



**Full Length Article**

## Late-Season Fluxes of Ammonium and Nitrate in Roots of Two Poplar Clones Pretreated with Nutrient Addition

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### Abstract

Nutrient uptake and storage at fall is important for improving tree stock quality. Fertilization during late-season has been proven to efficiently improve inherent nutrient storage in coniferous and some hardwood species, but information about poplar is limited. With the aim to quantify characteristics of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes in poplar roots at fall, two poplar clones of #26 and #46 with contrasting growing performances were obtained from hybrids generated from the hybridization (*Populus tomentosa* × *P. bolleana* and *P. alba* × *P. glandulosa* as male and female parents, respectively) and cultured as rooted grafted cuttings to receive pre-treatments of three-time fertilization (N,  $\text{P}_2\text{O}_5$ , and  $\text{K}_2\text{O}$ , 5, 4.5, and 0.5 g plant<sup>-1</sup>) at early May, June, and July 2008. Un-fertilized cuttings were labeled as the control and all cuttings were raised in pots for six months. In late September, shoot height of the 26 clone was greater by 6–71% relative to the 46 clone. In the pre-fertilization treatment, biomass increased by 21%, 23%, and 49% in leaves, stem, and root, respectively, in the 26 clone compared to the 46 clone. Pre-fertilized cuttings of the 26 clone had lower N concentration by 67% in stem but higher K concentration by 5% in leaves compared to the 46 clone. Vector diagnosis indicated that the pre-fertilization treatment countered N deficiency and induced steady-state loading of P relative to the control. At fall, pre-fertilization delayed the alternation of  $\text{NH}_4^+$  flux from efflux to influx for about 10 min in the 26 clone and resulted in a general  $\text{NH}_4^+$  influx in the 46 clone. Meanwhile the fertilization treatment converted the  $\text{NO}_3^-$  influx to efflux since the 6 min after experiment start. In conclusion, during the late-season, pre-fertilization resulted in a better nutrient status in hybrid poplar rooted cuttings of both clones, but  $\text{NH}_4^+$  influx and  $\text{NO}_3^-$  efflux were only promoted in the 46 clone. Although the 26 clone had a better natural ability for stem biomass production and  $\text{NH}_4^+$  uptake, the 46 clone may be more efficient in N uptake to receive fall fertilization. © 2017 Friends Science Publishers

**Keywords:** Non-invasive micro-test technique; Ion-selective microelectrodes; *Populus* spp. hybrid; Nutrient storage; Nitrogen

### Introduction

In face of the increasing concerns about contemporaneous challenges of climate change and energy security, more bioenergy is evoked from sources of short-rotation plantations at some high planting densities (~1,000–40,000 units ha<sup>-1</sup>). Poplar (*Populus* spp.) has been intensively developed as a bioenergy species due to abundant biomass production and easy vegetative propagation (Dillen *et al.*, 2011; Stobrawa, 2014). The general yield of a poplar short-rotation plantation was suggested to range 8–10 oven-dry tons ha<sup>-1</sup> year<sup>-1</sup>, which was larger than that of many other woody plants (Ai and Tschirner, 2010). The ability of biomass production by poplar trees mostly results from its fast-growing trait, which may also impact poplar stock

quality due to exhausted nutrient utilization when nutrient supply is usually limited (Schott *et al.*, 2013).

Nutrient loading is a practical technique during nursery culture whereby nutrients are loaded as storage to promote post-plant retranslocation and resistance (Oliet *et al.*, 2013). Exponential nutrient loading, or so-called exponential fertilization (EF), has been tested to be effective on the establishment of nutrient storage in juvenile coniferous (Duan *et al.*, 2013), hardwood (Schmal *et al.*, 2011; Li *et al.*, 2017), and some fast-growing trees (Chen *et al.*, 2012; Park *et al.*, 2012). However, EF usually failed to construct nutrient reserve in poplar (Schott *et al.*, 2013) unless the N supply strategy was modified (Zabek and Prescott, 2007). This is because the growth patterns of poplar species are indeterminate, and the fast growth of

poplar species at their early growing stage can be limited with exuberant nutrient demand.

Thus some tree species with multiple flushes may have null responses to pre-hardening EF fertilization in nutrient reserve establishment (Oliet *et al.*, 2011). Instead, fall fertilization (FF) can be an alternative fertilization strategy to improve their stock quality by supplying extra nutrients during the hardening stage at the late growing season (Oliet *et al.*, 2011). For example, the improvement of inherent nutrient status through EF was not apparent for Holm oak (*Quercus ilex* L.) seedlings (Villar-Salvador *et al.*, 2004; Oliet *et al.*, 2009; Uscola *et al.*, 2015a) due to the episodic growth pattern (Terradas and Savè, 1999), but FF achieved the nutrient storage (Oliet *et al.*, 2011). Therefore, the objective of FF is to increase nutrient reserves and to allow better new shoot growth in the following spring (Villar-Salvador *et al.*, 2015). Currently, FF has been successfully used to counter nutrient dilution in seedlings of Douglas-fir (*Pseudotsuga menziesii*) (Birchler *et al.*, 2001), black spruce (*Picea mariana* Mill. B.S.P.) (Boivin *et al.*, 2002, 2004), red pine (*Pinus resinosa* Ait.) (Islam *et al.*, 2009), Holm oak (Oliet *et al.*, 2011), and Changbai larch (*Larix olgensis* Henry.) (Wei *et al.*, 2014). The relationship between fast growing habit and nutrient reserve dependence has been demonstrated in some evergreen tree species (Uscola *et al.*, 2015b). However, no clear information has been reported about FF effect on poplar clones. Poplar trees continuously grow in height at fall, which challenges the success to enhance nutrient reserves in poplar at fall because growing tissues would exhaust most nutrients without significant accumulation (Schott *et al.*, 2013). Therefore, it is necessary to supply FF to poplar trees to compensate utilized ones. However, the flux characteristic of nutrients in poplar roots at fall is not clear, which hindered the formulation of FF dose and limited the knowledge about mechanism for nutrient uptake by poplar trees at fall.

Nitrogen (N) is the element mostly limiting tree growth in northern temperate and boreal coniferous forests (Rennenberg *et al.*, 2009). N reserve is often diluted with biomass accumulation in juvenile trees at fall, which generates the necessity of FF with N (Oliet *et al.*, 2011; Wei *et al.*, 2014). Ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) are two principal inorganic N sources for plant growth. The developments of the microelectrode technique and the non-invasive microelectrode ion flux measurement (MIFE<sup>®</sup>) system offer a reliable method for characterizing the concentration dependence of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> (Garnett *et al.*, 2003). Based on these techniques, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes can be determined in juvenile tree roots for their uptake kinetics (Garnett *et al.*, 2003), uptake rate (Miller and Hawkins 2007), flux rates along root hairs (Hawkins *et al.*, 2008), flux responses to pH (Hawkins and Robbins, 2010) and transporters' expression (Alber *et al.*, 2012) in minutes at the micro-scale. Information about the NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes in tree roots at the micro-scale contributes to a better understanding of root N uptake. The net NO<sub>3</sub><sup>-</sup> flux was

found to be inhibited by ca. 25–40% with the provision of NH<sub>4</sub><sup>+</sup> in root tip of poplar roots (Luo *et al.*, 2013a; Zhang *et al.*, 2014). Most of current studies detected inorganic N flux in juvenile tree roots in response to different solutions, but genotypic effect was also strong to affect N flux in poplar clones (Kalcsits and Guy, 2016). In addition, the variation among genotypes plus the variation of pre-conditions strengthen the uncertainty of N flux in poplar clones. In Douglas-fir and lodgepole pine (*Pinus contorta*) seedlings, Hawkins *et al.* (2008) reported that inorganic N flux was generally greater in N-starved seedlings than those under N-satisfied condition, suggesting that N flux in juvenile tree roots may have a response to combined factors of genotype and pre-condition of N status.

In the present study, grafted poplar cuttings of two genotypes with contrasting growth rates were practically cultured and pretreated with nutrient addition during growing season, after which net fluxes of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were measured. Simultaneous employment of these two clones can test the common hypothesis across different poplar clones. Comparisons of results between the two clones would indicate whether results were clone-type-specific. Also these two poplar clones are practically used for biomass production in Northern China. We hypothesized that: (1) inorganic N flux would be increased in roots of the poplar genotype with faster growth rate, (2) inorganic N flux would be lower in N-fed cuttings for both poplar genotypes, and (3) the interactive effects of genotype and preconditioned N were tested to be significant and the faster-growing genotype without N pretreatment would had highest rate of N flux.

## Materials and Methods

### Poplar Materials

All poplar materials used in the present study were produced and supplied by the National Nursery of High-Quality Poplar Varieties, Wei County, Hebei Province, China (37°02'33.45" N, 115°17'52" E). Two poplar clones, "46" and "26", were grafted to un-rooted of Thai cathay poplar (*Populus simonii* × *P. nigra* var. *italica*) stocks to create grafted cuttings. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. According to a pre-investigation, the 26 clone had higher growth rate than the 46 clone. Thai cathay poplar was used as un-rooted rootstock to enhance survival of hybrids after planting. The hybrid cuttings were 10–13 cm in length and 0.5–0.7 cm in basal diameter with rootstock of 5–7 cm in length and 1.5–2.0 cm in diameter.

### Poplar Culture

In mid-late March 2008, a number of 320 un-rooted grafted cuttings were planted in plastic pots (Diameter × Height, 37

cm × 28 cm) placed in a greenhouse of the experimental nursery of Beijing Forestry University (39°59'58.66" N, 116°20'20.90" E). Sunshade mesh and atomizing sprayers were employed to keep the highest temperature below 40°C. Throughout the period of poplar culture, average day/night air temperature was kept at 31/18°C. Average relative humidity and illumination were measured to be 37% and 700 μmol m<sup>-2</sup> s<sup>-1</sup>, respectively. Tree seedling growth lamps (Oudi® Illumination Ltd., Huzhou City, Zhejiang Province, China) were switched on under the necessary condition to maintain the illuminating intensity. Pots were filled with sandy loams collected from a poplar plantation nearby. Soils were dried in shade and passed the 5 mm screen before used as substrates. Each pot was filled with 16 kg of screened soils. Chemical analysis of the soils indicated that nutrient components included 0.303 g kg<sup>-1</sup> total N, 6.62 mg kg<sup>-1</sup> available phosphorus (P), and 34.6 mg kg<sup>-1</sup> potassium (K). Potted soils were determined to have pH of 8.77, volume-weight of 1.47 g cm<sup>-3</sup>, and field capacity of 14.89%. Before planting grafted cuttings were treated with ABT® rooting powder (Aibidi Biological Tech. Ltd., Beijing, China) by soaking rootstocks for 4 h. Grafted cuttings were planted by inserting into potted soils leaving Thai cathay poplar rootstocks underground and hybrids aboveground. Totally, grafted cuttings were planted in 320 pots in total. Both pesticide and herbicide were sprayed at a uniform dose (v/v, 1.2% and 19%, respectively) for each of potted cuttings.

### Experiment Design and Pretreatment of Nutrient

The experiment was conducted to investigate main and interactive effects of two poplar genotypes and two pre-hardening treatments of nutrient addition. All 320 potted cuttings were placed in four blocks as replicates ( $n=4$ ), within each of which included were cuttings from two poplar clones with or without nutrient supply. Therefore, before N flux was measured at fall 20 cuttings were available for the determination of cutting precondition in one of combined treatments of one block. Thereafter, half of the left cuttings were used for measures of either flux of NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>.

For the culture of poplar stocks, EF has been proven to have less efficiency on seedling quality and inherent nutrient storage than conventional loading at a constant rate (Zabek and Prescott, 2007). Three-stage of nutrient applications at a constant rate was also tested to be effective to hybrid poplar plantations (van den Driessche *et al.*, 2008). Therefore, grafted cuttings in our study were fertilized using three-time applications at a constant rate. Nutrient was delivered through the chemical mixture of urea, superphosphate, and potassium sulphate (46.4-21.1-50). The total amounts of supplied N, P, and K were 5, 1.96, and 0.42 g pot<sup>-1</sup>, respectively. Three nutrients were applied by watering pots using solute fertilizers on early May, June, and July, while potted soils of controlled cuttings were only wetted with distilled water at the same solution rate on the same days. Irrigation was conducted using atomized water sprayed out

through tiny apertures made by laser micro-beam on PVC pipelines along pots. Although herbicides were sprayed, some weeds still grew from late June on. These weeds were controlled manually once a week for all pots.

### Pretreated Cutting Sampling and Determination

On late September 2008, four cuttings were randomly chosen and sampled from each of 20 cuttings in one combined treatment (64 sampled cuttings in all). Roots were washed free from soils then cut off from shoots at the collar of rootstock. Shoot height was measured for length between rootstock top and apical tip. Collar diameter was measured at 3 cm over the base of hybrid cuttings. Shoot was divided into leaves and stem. The biomass of excised organ-samples was measured for dry mass after oven-dried at 70°C for 72 h.

Determination for total nutrient content in cutting tissues was adapted from the same method by Wei *et al.* (2012, 2014) and Duan *et al.* (2013). In brief, dried samples were ground to pass a 1 mm sieve; then a 0.2 g sample was digested in 5 mL of H<sub>2</sub>O<sub>2</sub>-H<sub>2</sub>SO<sub>4</sub>. The digestion solution was diluted to 50 mL. Nutrient concentration was determined using 5 mL of digested solutions for any of N, P, and K. Tissue N concentration was determined by an automatic N analyser (UDK 152 automatic N analyser, VELP Co., Usmate, Italy). Tissue P and K concentrations were determined by ICP-OES (Perkin Elmer Co., Waltham, United States). Tissue nutrient content was calculated by biomass multiplying nutrient concentration.

### Inorganic N Flux Measurements

On early October 2008, four cuttings were removed from pots out of one combined treatments to measure short-term inorganic N fluxes (64 sampled cuttings in all). NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes were measured by the MIFE® system (BIO-001A, Younger USA Sci. and Tech. Co., USA) in the Younger USA MIFE Service Center of China (Xuyue Co., Beijing, China). The three ion-selective microelectrodes were mounted vertically above the plant chamber in a modified, adjustable, three-electrode holder (Narashige). The ion-selective microelectrode consists of standard glass microelectrode, Ag/AgCl wires, liquid ion exchanger, and electrolyte solutions. Before measuring, microelectrodes for both NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were collaborated to keep Nernstian Slope > 56 mv/decade. Collaborating solutions for NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> performed components of 0.05 mM Ca (NO<sub>3</sub>)<sub>2</sub> and 0.5 mM KNO<sub>3</sub> with pH=6.0. The measuring solutions included 0.1 mM NH<sub>4</sub>NO<sub>3</sub>, 0.1 mM KCl, 0.2 mM CaSO<sub>4</sub>, and 1% sucrose, with pH=6.0, for NH<sub>4</sub><sup>+</sup>; while those included 0.25 mM KNO<sub>3</sub>, 0.625 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 0.25 mM Ca(NO<sub>3</sub>)<sub>2</sub>, and 1% sucrose, with pH=6.0, for NO<sub>3</sub><sup>-</sup>. These inorganic N concentrations in background solutions were employed at about 1/3 of the highest level adapted by Miller and Hawkins (2007) to supply a moderate N condition. After backfilling, electrode tips were filled

with  $\text{NH}_4^+$  selective liquid ion-exchange cocktails (#09879, Sigma); for  $\text{NO}_3^-$  electrode, the 10 mM  $\text{KNO}_3$  solution was employed to backfill, followed by a  $\text{NO}_3^-$  selective liquid ion-exchange cocktail (#7254, Sigma). The electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), whose tips were put together and positioned 20  $\mu\text{m}$  above the root surface.

Before the commencement of N flux measurement, all sampled cuttings were soaked in distilled water for root N starvation. Fine roots of 2–3 cm in length and 1–2 mm in diameter were randomly chosen, cut from the coarser root, and prepared for MIFE measures. These fine roots were cut off because it was quite difficult for measures if they were attached to the tap root, which is large and tough. Nevertheless, the excision was unlikely to affect the flux measurement in the elongation region since the MIFE recordings were performed at a root length of 200–300  $\mu\text{m}$  from the apex. Four to five fine roots were selected and three from them were randomly chosen and equilibrated in measuring solution for 30 min prior to measurement. Gradients of ions for both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  near to the root surface were measured by moving the ion-selective microelectrode between two positions between 10 to 35  $\mu\text{m}$  above root surface at the programmable frequency in the range of 0.3 to 0.5 Hz, so as to monitor the electrode voltages at the two positions, which were then digitized and recorded in a disk file for 25 min. This time course was confirmed because it may be long enough to present significant response of protons and ions (Shabala *et al.*, 2005). Recorded data were subsequently transferred into flux rates. The recording rate for the ion flux was one reading per 1 min. To investigate the net  $\text{NH}_4^+$  or  $\text{NO}_3^-$  fluxes the white fine roots were immersed in a petri dish containing 10–20 mL measuring solution and equilibrated for 30 min. Data were treated and analyzed using MageFlux® (<http://www.youngerusa.com/mageflux>). Fick's first law of diffusion was used to calculate ion fluxes:

$$J_x = K u_x C_x (V_2 - V_1)/r \ln (R_2/R_1)$$

Where,  $J_x$  is the net flux of ion  $x$ ,  $K$  is a unit conversion coefficient to express  $J_x$  in units of  $\text{nmol m}^{-2} \text{s}^{-1}$ ,  $u_x$  is the ionic mobility for ion  $x$  ( $\text{m mol N}^{-1} \text{s}^{-1}$ ),  $V_1$  and  $V_2$  are the electric potentials of the ion selective microelectrode at positions 1 and 2 (mV),  $r$  is the radius of the root ( $\mu\text{m}$ ). The ionic mobilities used were ( $\text{m mol N}^{-1} \text{s}^{-1}$ ):  $\text{NH}_4^+$ ,  $7.88 \times 10^{-13}$ ;  $\text{NO}_3^-$ ,  $7.66 \times 10^{-13}$ .

### Statistical Analysis

All data were examined for normality and variance homogeneity and no transformation was necessary. Parameters of shoot height, collar diameter, biomass, and nutrient status were averaged using the four sampled seedlings as one replicate observation; while those of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes were averaged using the two sampled seedlings. Results of pretreated cuttings were analyzed

following a full factorial design ( $2 \times 2$ ). A two-way ANOVA was conducted to test the main and interactive effects of poplar genotypes and pretreatment of nutrient addition on cutting growth and nutrient status. Another two-way ANOVA was conducted on  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes independently. When ANOVA indicated significant results ( $P < 0.05$ ) for any of effects, values were compared and ranged according to LSD test at  $\alpha = 0.05$  level. Statistical Package for the Social Science (SPSS) was used for data analysis. Vector diagnosis was employed to detect foliar nutrient status of two poplar genotypes in response to pre-fertilization treatment (Salifu and Timmer, 2003).

## Results

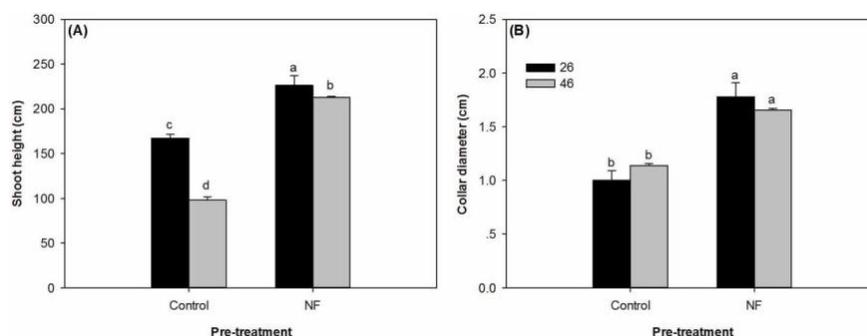
### Cutting Growth and Biomass Accumulation

Factors of poplar clones and pretreatment of nutrient fertilization had an interactive effect on shoot height and collar diameter (Table 1). Pretreatment of nutrient fertilization increased both shoot height and collar diameter relative to the control for either clone (Fig. 1). Relative to the control, the NF treatment increased shoot height by 35% and 117% for the 26 and 46 clones, respectively. Meanwhile, the NF treatment increased collar diameter by 45–78% compared to the control.

Factors of poplar clones and pretreatment of nutrient fertilization had an interactive effect on biomass in leaves, stem, and root (Table 1). Biomass in the 26 clone increased by 21%, 23%, and 49% compared to that in the 46 clone in leaves, stem, and root, respectively in the pretreatment of fertilization treatment (Fig. 2A). In the pre-fertilization treatment, the 26 clone allocated more biomass to stem and roots, while the 46 clone tended to allocate more mass to leaves. However, significant difference of biomass accumulation between poplar clones disappeared in the control. Also, no significant difference existed for root to shoot biomass ratio (R/S) between the two poplar clones in the control (Fig. 2B). In contrast, in the pretreatment of nutrient fertilization R/S in the 26 clone was lower by 45% than the 46 clone, while both of which were lower than those in the control.

### Nutrient Concentration and Content

Factors of poplar clones and pretreatment of nutrient fertilization had an interactive effect of N and K concentrations in leaves and stem (Table 1). N concentration was higher in both leaves and stem of the 46 clone by 20% and 67% in the pretreatment of nutrient fertilization than the control, respectively, but results of N concentration in stem of the 26 clone failed to show significant difference between the two pretreatments (Fig. 3B). Pretreatment of nutrient fertilization increased K concentration in both leaves and stem for both poplar clones relative to the control, except for the 26 clone response in leaves (Fig. 3C). N concentration



**Fig. 1:** Shoot height (A) and collar diameter (B) of two poplar clones of 26 and 46 in response to the control and pre-treatment of nutrient fertilization (NF) on late September 2008. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*Populus tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. Different letters indicate significant difference according to LSD test at the 0.05 level ( $n=4$ )

**Table 1:** *F* values from ANOVA analysis of effects of poplar clone (C), pre-fertilization (F) and their interaction (C × F) on parameters of morphology, biomass, nutrient content, nutrient concentration, and root N flux

Parameters and organs	Source of variation		
	C	F	C × F
— Morphology —			
Height	123.89***	547.43***	56.29***
RCD	0.03	178.55***	7.22*
— Biomass —			
Leaves	2.5	175.49***	60.56***
Stem	4.56	693.51***	22.23**
Root	18.58**	348.97***	56.97***
— N concentration —			
Leaves	11.62**	30.18***	15.56**
Stem	7.44*	32.67***	5.51*
Root	4.4	13.61**	2.48
— P concentration —			
Leaves	0.71	11.61**	0.03
Stem	9.03*	3.54	1.69
Root	15.61**	72.71***	2.03
— K concentration —			
Leaves	41.86***	9.03*	18.29**
Stem	0.35	22.22**	7.89*
Root	0.75	12.77**	1.11
— N content —			
Leaves	14.72**	359.29***	133.95***
Stem	0.73	125.06***	40.69***
Root	2.95	107.80***	16.05**
— P content —			
Leaves	0.58	125.06***	88.00***
Stem	0.39	105.16***	33.33***
Root	1.53	268.52***	14.35**
— K content —			
Leaves	0.15	152.71***	44.31***
Stem	4.59	820.36***	21.37**
Root	6.86*	208.86***	21.26**
— N flux —			
NH <sub>4</sub> <sup>+</sup>	21.26***	48.91***	74.90***
NO <sub>3</sub> <sup>-</sup>	0.22	3.58	0.01

Values of bold characters indicate a significant effect. Asterisks indicate significance: \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$

showed a generally higher level in the 46 clone compared to the 26 one in both leaves and stem, but K concentration was higher in the 26 clone in leaves than the 46 one and no

significant difference existed between the two poplar clones in stem (Fig. 3D).

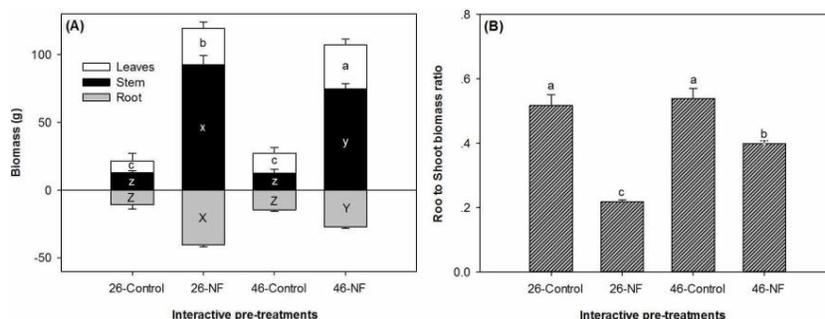
Both pre-fertilization and the clone type had a significant main effect on nutrient concentrations (Table 1). Relative to the control, pre-fertilization resulted in root nutrient concentration increase by 21, 33 and 13% for elements of N, P and K, respectively (Table 2). Also, pre-fertilization increased foliar P concentration by 20% compared to the control. The 46 clone had higher P concentration by 17% in both stem and root compared to the 26 clone.

Pre-fertilization and clone type had an interactive effect on nutrient content for all three elements (Table 1). In general, nutrient contents in pre-fertilized clones were greater than those in controlled clones for all organs no matter what clone type it was (Fig. 4). No significant difference can be found between the two clones in control.

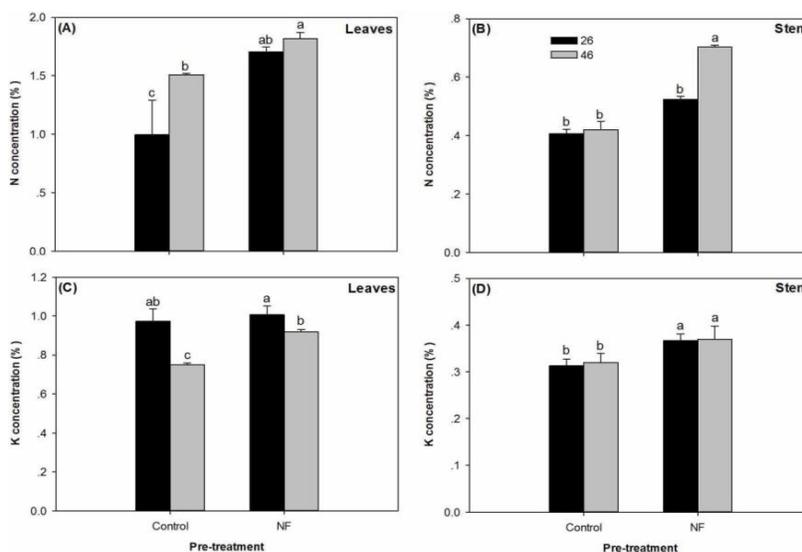
For example, foliar N content was increased in the NF treatment by 100–444% compared to the control in both clones (Fig. 4A). However, nutrient content in some organs of the clone in the NF treatment was different between the two clones. In clones receiving pre-fertilization, foliar N and P contents were greater in the 46 clone than those in the 26 clone by 30% and 20%, respectively. Root nutrient content in the NF treatment was less in the 46 clone than that in the 26 clone by 31, 33 and 27% for N, P and K, respectively. In addition, stem K in the NF treatment was less in the 46 clone than that in the 26 clone by 19% (Fig. 4C).

### Nutritional Status Diagnosed by Vector Analysis

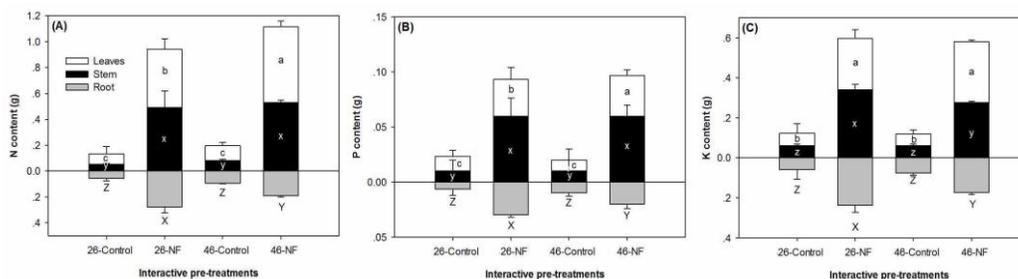
Two poplar clones had similar responses of nutrient statuses to pre-fertilization treatment diagnosed by vector analysis (Fig. 5). Relative to the control, all parameters of biomass, N content, and N concentration increased in the pre-fertilization treatment for both poplar clones, which were diagnosed to be a nutrient deficiency in the control caused by N limitation (Fig. 5A). Therefore, pre-fertilization countered this N limit and relieved the N deficiency.



**Fig. 2:** Interactive effects of poplar clones (26 and 46) and pre-treatment of nutrient fertilization (NF) on biomass accumulation in leaves, stem, and root (A) and root to shoot biomass ratio (B) on late September 2008. Controlled cuttings received no fertilizer in the pre-treatment. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. Different letters indicate significant difference according to LSD test at the 0.05 level ( $n=4$ ). Letters of a, b, and c for leaves; letters of x, y, and z for stem; letters of X, Y, and Z for root



**Fig. 3:** N (top) and K concentrations (bottom) in leaves (left) and stem (right) of two poplar clones of 26 and 46 in response to the control and the pre-treatment of nutrient fertilization (NF) on late September 2008. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. Different letters indicate significant difference according to LSD test at the 0.05 level ( $n=4$ )

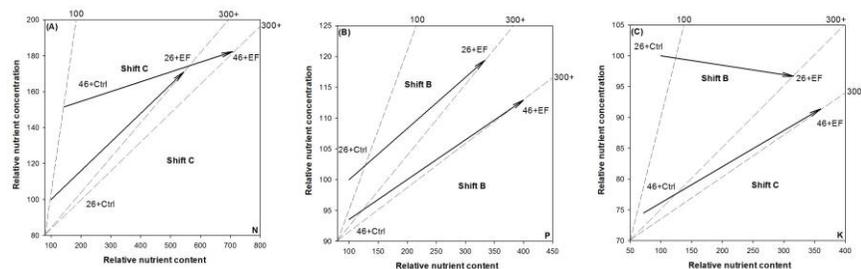


**Fig. 4:** Interactive effects of poplar clones and pre-treatment of nutrient fertilization (NF) on N (A), P (B), and K (C) contents in leaves, stem, and root. Controlled cuttings received no fertilizer in the pre-treatment on late September 2008. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. Different letters indicate significant difference according to LSD test at the 0.05 level ( $n=4$ ). Letters of a, b, and c for leaves; letters of x, y, and z for stem; letters of X, Y and Z for root

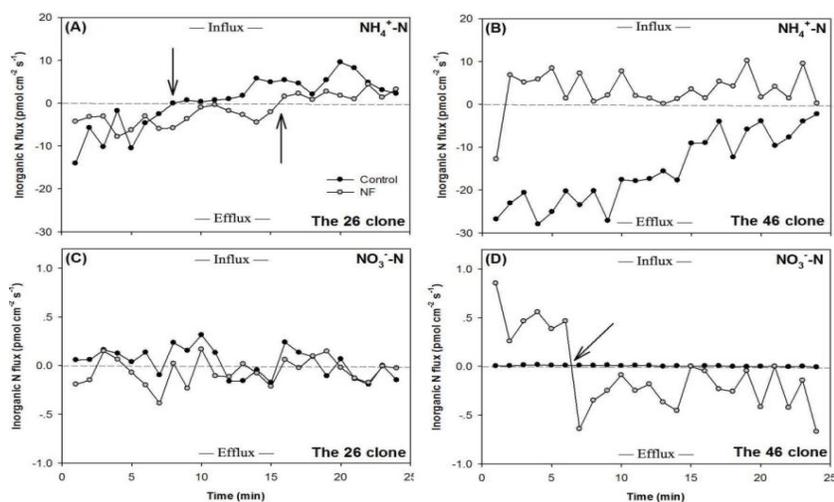
**Table 2:** Nutrient (N, P, and K) concentration in poplar organs of leaves, stem, and root in response to main effects of pre-fertilization and clone type

Parameters and organs	Main effects	
	Control	—pre-fertilization effect— Fertilization
Root N concentration (mg g <sup>-1</sup> )	0.57±0.06b	0.69±0.06a
Root P concentration (mg g <sup>-1</sup> )	0.06±0.01b	0.08±0.01a
Root K concentration (mg g <sup>-1</sup> )	0.54±0.01b	0.61±0.05a
Foliar P concentration (mg g <sup>-1</sup> )	0.10±0.01b	0.12±0.01a
	— Clone type effect —	
Stem P concentration (mg g <sup>-1</sup> )	The 26 clone 0.06±0.01b	The 46 clone 0.07±0.01a
Root P concentration (mg g <sup>-1</sup> )	0.06±0.01b	0.07±0.01a

Different letters in a row indicate significant difference at the 0.05 level



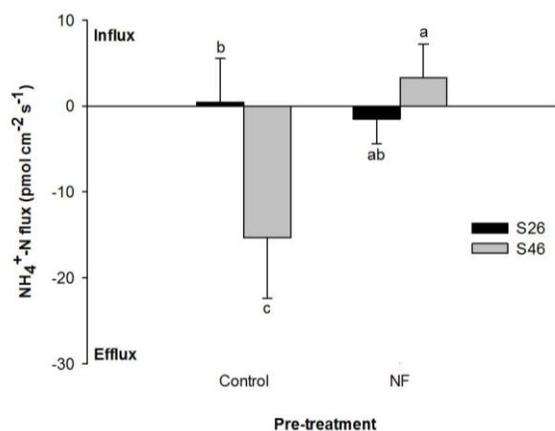
**Fig. 5:** Vector diagnosis of foliar nutritional statuses of the two poplar clones of (#26 and #46) in response to pre-fertilization treatment and the control on late September 2008. Interpretations for vector shifts as the diagnosis results were adapted from Salifu and Timmer (2003), wherein shift B and C indicate nutritional statuses of steady-state nutrient loading and limiting nutrient deficiency, respectively. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively



**Fig. 6:** Flux dynamics of NH<sub>4</sub><sup>+</sup> (top) and NO<sub>3</sub><sup>-</sup> (bottom) in the first 25 min of MIFE<sup>®</sup> measurement for roots of the poplar clones of 26 (left) and 46 (right) in response to the control and the pre-treatment of nutrient fertilization (NF) on early October 2008. Clones were obtained from hybrids generated from the hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively (n=3)

Due to the insignificant changes of P concentration in the pre-fertilization treatment relative to the control, the increase of foliar biomass resulted in the increase of foliar P content (Fig. 5B). Therefore, P statuses for both poplar clones were characterized to be sufficient in the control and pre-fertilization induced a steady-state loading of the P

element in leaves. Foliar K status was different for the two poplar clones (Fig. 5C). In the 26 clone, foliar K content was increased with the increase of foliar biomass in the pre-fertilization treatment relative to the control, but foliar K concentration did not change. Hence, foliar K status was suggested to be a steady-state one with K loading in leaves.



**Fig. 7:** Flux rate of  $\text{NH}_4^+$  in roots of two poplar clones of 26 and 46 in response to the control and pre-treatment of nutrient fertilization (NF) on early October 2008. Clones were obtained from hybrids generated from hybridization using Maoxin poplar (*P. tomentosa* × *P. bolleana*) and Yinxian poplar (*P. alba* × *P. glandulosa*) as male and female parents, respectively. Different letters indicate significant difference according to LSD test at the 0.05 level. Data are obtained from the same set of Fig. 6 ( $n=24$ )

In the 46 clone, parameters of foliar K content and concentration and biomass all increased in the pre-fertilization treatment, which was suggested to be the symptom of nutrient deficiency in the control.

### Dynamics of $\text{NH}_4^+$ and $\text{NO}_3^-$ Fluxes

$\text{NH}_4^+$  flux showed an increased trend with time in the first 25 min of MIFE<sup>®</sup> measurement for 26 clone in both control and the pre-fertilization treatment (Fig. 6A). However, pre-fertilization “delayed” the inversion of  $\text{NH}_4^+$  from efflux to influx by about 10 min (as arrows indicated in Fig. 6A). For the 46 clone, controlled cuttings showed a decline of  $\text{NH}_4^+$  efflux with time, while pre-fertilization altered  $\text{NH}_4^+$  flux from efflux to influx since the 2 min on (Fig. 6B).

No clear dynamic of  $\text{NO}_3^-$  flux could be captured for the 26 clone in both the control and the pre-fertilization treatment (Fig. 6C).  $\text{NO}_3^-$  flux showed alternative influx and efflux patterns for both the control and the pre-fertilization treatment throughout the first 25 min. However, controlled cuttings of the 46 clone had a near-balance  $\text{NO}_3^-$  flux dynamic with time between influx and efflux (Fig. 6D). Nevertheless, in the pre-fertilization treatment the  $\text{NO}_3^-$  flux was converted from influx to efflux at about 6 min.

Factors of poplar clones and pretreatment of nutrient fertilization had an interactive effect on  $\text{NH}_4^+$  flux (Table 1).  $\text{NH}_4^+$  flux in controlled cuttings of the 46 clone was negative and lower than those of the 26 clone, both of which were lower than  $\text{NH}_4^+$  flux in cuttings of the 46 clone receiving pre-fertilization treatment (Fig. 7).

### Discussion

The clone types used in our study are widely used in poplar afforestation at Northern China, but rarely compared for growing rate among other poplar clones. However, height of the 26 and 46 clones was about 9-fold higher than Trembling aspen (*P. tremuloides*) seedlings (Schott *et al.*, 2013) and 40% higher than poplar clones in France (Ciadamidaro *et al.*, 2017), but both height and diameter were lower by 35–40% compared to the 74/76 (*P. × euramericana* cv. ‘74/76’) clones (Wang *et al.*, 2016). These results demonstrated that the poplar clones employed in our study have moderate growing rate as grafted cuttings. Continuously height growth until late-season is a characteristic for poplar, which is tended to be stimulated by pre-fertilization and suggested to be controlled under necessary condition (Schott *et al.*, 2013).

Whole-plant biomass ranged from 30 g cutting<sup>-1</sup> to 160 g cutting<sup>-1</sup>, which was much greater than cuttings of three *P. trichocarpa* × *P. deltoies* clones (49-177, DTAC-7, and 15-29) (Zabek and Prescott, 2007), but was comparable with cuttings of Zhonglin 46 (*P. euramericana* [Dode] Guinier CL.), 107 (*P. euramericana* [Dode] Guinier CV. ‘Neva’) (Yan *et al.*, 2013) and 74/76 (Wang *et al.*, 2016). The 26 clone had greater stem biomass in response to pre-fertilization, which indicated that this species can be more efficient in bioenergy production under the condition of pre-autumn management. Results about root to shoot biomass ratio (Fig. 2B) revealed that pre-fertilization resulted in more biomass allocated to the shoot part. This is a good because both clones in this study are practically used in afforestation at Northern China due to their high utilization of nutrients for stem biomass accumulation. Also, sensitive response of biomass allocation to shoot part to nutrient addition was also found in trembling aspen seedlings (Schott *et al.*, 2013).

Because nutrient content was calculated by biomass timing nutrient concentration, most results of nutrient contents in our study mainly followed the ones of biomass and partly followed the ones of nutrient concentration. For instance, in the control, although foliar N concentration was different between the two clones, their foliar biomass was not different, neither were their foliar N contents as well. Foliar P concentration did not show any responses, foliar P content was greatest in the 46 clone receiving pre-fertilization due to the greatest biomass accumulation. At the whole-plant scale, pre-fertilization resulted in greater nutrient content no matter what clone type it was. This suggested that both clones in this study were nutrient-sensitive but their responses in shoot growth and biomass accumulation varied.

Foliar nutrient concentration could be taken as a parameter to evaluate the ability of nutrient uptake during tree stock growing season (Salifu and Timmer, 2003). The 26 clone had lower N concentration in both leaves and stem in the control and in the pre-fertilization treatments, respectively (Fig. 3B). Hence, decline of foliar N concentration indicated the stem N dilution as well, and both resulted from faster biomass accumulation in the 26 clone.

Also, the 46 clone may harbor a better ability of nutrient uptake to leaves and accumulation in stem than the 26 one. The dilution effect of N accumulation by fast biomass accumulation was also found on trembling aspen (Schott *et al.*, 2013). In contrast, the 26 clone had higher foliar K concentration than the 46 one in the pre-fertilization treatment. Contrasting concentrations of N and K have been reported for several times (e.g., Wei *et al.*, 2014), which can be interpreted by the influence of  $\text{NH}_4^+$  on  $\text{K}^+$  uptake. In addition, at fall time, this may also be the result of  $\text{K}^+$  translocation, although this surmise cannot be verified by our study because we only determined K concentration for one time. Also, we did not employ the isotope technique for the analysis of K composition, which can be available to determine nutrient translocation with one-time data.

Vector diagnosis clearly indicated the N deficient status in the control for both clones and the K deficient status in the control for the 46 clone, which were all countered by the pre-fertilization. This demonstrated that the three-time fertilization were efficient to counter nutrient efficiency in poplar clone cuttings. The fertilizer regime of three-time applications has also been employed for the culture of other juvenile fast-growing tree stocks (Schott *et al.*, 2013; Wei *et al.*, 2013; Wang *et al.*, 2016). In other studies on fertilization to fast-growing poplar cuttings, fertilizers were delivered at the rate of 8–10 g N plant<sup>-1</sup> (Yan *et al.*, 2013; Wang *et al.*, 2016), but other evidence reported that juvenile poplar stocks can be cultured with 1–7 g N plant<sup>-1</sup> (Zabek and Prescott, 2007; Schott *et al.*, 2013). Therefore, the N dose in our study (5 g plant<sup>-1</sup>) equaled to the medium value among relevant studies. Therefore, pre-fertilization had changed nutrient status for both clones. This is an important premise to determine the following N fluxes in clones at fall with contrasting initial nutrient statuses.

The *Populus* spp. contains many species adapting to riparian ecosystems, where  $\text{NH}_4^+$  may become rapidly oxidized to  $\text{NO}_3^-$ , and both ion fluxes can be absorbed by poplar roots efficiently (Luo *et al.*, 2013a). In roots of the 26 clone, pre-fertilization lengthened the time of  $\text{NH}_4^+$  efflux for about 10 min relative to the control without significant impact on  $\text{NO}_3^-$  flux. In roots of the 46 clone, pre-fertilization resulted in a general  $\text{NH}_4^+$  influx throughout the first 25 min but altered  $\text{NO}_3^-$  efflux to influx since the first 6 min on, while roots of controlled cuttings all had  $\text{NH}_4^+$  efflux without any influx (Fig. 6). These results demonstrated that pre-fertilization had a clone-type-specific effect on root N flux in poplar cuttings at fall, which concur with Kalcsits and Guy (2016). For the 46 clone, pre-fertilization stimulated a selective uptake of  $\text{NH}_4^+$  with exudates of  $\text{NO}_3^-$ . According to Luo *et al.* (2013a) the selective uptake of N ions was related to the movement of proton, which may have been exhausted from the root cell and promoted the efflux of  $\text{NO}_3^-$ . In contrast, pre-fertilization can promote nitrate uptake by *Suaeda salsa* (Bai *et al.*, 2017). In addition, data of  $\text{NH}_4^+$  flux in the control indicated that the two clones had different natural efflux course times, which was about 8 min for the 26

clone and at least 25 min for the 46 clone. Hence, the 26 clone can have a better nature of utilization of  $\text{NH}_4^+$  than the 46 one. The near-balance dynamic of  $\text{NO}_3^-$  flux in cuttings of the 46 clone in the control indicated that this clone did not manage the flux  $\text{NO}_3^-$  in the first 25 min. Luo *et al.* (2013b) reported the similar near-balance of proton in fine roots of poplar.

Hawkins *et al.* (2008) ever reported that both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  uptakes were higher in roots in absence of N addition to Douglas-fir and lodgepole pine seedlings. In contrast, in our study pre-fertilization promoted N influx by converting N efflux in the 46 clone (Fig. 7). However, throughout the first 25 min, we failed to get the equilibration status of N flux. Thus the excision process may cause stresses impacting the equilibration. Also, our study term may be not long enough to obtain the equilibration status for the two poplar clones. Some other studies revealed that the equilibration status occurred at the stationary phase of  $\text{H}^+$  which did not appear until the first 60 min after the commencement (e.g., Shabala *et al.*, 2001; Ruan *et al.*, 2016). Due to this issue, the effect of pre-fertilization on N flux can be more correct if the equilibration occurred.

Relative to the 46 clone, the 26 one showed faster-growing rate and earlier demand for  $\text{NH}_4^+$  influx in the first 25 min. These results together revealed that the 26 clone had a better ability to obtain N for growth at fall. However, N flux in its roots was stationary to some extent and seemed hard to be adjusted by pre-fertilization. In contrast, the 46 clone was more sensitive to pre-fertilization and more efficiently to regulate the selective uptake of  $\text{NH}_4^+$  compared to the 26 clone. If fall fertilization is necessary for the culture of poplar stock, the 46 clone can be a better candidate than the 26 one and  $\text{NH}_4^+$  can be more effective than  $\text{NO}_3^-$ .

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