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RESEARCH PAPER

Uptake of nitrogen forms by diploid and triploid white poplar depends on seasonal carbon use strategy and elevated summer ozone

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Abstract

The ability of plants to acquire soil nitrogen (N) sources is plastic in response to abiotic and biotic factors. However, information about how plant preferences among N forms changes in response to internal plant N demand through growth phases, or to environmental stress such as ozone (O_3), is scarce. Diploid and triploid Chinese white poplar were used to investigate N form preferences at two key developmental periods (spring, summer) and in response to summer O_3 (ambient, 60 ppb above ambient). We used stable isotopes to quantify NH_4^+, NO_3^- and glycine N-uptake rates. Carbon acquisition was recorded simultaneously. Both ploidy levels differed in growth, N form preferences, and N and C use strategies. Diploid white poplars grew faster in spring but slower in summer compared with triploids. Diploid white poplars also showed plasticity among N form preferences through the season, with no preferences in spring, and NO_3^- preferred in summer, while triploids showed an overall preference for NO_3^-. Carbon acquisition and NO_3^- uptake were inhibited in both ploidy levels of poplar at elevated O_3, which also reduced diploid total N uptake. However, triploid white poplars alleviated N uptake reduction, switching to similar preferences among N forms. We conclude that N form preferences by white poplar are driven by internal C and N use in response to nutrient demands, and external factors such as O_3.

Keywords: Amino acids, ammonium, nitrate, N preferences, photosynthesis, relative growth rate.

Introduction

Nitrogen (N) is an important limiting factor for plant growth (Rennenberg *et al.*, 2009; Millard and Grelet, 2010; Villar-Salvador *et al.*, 2015) and is a common limiting factor in many terrestrial ecosystems (LeBauer and Treseder, 2008). Nitrogen in the soil is available in multiple chemical forms including inorganic N (nitrate- NO_3^-, and ammonium- NH_4^+) and organic N (amino acids or proteins; Schimel and Bennett, 2004;Näsholm *et al.*, 2009). Nowadays it is widely recognized that plants are able to take up all types of N forms, including intact organic N (Näsholm *et al.*, 2009). However, preference among N forms, or uptake rates of each N form, are species-specific; while some plant species prefer NH_4^+ over other N forms, others prefer amino acids, or NO_3^- (Ashton *et al.*, 2010; Schulz *et al.*, 2011; Uscola *et al.*, 2017). Furthermore, plant species show plasticity in their preferences among N forms in response to abiotic (BassiriRad *et al.*, 1997; Warren, 2009a,b; Boczulak *et al.*, 2014; Zhang *et al.*, 2014) and biotic factors (Ashton *et al.*, 2010; Fraterrigo *et al.*, 2011; Leberecht *et al.*, 2016). However, there is still a lack of knowledge on the mechanisms that modulate plant species preferences among N forms.

At an intraspecific level, within the annual growing period, growth rates are altered through plant developmental phases, driving changes in plant nutrient demands (Lambers *et al.*, 2008). Consequently, plants can respond to growth rate changes by modifying resource acquisition rates through time (Lambers *et al.*, 2008; Nacry *et al.*, 2013). However, there is little information about whether preferences among N forms vary with plant developmental phases as a response to changes in growth rate and N demands. Previous studies have found contrasting trends in this relationship between plant phenology and the uptake rate of each N form. While some temperate heath coastal species such as *Salix arenaria* and other graminoids maintain their preferences between NH_4^+ and organic N through the growing season and off season (winter), others, such as *Calluna vulgaris*, modify its N form preferences (Andresen and Michelsen, 2005). Similarly, alpine crop species have either changing preferences between inorganic N forms (i.e. grasses such as *Hordeum vulgare* and *Avena sativa*) or constant preferences (i.e. legumes such as *Medicago sativa* and *Vicia sativa*) from early to late growth stages (Cui *et al.*, 2017). However, none of these studies have evaluated all the N forms simultaneously, or the dependence on plant growth rate. Furthermore, carbon (C) and N acquisition and metabolism are closely related processes (Gruffman *et al.*, 2013; Uscola *et al.*, 2015; Villar-Salvador *et al.*, 2015). For instance, limitation on C acquisition might compromise inorganic N form uptake, amino acid transport and synthesis as their requirements of energy and C skeletons from photoassimilates and/or photorespiration (Masclaux-Daubresse *et al.*, 2010; Gruffman *et al.*, 2013; Franklin *et al.*, 2017; Perchlik and Tegeder, 2017). However, N uptake limitation might reduce C acquisition by driving a decrease of photosynthetic machinery, as chlorophylls and photosynthetic enzymes are highly enriched in N (Lambers *et al.*, 2008; Perchlik and Tegeder, 2018). Besides, plant endogenous N status can also modify C and N acquisition through feedback signals of internal amino acid metabolism (Masclaux-Daubresse *et al.*, 2010; Perchlik and Tegeder, 2018). Therefore, to fully understand N form uptake, both C and N acquisition processes must be studied simultaneously.

In general, the assimilation costs of amino acids biochemically calculated is half of that for NH_4^+ and three times lower for NO_3^-, according to Zerihun *et al.* (1998). Consequently, the strongest preference for the most reduced N forms (amino acids and NH_4^+), with lower energetic and C cost, would be advantageous, allowing higher growth rates (Boudsocq *et al.*, 2012; Gruffman *et al.*, 2013; Franklin *et al.*, 2017). Nevertheless, we assume that plant preferences among N forms might vary through the growing season as a response to soil N form availability, photosynthesis rate, and plant N demand driven by growth rate. In this sense, in the spring, low soil temperatures might reduce plant N uptake and mineralization (Uscola *et al.*, 2015; Villar-Salvador *et al.*, 2015), together with high plant N demand and low photoassimilates for metabolization of the N taken up (Millard and Grelet, 2010; Uscola *et al.*, 2015; Villar-Salvador *et al.*, 2015), thus, favoring organic N plant uptake, while in summer, inorganic N forms must be more accessible and taken up.

In addition, other external factors might also affect seasonal N form uptake as the phytotoxic air pollutant, ozone (O_3). Ozone has reached high concentrations all around the world due to urbanization and industrialization, and is still increasing (Paoletti *et al.*, 2014; Feng and Li, 2017). For instance, daytime summer O_3 concentrations have exceeded 40 ppb in many parts of the Northern hemisphere (Vingarzan, 2004), and it is predicted that values will increase over 70 ppb by 2100 (Sitch *et al.*, 2007). Elevated O_3 concentration (above 40 ppb) can inhibit photosynthesis, decrease carbohydrate allocation to below-ground and reduce the uptake of nutrients, such as N (Luedemann *et al.*, 2005; Wittig *et al.*, 2009; Weigt *et al.*, 2012; Feng *et al.*, 2019). To our best knowledge, only few studies have examined the effects of O_3 on the uptake of N forms (Jung *et al.*, 1994; Haberer *et al.*, 2007). For instance, Haberer *et al.* (2007) found that, in *Fagus sylvatica*, the exposure to O_3 decreased the uptake of NH_4^+ in the first year but decreased NO_3^- uptake in the next year, indicating a strong plasticity of N preference in this species under O_3 fumigation, at least at mid-long term scale. Nonetheless, when evaluating O_3 effects, no studies have investigated the uptake of organic N form, one of the most important components of the N cycle.

Chinese white poplar (*Populus tomentosa* Carr.), a deciduous broadleaf tree native to northern China, is extensively used in afforestation for ecological and commercial purposes (Zhu, 2006). Polyploidization is a useful practice to promote growth and nutrient uptake (Guignard *et al.*, 2016; Wu *et al.*, 2019). Compared with natural diploids, triploid hybrid *P. tomentosa* improved cell and leaf size, triggering faster growth rate and

shorter-rotation periods (Zhu, 1995). Due to the greater wood and fiber properties and the highest disease-tolerance, triploids are of great importance in the timber, pulp and paper industry (Zhu, 2006; Zhang *et al.*, 2015). However, there is little information about N form uptake differences between diploid and triploid Chinese white poplar, and no connection with developmental phase. Furthermore, there is limited information about the ability of poplars to uptake intact amino acids (but see for example Zhang *et al.*, 2014) and if this ability differs among ploidy levels. In addition, mean values of summer O_3 concentration of the maximum daily 8 h average (MDA8) above 60 ppb have been frequently reported in China (Li *et al.*, 2019; Sahu *et al.*, 2021); this has already impacted on tree fitness and the ecological and economic benefits that they provide (Li *et al.*, 2017; Feng *et al.*, 2019). Although tolerance to O_3 among Chinese white poplar ploidy levels has never been studied, in other plant species, polyploidy, linked to faster growth rate, is also generally linked to highest O_3 sensitivity (Biswas *et al.*, 2008; Zhang *et al.*, 2010). We used a soil applied ^{15}N labeling of NO_3^-, NH_4^+ and dual labeled ^{15}N-^{13}C glycine in 1-year-old plants of diploid and triploid Chinese white poplar to investigate: (i) the uptake rate of each N form and the ability to uptake intact amino acids; and (ii) the plasticity in N form preferences through developmental phases, and in response to an O_3-enriched atmosphere during summer. Here, we mainly focus on summer O_3, because in this season the values exceed the threshold to be considered contamination, while generally in spring the values are below 40 ppb (no contamination; Li *et al.*, 2019; Sahu *et al.*, 2021). The initial hypotheses were: (i) in general, triploid white poplars will have higher total N uptake rate than diploids due to faster growth rate, higher nutrient demands, and the higher uptake rate of NH_4^+ and intact amino acids; (ii) total N uptake will be higher in summer for both ploidy levels, with an increase in NO_3^- uptake but a decrease in amino acids and NH_4^+ N uptake towards the summer; and (iii) O_3 will reduce N uptake of both ploidy levels, especially NO_3^--N uptake which will be reduced due to the inhibition of C assimilation; and (iv) the reduction of total N uptake rate, as well as the plasticity in N form preference might be larger in triploids, as it is expected to be more sensitive to O_3.

Materials and methods

Plant material and cultivation in greenhouse

Two ploidy levels of tissue-cultured Chinese white poplar (*Populus tomentosa* Carr.) were selected: diploid 'Lumao 50' (*Populus tomentosa*) and triploid 'Beilinxiongzhu1' [(*P. alba* ×*P. glandulosa*)×(*P. tomentosa* ×*P. bolleana*)]. The propagation of diploid plantlets started in August 2018, and one month later for triploids. Propagation was carried out at a state-owned nursery of Chinese white poplar in Guanxian county, Shandong Province (36°30'N, 115°22′E, 37 m.a.s.l.), following the *in vitro* propagation technique described in Wang *et al.* (2014). On 20 October 2018, 120 plantlets of each ploidy level were transplanted into individual 1.5 l plastic pots filled with peat ('Xinyuan', Yinong nursery substrates Co., Ltd, Shandong, China) and kept under greenhouse conditions until transplanting was required.

On 14 March 2019, 226 plants in total, 113 plants per ploidy level, were transplanted into 10 l circular plastic pots (230×240 mm; height×top diameter) filled with a 3:1 (v:v) mixture of sand and vermiculite (pH=7.08, Xinyang Jinhualan Mining Co., Henan, China). Before transplanting, trace N from previous growth medium was removed by immersing roots in a 0.5 mM KCl solution for 15 s and then repeatedly washing with deionized water. Plants at transplanting were 6.08±0.481 and 4.58±0.373 cm in height, and 1.92±0.140 and 1.21±0.104 mm in diameter, for diploids and triploids, respectively (n=5).

Plants were randomly allocated in the greenhouse of Beijing Forestry University near Jiufeng Mountain, Beijing (39°54'N, 116°28′E, 450 m.a.s.l.). Fertilization started on 31 March 2019. A water-soluble fertilizer at 8.4 ppm N (i.e. 0.6 mM) was used twice a week with 200 ml per plant (pH=6.38). The fertilizer had three N forms in equimolar mixture, 0.2 mM KNO_3, 0.1 mM $(NH_4)_2SO_4$, 0.2 mM glycine, and completed with 0.2 mM KH_2PO_4, and micro-nutrients 5.1 μM EDTA and 1.1 μM DTPA (Macklin Biochemical Co., Ltd, Shanghai, China). We used glycine as a surrogate of amino acids because it is widely used in organic N uptake experiments and it is an abundant amino acid in forest soils (Yu *et al.*, 2002; Näsholm *et al.*, 2009). Inorganic N concentration was similar to the soil inorganic N in poplar forests (Shang *et al.*, 2019). Plants were irrigated to full saturation twice a week with deionized water. A JL-18 Series thermometer (Huayan Instrument and Equipment, Shanghai, China) was used to measure temperature and air relative humidity at 15 min intervals. The monthly average temperatures of March, April, and May were 14.4, 17.2, and 20.0 °C, respectively, and monthly average air relative humidity was 47.6, 58.1 and 64.9%, respectively. The greenhouse had an automatic black shade cloth unfolded in the central hours of the day (11.00 h to 14.00 h) to avoid overheating, with a daily average light intensity of 435 μmol m^{-2} s^{-1}.

Ozone treatments

On 27 May 2019, plants were transported to Yanqing county (40°29'N, 115°60′E, 500 m a.s.l.), northwest of Beijing, with a continental monsoon climate. After 24 d acclimation to the chamber environment, O_3 fumigation was initiated. There were two O_3 treatments: non-filtered ambient air (NF), and NF with targeted ozone addition of 60 ppb (NF60); this concentration is expected to be reached by the middle of this century (Li *et al.*, 2019). Each O_3 treatment had three replicate open-top chambers (OTCs, octagonal base, 12.5 m^2 of growth space and 3.0 m of height, covered with toughened glass), and each OTC had twelve plants per ploidy level. In total, there were six OTCs and 144 plants (36 plants per ploidy level and ozone treatment). The same fertilizer solution as described above was used twice a week, and irrigation was provided to full saturation twice a week with deionized water.

Ozone was generated from pure oxygen using an O_3 generator (HY003, Chuangcheng Co., Jinan, China) and mixed with ambient air by a fan (1.1 KW, 1080 Pa, 19 m^3 min^{-1}, CZR, Fengda, China) to achieve the target O_3 concentration at the top of the canopy. Ozone concentrations within the OTCs were continuously monitored using an ultraviolet (UV) absorption ozone analyzer (Model 49i; Thermo Scientific, Franklin, MA, USA), via a Teflon solenoid valve switch system, which collected air from sampling points at approximately 10 cm above the plant canopy during the experiment. The monitors were calibrated by a 49iPS calibrator (Thermo Scientific, USA) before the experiment, and once a month during the experiment. The daily fumigation period was 10 h (from 08.00 h to 18.00 h) and lasted 42 d from 20 June to 31 July, 2019 when there was no rain, fog, mist, or dew. During the summer experimental period the average O_3 concentrations (from 08.00 h to 18.00 h) were 64.9±0.9 and 119.3±1.1 ppb in NF and NF60, respectively (n=3), and values of AOT40 (accumulated O_3 exposure over an hourly threshold concentration of 40 ppb) were 11.6±0.3 and 33.5±0.4 ppm h in NF

and NF60, respectively (n=3). Monthly average temperature inside the OTCs of May, June and July were 19.7, 23.0 and 25.3 °C, respectively and monthly average air relative humidity 48.0, 66.6 and 77.0%, respectively.

Nitrogen labeling

Five plants per ploidy level were harvested at transplanting for initial morphology characterization (T_0). Plants at this stage were 0.167±0.021 and 0.275±0.059 g shoot and root biomass, respectively, for diploids, and 0.054±0.015 and 0.177±0.100 g shoot and root biomass for triploids (n=5). First evaluation of the uptake of each N form was carried out on 6–7 May 2019 before moving the plants to the OTCs and to the O_3 treatments, corresponding with the end of the first rapid growth phase of poplar in spring (T_1; Li *et al.*, 2012). Second evaluation of the uptake of each N form was done on 1–2 August 2019 corresponding with the middle of the second rapid growth phase of poplar and after exposition to O_3 treatments, in summer (T_2; Li *et al.*, 2012; Feng and Li, 2017). Thirty-six plants from each ploidy level were randomly selected in spring, and the remaining 36 additional plants from each ploidy level and ozone treatment were used in summer.

To allow for acclimation to N forms, 28 d prior to labeling (starting on 8 April 2019 for spring labeling and on 3 July for summer labeling), selected plants were fertilized three times per week with the same fertilizer solution described above. Last fertilization and last watering were carried out 3 d and 2 d before the labeling, respectively.

For the labeling, we used four different solutions at 8.4 ppm N (i.e. 0.6 mM). All fertilizer solutions included the three N sources in equimolar proportions, but differed from one another in the labeled N source: (i) nitrate labeled: $K^{15}NO_3$, $(NH_4)_2SO_4$, and glycine, (ii) ammonium labeled: KNO_3, $(^{15}NH_4)_2SO_4$, and glycine; (iii) glycine labeled: KNO_3, $(NH_4)_2SO_4$, and 2-$^{13}C^{15}N$ glycine and (iv) control solution with all the N forms in natural abundance. Uptake rates of different N forms are different when the N forms are applied individually or in mixtures (Näsholm *et al.*, 2009). Thus, preferences among N forms should be measured by applying all N forms in equimolar amounts. Abundance of ^{15}N in $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$ was 60%, (Shanghai Engineering Research Center of Stable Isotope, Shanghai, China) and abundance of 2-$^{13}C^{15}N$ glycine was 2-^{13}C, 99%, ^{15}N, 98% (Cambridge Isotope Laboratories, London, UK), while abundance in unlabeled N forms was 0.366% and 1.082% for ^{15}N and ^{13}C, respectively. In spring, nine plants per ploidy level were used for each labeled solution, and nine additional ones for the control solution. In summer, three plants of each ploidy level and chamber were supplied with one of the four solutions, and consequently, in total from all OTCs nine plants of each ploidy level were applied in one of the four solutions.

For the labeling, 200 ml of the described solutions were applied individually to each plant at 8.00 h–9.00 h (solar time). Net photosynthesis rate (A_{net}) was measured at the end of the labeling period (10.00 h–12.00 h solar time) using an open gas exchange system LI-6400XT (LICOR Corp, USA). In order to quantify only internal and not abiotic limitations, and for comparative purposes between labeling dates, parameters were set equally in both dates, photosynthetic active radiation (PAR) was set at 1200 μmol m^{-2} s^{-1}, CO_2 concentration at 400 ppm, block temperature at 30 °C, and ambient relative humidity (RH), 45–55% in spring and 55–65% in summer. Plants were harvested 3 h after labeling. Both the time of application of the fertilizer and time of harvesting were recorded for each plant, and the difference was considered as labeling time. After labeling, the plants were harvested. Each plant was separated into fine roots, coarse roots, stems and leaves. At harvesting, all fine roots (<2 mm) were washed in 0.5 mM KCl for 15 s to remove traces of the fertilizer in the root surface and three times in deionized water to eliminate the salt. Finally, all plant organs were oven-dried at 65 °C until the weight was stable and weighed. Fine roots were ground in a ball mill (MM400, Retsch, Haan, Germany), and N and C concentration, and ^{15}N and ^{13}C abundance were determined by isotope ratio mass spectrometry (EF-IRMS Isochrom, Micromass, UK) at the UC Davis Stable Isotopes Laboratory (Davis, California, USA).

Mathematical calculations

Absolute biomass growth (in g) between T_0 and T_1 or between T_1 and T_2 was calculated as:

$$\text{Absolute growth}_{T_i} = M_{T_i} - M_{T_{i-1}} \quad (1)$$

where M is the biomass of each organ or plant; and T_i is either T_1 or T_2. Relative growth rate (RGR; in mg g^{-1} d^{-1}) at plant level between T_0 and T_1 or between T_1 and T_2 was calculated as:

$$\text{RGR} = \frac{\ln(\text{plant mass}_{T_i}) - \ln(\text{plant mass}_{T_{i-1}})}{T_i - T_{i-1}} \quad (2)$$

where, ln is natural logarithm and T_i is either T_1 or T_2.

Due to the short-term labeling (3 h), the isotopic signal provided by each N form could be considered stable. Through dilution equations (Deléens *et al.*, 1994), we can calculate the N taken up from a specific label source from the soil independently if it is the heavy or light isotope. The amount of N taken up ($N_{uptaken}$; in μg) by fine roots from a specific labeled N form was calculated as:

$$N_{\text{uptaken}} = X_N \times [N_{\text{fine root}}] \times DM \times 1000 \quad (3)$$

where $[N_{\text{fine root}}]$ is the N concentration in fine roots (mg g DM^{-1}); DM is the biomass of fine roots (g); and X_N is the proportion of N in fine roots that came from the specific labeled N form (either NO_3^-, NH_4^+ or glycine), which was calculated as:

$$X_N = \frac{A_{LO} - A_{UO}}{A_{LF} - A_{UF}} \quad (4)$$

where, A_{LO} is the ^{15}N abundance (atom%) in fine roots in each labeled plant, A_{UO} is the average ^{15}N abundance of fine roots in the unlabeled plants; and A_{LF} and A_{UF} are the ^{15}N abundance of the labeled and unlabeled fertilizer, respectively. The amount of C uptake in fine roots from the dual labeled 2-$^{13}C^{15}N$ glycine ($C_{uptaken}$) was calculated using the same equations, but substituting X_N, N_{root} and ^{15}N abundance with X_C, $[C_{\text{fine root}}]$ and ^{13}C abundance, respectively (Supplementary Table S1).

Nitrogen uptake rate (in μg g^{-1} h^{-1}) from each N form was calculated as:

$$N_{\text{uptake rate}} = \frac{N_{\text{uptaken}}}{\text{DM} \times \text{time}} \times 1000 \quad (5)$$

Labeling time (time) and fine roots dry biomass of each single plant (DM) were used to standardize calculations and avoid differences among plants due to small differences in labeling duration and root development, respectively.

The proportion of intact glycine taken up by fine roots was estimated by comparing the slope of the regression line between $C_{uptaken}$ against $N_{uptaken}$ determined by IRMS in fine roots from the regression line of slope=1 predicted from the stoichiometry of intact dual labeled glycine uptake (i.e. 1 mol of ^{13}C per mol of ^{15}N; Warren, 2009a).

Statistical analysis

To avoid incomplete factorial design, two different analysis were carried out. In the first one the effect on biomass and photosynthesis rate (or N uptake rate) of ploidy levels and developmental phase (in season and N form) were assessed using a two-way ANOVA (or by three-way ANOVA) by comparing data of plants in spring with data of plants in summer under ambient ozone concentration (NF). The second analysis evaluated the effect on biomass, photosynthesis rate and N uptake rate of ozone contamination and ploidy levels by a generalized mixed model, by comparing data of plants in summer in NF and NF60 ozone treatments and considering chamber as a random

factor. When normality assumption was not met, data were $\log_{10}$ or square root transformed. When ANOVA showed a significant effect, a post-hoc Fisher LSD test was carried out for multiple comparisons among treatments at α=0.05. Additionally to the analysis of C and N uptake correlations for each treatment, differences between ploidy levels in the slope of the regressions between $C_{uptaken}$ versus $N_{uptaken}$ were conducted by checking the significance of regression coefficient β_3 from the model:

$$N_{uptaken} = \beta_0 + \beta_1 \times C_{uptaken} + \beta_2 \times \text{species} + \beta_3 \times C_{uptaken} \times \text{species} + \varepsilon \quad (6)$$

where β_i is the regression coefficient and ε is the random term of the model (Doménech, 1999). The same approach was used to detect differences between slopes of seasons/ozone concentrations within the same ploidy level. Statistical analyses were performed using STATISTICA 7.0 (StatSoft, Tulsa, USA).

Results

Plant growth on different seasons and under summer-enriched ozone

Despite initial differences in organ and plant biomass between ploidy levels, with triploid white poplars being half the size of diploids at transplanting and in spring, both ploidy levels had the same final plant biomass in summer (Supplementary Table S2). Elevated O_3 reduced total plant biomass of triploids, with the biggest reduction in perennial organs (stems and roots), while the plant biomass of diploids was unaffected.

Diploid white poplars had higher organ and plant absolute growth in spring, but lower in the summer than triploids (interaction season×ploidy levels; F=5.7, P=0.018; F=158, P<0.001; F=68.0, P<0.001, and F=85.3, P<0.001 for leaves, stems, roots and whole plant, respectively; Fig. 1A). Consequently, both absolute and relative growth rates, RGR (interaction season×ploidy levels; F=68.9, P<0.001; Fig. 2), changed depending on the season. While diploids maintained mostly the same absolute growth in spring and in summer, the absolute growth of triploids increased around four times from spring to summer (RGR reduced to half in summer). Although the biomass of all organs increased in both seasons, summer absolute growth was more intense in perennial organs (stems and roots) than in deciduous organs (leaves).

Additionally, both ploidy levels decreased plant absolute growth under NF60 compared with NF (15.8% and 19.3% reduction for diploids and triploids, respectively) (interaction ozone×ploidy levels; F=0.17, P=0.67, Fig. 1B). The decrease of absolute growth in diploids was exclusively due to the reduction of leaf biomass. While in triploids the absolute growth reduction was primarily associated with perennial organs growth (stems and roots). However, while triploids decreased their RGR in response to NF60, diploids had no change in RGR (interaction ozone × ploidy levels; F=3.1, P=0.082; Fig. 2).

Effects of season and summer ozone on N forms, total N uptake rates and C acquisition

Total N uptake in diploid white poplars was lower in spring than in summer, while in triploids the pattern was inverse (interaction season × ploidy levels; F=29.7, P<0.001; Fig. 3A). Consequently, total N uptake rate in diploids was lower than triploids in spring, but higher than triploids in summer. The N uptake from each N form also changed with the season, and depended on the ploidy levels (interaction season × ploidy levels × N form; Table 1). Diploids had similar N uptake rates from each N form in spring. However, in the summer, NO_3^--N uptake rate doubled, while it remained unchanged for NH_4^+ and glycine. In contrast, for triploids NO_3^- was the N form with the highest N uptake rate independent of the season. In spring, triploid NH_4^+-N uptake rate was higher than glycine. In summer, NO_3^- and NH_4^+ uptake rate decreased to half, but it remained unchanged for glycine-N uptake. Consequently,

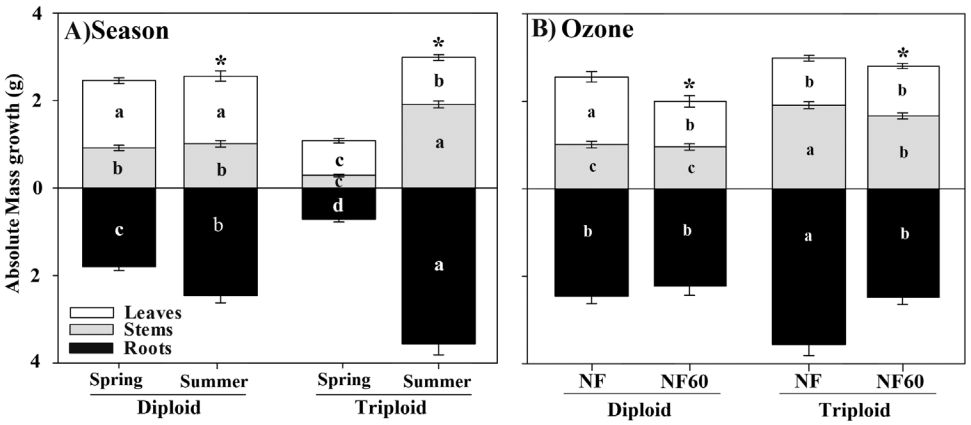

Fig. 1. Absolute biomass growth in two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants. (A) Effect between seasons: spring (T_0-T_1), from transplanting time (T_0) to the end of first rapid growth phase (T_1), or summer (T_1-T_2), from spring (T_1) to middle of the second rapid growth phase of the species (T_2). (B) Effect of O_3 concentrations during summer: ambient O_3 (air non filtered, NF), or with an increase of 60 ppb O_3 over ambient (NF60). Different lowercase letters indicate statistically significant differences in organs, while "*" indicates statistical differences in total plant biomass. Data are means ±SE (n=36).

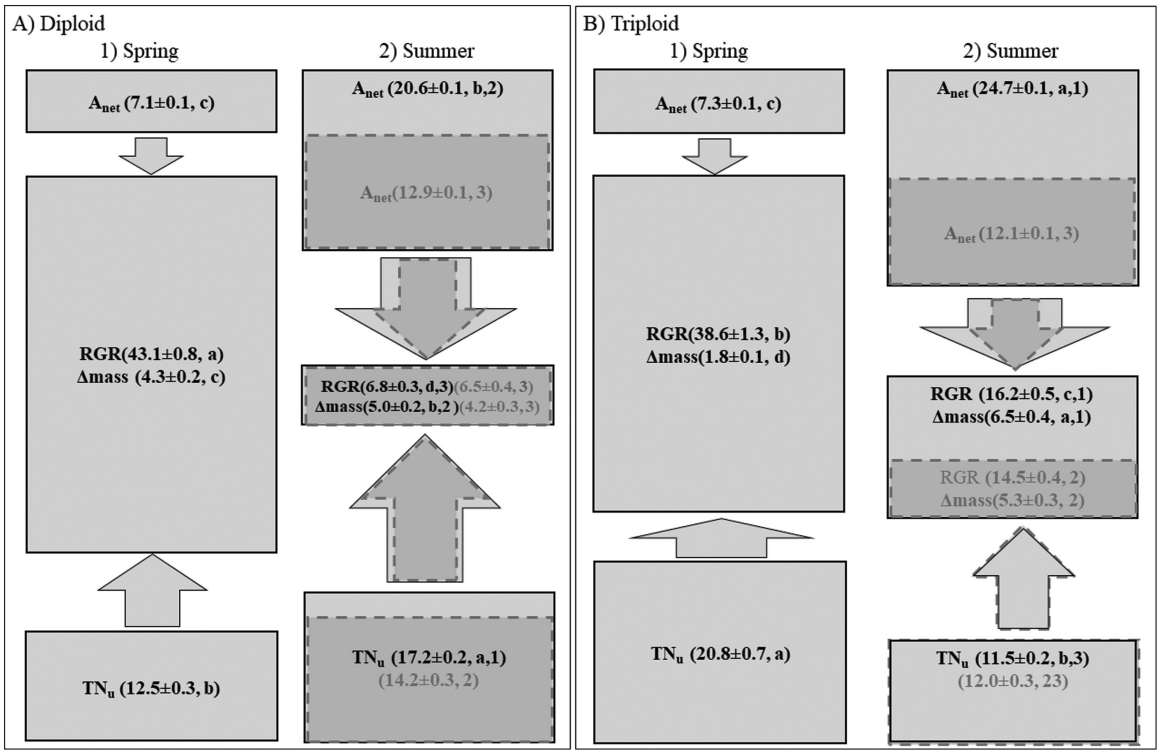

Fig. 2. Conceptual diagram of the effects of season and O_3 on photosynthesis rate (A_{net}, μmol CO_2 m^{-2} s^{-1}, arrows), relative growth rate (RGR, mg g^{-1} day^{-1}, boxes), absolute biomass growth (g, inserted numbers) and total N uptake rate (TN_u, μg N gDM^{-1} h^{-1}, arrows) in two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants. (A) Diploid plants and (B) triploid plants at (1) spring, the end of the first rapid growth phase (T_1); and (2) summer, at the middle of the second rapid growth phase of the species (T_2). During summer (starting on 20 June), plants were exposed to two different O_3 concentrations: ambient O_3 (air non filtered, NF) or with an increase of 60 ppb O_3 over ambient (NF60). Solid medium-grey boxes and black numbers indicate the values of variables in response to seasons at spring (1) and summer (2), long dashed dark grey boxes and grey numbers at summer indicate the values of variables in response to elevated O_3. Arrows show the expected flux direction of C and N. Note that the height of boxes and width of arrows are proportional to the magnitude of the process indicated. Different letters (or numbers) indicate statistical difference between seasons (or O_3 concentrations at summer).

in summer for triploids NH_4^+ and glycine-N uptake rates were similar, but lower than NO_3^-. Triploid NO_3^--N uptake rate was more than two times faster than diploids in spring, but half of that of diploids in summer. Triploid NH_4^+ and glycine-N uptake rates were similar to diploids, independent of the season.

In response to NF60, both ploidy levels decreased mainly the N uptake rate of NO_3^- (interaction N form×ozone; Fig. 3B; Table 1). Additionally, diploids showed a higher reduction in N form uptake than triploids (interaction ploidy levels×ozone). However, post-hoc test indicated differences at the triple interaction level (ploidy levels×ozone×N form). In diploids under NF60, together with 22% of NO_3^--N uptake reduction, glycine-N uptake was decreased by about 36%. These changes did not alter the ranking of diploid N form uptake rates; despite the O_3 concentration, diploid NO_3^--N uptake was faster than NH_4^+ and glycine. In contrast, in triploids, the NO_3^- N uptake reduction under NF60 was not enough to produce significant differences compared with NF. However, the non-significant reduction in NO_3^--N uptake and a non-significant increase in NH_4^+-N and glycine-N uptake rate under NF60 were enough to eliminate differences among N forms. The ranking of N uptake rates among N forms changed from higher NO_3^--N uptake than the other two N forms under NF, to similar N uptake rates among N forms in NF60 (Fig. 3B; Table 1). Finally, the total N uptake rate of diploids decreased in NF60 compared with NF plants, while it remained unchanged for triploid plants (interaction ozone×ploidy levels; $F=5.2$, $P=0.029$; Fig. 3B).

There was a significant correlation between $N_{uptaken}$ and $C_{uptaken}$ for both ploidy levels in each season or O_3 concentration, except for diploids in spring in which this correlation was only marginally significant (Table 2; Supplementary Fig. S1). Based on season, the slope of $N_{uptaken}$ and $C_{uptaken}$ was steeper in spring than in summer, and steeper in triploids than in diploids ($P<0.01$ in all the cases). In response to O_3, both ploidy levels reduced the regression slope, but the decrease in triploids was sharper than that of diploids ($P<0.01$ in all the cases).

There was no significant difference in A_{net} between ploidy levels in spring, but in the summer, even though both showed increased A_{net}, the increase in diploids was lower than that for triploids (188% and 239% increase, for diploids and triploids, respectively; Fig. 3). Consequently, in the summer, triploids had higher A_{net} than diploids (interaction ploidy levels×season; F=12.9, P<0.001). Compared with NF, A_{net} of diploid plants decreased by 38%, while triploid plants decreased by 51% at NF60. These reductions eliminated A_{net} differences between ploidy levels under NF60 (interaction ploidy levels×ozone F=20.3, P<0.001). Across seasons, ploidy levels and O_3 concentrations, only NH_4^+ -N uptake rate was marginally and negatively correlated with A_{net} (y=−0.13x+6.2; r^2=0.65; $F_{1,5}$=7.5; P=0.051). In contrast, neither total N uptake (y=−0.15x+16.8; r^2=0.09; $F_{1,5}$=0.40; P=0.56) nor NO_3^- (y=−0.037x+7.17; r^2=0.09; $F_{1,5}$=0.05; P=0.83) or glycine (y=0.017x+3.4; r^2=0.03; $F_{1,5}$=0.14; P=0.72) N uptake rates were correlated with A_{net} (Supplementary Fig. S2).

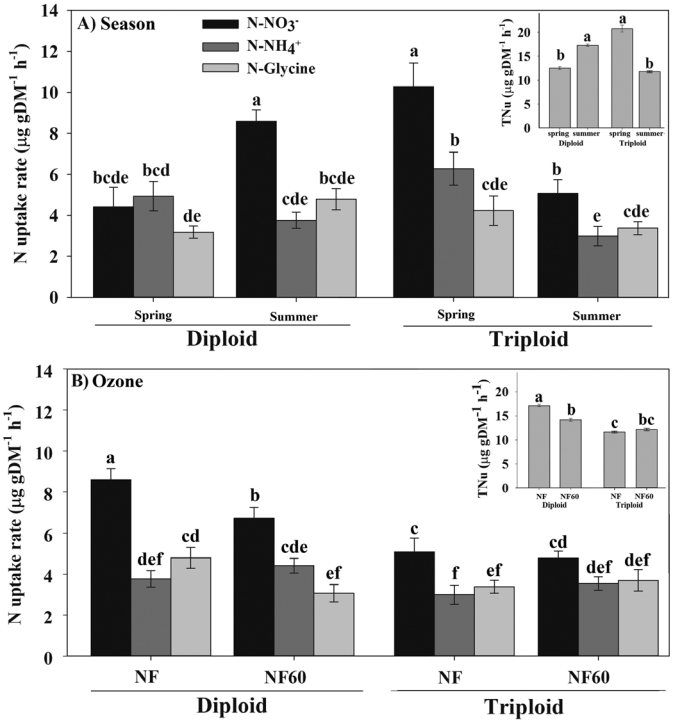

Fig. 3. Nitrogen uptake rate from NO_3^-, NH_4^+, and glycine in two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants. (A) Uptake rate between seasons: spring, end of first rapid growth phase (T_1), or summer, middle of the second rapid growth phase of the species (T_2). (B) Uptake rate between O_3 concentrations: ambient O_3 (air non filtered, NF), or with an increase of 60 ppb O_3 over ambient (NF60). Bars marked with different letters indicate statistically significant difference between seasons or O_3 concentrations at summer. Inserted figures indicate total N uptake rate (TNu). Data are means±SE (n=9).

Discussion

Preference for N forms in diploid white poplar shows greater plasticity in response to seasons

According to our hypothesis (i), both ploidy levels were able to take up intact glycine in both seasons, demonstrated by the high correlation between $N_{uptaken}$ and $C_{uptaken}$ (Table 2; Supplementary Fig. S1). Furthermore, slopes indicated that more than 60% of the glycine was absorbed intact in spring, and around 40% in summer. Additionally, according our first hypothesis, triploids had generally a higher N uptake of intact glycine than diploids, especially in spring, which would match with a strategy to reduce C and N assimilation cost during spring and improve growth rate of the latter (Boudsocq *et al.*, 2012; Gruffman *et al.*, 2013; Franklin *et al.*, 2017). However, the proportion of intact glycine uptake in white poplar is lower than the 86–95% reported for most of the forest tree species (Warren, 2009a; Gruffman *et al.*, 2013; Uscola *et al.*, 2017). Differences between ploidy levels and with other tree species might be due to differences in abundance or activity of root cellular transporters for amino acids or by different experimental conditions (Näsholm *et al.*, 2009; Nacry *et al.*, 2013; Zhang *et al.*, 2014). However, the estimation method has some limitations, i.e. amino acids are mineralized in the soil and ^{13}C and ^{15}N might be taken up independently; or the ^{13}C:^{15}N molar ratio of labeled roots can be affected by post-uptake loss of $^{13}CO_2$ via respiration, photorespiration and internal remobilization of ^{15}N and/or ^{13}C, leading to over or underestimation of intact glycine (Warren, 2012). Additionally,

Table 1. F and *P* values of N uptake rates for NO_3^-, NH_4^+, and glycine in two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants with season (or O_3 concentration at summer) on a three-way ANOVA.

Variable	F	*P*	Variable	F	*P*
Season	4.032	**0.047**	Ozone	2.268	0.135
Ploidy levels	1.192	0.278	Ploidy levels	23.943	**<0.001**
N Form	25.315	**<0.001**	N Form	41.582	**<0.001**
Season×Ploidy levels	35.404	**<0.001**	Ozone×Ploidy levels	4.762	**0.032**
Season×N Form	3.847	**0.024**	Ozone×N Form	3.640	**0.030**
Ploidy levels×N Form	1.035	0.359	Ploidy levels×N Form	7.166	**0.001**
Season×Ploidy levels×N Form	9.156	**<0.001**	Ozone×Ploidy levels×N Form	1.469	0.235

Statistical analysis for O_3 effects OTC chamber was included as a random variable.

Table 2. F-, *P*-, and R^2- values of regressions between $C_{uptaken}$ and $N_{uptaken}$ in fine roots of two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants after soil application of dual ^{13}C and ^{15}N labeled glycine at spring, the end of the first rapid growth phase; and summer, at the middle of the second rapid growth phase of the species.

Variable	df	F	*P*	R^2	slope
Spring-Diploid	8	3.7649	0.0935	0.3497	0.6292 b
Spring-Triploid	8	66.8073	**<0.0001**	0.9052	0.8387 a
Summer-NF-Diploid	8	8.9732	**0.0201**	0.5618	0.4237 d
Summer-NF-Triploid	8	21.9957	**0.0022**	0.7586	0.4806 c
Summer-NF60-Diploid	8	15.7893	**0.0054**	0.6928	0.4179 e
Summer-NF60-Triploid	8	9.1652	**0.0192**	0.567	0.3921 f

During summer (starting on 20 June), plants were exposed to two different O_3 concentrations: ambient O_3 (air non filtered, NF) or with an increase of 60 ppb O_3 over ambient (NF60) (n=9).

we cannot discard that part of the N taken up and/or the N forms were translocated from roots to other plant organs, affecting the estimation of N uptake rates.

Preference for N forms in diploids shows greater plasticity in response to seasons compared with triploids (Fig. 3A). Diploids had no specific preferences among N forms in spring, while in summer, they preferred NO_3^- over NH_4^+ and glycine. However, triploids did not modify their N form preferences in response to season, and showed an overall preference for NO_3^-. The plasticity in N form preferences of diploids was mainly due to the plasticity in NO_3^--N uptake, which increased in summer, rather than to other changes in N uptake rates in other N forms, as claimed in our hypothesis (ii). As NO_3^- uptake is highly dependent on current photoassimilates (Gruffman *et al.*, 2013; Franklin *et al.*, 2017), low NO_3^--N uptake rates in spring were expected due to internal limitations on photosynthesis rates in our experiment (Fig. 2A). The lack of correlation between NO_3^--N uptake and photosynthesis rate might reflect the utilization of soluble sugars from remobilization of reserves as an alternative pathway. Additionally, according to our hypothesis (ii), both ploidy levels tended to reduce the intact glycine and NH_4^+-N uptake in summer. Both N forms and especially glycine are less dependent on current photoassimilates (Gruffman *et al.*, 2013; Franklin *et al.*, 2017). Consequently, glycine N uptake was not correlated with photosynthesis, and NH_4^+-N uptake decreased with increased photosynthesis (Supplementary Fig. S2). Preferences among N forms in both ploidy levels might also respond to changes in N form availability. In the summer, microbial activity increases, triggering high mineralization rates and increasing NO_3^- availability (Schimel and Bennett, 2004; Rennenberg *et al.*, 2009).

Generally it is assumed that N uptake matches growth (Lambers *et al.*, 2008). In our study, differences between ploidy levels on RGR, absolute growth, and C assimilation did not keep pace with total N uptake rate and preferences among N forms. One possible explanation might be the different C and N use strategies between ploidy levels. In spring, diploids grew faster (absolute growth and RGR) and had similar C assimilation but lower N acquisition than triploids (Fig. 2A), suggesting more C directed to support growth and less to fuel the metabolism of N taken up (Gruffman *et al.*, 2013; Uscola *et al.*, 2015; Villar-Salvador *et al.*, 2015). In order to achieve a reduced cost in the metabolism of N uptake, diploids increased the proportion of low metabolic cost N forms taken up, i.e. amino acids and NH_4^+ (Boudsocq *et al.*, 2012; Gruffman *et al.*, 2013; Franklin *et al.*, 2017), and used N form as a function of its soil availability, as they were applied in equimolar mixtures in this study. This result could also suggest that diploids rely more on C and N reserve remobilization in early growth phases than triploids, as usually do fast-growing species (Uscola *et al.*, 2015; Villar-Salvador *et al.*, 2015). In contrast, the lower growth but higher total N and NO_3^- uptake rates of triploid white poplars compared with diploids suggest that C might be mainly used for growth together with metabolism of the NO_3^- taken up (Fig. 2B). However, due to low C acquisition, C reserves may also play an important role at this moment. In summer, even diploids maintain similar absolute growth compared with spring growth; it had six times less RGR, and increased to double N uptake rates, mediated by an increase in NO_3^- preference (Figs 2A; 3A), which has high acquisition and metabolism cost (Boudsocq *et al.*, 2012; Gruffman *et al.*, 2013; Franklin *et al.*, 2017). For now, as photosynthesis results suggest no limitation on C assimilation, the current acquired C could be enough to fulfill the C demands, i.e. growth and NO_3^- uptake metabolism. However, triploids had noticeable absolute growth increment in summer (around four times with respect to spring) but two times lower RGR than in spring, and N uptake decreased to half (Figs 2B; 3A). These results, together with the high photosynthesis rate in summer, suggest that newly acquired C might be mainly used to fuel growth. Consequently, triploids decreased the C and energy cost of N metabolization by decreasing NO_3^- and total N uptake. Additionally, under our experimental conditions, as summer ambient O_3 concentration (64.9 ppb) exceeded 40 ppb, both ploidy levels would consume part of the acquired C for detoxification and repairing reactive oxygen species damage (Andersen, 2003; Sitch *et al.*, 2007; Sahu *et al.*, 2021).

Plasticity in N form preferences of triploids allows maintenance of total N uptake under elevated summer O_3

According to our hypothesis (iii), elevated O_3 concentration negatively affected both ploidy levels. Though they had some similar responses, each ploidy level was differently impacted upon. In response to an enriched atmosphere of O_3, both ploidy levels decreased photosynthesis and decreased NO_3^--N uptake rates, especially in diploids (Figs 2; 3B), as we expected. The reduction of NO_3^--N uptake, considering its high dependence on photoassimilates (Gruffman *et al.*, 2013; Franklin *et al.*, 2017), was likely related to the significant decrease in C assimilation under elevated O_3. Reductions in C assimilation are also common negative effects under elevated O_3 (Li *et al.*, 2017; Feng *et al.*, 2019). Additionally, both ploidy levels decreased the proportion of intact glycine uptake, implying that N from glycine was taken up as NH_4^+ (Table 2; Fig. 3B). This effect was significantly stronger in triploids, with a reduction of 2% in uptake of intact glycine in diploids versus 18% in triploids. However, diploids decreased NO_3^- and also glycine-N uptake rates and, as a consequence, total N uptake was reduced by ~17%, although these changes did not modify its N form preferences (Fig. 3B). Additionally, the reduction in N acquisition ability of diploids under elevated O_3 suggests a limitation in acquiring other soil resources, such as water, that under other conditions would make them more susceptible to other stress conditions, like drought stress (Pollastrini *et al.*, 2014). On the contrary, in triploid white poplars, elevated O_3 unaffected total N uptake, indicating that these plants were able to alleviate or compensate for the negative effects of O_3 on NO_3^--N uptake decline by modifying their N form preferences and slightly increasing NH_4^+ and intact glycine N uptake rates (Fig. 3B).

Previous studies reported that the negative impact of O_3 on plant growth and total N uptake are simultaneous (Luedemann *et al.*, 2005; Weigt *et al.*, 2012; Zak *et al.*, 2012). However, our results showed that O_3 damage on growth was not always coupled with total N uptake or the uptake of each N form. The differential effects of O_3 by ploidy levels on growth and/or N uptake might be linked with C use strategy, similar to the performance between different seasons. In diploids, under elevated O_3 concentrations, inhibition in C assimilation did not drive intense reductions of growth, but N uptake rate was reduced, indicating that the inhibition of elevated O_3 on C assimilation mainly impaired N uptake (Fig. 2A). In contrast, under elevated O_3, triploids showed a strong decrease in C assimilation, leading also to a significant decrease in growth without decreasing N uptake (Fig. 2B) (Andersen, 2003; Sitch *et al.*, 2007). Furthermore, both ploidy levels also had to invest more C for defense under elevated ozone (Sitch *et al.*, 2007; Li *et al.*, 2017; Feng *et al.*, 2019). Considering the higher decrease of net photosynthesis (51% versus 38%), and absolute biomass growth (20% versus 16%) in triploids compared with diploids, triploids were intensely impacted by elevated O_3, as stated in our hypothesis (iv).

However, besides growth and N uptake, newly assimilated C can also be allocated to support respiration, reserve, defense or other sink demands (Andersen, 2003; Huang *et al.*, 2019). Further studies should examine differences of C and N acquisition between ploidy levels (or species) in response to different time scales and stress conditions, and studied together with C and N allocation strategies among different sinks.

To the best of our knowledge, for the first time, we have investigated the plasticity of preference among N forms in response to season (with different growth rate), and O_3 contamination. Chinese white poplar had the ability to uptake intact amino acids, with triploids having higher intact amino acid uptake ability compared with diploids. Preferences among N forms of each ploidy level shifted in response to seasons and/or O_3. While diploids changed from no preferences among N forms in spring to high NO_3^- preference in summer, triploids had an overall preference for NO_3^-. Furthermore, growth rate was asynchronous with N uptake. Differences between both processes might be explained by different strategies on C and N use. In spring, when C and N acquisition were limited, diploids probably used newly acquired nutrients and reserve remobilization to fuel its fast growth. Triploids in spring with low growth rates likely used C to support N uptake metabolism. In summer, C assimilation was sufficient for diploids to fuel growth and metabolize N uptake, but in triploids C was probably primarily used to fuel fast growth. Under elevated O_3 fumigation, C acquisition and NO_3^--N uptake decreased in both ploidy levels. However, in diploids O_3 effects resulted in growth and total N uptake reductions, without changes in N form preferences. In contrast, in triploids, the effect of O_3 was a reduction in growth; furthermore, triploids were able to avoid O_3 impact on N uptake shifting preferences from an overall preference for NO_3^- to similar preferences among N forms. Our study provides a new direction to explore plant N form uptake patterns combining its own growth rhythm with C and N use strategies, and to explore plant behavior under stress conditions.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. ^{15}N and ^{13}C abundance (atom%) in unlabeled (A_{UO}) and labeled (A_{LO}) new roots in two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants.

Table S2. Organ and plant biomass at different harvesting moments of two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants.

Fig. S1. ^{13}C-^{15}N molar correlations after uptake of dual-labeled glycine in roots of two ploidy levels of *Populus tomentosa* Carr. 1-year-old plants.

Fig. S2. Relationship between the photosynthesis rate (Anet) across ploidy levels, seasons and ozone treatments and the N uptake rates from glycine, NH_4^+ and NO_3^-.

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Author contributions

MMW and MU designed the experiments; MMW cultivated the plants, performed the labeling treatments, harvesting and sample processing; YSX performed the ozone treatments; MMW and MU performed the data calculations, statistical analysis, and wrote the manuscript; GLL, YL and ZZF provided the financial support and supervised the writing of the final manuscript.

Data availability

The data that support the findings of this study are openly available at Dryad Digital Repository https://doi.org/10.5061/dryad.qfttdz0gd; Wang *et al.*, (2021)

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