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Research paper

Poplar leaf rust reduces dry mass accumulation and internal nitrogen recycling more markedly under low soil nitrogen availability, and decreases growth in the following spring

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Rust is one of the most important biotic stress factors that affect poplars. The aims of this work were: (i) to analyze the changes in growth and nitrogen (N) accumulation in *Populus deltoides* W. Bartram ex Marshall plants infected with rust (*Melampsora medusae* Thümen.) and to determine how internal N stores are affected by the disease, in plants growing under two N availabilities in the soil; and (ii) to evaluate the impact of rust in the early sprout in the following growing season and the cumulative effect of the disease after repeated infections. Two clones with different susceptibility to rust were analyzed. At leaf level, rust reduced gas exchange capacity, water conductance in liquid phase and photosynthetic rate in both clones. At plant level, rust reduced plant growth, accelerated leaf senescence and abscission occurred with a higher concentration of leaf N. Even though N concentration in stems and roots were not significantly reduced by rust, total N accumulation in perennial tissues was reduced in infected plants. The vigor of the early sprout of plants infected by rust in the previous season was lower than that of non-infected plants. Therefore, rust affects plant growth by reducing the photosynthetic capacity and leaf area duration, and by decreasing internal nutrient recycling. As nutrient reserves in perennial tissues are lower, rust infection reduces not only the growth of the current season, but also has a cumulative effect on the following years. The reduction of growth was similar in both clones. High availability of N in the soil had no effect on leaf physiology but increased plant growth, delayed leaf senescence and abscission, and increased total N accumulation. If fertilization increases plant growth (stem and root dry mass) it can mitigate the negative effect of the pathogen in the reduction of nutrient storages and future growth.

Keywords: fertilization, leaf fungus disease, *Melampsora medusae* (rust), plant–fungi interaction, poplar clones, *Populus deltoides* (poplar).

Introduction

Plant growth is adversely affected by different types of stress factors, biotic and abiotic in origin. Negative biotic interactions (e.g., intra- and interspecific competition, pests and pathogens) can cause large yield losses in economically important species, and therefore, efforts are directed at understanding these interactions to mitigate their negative effects. The deleterious effects of pathogen infections can be linked to pathogen toxins causing cell death (Navarre and Wolpert 1999), competition for nutrients (Horst et al. 2010), decreased plant leaf area and reduced photosynthesis (Serrago et al. 2009, Carretero et al. 2011), among others. The physiological processes underlying the damage caused by diseases can differ between biotrophic and necrotrophic pathogens.

Apart from physiological processes related to carbon balance and growth, internal nutrient recycling can be affected by biotrophic

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pathogens (e.g., rust). Nitrogen (N) is quantitatively the most important mineral nutrient for plants, and, therefore, the most studied by physiological and molecular approaches (Coleman et al. 2004, Cooke et al. 2005, Rennenberg et al. 2010). Several aspects of N metabolism are affected by biotrophic infections (Fagard et al. 2014), and, reciprocally, N nutrition can also impact on the development of the disease, both at the organ and population level (Neumann et al. 2004).

In many perennial species, the internal redistribution of N can be divided into four phases: 1—primary uptake of N by roots and assimilation in sink organs (new roots, leaves and stems) during the growing season; 2—reallocation of N with metabolites from senescent organs to new organs during the growing season; 3—redistribution of N during autumn senescence from senescing leaves to be stored in stems and roots during winter; and 4—remobilization of N from stems and roots to new roots, leaves and twigs during spring flush (Cooke and Weih 2005). In infected plants, senescence (and abscission) of leaves could occur with higher concentrations of nutrients that are retained by the fungal biomass as observed in wheat (van den Berg et al. 2007), thereby decreasing the redistribution and re-translocation of nutrients to growing or reserve organs (Silla and Escudero 2004). These losses of nutrients will constrain growth at the beginning of the next spring, since nutrients accumulated in woody structures support growth at sprouting (Cooke and Weih 2005).

Poplars cover around 80 million hectares of the Earth's surface, of which 7 million are plantations (Ball et al. 2005). Poplar plantations are widespread because it is very easy to establish a new plantation from un-rooted cuttings, poplars have high growth rates (Ault et al. 2016) and its wood has many uses (Ball et al. 2005). However, plantation productivity is limited by several biotic and abiotic stresses. Rust is one of the most important biotic stress factors that affect poplars around the world (Tabor et al. 2000). This disease causes important yield losses. Early defoliation caused by rust reduces leaf area; therefore, light interception and photosynthetic capacity of the canopy are reduced. Poplars affected by rust present a 50% reduction in aboveground biomass (May De Mio and Ruaro 2008). The growth reduction is closely related to disease severity (May De Mio et al. 2006). In addition, the extent of damage strongly depends on the susceptibility of the genetic material. Yield losses range from nearly zero in tolerant clones to around 60% in susceptible ones (Widin and Schipper 1981).

On the other hand, N fertilization acts contrary to rust. High soil levels of N increase growth and accumulation of N in poplar stems (Lasa et al. 2016). Poplars fertilized with N grew 40% more during the growing season than non-fertilized ones (Coleman et al. 2004). In fertilized plants, there is an increase in the number of leaves per plant, senescence is delayed (Cooke et al. 2005) and higher accumulation of N in reserve tissues is observed (Zak et al. 2000, Coleman et al. 2004, Cooke and Weih 2005). Therefore, the increase in N availability could compensate for the negative effect on growth of the presumptive retention of nutrients in rust-infected plants. However, this increase in availability of N could also enhance fungal growth due to the increased availability of nutrients. In this way, fertilization could increase the incidence of the disease (Jensen and Munk 1997, Robert et al. 2002) and could affect negatively plant growth. However, plants under higher nutrient availability, presumably with higher photosynthetic activity, might have more carbon skeletons to activate defense mechanisms, thus limiting fungal growth. In summary, it is not possible to determine the consequences of multiple factors that occur at the same time only by adding up their presumably separate effects (Bansal et al. 2013). As a result, it is difficult to predict the results of the interaction between fertilization and rust, i.e., will plants with high N availability be more or less affected by rust than plants with low N availability? Also, has the presumed retention of N in abscising rust-infected leaves any relevant impact in N stores and future plant growth?

The aims of this work were: (i) to analyze the changes in growth and N accumulation in *Populus deltoides* W. Bartram ex Marshall plants infected with rust (*Melampsora medusae* Thümen.) and to determine how internal N stores are affected by the disease, in plants growing under two N availabilities in the soil; and (ii) to evaluate the impact of rust in the early sprout in the following growing season and the cumulative effect of the disease after repeated infections.

Materials and methods

Plant material

Two rust-susceptible *Populus deltoides* W. Bartram ex Marshall clones were used. Clone Onda (hereinafter abbreviated O) has few branches, big leaves and is very susceptible to rust. The other clone used is 'Australiano 106/60' (hereinafter abbreviated A), which has many branches, smaller leaves and it is less susceptible to rust than clone O. Rust affects leaf physiology of both clones by similar mechanisms but the magnitude of the damage is directly correlated with the level of infection: the extent of the disease at leaf level (number of pustules and fungi mass) is higher in clone O than in clone A (Gortari et al. 2018). Along a growing season, the total leaf area produced by each clone was 7.5 m^2 in clone A and 7.1 m^2 in clone O, with average individual size of leaves of 176 cm^2 in clone A and 296 cm^2 in clone O. Both clones have similar dates of flushing and autumnal senescence, produce new leaves until the end of March and lose 5% of leaves before autumnal senescence if they are healthy. However, in both clones the drop of leaves is around 25% until the end of March if plants are infected by rust (Cortizo 2014).

Experiment 1: effects of rust during the growing season

Experimental setup In the first week of August 2012 (winter) a pot experiment was installed at INFIVE facilities (34°54′

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45.64′S; 57°55′51.01′W). Forty-liter pots were filled with a mix of loamy soil:sand (1:10 v/v). Forty-cm length un-rooted cuttings of clones A and O were planted, one cutting per pot. Half of the plants were sprayed with a systemic fungicide (0.258 g tebuconazole l^{-1}) every 15 days, since early sprouting to autumnal abscission (R–). The other half was naturally infected by rust (R+). At the same time, half of R– and R+ plants were fertilized with two additions of N, as 2.5 g of urea (46-0-0) per pot each, on 26 October and 21 December (N+) totaling 5 g per pot, while the other half of the plants were not fertilized (N–). Therefore, eight treatments were applied: clone O without rust, without N fertilization (O R– N–); clone O without rust, with N fertilization (O R– N+); clone O with rust, without N fertilization (O R+ N–); clone O with rust, with N fertilization (O R+ N+); clone A without rust, without N fertilization (A R– N–); clone A without rust, with N fertilization (A R– N+); clone A with rust, without N fertilization (A R+ N–); and clone A with rust, with N fertilization (A R+ N+). Five randomly distributed plants per treatment were used. Pots were watered every other day to ensure adequate water availability.

Development of the disease and leaf physiology during summer The first day rust symptoms were observed and were registered. Disease evolution was registered every 7 days, through visual inspection of all plants. Incidence (number of leaves with rust symptoms with respect to the total number of leaves of each plant) and severity (percentage of the leaf surface affected by pustules) was estimated with a visual scale during disease development by analyzing all the leaves of each plant.

Leaf physiological traits were measured to describe the effects of rust in interaction with N availability on leaf assimilatory capacity. Measurements were made on one leaf per plant, in all plants. In the R+ treatments the fully expanded mature leaf with the highest rust severity in each plant was measured, i.e., leaf 5–10 starting from the apex. In R– treatment, leaves in an equivalent position were measured. Measurements were done on three sunny days, at midday in February when the incidence of rust was 100% and the severity was the highest for each treatment, and plants were still in active growth. All plants were measured each day. Light-saturated net photosynthetic rate (A_{sat}) was measured with an Infra Red Gas Analyzer (IRGA, CIRAS 2, PP Systems, Amesbury, MA, USA). The IRGA leaf chamber was set at 1500 μm photons m^{-2} s^{-1}, 25 °C and 360 ppm CO_2. After measuring A_{sat} light was turned off, the leaf was left to acclimate to dark during 5 min and respiration (Resp) was recorded when the rate of CO_2 evolution was stable at least for 5 min. As A_{sat} measurement is affected by the CO_2 released by fungal respiration, direct effects of rust infection on photosynthesis were probed by examining photosystem 2 electron transport rate (ETR) with the modulated chlorophyll fluorescence method (FMS2, Hansatech, Norfolk, UK), under natural irradiance conditions, in the same portion of leaf in which A_{sat} was measured. Stomatal conductance (g_s) was measured with a porometer (Decagon SC1, Pullman, WA, USA) on sunny days between 10:00 and 11:00 h, in the adaxial side of the leaves, because both leaf sides had the same g_s.

After these measurements, a leaf disc was cut and stored at −80 °C to determine glucosamine concentration (see methodology below). Glucosamine is a component of fungi cell walls and it is not present in healthy plant tissues, so it can be used to estimate fungi biomass (Wallander et al. 2013). Determination of glucosamine concentration was made in leaf discs (35 mm diameter) with a spectophotometric method (Ride 1972). A calibration curve was made with D-glucosamine (Sigma-Aldrich, MO, USA).

In another set of leaves, hydraulic conductance (K_{leaf}) was measured with the pressure drop method (Melcher et al. 2012, Graciano et al. 2016). During measurement, leaves were illuminated to stimulate stomatal opening. After flux measurement, the leaf was enclosed in a plastic bag in darkness, and water potential was measured with a pressure chamber after 5 min of stabilization.

Autumnal leaf senescence and dry mass accumulation at the end of the growing season From 15 April until complete autumnal senescence, the number of leaves per plant was registered weekly. After complete autumnal leaf fall, in June 2013, total height and stem collar diameter (CD) were measured in all plants. Aerial parts were cut, and roots were washed of soil with tap water. After that, stems, fine roots (<5 mm in diameter) and coarse roots (≥5 mm in diameter) were separated. Small samples of each compartment from each plant were taken and stored at −80 °C to determine reserve protein contents (details are below). The material was dried in an oven (60 °C) until constant weight. Dry mass by compartment and total plant dry mass were determined.

Determinations in plant tissues in autumn Nitrogen concentration was analyzed in fully expanded leaves of each treatment, after natural autumnal yellowing and senescence. As leaves fell down, they were collected: a leaf disc (diameter: 35 mm) from each plant was stored at −80 °C to determine glucosamine concentration as an estimate of fungal biomass as described above. To determine N concentration, another sample was dried (60 °C) to constant weight. For each plant, a composite sample was taken, with at least five leaves; one of those was used to extract the disc to determine glucosamine. Dry samples of stems, fine and coarse roots were also analyzed. Total N was determined with a semi-micro Kjeldahl method (determinations were made by LANAIS N-15, CONICET-UNS Agronomía, 8000 Bahía Blanca, Argentina). As dry mass for each compartment was known, the N content for each compartment was calculated as the product of N concentration and dry mass of each compartment. Total N content was the sum of the N contents of roots and stems, as poplars are deciduous.

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Determination of soluble protein concentration in stems and roots was done by an adaptation of the Lowry colorimetric method. Stem was manually separated into xylem (excluding the pith) and phloem (bark), and both parts were analyzed separately. Each sample (30 mg dry mass) was ground and homogenized with liquid N_2 and proteins were extracted following the Lowry method (Lowry et al. 1951). A calibration curve was prepared with bovine serum albumin (BSA) as standard.

Experiment 2: effect of rust in the following growing seasons

To evaluate the effect of rust infection on the growth during the following season, a field experiment was installed in a cutting nursery, with 0.80 × 0.80 m distance between plants. The experiment was installed in INTA EEA Delta del Paraná facilities (34°10′31″S, 58°51′43″W). Six plots of each clone (Onda and 'Australiano 106/60') were installed, with 4 × 10 plants per plot. Three plots of each clone were sprayed every 15 days with a systemic fungicide (0.258 g tebuconazole l^{-1}) throughout the growing season (R–). The other three plots were naturally infected by rust (R+). Therefore, the design was random complete blocks, with three replicates. Treatments were: A R–, A R+, O R– and O R+. The first symptom of rust appeared on 5 January. The maximum number of pustules registered during the summer was 19 pustules cm^{-2} in clone A R+, and 38 pustules cm^{-2} in clone O R+. In O R– and A R– no pustules were observed. After the autumn, all the stems were harvested and only the stump with the roots remained during the winter. The following spring, in December, previously to the next rust appearance, stems of two plants per plot were sampled, the number of leaves was counted and leaves and stems were dried at 60 °C to constant weight (Figure 1). Chlorophyll determination was done with a SPAD-502 Minolta (Spectrum Technologies Inc., Plainfield, IL, USA) in the upper fully expanded leaf of four plants per plot; thereafter leaf size was determined on digital photographs with the Image Tool CMEIAS update software (Liu et al. 2001) and specific leaf area was calculated after drying the leaves to constant weight. Total leaf area was calculated by multiplying the number of leaves of each tree by the mean leaf size of the treatment. Therefore, in these results differences between R+ and R– were only due to the rust infection in the previous year.

Next, the number of leaves of two plants per plot was counted every month from the end of December to final autumnal abscission. When the growing season finished, the stems of all the plants were harvested and dry mass was determined. The experiment continued during 2 years, applying fungicide every 15 days in the R– plots during the growing seasons. Stems were harvested every winter. After the end of the third growing season, the stems of four plants per plot were sampled; then four soil samples were taken from each side of each plant, 20 cm apart from the plant neck. Soil samples were taken from the upper 15 cm with a core of 8.5 cm diameter. Soil samples were washed to separate roots. Dry mass of roots and stems were determined, as well as N concentration of abscising leaves, stems and roots.

Statistical analysis

In Experiment 1, ANOVA was performed considering clone (O or A), rust (R+ or R–) and nitrogen (N+ or N–) as factors. Complete interactions between the three factors were analyzed. If any interaction was significant, means were compared by Duncan test ($P < 0.05$). In Experiment 2, the ANOVA was performed considering clone (O or A) and rust (R+ or R–). The same post hoc comparison of means was done, if the interaction was significant.

Results

Experiment 1: Effects of rust during the growing season

Rust symptoms (i.e., chlorotic areas and yellow pustules in the abaxial side of the leaf) appeared at the beginning of January 2013. Fungicide-sprayed plants of both clones had no rust symptoms (0% incidence), whereas all non-sprayed plants were infected by rust (100% incidence). Clone O reached 100% incidence at the beginning of March, while clone A reached 100% incidence at mid April. Nitrogen availability did not change rust incidence in any clone. In autumn, clone A severity reached 30%

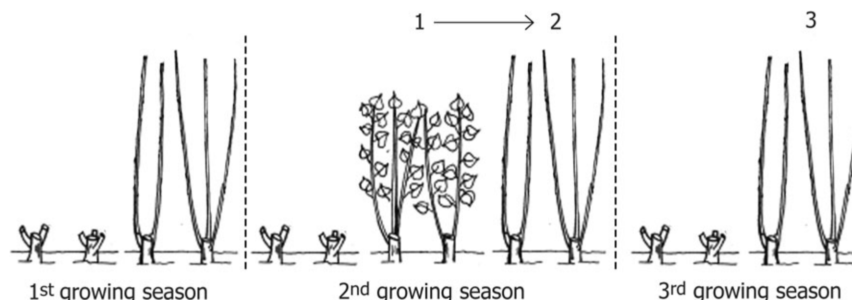

Figure 1. Scheme of Experiment 2. Measurements done in December before rust appearance in second growing season, (1): chlorophyll content, leaf size, number of leaves, total leaf area and stem dry mass. Measurements done at the end of the second growing season, (2): stem dry mass. Measurements done between 1 and 2 (arrow): number of leaves. Measurements done at the end of the third growing season, (3): stem and root dry mass, [N] in stems, roots and abscising leaves.

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in both N availability treatments, while severity in clone O reached higher values, with peaks of 53% and 78% for high and low N respectively (Figure 2c and d). In both clones the highest severity was reached at the end of February and stayed high until the beginning of autumnal senescence. Thereafter, severity decreased because the first leaves to fall were those with higher severity, so the remaining leaves had a lower amount of pustules.

Rust reduced leaf assimilation capacity, because net carbon exchange rate at saturating light (A_{sat}) and the photosynthetic ETR were lower in R+ than in R– plants. Rust also increased respiration rate (Resp). In R+ plants, intercellular CO_2 concentration (Ci) was higher in N+ plants than in N– plants. However, in R– plants Ci was similar irrespective of N fertilization. Water movement in liquid phase (K_{leaf}) and water exchange in vapor phase (g_s) were also reduced by rust. Glucosamine concentration, an estimation of rust severity, was higher in R+ than R– plants of both clones. Except for Ci, none of the other traits was affected neither by N fertilization nor by clone (Table 1).

The number of leaves per plant was modified by N availability and rust infection in clone O (Figure 2a). At the beginning of autumn, O R–N+ plants had >100 leaves whereas the other clone O treatments had <45 leaves. Leaf number in O R–N– plants was constant until May 13, while O R–N+ retained leaves for a week longer; thereafter the number of leaves decreased remarkably in both treatments. In O R+ treatments, leaf number decreased constantly from the beginning of autumn (Figure 2a). In clone A there were only effects of rust infection (Figure 2b). At the beginning of measurements, A R– plants had 100 leaves per plant, which persisted until mid-May, when leaf number started to decrease with a time-course similar to that in treatment O R–. In treatment A R+, the number of leaves was lower and decreased constantly from the start of measurements (Figure 2b).

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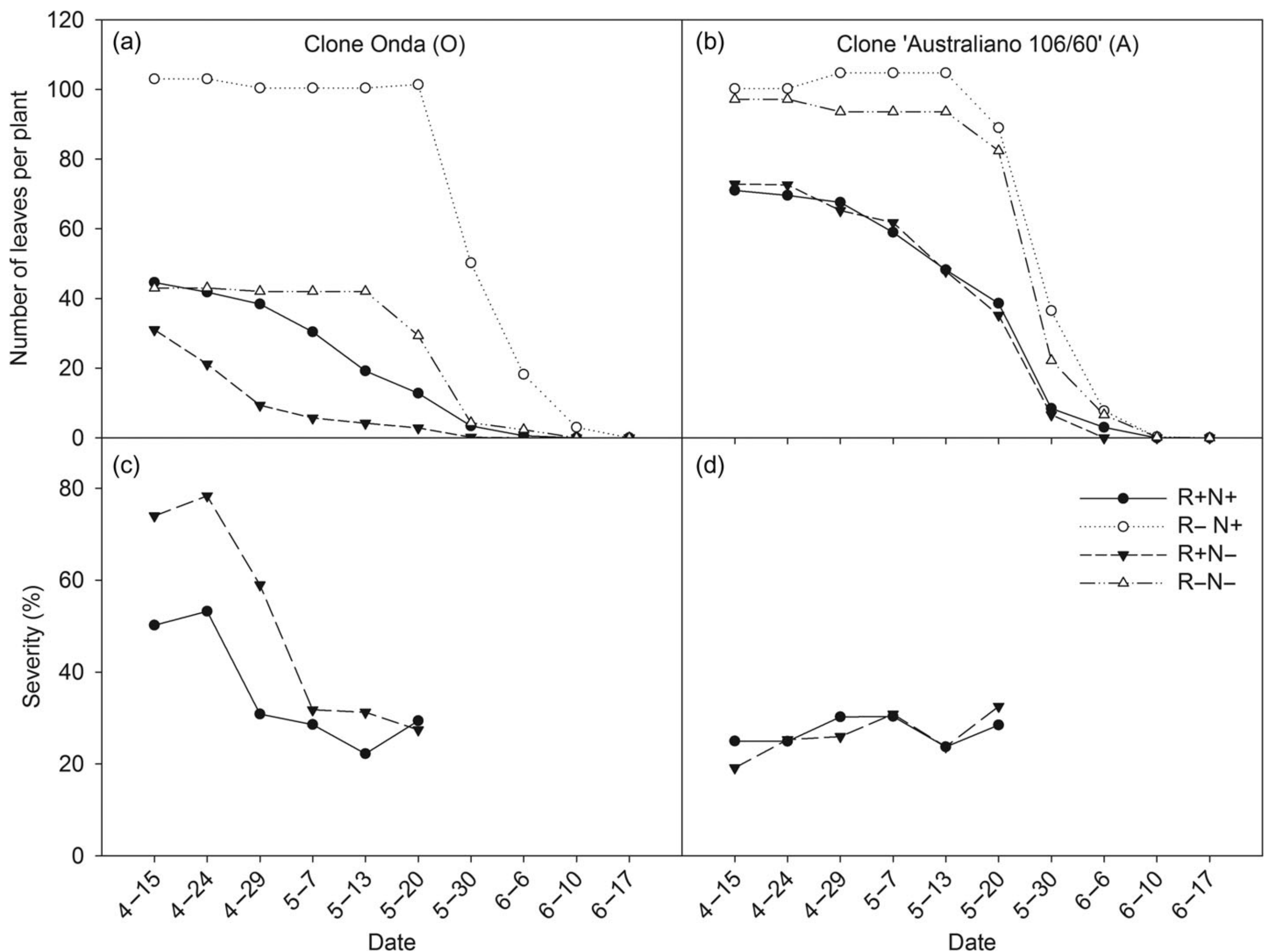

Figure 2. Number of leaves per plant at the beginning of the autumn and level of rust infection in clone O (a and c) and clone A (b and d), with rust (R+), without rust (R–), with N fertilization (N+) and without N fertilization (N–). In all R+ treatments incidence was 100% since mid-April.

The concentration of N in abscising leaves showed an interaction between clones and rust infection, but there were no effects of N availability, nor interaction with the other factors. R– abscising leaves of both clones presented similar N concentrations, whereas R+ abscising leaves had higher (two- to threefold) N concentrations in both clones (Table 2). Associated with this higher N concentration in R+ abscising leaves we found an increase in fungal mass, expressed by glucosamine concentration (Table 2), but there were no effects of other factors or interactions for glucosamine. Glucosamine concentration in abscising leaves was lower in R– than in R+, with higher concentrations in clone O, consistently with higher disease severity in this clone (Table 2).

Table 1. Net photosynthesis at saturating light (A_{sat}, µmol CO_2 m^{-2} s^{-1}), intercellular CO_2 concentration (Ci, ppm), dark respiration rate (Resp, µmol CO_2 m^{-2} s^{-1}), electron transport rate (ETR, µmol e^- m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), leaf hydraulic conductance (K_{leaf}, g MPa^{-1} s^{-1} 10^{-3}) and glucosamine concentration (µg cm^{-2}) in full expanded summer leaves of clone O and clone A, without rust (R–) or with rust (R+), without N fertilization (N–) or with N fertilization (N+). Measurements were done on three sunny days, at midday in February when the incidence of rust was 100% and the severity was the highest for each treatment. ANOVA *P*-values are shown below each factor: C (clone), R (rust), N (N fertilization) and the interactions. Bold type highlights *P*-values ≤0.05. Duncan test is included only if any interaction is significant; different means are marked with different letters ($P \leq 0.05$).

Clone	Rust/N	A_{sat}	Ci	Resp	ETR	g_s	K_{leaf}	[Glucosamine]
O	R–N–	25.35	221 ab	0.38	247	227	4.27	0.38
	R+N–	15.21	196 a	2.81	192	177	2.73	4.05
	R–N+	24.20	213 ab	0.80	242	264	3.71	0.26
	R+N+	11.70	275 b	2.72	194	186	3.20	5.11
A	R–N–	24.10	227 ab	1.24	253	275	4.56	0.02
	R+N–	18.57	178 a	2.65	220	168	2.85	1.36
	R–N+	23.50	216 ab	0.96	250	262	5.73	0.29
	R+N+	16.74	229 b	1.80	206	152	1.83	1.86
P-value	C	0.24	0.30	0.96	0.17	0.95	0.81	0.061
	R	**<0.01**	0.98	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	N	0.20	**0.04**	0.57	0.48	0.70	0.74	0.60
	C × R	0.06	0.16	0.13	0.49	0.06	0.14	0.09
	C × N	0.68	0.55	0.30	0.63	0.11	0.68	0.95
	R × N	0.51	**<0.01**	0.44	0.86	0.50	0.38	0.66
	C × R × N	0.83	0.65	0.96	0.57	0.59	0.06	0.76

Table 2. Height (cm) and stem collar diameter (CD, mm) of the plants at the end of the growing season, N concentration (%) and glucosamine concentration (µg cm^2) in abscising leaves; and protein concentration in xylem (µg g^{-1} FW) and bark (mg g^{-1} FW) of stems after complete leaf senescence for each treatment at the end of the growing season. Clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) without rust (R–), low availability of N (N–) and high availability of N (N+). ANOVA *P*-values are shown below each factor: C (clone), R (rust), N (N fertilization) and the interactions. Bold type highlights *P*-values ≤0.05. Duncan test is included only if any interaction is significant; different means are marked with different letters ($P \leq 0.05$).

		Size of the plants after complete senescence		Abscising leaves		Stems after complete senescence	
Clone	Rust/N	Height (cm)	CD (mm)	[N]	[glucosamine]	[protein] in xylem	[protein] in bark
O	R–N–	172	13.3	0.61 a	1.02	310	6.76
	R+N–	165	14.1	1.69 c	14.30	230	6.47
	R–N+	225	21.4	0.66 a	0.48	355	8.01
	R+N+	207	17.7	1.99 c	11.77	232	4.82
A	R–N–	224	18.0	0.75 a	1.02	267	8.19
	R+N–	166	13.7	1.50 b	10.34	218	6.45
	R–N+	293	23.8	0.74 a	1.36	283	7.56
	R+N+	218	17.1	1.49 b	8.41	185	6.61
P-value	C	**0.03**	0.32	0.13	0.42	0.07	0.22
	R	**0.01**	**0.03**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	N	**<0.01**	**0.01**	0.26	0.26	0.83	0.69
	C × R	0.07	0.20	**<0.01**	0.46	0.55	0.72
	C × N	0.66	0.68	0.21	0.79	0.58	0.97
	R × N	0.62	0.27	0.40	0.16	0.39	0.34
	C × R × N	0.73	0.74	0.42	0.96	0.92	0.10

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Effect of rust on plant growth at the end of the growing season Plant height and CD increased with higher N availability and decreased with rust infection (Table 2). Plant height differed between clones: clone A plants were taller than clone O plants. Clones did not differ in CD. There were no interactions between factors (Table 2).

Total dry mass was higher in N+ plants than in N–, in R– plants than in R+ and in clone A than in clone O, but there were no interactions between factors (Figure 3). Belowground dry mass (fine and coarse roots) changed in the same direction as total dry mass. However, the difference between clones in aboveground dry mass was marginally significant ($P = 0.06$), although N and rust effects were similar to total dry mass. ANOVA for belowground dry mass (fine and coarse roots) was: clone (C), $P = 0.01$; rust (R), $P < 0.01$; N fertilization (N), $P < 0.01$; C × R, $P = 0.30$; C × N, $P = 0.49$; R × N, $P = 0.06$; C × R × N, $P = 0.96$. ANOVA for aboveground dry mass (stem and cutting) was: C, $P = 0.06$; R, $P < 0.01$; N, $P = 0.02$; C × R, $P = 0.30$; C × N, $P = 0.58$; R × N, $P = 0.14$; C × R × N, $P = 0.97$. Therefore, high N availability increased dry mass compared with low N availability. In contrast, rust infection produced a reduction of dry mass compared with non-infected plants (Figure 3).

The N concentration in fine roots was modified by the interaction between clone and rust infection (Table 3). In fine roots of clone A, N concentration was similar in R+ and R– plants. By contrast, in fine roots of clone O, the N concentration decreased in plants infected by rust. The N content of fine roots (the product of N concentration and dry mass) was modified only by N availability. High N availability produced an average increase in total N from 0.12 g to 0.28 g per plant.

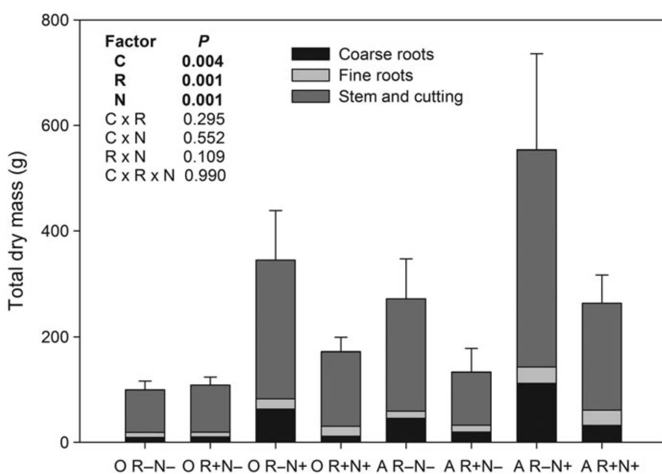

Figure 3. Total dry mass (g) is the sum of belowground dry mass (fine and coarse roots) and aboveground dry mass (cutting used to plant plus the stem produced during the growing season). Clone Onda (O) and clone 'Australiano 106/60' (A), with rust (R+), without rust (R–), without N fertilization (N–) and with N fertilization (N+). Bars indicate standard errors of total dry mass.

The N concentration of coarse roots was modified by the interaction between clone and rust ($P < 0.01$); and by the interaction between clone and N availability (Table 3). From the C × R interaction we observed in clone O a reduction in N concentration in plants infected by rust, but there were no significant differences in N concentration of coarse roots in clone A. The interaction C × N indicated that N concentration of coarse roots in clone O was not modified by N availability; by contrast, in clone A the N concentration increased with higher N availability. Nitrogen content of coarse roots was higher in clone A compared with clone O, in R– compared with R+ plants and in N+ plants compared with N– plants.

There was an interaction between clone, rust and N availability in the N concentration of stems (Table 3). Clone O with high N availability had the same N concentration regardless of the presence or absence of rust symptoms. When clone O plants grew with low N availability, rust infection caused a decrease in N concentration. However, the N concentration of stem in clone A was not modified by N availability in plants R– or R+. Total N content of stems (N concentration multiplied by dry mass) was only modified by rust infection (Table 3). R– plants had higher N content compared with R+ plants.

The sum of N contents of stems and fine and coarse roots allowed us to calculate the total N content per plant after leaf fall. Total N was higher in clone A than O, was lower in R+ than in R– plants and was higher in plants fertilized with N (N+) than non-fertilized plants (N–). There were no interactions between factors (Table 3).

Protein concentration in stems, both in bark and xylem, was modified only by rust infection and there were no interactions between factors. The bark protein concentration in R+ plants was reduced by 20% compared with R– and the reduction was about 35% in xylem protein concentration (Table 2). There was no change in protein concentration in fine and coarse roots caused by clone, rust infection, N availability or interaction between factors (fine roots: C, $P = 0.929$; R, $P = 0.480$; N, $P = 0.445$; C × R, $P = 0.218$; C × N, $P = 0.421$; R × N, $P = 0.608$; C × R × N, $P = 0.893$; coarse roots: C, $P = 0.660$; R, $P = 0.294$; N, $P = 0.661$; C × R, $P = 0.656$; C × N, $P = 0.803$; R × N, $P = 0.580$; C × R × N, $P = 0.190$) (data not shown).

Experiment 2: effect of rust in the following growing seasons

The number of leaves in the spring was higher in plants that had not been infected with rust the previous season (R–) compared with plants that had been infected (R+), regardless of the differences in the number of leaves between clones (Table 4). Differences between R– and R+ plants were evident in a visual observation, both in the size of plants as in the green color of the leaves in clone O. The chlorophyll content and leaf size were lower in O R+ plants than in O R–, while there were no differences between A R+ and A R–. Total leaf area and dry mass of the stems were lower in plants of both clones that had been infected with rust the previous growing season (Table 4).

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Table 3. Nitrogen concentration (%), N content by compartment (g) and total N per plant (g) for each treatment at the end of the growing season. Clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) without rust (R–), low availability of N (N–) and high availability of N (N+). ANOVA *P*-values are shown below each factor: C (clone), R (rust), N (N fertilization) and the interactions. Bold type highlights *P*-values ≤0.05. Duncan test is included only if any interaction is significant; different means are marked with different letters ($P \leq 0.05$). Upper-case letters compare means in CxR interaction, whilelower-case letters compare means in CxN interaction.

		Stem		Fine roots		Coarse roots		Total N content
Clone	Rust/N	[N]	N content	[N]	N content	[N]	N content	
O	R–N–	0.91 c	0.72	1.24 b	0.10	1.77 A-ab	0.18	1.00
	R+N–	0.57 a	0.47	1.21 a	0.09	1.27 B-ab	0.13	0.69
	R–N+	0.54 a	1.53	1.37 b	0.32	1.49 A-a	0.82	2.66
	R+N+	0.54 a	0.74	1.13 a	0.19	0.95 B-a	0.08	1.01
A	R–N–	0.62 ab	1.68	1.03 a	0.13	1.28 A-a	0.88	2.70
	R+N–	0.82 bc	0.64	1.07 a	0.14	1.59 A-a	0.17	0.95
	R–N+	0.65 ab	3.64	1.13 a	0.33	1.76 A-b	2.49	6.47
	R+N+	0.54 a	1.04	1.16 a	0.26	1.78 A-b	0.57	1.87
P-value	C	0.79	0.07	**<0.01**	0.16	**0.03**	**<0.01**	**0.02**
	R	0.23	**0.02**	0.15	0.10	0.09	**<0.01**	**<0.01**
	N	**<0.01**	0.08	0.08	**<0.01**	0.86	**0.01**	**0.02**
	C × R	0.06	0.17	**0.03**	0.53	**<0.01** (A-B)	0.06	0.10
	C × N	0.50	0.49	0.35	0.98	**<0.01** (a-b)	0.13	0.31
	R × N	0.89	0.27	0.14	0.11	0.44	0.06	0.12
	C × R × N	**<0.01**	0.58	0.15	0.72	0.55	0.57	0.56

Table 4. Effect of rust infection on the growth in the Spring of the following season, reflected by the number of leaves, chlorophyll content (Spad units) and shoot dry mass (DM, g) and after a complete growing season, i.e., 2 years with or without rust infection. Clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) without rust (R–). ANOVA results are shown below each variable. Bold type highlights *P*-values ≤0.05. Duncan test is included only if the interaction is significant; different means are indicated by different letters ($P \leq 0.05$).

		Beginning of the second year					End of the second year
Clone	Rust	Chlorophyll content	Leaf size (cm^2)	Number of leaves	Total leaf area (m^2)	Stem DM (g)	Stem DM (g)
O	R–	37.8 b	327 b	215	7.0	622	1406
	R+	34.4 a	252 a	173	4.4	459	816
A	R–	34.94 a	282 a	317	8.9	687	1711
	R+	35.17 a	288 a	221	6.4	511	1242
P-value	C	0.07	0.71	**0.02**	**0.04**	0.32	**0.03**
	R	**<0.01**	**0.01**	**0.03**	**<0.01**	**<0.01**	**0.02**
	CxR	**<0.01**	**<0.01**	0.38	0.95	0.91	0.06

During the second growing season, R+ plants had lower number of leaves than R– plants, irrespective of the differences in number of leaves between clones (Figure 4). The maximum number of leaves was reached by the end of March in both clones. After that date, the number of leaves present in the plants decreased, at a higher rate in R+ than in R– plants. Both clones completely lost their leaves in the beginning of June. By the end of the second growing season, plants of both clones that had rust during 2 years (R+) produced less stem dry mass than plants that had been never infected with rust (R–) (Table 4).

After 3 years of rust infection shoot and root dry mass and concentration of N in roots were lower in R+ plants than in R– plants (Table 5). However, there was no reduction in the concentration of N in the stems. The concentration of N in abscissing leaves was higher in R+ in both clones.

Discussion

Effects of rust during the growing season

Rust developed with different intensity in both clones, and this is reflected in the extent of defoliation as well as in symptom severity (Figure 2). In clone O there was a clear effect of high soil N availability in delaying rust-associated defoliation, and decreasing severity (Figure 2). However N fertilization had no effect on fungal proliferation at leaf level, because fungi biomass (glucosamine content) was similar in infected leaves of plants with low or high soil N availability (Tables 1 and 2). Consistently, photosynthetic rate (A_{sat} and ETR), stomatal conductance and leaf water transport capacity (K_{leaf}) were equally reduced by rust in both clones, under low or high soil N availability. Similarly, respiration rate increased in rust-infected leaves by the same magnitude irrespective of soil N availability or clone (Table 1). A_{sat} can be

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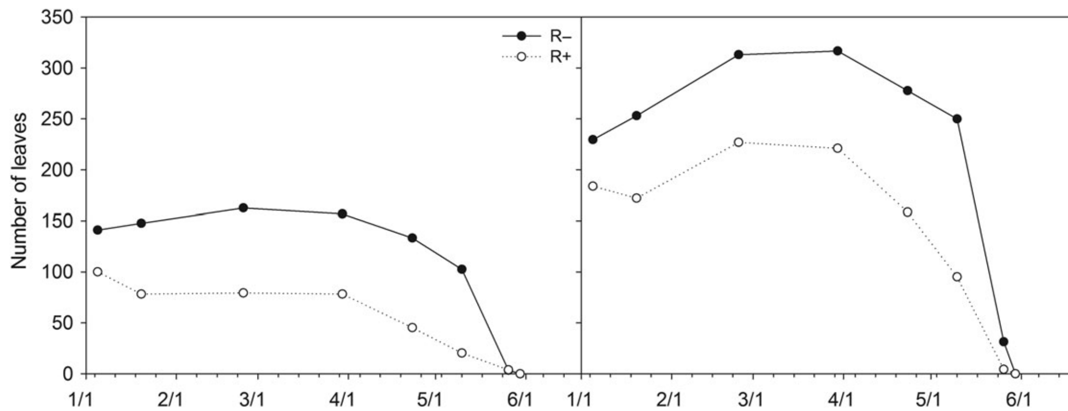

Figure 4. Evolution of the total number of leaves present in the plants along the growing season in plants that had been infected with rust (R+) or not (R–) the previous year and the current season, i.e., the second year of rust infection. Rust infection appeared at the beginning of January. The highest number of pustules was registered on 15 January in both clones: 15 pustules cm^{-2} in clone A and 40 pustules cm^{-2} in clone O.

Table 5. Dry mass (DM, g) and N concentration (%) of stems and roots and N concentration (%) of abscising leaves in poplar clones in nursery plantation at the end of three seasons with rust infection. Roots were taken from 850 cm^3 soil samples. Clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) without rust (R–). ANOVA results are shown below each variable. Bold type highlights *P*-values ≤0.05.

		End of the third year with/without rust				
Clone	Rust	Stem DM	Root DM	[N] in stems	[N] in roots	[N] in abscising leaves
O	R–	801	1.47	0.37	2.34	0.92
	R+	362	0.67	0.37	1.32	1.38
A	R–	808	3.15	0.28	2.38	0.82
	R+	515	1.42	0.38	1.31	1.20
P-value	C	0.55	**<0.01**	0.54	0.50	**<0.01**
	R	**<0.01**	**<0.01**	0.89	**<0.01**	**<0.01**
	C × R	0.55	0.06	0.10	0.65	0.26

reduced by a decrease in Ci. For R+ N– plants, both A_{sat} and Ci are lower than for R–N– plants. However, since Ci does not decrease like A_{sat} for R+N+ plants, rust appears to directly influence photosynthesis. The effect of rust on leaf physiology was similar to reports in other poplar–fungi interactions (Jiang et al. 2016, Zhang et al. 2016) and in the same pathosystem (Gortari et al. 2018). Although during summer there were no differences at leaf level between clones and soil N availability, at the end of the growing season there were differences in growth (i.e, biomass accumulation, final height and basal stem diameter) (Figure 2, Table 2). In both clones, growth was reduced by rust and increased with N fertilization.

As N concentration in leaves is expected to increase if the availability of N in the soil is higher (Pokharel and Chang 2016), rust reproductive cycles might be shortened in fertilized plants (Jensen and Munk 1997, Robert et al. 2002). As fungal spores probably germinate and grow faster in leaves with higher nutrient contents, they might produce infection cycles more frequently and therefore the severity of rust might be higher. In fact, N fertilization generally increases susceptibility towards biotrophic pathogens (Fagard et al. 2014). On the other hand, it is known that the synthesis of defense compounds and its relationship with nutrient availability is different between poplar clones (Donaldson and Lindroth 2007). However, in plants of clone O the severity of rust infection was higher with low than with high availability of N, and in clone A severity was similar under both N availabilities (Figure 2c and d). Unfortunately, we did not measure N concentration or poplar defenses in green leaves. However, the similar A_{sat} in N+ and N– leaves and of N concentration in senescing R–N+ and R–N– leaves may suggest that foliar N concentration, and thus N availability to rust, was not different in green leaves of N+ and N– plants. In both clones the higher availability of N partially counteracted the negative effect of rust on biomass accumulation, i.e., infected plants of both clones grew more if soil N was higher.

Rust-infected plants had fewer leaves at the beginning of autumn and they fell down earlier than in healthy plants (Figure 2a). In two *Populus* hybrids it was demonstrated that leaf area duration correlates negatively with the damage caused by rust (May De Mio et al. 2006). In both clones, healthy leaves abscised with lower N concentration than infected leaves (Table 2). The higher N concentration in rust-infected fallen

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leaves might be due to N retention in the fungal mass (hyphae, reproductive structures and spores). Fungal nutritional requirements are higher than leaf requirements, e.g., N concentration for *Puccinia triticina* spores is 3.5% (Robert et al. 2004) so rust-infected leaves became a strong sink for N (Hall and Williams 2000). The presence of fungal mass in abscising leaves was demonstrated by glucosamine concentration (Table 2), a carbohydrate that is only present in fungal cell walls but almost absent from healthy plant cells (Ekblad and Nasholm 1996, Wallander et al. 2013). This premature leaf abscission with high N concentrations reduced the amount of N that could be accumulated in storage tissues, such as stems and roots.

The retranslocation of N from abscising leaves to new tissues, or to overwintering organs, is very important in the N metabolism of trees (Killingbeck 2003). In young trees, most of the N is located in leaves. Thus, rust infections on young trees have a strong influence on plant N economy, because this disease affects three steps in N recycling (Cooke and Weih 2005): (i) the remobilization of N from senescing leaves to new tissues during the growing season is reduced by rust because the fungi retain N in senescing leaves that otherwise would be remobilized to sink tissues; (ii) reallocation of N between metabolites is also affected by rust, because the fungi consumes some plant metabolites and also rust elicits the synthesis of defense compounds, diminishing the N available to other metabolites, for example those related to photosynthesis; and (iii) the remobilization of N from senescing tissues to perennating tissues in autumn is also reduced by rust, because the N retained in the fungi biomass falls down with the leaves instead of being remobilized to stems and roots. This last point is related to the next subject to be discussed.

Effect of rust on plant growth at the end of the growing season

In healthy plants, fertilization with N increased plant biomass by 100% in clone A and 250% in clone O, compared with healthy plants with low availability of N in the soil (Figure 3). Unlike N fertilization, rust infection reduced growth. Above- and below-ground biomass had similar changes as total biomass (Figure 2). Reductions in growth and aboveground biomass in plants infected with rust compared with non-infected plants were observed for the same clones in a field experiment (Gortari et al. 2015) and in several clones of *Populus nigra* infected by *Melampsora larici-populina* (Stochlová et al. 2016).

Focusing on roots, N concentration only decreased in clone O in plants with rust with respect to healthy plants (Table 3). Furthermore, coarse roots had higher N concentration than fine roots, probably due to the nutrient reserve function for coarse roots (Millard et al. 2006, Millard and Grelet 2010), while the main function of fine roots is nutrient absorption (Hawkins et al. 2014). Regarding N retained overwinter in aboveground biomass, rust reduced N concentration in clone O under low N availability with respect to healthy plants, while in the other treatments differences were not important. As differences in N concentrations were slight, if any, biomass production was the main driving factor of N content.

A particular feature was observed in clone O with low availability of N: there were no differences in biomass production between plants with or without rust, but there was a reduction in N concentration. Hence, N accumulation was lower in rust-infected plants due to a decrease in N concentration, not in biomass. Therefore, in sites with low availability of N in the soil, the negative effect of rust would be more severe than in more fertile sites or in fertilized plantations. In this sense, fertilization can help to counteract the reduction in nutrient storage and in growth produced by rust.

Nitrogen accumulates as vegetative storage proteins (VSP) until the next growth season (Rennenberg et al. 2010). In *Populus* sp., 70% of the N present in stems in autumn is in the form of VSP, mainly concentrated in bark parenchymal cells, so these proteins are referred to as bark storage proteins (BSP) (Cooke and Weih 2005). In our results, bark protein concentration in healthy plants was similar to that previously reported for *Populus trichocarpa* (Langheinrich and Tischner 1991), and it decreased in infected plants. This reduction was up to 30% in rust-infected and fertilized plants of clone O compared with non-infected ones (Table 2). The same pattern was observed in xylem tissue with a 10–30% decrease of protein concentration in R+ with respect to R–. In any event, protein concentration in the bark was 20 times higher than in xylem, demonstrating the capacity of bark to act as a storage tissue. The N concentration of stems only changed between rust-infected and healthy plants in non-fertilized plants of clone O (Table 3). However, bark protein concentration decreased in all cases when plants were infected (Table 2). Those results allow us to surmise that even if rust does not have a marked impact on stem N concentrations, rust might cause a shift in the form of N stored. Possibly, BSP synthesis is significantly reduced, and these proteins are replaced by other molecules with lower biosynthetic costs, such as amino acids (Rennenberg et al. 2010).

As plants get bigger, the concentrations of nutrients in the tissues decrease (Barron-Gafford et al. 2003). Low N concentration in stems of fertilized plants is expected if a dilution effect is considered: plants fertilized with N grew much more than unfertilized plants, and the proportion of active xylem near the phloem respect to non-active xylem is reduced. Active xylem near the phloem has more N concentration than xylem near the pith. Also, as in a bigger stem there are more cells to store nutrients, tissue concentration is lower. That is why N concentration in stem is lower in bigger plants (i.e., fertilized plants) (Table 3).

A final remark from our results is that total N accumulated in biomass decreased noticeably when plants were infected by rust. This reduction was at least of 30% and up to 70% depending on the clone and soil N availability (Table 3). Fertilization

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increased N content in both clones, two fold in infected plants and to a higher extent in healthy plants. Consequently, one strategy to counteract the reduction in N storage capacity in poplars infected by rust is to fertilize plants during the growing season. If fertilized plants grow more, N storage in coarse roots and stems will be higher, with positive effects on the early sprout in the following season.

Effect of rust in the following growing seasons

Nitrogen stored during autumn is remobilized during spring sprouting, thereby making new growth relatively independent of environmental conditions (Rennenberg et al. 2010). Stems and roots are the main tissues that contribute to N and carbon storage capacity in most trees, although the influence of root storage capacity in poplars was not determined (Millard and Grelet 2010). In both clones, restricted sprouting (i.e., smaller leaves, lower stem size and smaller total leaf area) was observed in plants infected by rust in the previous season (Table 4). As stems were removed, plants had only the reserves stored in roots and the stump to sustain the new growth, reserves that were reduced in infected plants (Table 5). In apple trees (*Malus domestica* L.), N remobilized to new tissues in spring was positively and linearly related to N content in reserve organs accumulated during the previous growing season. Moreover, as N reserves increase, root uptake contribution to next spring sprouting decreases (Cheng and Fuchigami 2002), so the opposite is also expected to occur. After 2 years of rust infection, the production of stems was 30% lower in R+ than in R– plants (Table 4), while after 3 years of rust infection, this reduction was close to 50% (Table 5). These results demonstrate that rust has a cumulative negative effect on plant growth in both clones, irrespective of their different sensitivity to rust infection.

Our experiments highlight the importance of the reserves accumulated in the roots in the spring growth. Thus, a negative influence on sprouting capacity in the following growing season due to the decrease of N storage in rust-infected plants was demonstrated.

Conclusions

Both N fertilization and rust infection produced strong shifts, in opposite directions, in growth and internal autumnal N recycling; however, there was no interaction between these factors. On one hand, N fertilization (high N availability in the soil) increased height, stem collar diameter and hence, total dry mass. Leaf physiology was similar under low and high soil N availability. However, N fertilization delayed leaf senescence and abscission, and increased total N accumulation.

On the other hand, rust infection had an important effect on leaf physiology; it also reduced plant height, stem collar diameter and total dry mass. Rust also accelerated leaf senescence, and leaf abscission occurred with higher N concentrations associated with fungal mass. Even though N concentration in stems and roots were not highly affected by rust, total N accumulation was lower in plants infected by rust with respect to healthy plants.

Therefore, rust affects plant growth (height and stem diameter) mainly by the reduction in the photosynthetic capacity, leaf area duration and dry mass accumulation, as well as by decreasing internal nutrient recycling. The negative effect of rust continues in the sprouting during the following growing season, even before the new cycle of rust infection. The cumulative reduction of growth was observed in both clones, although they have different sensitivity to rust. Fertilization with N can counteract the negative effect of rust, especially if it promotes stem and root growth.

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Conflict of interest

None declared.

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