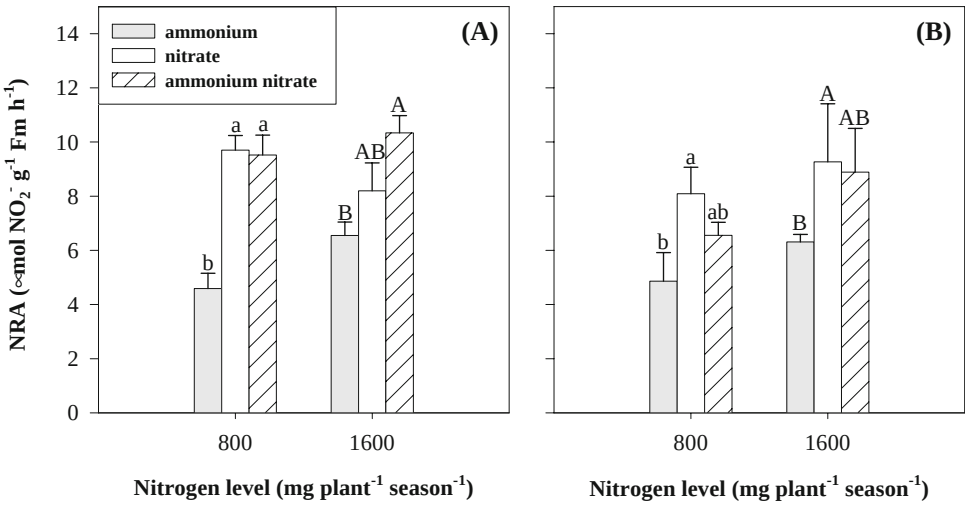

Fig. 6 Interaction effects of N application level by source on specific nitrate reductase activity in greenhouse grown 1-year-old black walnut leaves (**a**) and roots (**b**). *Letters above bars* indicate significant differences (Tukey, $\alpha = 0.10$). *Bars* represent standard error associated with mean estimates

(Juglandaceae), NH_4^+ was taken up preferentially to NO_3^-, but best growth occurred with a mixed N source (Kim et al. 2002). Similarly, NH_4NO_3 increased total N content (13–16%) and promoted net photosynthesis in black walnut than in plants fed with NO_3^- or NH_4^+ (Nicodemus et al. 2008). These results strongly suggest that black walnut seedlings respond favorably to NH_4NO_3. Therefore, fertilizing walnut with NH_4NO_3 may result in lower energy cost to the plant thereby directing available resources to increase productivity rather than channeled to metabolic processes.

Nitrate reductase activity may differ with N source. For example, leaf NRA was lower with NO_3^- than with NH_4NO_3 in walnut (Nicodemus et al. 2007), contrasting markedly with results of our current study that suggest similarity in leaf NRA in NO_3^--fed compared with those fertilized with NH_4NO_3. European ash (*Fraxinus excelsior* L.) and English oak exhibited similar NRA when fertilized with NH_4NO_3 or NO_3^- (Stadler et al. 1993). One-year-old rose (*Rosa hybrida* L.) seedlings treated with NO_3^- had more NRA in leaves than controls, but less NRA for similar plants treated with NH_4^+ (Lorenzo et al. 2001), which suggests NRA was NO_3^--induced and NH_4^+-repressed. Raspberry (*Rubus idaeus* L.) had greater NRA with NO_3^- than NH_4^+ in roots, but had comparable rates of NRA in leaves (Claussen and Lenz 1999).

It is likely that NRA would be induced by exogenously supplied NO_3^-, whereas in NH_4^+-fed plants, the only NO_3^- available to induce NRA would originate from storage. Another explanation for NRA in NH_4^+-fed plants in our study is the presence of exogenous NO_3^- in the growing medium. The specific NRA of the roots was also higher with the NO_3^- than the NH_4^+ source, indicating a greater level of NRA induction with exogenously supplied NO_3^-. Xylem exudate analysis did not show the same trends as NRA in regard to N source, suggesting the limitation of xylem exudate analysis for estimating NRA in black walnut. We would expect a strong relationship

between the relative proportion of NO_3^- in xylem exudate and that of NRA in leaves compared to roots, as NO_3^- that is transported in xylem is generally not transported back to roots, but must be assimilated or stored. However, the relationship could be affected by storage of NO_3^- in leaves, or by remobilization of stored N, or cycling of AA in phloem (Malaguti et al. 2001; Guak et al. 2003).

Nitrogen source × level interaction effects

Interaction effects of N source × level on leaf N concentration and C:N demonstrate that the merit in increasing fertilization from 800 to 1,600 mg N level may be limited to NH_4^+ or the mixed N source. This increase would mainly be valuable in terms of stored N reserves as dry mass was similar between 800 and 1,600 mg N. Nitrate reductase activity in leaves actually decreased with increasing N level whereas NRA increased with fertilization in NH_4^+-fed and NH_4NO_3-fed plants. In roots, and for all N sources, NRA increased with an increase in fertilizer level, but this effect was more pronounced in NH_4NO_3 than in NO_3^--fed plants. The interaction effects in this study suggest that, if the higher N level is to be used, more benefit may be gained from using NH_4^+ or NH_4NO_3 compared to NO_3^-.

Some studies have suggested that a NRA assay as used in this study provides only maximal rates of NRA (Gibon et al. 2004), which would still lend credence to understanding the location of NO_3^- assimilation to help evaluate what N source may be best suited to black walnut growth. An alternative method uses an assay mixture with or without Mg^{2+} in medium to determine the phosphorylation state of the protein and separate the maximal level of NRA from the selective activity (Gibon et al. 2004). Future studies should test this alternative approach in black walnut under varied N source and level and evaluate how they relate to results of the current study. Our study results supplement the relative lack of information for woody angiosperms regarding plant responses to N source and level, and how these affect location of NRA. Our findings suggest that generalizations relevant to woody gymnosperms or herbaceous angiosperms may not apply to woody angiosperms. In the current study, a woody angiosperm was able to use NO_3^-, as similarly suggested by Min et al. (1999). This separates black walnut from most woody gymnosperms that preferentially use NH_4^+ because of growth on acidic substrates and adaptations to store NH_4^+ (Aarnes et al. 2007). Most total NRA occurred in roots in the current study, which contrasts markedly with results noted for herbaceous angiosperms (Hageman 1980). This suggests N source preference of woody angiosperms is unlike that of most woody gymnosperms and herbaceous angiosperms.

Conclusions

We have shown that N uptake and assimilation processes in black walnut are influenced by N source, N level, and NRA. A mixed N source (NH_4NO_3) improved growth and physiological responses over NO_3^- (as $NaNO_3$) or NH_4^+ [as $(NH_4)_2SO_4$]. Addition of N increased the proportion of AA in xylem exudates in confirmation of results noted by Kim et al. (1987). Nitrate reductase activity was stimulated by addition of NH_4NO_3 and NO_3^--N compared with NH_4^+-N source. Contrary to our expectations (see third objective), our results suggest that increasing N addition decreases the proportion of exogenous NO_3^- transported in xylem exudate, leading to assimilation of NO_3^- mostly in roots, which is associated with greater energy cost to plants. This suggests limited advantage in using only NO_3^- over other N sources from an energetic standpoint, which may have been reflected in decreased leaf and root biomass with the NO_3^-–N species. Proportion of NO_3^- in xylem exudate decreased with N addition consistent with results noted in other studies (e.g., Muller et al. 1996), signifying greater energy cost to plants because NRA in roots would require respiration energy as opposed to NRA in leaves, which can use direct products of photosynthesis. Though N source preference has been studied comprehensively in herbaceous angiosperms and woody gymnosperms, little research has been conducted with woody angiosperms, and especially *Juglans* spp. The current study provides insight to help improve understanding of how woody angiosperms respond to different N sources and levels, and the location of NRA. This knowledge will be useful toward selecting desired N sources for varying species and formulating fertilizer regimes that minimize plant energy costs associated with N uptake and assimilation.

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