

Source–sink relationships for carbon and nitrogen during early growth of *Juglans regia* L. seedlings: analysis at two elevated CO₂ concentrations

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Abstract – Assimilation and allocation of carbon (C) and nitrogen (N) were studied in seedlings (*Juglans regia* L.) grown for 55 days under controlled conditions (22 °C, 12 h, 90 % relative humidity [RH]) using two CO₂ concentrations (550 and 800 μL L⁻¹ CO₂). C and N decrease in seeds was unaltered by CO₂. At the end of seed contribution (day 35), C and N accumulation in seedlings was favoured under 800 μL L⁻¹ [CO₂], resulting in an increase of about +50 % for C and +35 % for N. Growth enhancement was larger in roots than in shoot, resulting in a higher root:shoot ratio (R:S = 0.62) with respect to 550 μL L⁻¹ CO₂ (R:S = 0.40) at day 55. These results were due, in order, to: 1) a shoot respiration temporarily depressed by [CO₂], 2) a reduction by 46 % of the root + soil respiration, 3) a stimulation by 14 % of the C assimilation and 4) an increased uptake and assimilation of N coming from the rooting medium. An increased use of N originated from the seed was observed in leaves and lateral roots, suggesting optimisation of distribution of stored N pools by seedlings. These changes finally gave rise to an increased C:N ratio for taproot (+27 %), roots (+20 %), stem (+28 %), and leaves (+12 %), suggesting a N dilution in the tissues. (© Inra/Elsevier, Paris.)

Juglans regia / CO₂ / C balance / ¹⁵N / shoot / root

Résumé – Relations source-puits pour le carbone et l'azote durant les premiers stades de croissance de semis de *Juglans regia* L. : analyse à deux concentrations en CO₂ atmosphérique élevées. L'assimilation et la répartition du carbone (C) et de l'azote (N) ont été étudiées chez des semis de *Juglans regia* L. cultivés 55 j en conditions contrôlées (22 °C, 12h, 90 % H. R.) à deux teneurs en CO₂ atmosphérique (550 et 800 μL L⁻¹ CO₂). La diminution en C et N des graines n'est pas modifiée par la teneur en CO₂. L'accumulation de C et N dans les plants est augmentée de 50% et 35% respectivement à 800 μL L⁻¹ CO₂, dès l'arrêt de la contribution de la graine (j 35). Sous la plus forte teneur en CO₂, le gain de croissance observé est plus important pour le système souterrain qu'aérien aboutissant à un rapport tige-racine augmenté (0,62) à 800 μL L⁻¹ CO₂ comparé à 550 μL L⁻¹ CO₂ (0,40). Ces résultats sont dus à (1) une respiration temporairement déprimée par le CO₂, (2) une diminution par 46 % de la respiration sol + racines, (3) une stimulation par 14 % de l'assimilation du C, et (4) une augmentation de l'absorption et de l'assimilation de l'azote du sol. Une augmentation de l'utilisation de l'azote originare de la graine est observée dans les feuilles et les racines latérales suggérant une optimisation de l'utilisation et de la répartition de l'azote stocké par les plants. Ces changements aboutissent à une augmentation du rapport C/N pour le pivot (+27 %), les racines (+20 %), la tige (+28 %), et les feuilles (+12 %), suggérant une dilution de l'azote dans les tissus. (© Inra/Elsevier, Paris.)

Juglans regia / CO₂ / C balance / ¹⁵N / tige / racine

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1. INTRODUCTION

Growth and survival of young plants, particularly during the transition to an autotrophic existence, depend on both efficient use of seed reserves and new photosynthates [15, 25, 33]. In this context, environmental conditions and changes in resource availability will notably influence trophic relationships between the seed and its emerging seedling, and the chances of a successful establishment [8, 15, 29]. For tropical and temperate forest ecosystems it was shown that steep CO_2 gradients exist between the forest floor and the top of the canopy [3, 4]. Elevated CO_2 concentration (400 to $550 \mu\text{L L}^{-1}$) near the soil surface particularly, due to intensive soil respiration, is very frequent in forests [3, 4], suggesting that in natural regeneration systems emerging seedlings frequently grow under elevated CO_2 concentrations. Nevertheless, little work has focused on the influence of elevated CO_2 concentration on seed germination and emergence [42], even though, in light of experiments on tobacco [28], principal changes of metabolism and growth under elevated CO_2 would occur early after germination. Moreover, understanding how heterotrophic seedlings respond to elevated CO_2 can be of importance regarding biomass and plant production in field or greenhouse situations, as shown by Kimball [21, 22].

In order to gain a better understanding of the fate of carbohydrates and nitrogen (N) nutrients in young heterotrophic walnut trees (*Juglans regia* L.), carbon (C) and N partitioning between organs and physiological functions (growth, respiration and reserve storage) were previously investigated under $550 \mu\text{L L}^{-1} \text{CO}_2$ [25, 26]. After this initial investigation, interactions between sink organs and the two source organs (seed, leaves) to the translocation and distribution of assimilates in the seedling remained unclear even though the use of a deterministic and dynamic model of carbon allocation [17] indicated that an intensive competition for carbohydrates dominates the relations among organs during transition to autotrophy.

Experimental changes of source–sink balance in plants by organ removing or light treatment can help considerably in changing the distribution pattern of photoassimilates compared with control plants and the study of possible mechanisms controlling source–sink relationships [18, 35]. In the present study, we attempted to alter both photosynthetic supply and source–sink relationships for C and N of heterotrophic walnut seedlings growing under $550 \mu\text{L L}^{-1} \text{CO}_2$ by increasing the CO_2 concentration. In fact, manipulating the photosynthetic supply of plants by CO_2 to alter source–sink relationships for C and N prevent complex morphogenetic responses generated by organ removing or environmental light changes [1, 23,

39]. We examined the consequences of the expected gain in photoassimilated C on growth and on the patterns of C and N partitioning between sources and sinks of seedlings, and specifically addressed the following set of questions. To what extent might changes in C assimilation alter 1) the import of maternal C and N, 2) N uptake and assimilation, 3) partitioning of C and N between shoot and roots and 4) the time lapse prior to a complete independence of the seedling from seed reserves? The relative contributions of the two sources of organic N (seed reserves, plant assimilation) available during the early stages of seedling growth were investigated by using the natural differences in the abundance of the stable isotopes ^{15}N and ^{14}N in the nutrient solution and the seed.

2. MATERIALS AND METHODS

2.1. Plant material and culture conditions

Seeds of *Juglans regia* L. (c.v. Franquette) were obtained from Inra (Bordeaux, France). For each CO_2 treatment, 200 seeds were soaked for 48 h under running water at room temperature. The seeds were planted in pots filled with vermiculite and maintained under controlled conditions for 60 days in an automatically controlled climatic chamber ($22 \pm 1^\circ\text{C}$, 12 h, 90 % relative humidity [RH]). The chamber (1 000 L) which held 20 containers, was divided into tightly sealed compartments: the upper compartment (750 L) contained the canopy of the plants, and the lower one (250 L) the soil containers. The two parts were separated by an opaque plastic cover with 20 holes (one for each container). Access to the inside of the chamber could be obtained through three doors sealed hermetically during measurements of CO_2 exchange. Ambient CO_2 concentration was maintained at $550 \mu\text{L L}^{-1}$ in accordance with Maillard et al. [25–27] or at $800 \mu\text{L L}^{-1}$ with an industrial CO_2 flow (5 % CO_2 , 19.1 % O_2 and 75.9 % N_2) controlled by an infrared gas analyzer (IRGA; ADC 225 MK 3, The analytical Development Co., Ltd., Hoddesdon, Hertfordshire, UK) and an automated regulation system as described previously by Maillard et al. [25]. Gas exchange rates, i.e. shoot, root + soil respiration, and net CO_2 assimilation, were measured and calculated from the time course of CO_2 [25].

Light was supplied by a bank of 12 mercury vapour discharge lamps (OSRAM HQITS 250 W) which provided the plant chamber with $420 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) at plant level. For 2 months, the plants were watered automatically four times a day with a nutrient solution [24] which contained 2.0 mM KNO_3 , 2.1 mM $\text{Ca}(\text{NO}_3)_2$ and 0.6 mM $(\text{NH}_4)_2 \text{SO}_4$.

2.2. C and N analyses

Five to ten seedlings were sampled twice a week at the end of the photoperiod for C, N and $^{15}\text{N}/^{14}\text{N}$ isotope ratio analyses. Due to their small weight, the different organs (leaves, stem, taproot, lateral roots and kernel) of the harvested seedlings were pooled respectively, frozen quickly in liquid N_2 , freeze-dried, weighed and ground to a fine homogeneous powder with a laboratory mill. Samples were stored at -20°C before analysis of biochemical content and isotope composition.

Total C and N contents and isotope ratio $^{15}\text{N}/^{14}\text{N}$ in plant material were measured using the corresponding gases derived from the combustion of aliquots of plant tissues, and analysed in an elemental analyser (CNRS, Service Central d'Analyses, Lyon, France) coupled with a mass spectrometer (Delta S, Finnigan, USA). All samples were analysed at least twice. Isotopic composition was expressed in δ units versus N_2 of ambient air as a standard:

$$\delta^{15}\text{N} = \frac{\frac{^{15}\text{N}}{^{14}\text{N}} \text{ sample} - \frac{^{15}\text{N}}{^{14}\text{N}} \text{ standard}}{\frac{^{15}\text{N}}{^{14}\text{N}} \text{ standard}} \times 10^3$$

The error (standard deviation) between repeated analyses of the same plant sample was between 0.03 and 0.14 ‰. Nutrient solution used exhibited values of $\delta^{15}\text{N}$ at -3‰ and kernel values of $\delta^{15}\text{N}$ at 5.

The proportion of N assimilated from the nutrient solution in total N of the plant sample was calculated as follows [11]:

$$N_p = \frac{\delta^{15}\text{N organ} - \delta^{15}\text{N kernel}}{\delta^{15}\text{N nutrient solution} - \delta^{15}\text{N kernel}} \times 10^2$$

with $100 - N_p = N_k$ corresponding to the proportion of N coming from seed reserves.

3. RESULTS

3.1. Time course of cumulated C exchanges in whole seedlings

Figure 1 shows cumulated CO_2 exchanges from day 21 to day 55 (end of experiment). Day 21 corresponded to the beginning of a measurable net CO_2 assimilation, i.e. 6 days after emergence of the first two leaves. Photosynthetic C accumulated exponentially until day 55 (figure 1A). Differences in the photosynthetic C accumu-

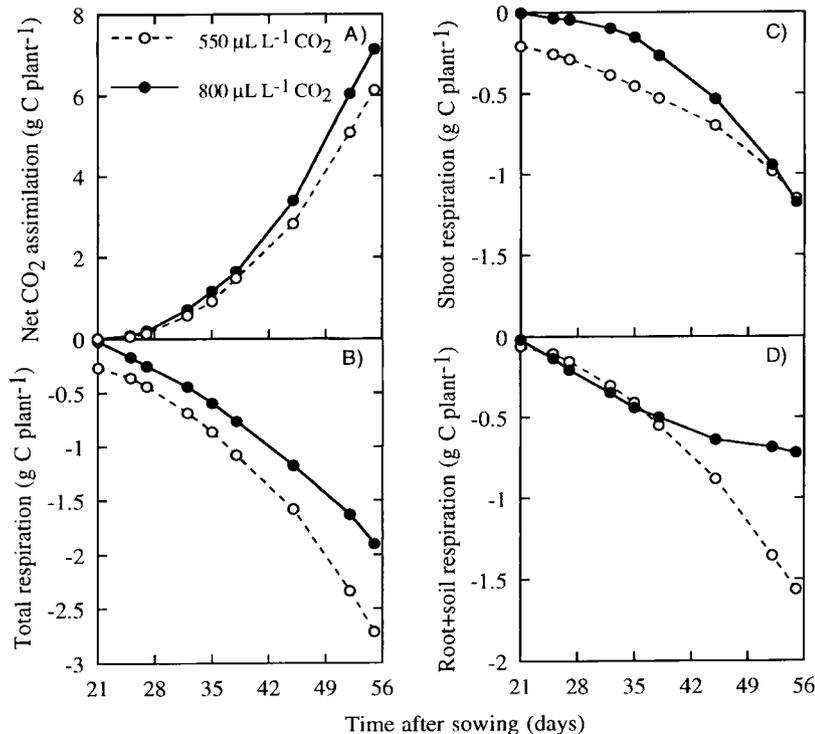


Figure 1. Time course of cumulated respiration and net CO_2 assimilation of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22°C , 12 h) with 550 or $800\ \mu\text{L L}^{-1}$ CO_2 in the atmosphere. Average from 50 to 200 plants. (A) Net CO_2 assimilation; (B) total respiration; (C) shoot respiration; (D) root + soil respiration.

lation between both CO_2 treatments appeared after day 27 and were obvious at day 45, ending with a notable stimulation by 14 % on day 55 at 800 compared with $550 \mu\text{L L}^{-1} \text{CO}_2$ (figure 1A).

Comparison of dark shoot respiration revealed marked differences at the occurrence of measurable net CO_2 assimilation (figure 1C). At day 21, it was negligible under 800 but already noticeable under $550 \mu\text{L L}^{-1} \text{CO}_2$. Then, the shoot respiration was strongly stimulated under elevated CO_2 , ending in a cancellation of initial differences on day 55 (figure 1C).

Subterranean respiration increased gradually with growth, and no differences were observed between the two CO_2 treatments until day 37 (figure 1D). Then, subterranean respiration continued to increase under $550 \mu\text{L L}^{-1} \text{CO}_2$, whereas it was markedly depressed by 46 % on day 55 under $800 \mu\text{L L}^{-1} \text{CO}_2$ (figure 1D). As a result, after the first 2 months, both increased C assimilation and depressed total respiration (figure 1A, B) ended in a gain in C for seedling growth of about 54 % under $800 \mu\text{L L}^{-1} \text{CO}_2$ compared to $550 \mu\text{L L}^{-1} \text{CO}_2$.

3.2. C and N changes in the seedling–seed system

The C and N content of seeds decreased gradually until day 40, and then stabilized after this date. A loss of about 78 % of C and of about 86 % of N was recorded on day 55 (figure 2). These changes were similar under both CO_2 treatments. The time course of C or N content was similar in the seeds under both CO_2 treatments suggesting no effect of CO_2 on these parameters.

The C content in the whole seedlings increased exponentially and similarly from day 4 to day 39 under the two CO_2 treatments (figure 2). After day 39, corresponding to the end of C and N loss by the seed, and 18 days after beginning of the photosynthetic activity, C accumulation was favoured under 800 compared to $550 \mu\text{L L}^{-1} \text{CO}_2$, ending in a doubled C accumulation on day 55 (figure 2). This increase was observed in the taproot (+63 %), roots (+64 %), stem (+18 %) and leaves (+39 %) (figure 3). Growth enhancement was larger under $800 \mu\text{L L}^{-1}$, in roots than shoot, resulting in a higher root:shoot ratio (R:S = 0.62) relative to $550 \mu\text{L L}^{-1} \text{CO}_2$ (R:S = 0.40).

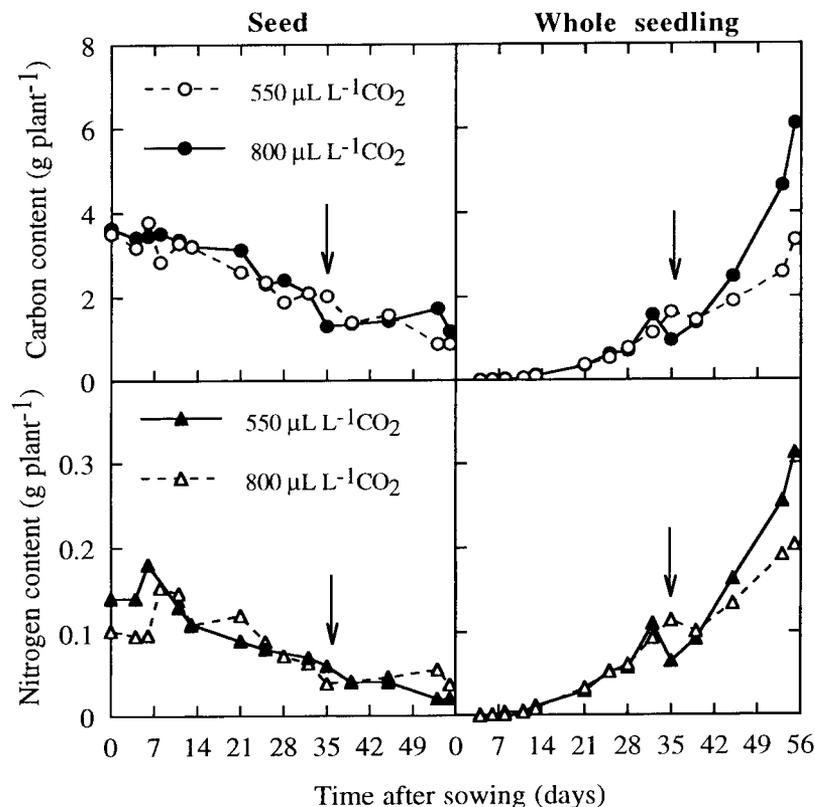


Figure 2. Time course of carbon and nitrogen contents of the walnut seedling–seed systems (*Juglans regia* L.) grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or $800 \mu\text{L L}^{-1} \text{CO}_2$. The arrows show the date of the complete depletion of carbon and nitrogen reserves of seeds. Each value is the mean of three replicates of a sample originating from five to ten seedlings or seeds.

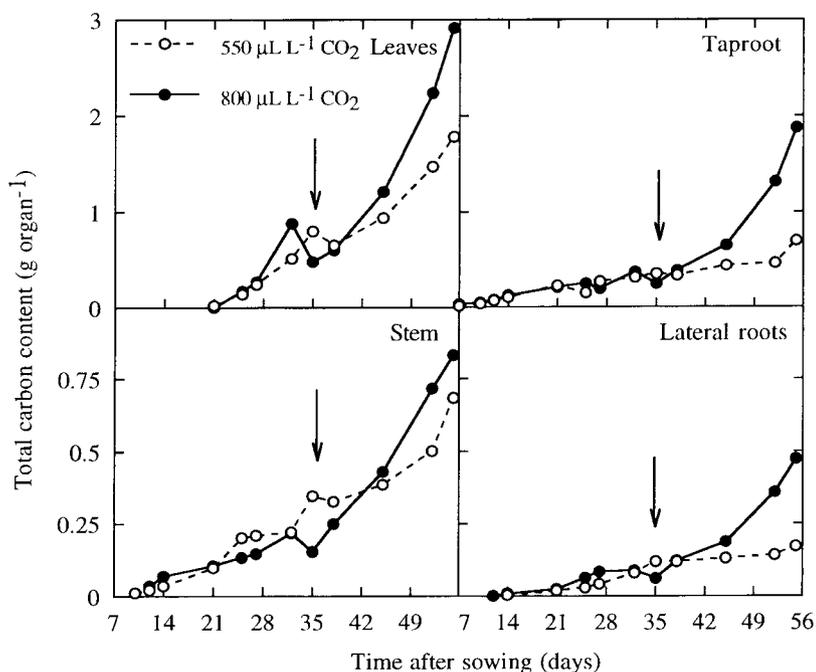


Figure 3. Accumulation of carbon in the various organs of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or 800 $\mu\text{L L}^{-1}$ CO_2 . The arrows show the date of the complete depletion of carbon and nitrogen reserves of seeds. Each value is the mean of three replicates of a sample originating from five to ten seedlings.

The N content in the whole seedlings increased exponentially and similarly under the two CO_2 treatments from day 4 to day 39 (figure 2). After the end of loss of C and N by the seed, and 18 days after the beginning of the photosynthetic activity, N accumulation was more favoured under 800 than under 550 $\mu\text{L L}^{-1}$ CO_2 , resulting in a N accumulation increased by about 35 % on day 55. Differences in the N content of the subterranean system occurred only after day 38 (figure 4). There was more N accumulated in the taproot and lateral roots under 800 than under 550 $\mu\text{L L}^{-1}$ CO_2 . As a result, N in the taproot and lateral roots was increased by +49 and +54 %, respectively, on day 55 (figure 4). Differences in the N content of the aerial system were less pronounced than in the subterranean one for both CO_2 treatments (figure 4). N content of the stem was notably depressed at 800 $\mu\text{L L}^{-1}$ CO_2 from day 20 to day 46 (figure 4). After this period, the stem N content reached values near that observed at 550 $\mu\text{L L}^{-1}$ CO_2 . The N content of leaves varied similarly until day 38 for both CO_2 conditions, then, increased faster at 800 $\mu\text{L L}^{-1}$ CO_2 , resulting in a final value of +31 % in excess with respect to 550 $\mu\text{L L}^{-1}$ CO_2 on day 55 (figure 4).

3.3. C:N ratio variations

C:N ratios were similar in the two treatments until day 38 but diverged thereafter, and were higher for taproot

(+27 %), lateral roots (+20 %), the stem (+28 %) and leaves (+12 %) under 800 $\mu\text{L L}^{-1}$ CO_2 compared with 550 $\mu\text{L L}^{-1}$ CO_2 (figure 5). C:N ratio in the shoot increased before that in roots.

3.4. Assimilation and allocation of N in the whole seedling

Assimilated N appeared first in the taproot after day 14. Differences between the two CO_2 treatments appeared after day 35, corresponding to the end of the N supply by the seed (figure 6). After this date, the percentage of N assimilated from the nutrient solution (N_p) by the taproot increased strongly, particularly under 800 $\mu\text{L L}^{-1}$ CO_2 . As a result on day 55, the taproot contained only recently assimilated N under 800 $\mu\text{L L}^{-1}$ CO_2 , whereas 20 % of N in the taproot was derived from seed reserves under 550 $\mu\text{L L}^{-1}$ CO_2 . In lateral roots, N_p increased strongly after day 21 and similarly under the two CO_2 treatments until day 35. After this date, N_p stabilized at about 60 % under 550 and 50 % under 800 $\mu\text{L L}^{-1}$ CO_2 .

N_p increased strongly in the stem after day 14 to stabilize at about 70 % on day 55 and seemed unaltered by CO_2 (figure 6). In contrast, from day 24 to day 55, the N_p of leaves was always slightly higher under 800 than with 550 $\mu\text{L L}^{-1}$ CO_2 . Note that this percentage decreased after

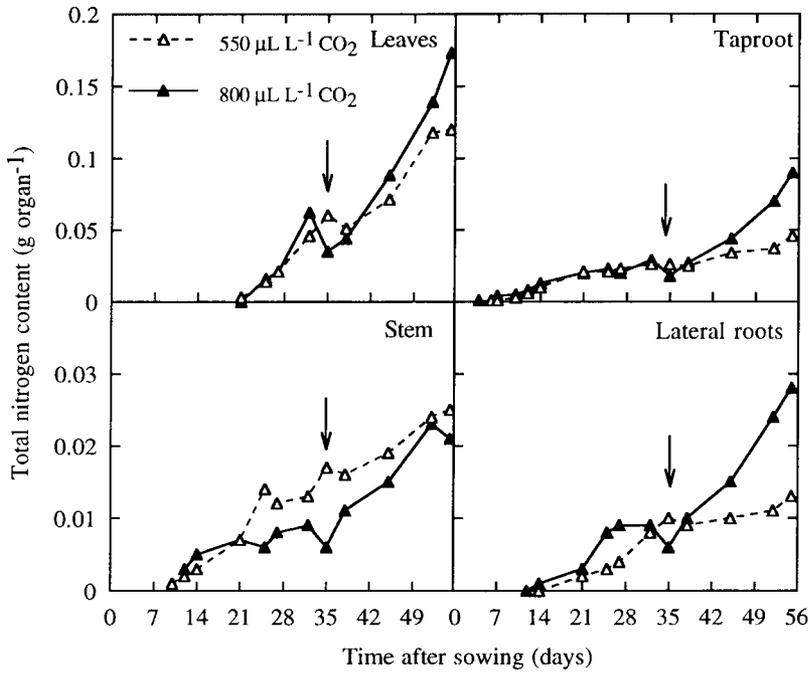


Figure 4. Accumulation of nitrogen (N) in the various organs of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or 800 $\mu\text{L L}^{-1}$ CO_2 . The arrows show the date of the complete depletion of carbon and nitrogen reserves of seeds. Each value is the mean of three replicates of a sample originating from five to ten seedlings.

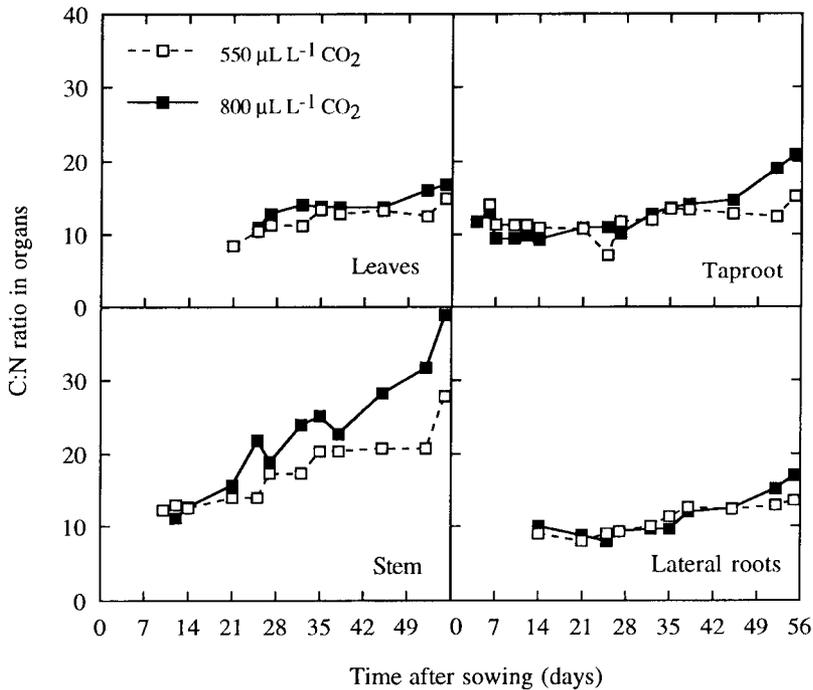


Figure 5. Variation of carbon:nitrogen (C:N) ratio of the various organs of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or 800 $\mu\text{L L}^{-1}$ CO_2 .

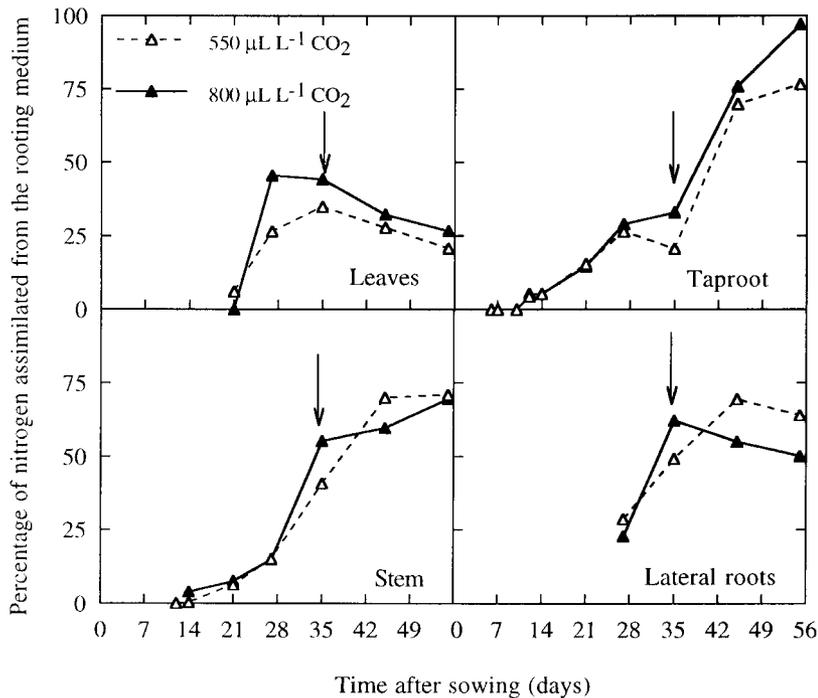


Figure 6. Time course of the percentage of nitrogen assimilated from the rooting medium (N_p) in the various organs of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or 800 $\mu\text{L L}^{-1}$ CO_2 . The arrows show the date of the complete depletion of carbon and nitrogen reserves of seeds. Each value is obtained from two replicates of a sample originating from five to ten plants.

day 38 and remained at a low level (about 25 %) compared to the other organs (70–100 %).

Newly assimilated N on a content basis (N_n) accumulated strongly in the taproot in response to CO_2 (figure 7). In contrast, N content originating from reserves (N_a) was not altered. N originating from both sources increased strongly in lateral roots.

Variations of the N content of both origins were also notably altered in the shoot in response to CO_2 . Accumulation of N_n decreased in the stem without notable alteration of N_a at the end of experiment in the two treatments. Increased total N content in leaves observed above under 800 $\mu\text{L L}^{-1}$ CO_2 was due to increased accumulation of N of both origins. It was noted that the leaves contained the most important part of N_a .

4. DISCUSSION

Our results indicate a marked sensitivity of walnut seedlings to CO_2 concentration, particularly noticeable at two specific stages during the course toward autotrophy: at the beginning of their ability to photosynthesize (about day 21) and at the time of complete depletion of seed reserves (about day 38). This sensitivity was observed initially on respiration and C assimilation, whereas alter-

ations of C and N accumulation in seedlings began to be noticeable only after complete depletion of seed reserves.

4.1. Effect of $[\text{CO}_2]$ on gas exchanges

The first noticeable alterations induced by elevated CO_2 were encountered in shoot respiration and C assimilation. This observation differs from that made using young oak seedlings that display a low sensitivity to elevated CO_2 concentrations, probably due to the trophic preponderance of the seed for this species during the course toward autotrophy [32].

The observed depressed shoot respiration (figure 1C) has been reported before for several woody plants such as oak [40] or chestnut [30]. Reasons for this alteration of metabolism and changes of tissue N concentrations observed before the complete acquisition of photosynthetic ability by seedlings, remain largely unknown but could be related to a direct effect of CO_2 on enzymes of the respiratory pathways [5, 4, 16, 20]. Moreover, Curtis [12] suggested that the accumulation of non-structural carbohydrates could account for the decreased dark respiration of leaves of tree species grown under elevated CO_2 . In the case of heterotrophic walnut seedlings, sensitivity of dark aerial respiration to elevated $[\text{CO}_2]$ was not sus-

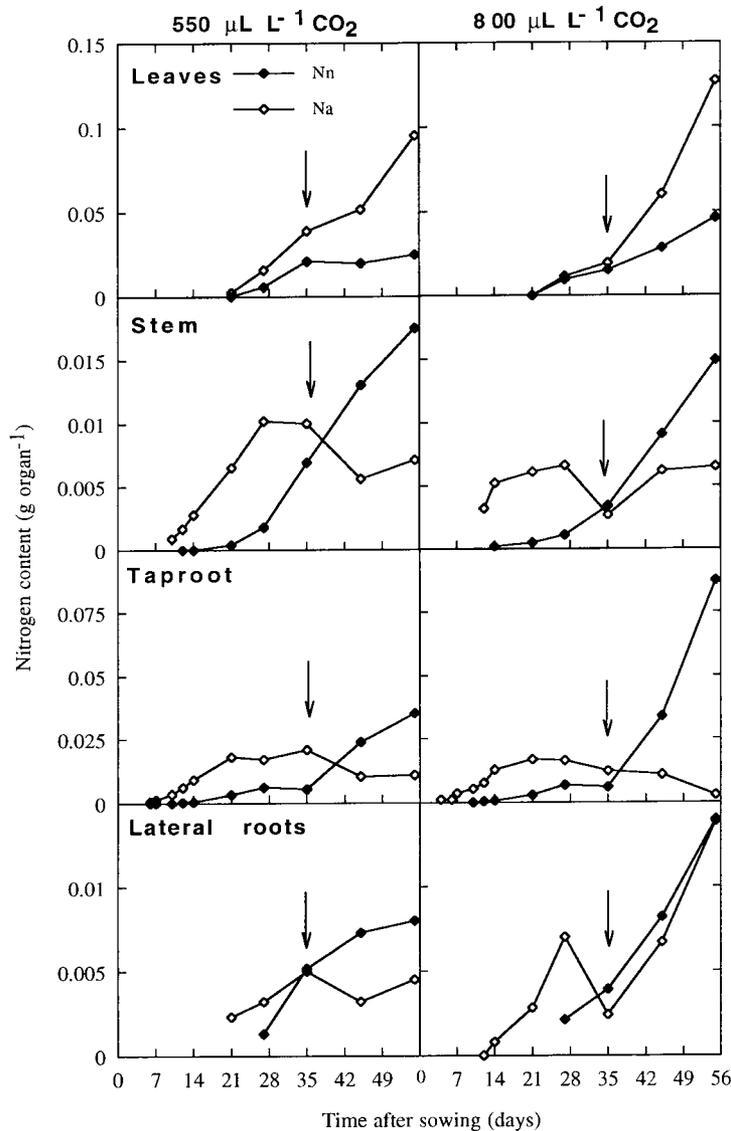


Figure 7. Time course of the nitrogen pool coming from seed reserves (Na) and of assimilation (Nn) in the various organs of *Juglans regia* L. seedlings grown for 55 days under controlled conditions (22 °C, 12 h) with an atmospheric concentration of 550 or 800 $\mu\text{L L}^{-1}$ CO_2 . The arrows show the date of the complete depletion of carbon and nitrogen reserves of seeds. Each value is obtained from two replicates of a sample originating from five to ten plants.

tained after complete depletion of seed reserves (day 39), the time when net C assimilation was markedly increased by CO_2 . In fact, aerial respiration was then strongly increased concomitantly with an increased import of N recently assimilated by leaves and the beginning of an export of recently assimilated C toward roots [27]. These observations suggest that changes in carbohydrate metabolism may occur in the response of aerial respiration of walnut to CO_2 , in agreement with Curtis [12].

The subterranean respiration of walnut seedlings remained insensitive to the increase of $[\text{CO}_2]$ even after 24 days of photosynthetic activity (figure 1D). It then

decreased markedly under 800 $\mu\text{L L}^{-1}$ CO_2 , suggesting in this case an indirect effect of elevated $[\text{CO}_2]$ on this component. Many authors report such CO_2 effects on trees [6, 30, 37]. As for the alteration of aerial respiration of plants, the mechanisms are largely unknown, but changes in growth: maintenance respiration balance are generally hypothesised [7, 41]. Many causes could be involved in the case of walnut seedlings:

- The dilution of N recorded in tissues under elevated $[\text{CO}_2]$ could lead to decreased respiration needs.
- The excess of C assimilated under elevated $[\text{CO}_2]$ would be allocated to the taproot mainly for storage rather

than for growth, inducing an alteration of the maintenance: growth respiration balance. In fact, the taproot is the main storage organ very early under $550 \mu\text{L L}^{-1}$ (40 % of total stored starch in the plant; unpublished data) and root respiration begins to be depressed as soon as the roots start to be supplied by C imported from the leaves ([26] *figure 1*).

– Modifications of the energy cost for ion uptake and nutrient acquisition are also reported in this response of roots to elevated CO_2 [14].

4.2. Effect of $[\text{CO}_2]$ on assimilation and partitioning of C and N

C and N accumulated more in roots than in shoots in response to elevated CO_2 , causing an increased root:shoot ratio in walnut seedlings. Increased accumulation of C in roots was also reported in many studies at elevated CO_2 [9] but much less information is available in the literature concerning N accumulation in roots. As previously described for other species [9, 37], an increase in root:shoot ratio was observed in heterotrophic seedlings, suggesting that elevated $[\text{CO}_2]$ induced extra root storage preferentially to shoot storage. Many reports on trees show that elevated $[\text{CO}_2]$ leads to decreased plant N concentration despite a high N content of the growth media [31, 36]. Our results show that whole plant N pools were increased under the highest CO_2 for walnut seedlings but were not high enough to compensate for the increase of C incorporation. Consequently, C:N increased more in the roots, as soon as they imported photosynthates from leaves [26], than in the leaves, probably due to the intensive leaf metabolism at this time.

Despite dilution of N in walnut seedlings under CO_2 enrichment, both an increased assimilation of N originating from the nutrient solution by taproot and a modified relative distribution of N were observed. These changes induced by elevated $[\text{CO}_2]$ could be linked both to increased root biomass and to an alteration of root function. This latter point needs further confirmation but differs from results reported for older trees such as oak, where the allocation of ^{15}N originated from a fertilised soil was not altered by CO_2 [36]. In trees, the role of buffer played by the mobilisation of N reserves in case of temporary depletion can be significant [34]. In very young walnut seedlings such a trophic strategy seems unlikely due to the very intensive growth of the whole plant and to the low level of N seed reserves at this developmental stage [26, 27]. In this case, the observed increase of ^{15}N allocation could be an alternative to the effects of the elevated CO_2 .

In very young walnut seedlings grown under the highest CO_2 treatment, increased use of N coming from both origins was observed in the leaves and lateral roots, suggesting, at this developmental stage, both optimisation of N assimilation and distribution of stored N pools in metabolically active organs [25, 26]. Surprisingly, compared to other organs, high Na was noted in leaves a long time after seed reserve depletion (*figure 7*), indicating a late and high use of ancient N for current metabolism. The permanent and high turnover of proteins in leaves [13] could be responsible for this phenomenon. The fact that the ancient N content of leaves was increased under 800 compared to $550 \mu\text{L L}^{-1}$ CO_2 could be due to the mobilization and import of old N reserves from neighbouring organs such as the stem, for example.

In conclusion, our data under 800 compared to $550 \mu\text{L L}^{-1}$ CO_2 confirm that, during the heterotrophy–autotrophy transition, a strong C supply limitation exists due both to seed and photosynthetic leaves of walnut seedlings and suggest that the root was more affected by the C supply limitation than shoot growth, in accordance with Escobar-Gutierrez et al. [17]. On the other hand, assimilation and use of C and N by very young trees such as walnut seedlings are interrelated and changes in availability or acquisition of one at autotrophy often lead to changes in availability and acquisition of the other as reported by Bassirirad et al. [2] on loblolly and ponderosa pine. Due to the demand for photosynthates, N assimilation is closely related to the C metabolism and it was shown, mainly on herbaceous plants, that a surplus of N can divert photosynthates away from the formation of storage or transport carbohydrates such as starch or sucrose to amino acid or protein synthesis by modifying the activity of some enzymes connecting carbohydrate and amino acid metabolisms [10, 19, 38]. Analysis of changes in amino acid pools and enzyme activities involved in the interaction of carbohydrate and N metabolism of walnut seedlings could be a useful tool for understanding by which mechanisms the carbon assimilated in excess by leaves under the highest CO_2 treatment resulted in 1) an improved efficiency in N assimilation, 2) an increased use of N originating from both origins and 3) a strongly depressed root respiration. However, our results were obtained under conditions in which N supply was non-limiting. Whether photosynthesis and growth stimulation of walnut seedlings by CO_2 would also be maintained under limited N supply conditions remains an open question.

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