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Biological inhibition of denitrification (BDI): an early plant strategy for *Fallopia x bohemica* seedling development.

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Abstract

- Background and Aims

The successful plant *Fallopia x bohemica* presents interesting capacities for the control of soil nitrogen cycle at the adult stage, named the biological denitrification inhibition (BDI). BDI strategy allows the plant, through the production of secondary metabolites (procyanidins), to compete with denitrifying microbial community and to divert, to its benefit, the nitrate from soil. This study aims to analyze whether seedlings of *F. x bohemica*, can implement BDI at the seedling stage. We also determined whether soil nitrogen availability influence the implementation of BDI and seedling growth.

- Methods

We sowed achenes of *F. x bohemica* in soils representing a nitrogen gradient (6 treatments) and harvested seedlings after twenty and forty days of growth. The denitrification and related microbial communities (i.e., functional gene abundances of *nirK* and *nirS*), soil parameters (nitrate content and humidity) and plant performances (biomass, growth and root morphology) were determined.

- Key Results

On soil without nitrogen addition, BDI was observed after twenty days of growth, whereas a stimulation of denitrification was found after forty days. The increase of soil N content had few effects on activity and structure of soil denitrifying community and on the plant biomasses or the relative growth rates. Correlations between plant and microbial parameters were observed after 20 days of growth reflecting early and strong chemical interactions between plants and denitrifying community, which decreased with plant growth after 40 days.

- Conclusions

This study shows that an early in the first weeks of growth, and then to a change to a root conservative strategy after 40 days. This switch to a conservative strategies involved resource storage, an altered allocation to aboveground and belowground parts and an investment in fine roots. It now seems clear that this storage strategy starts very young with an early BDI establishment, allowing this clonal plant exceptional storage and multiplication capacities.

Keywords:

Biological Denitrification Inhibition (BDI), microbial denitrifying activity and community, plant traits, plant seedling, *Fallopia x bohemica*.

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INTRODUCTION

Some plants have exceptional growth and colonization capabilities and fall into the category of exploitative or acquisitive plants (Gross *et al.*, 2007). Due to their functional traits (high photosynthesis rates, leaf span, leaf N or root N contents), these plants are fast-growing species with high nutrient capture ability and utilization efficiency (Grime, 2001). Among nutrients, nitrogen (N) is one of the major factors that limit plant growth in many ecosystems (Vitousek & Howarth, 1991; LeBauer & Treseder, 2008). Thus, plant growth strongly depends on N cycle processes that drive N transformation and availability in soils (Bothe *et al.*, 2006). The N uptake by plants is mainly in the form of nitrate (NO_3^-) and ammonium (NH_4^+) and the competition between roots and microorganisms for these two N forms can be highly intense (Hodge *et al.*, 2000; Boudsocq *et al.*, 2012; Kuzyakov & Xu, 2013). Microorganisms often out-compete plant roots to acquire this limited resource because of their large area/volume ratio and rapid growth rate (Hodge *et al.*, 2000; Kuzyakov & Xu, 2013). However, some plants bypass this competition by taking control of the N cycle in their rhizosphere through the release of secondary metabolites that inhibit N cycle processes (i.e. N mineralization (Dietz *et al.*, 2013), nitrification (Subbarao *et al.*, 2009) and denitrification (Bardon *et al.*, 2014)). According to plant-soil feedback theories (Ehrenfeld *et al.*, 2005; van der Putten *et al.*, 2013), plants that modify N availability in rhizosphere soil may in turn influence their own growth (Subbarao *et al.*, 2009; Bardon *et al.*, 2014; Bardon *et al.*, 2017). Thus, biological control of microbial activities of the nitrogen cycle in soils by plants is an important plant strategy allowing nitrogen acquisition and thus participating in plant growth and performance (Cantarel *et al.*, 2015; Moreau *et al.*, 2015; Pommier *et al.*, 2018).

The successful plant model *Fallopia* spp. species complex (Japanese knotweed in the broad sense) presents very interesting capacities for the control of N cycle microorganisms. Indeed, this model has allowed us for the first time to demonstrate the biological inhibition of denitrification (BDI) (Bardon *et al.*, 2014). This inhibition allows the plant, through the production of secondary metabolites (procyanidins), to compete with the denitrifying microbial community of the nitrogen cycle and to divert, to its benefit, the nitrate from the microbial pathway (Dassonville *et al.*, 2011; Bardon *et al.*, 2014, 2016, 2017). In Japan, *Fallopia* spp. has also been shown to influence N cycling in the soil. On Japanese volcanoes, *F. japonica* is a common perennial pioneer species. In this habitat, nitrogen is a major limiting factor of plant growth. The concentration of N in soil increases in the course of patch development, accumulating in organic and mineral forms (Hirose & Tateno, 1984; Tateno & Hirose,

1987) available for other plants in plant succession. These results show that *Fallopia* spp. are very important primary colonizers and a particularly pertinent plant model in the studies of the interactions between plant-soil and N associated microorganisms.

BDI has been highlighted on established *Fallopia* spp. in the field (Dassonville *et al.*, 2011) or in controlled greenhouse conditions on plants regenerated from vegetative propagules (rhizome fragments) (Cantarel *et al.*, 2020). For adult plants, BDI leads to an increase in available soil nitrate and a change in plant root traits stimulating root system growth and their competitive capacity (Bardon *et al.*, 2017). However, plant N demand is known to vary along the plant life cycle associated with the changes in dry matter production and light interception (Perthame *et al.*, 2020), which are strongly linked to life and phenological stages (i.e., germination compared to the reproductive stage) and/or soil N-availability. For high productive plants, as *Fallopia* spp., previous studies have reported a higher germination rate (Engler *et al.*, 2011; Lamberti-Raverot *et al.*, 2019), photosynthetic activity, a greater N uptake and N accumulation in seedling than in adult plants (Agren & Franklin, 2003). These results suggested that seedling could compete more strongly with soil N microorganisms for N and could develop BDI strategies at early growth stages. No study has yet shown the seedling impacts on the soil N-microorganisms functioning and even less for the seedlings of BDI plants like *Fallopia* spp.

This study aims to analyse whether seedlings of *F. x bohemica*, known to develop a BDI strategy at the adult stage, can do so at early plant life stages and whether the N nutrition conditions influence the establishment of BDI and the growth of seedlings (i.e., plant performances). Currently, soil procyanidin concentration is difficult to measure directly in soils, making root procyanidine exudation inputs particularly difficult to quantify (Kraus *et al.*, 2003), notably because procyanidins strongly bind to soil components (Kraal *et al.*, 2009). In our study, BDI will be highlighted with a significant decrease of denitrification under *F. x bohemica* planted conditions relative to unplanted conditions, presented as a percentage (Dassonville *et al.*, 2011; Cantarel *et al.*, 2020). Through an experiment in microcosms under controlled conditions, we tested the following hypotheses: 1) *F. x bohemica* seedlings develop a BDI strategy as early as 20 or 40 days after emergence, 2) a low soil N content triggers BDI establishment and 3) the BDI strategy allows the seedlings to improve its performance with growth advantages, such as higher growth rate and biomass. To test these hypotheses, measurements on soil denitrifying microbial community (denitrification and denitrifying gene abundances) and on plant traits (biomasses and root morphology) have been made at early stages of plant development (seedling at 20 days and 40 days of

growth after emergence, respectively 12 and 13 stages on BBCH scale) along a nitrate/ammonium mixture gradient.

MATERIALS AND METHODS

Biological material and N gradient

Fallopia x bohémica is rhizomatous plant of the *Polygonaceae* family. *F. x bohémica* was chosen in this experiment as plant model adopting a biological inhibition of denitrification (BDI) strategy (Bardon *et al.*, 2014). The *Fallopia* complex includes *F. japonica*, *F. sachalinensis*, the complex of polyploid *F. x bohémica* hybrids (Chrtek & Chrtková), originating from the hybridization of *F. japonica* and *F. sachalinensis*, backcrosses and F2 and other crosses involving *F. baldschuanica* (Bailey *et al.*, 2009). Within the complex, *F. x bohémica* presents the highest genetic and morphological variation (Mandák *et al.*, 2005; Tiébré *et al.*, 2007). Although the spread of *F. x bohémica* has been mainly considered as clonal, recent sexual spread has been shown in Europe (Rouified *et al.*, 2011; Lamberti-Raverot *et al.*, 2017, 2019). Sexual propagules consist of a single-seeded achene that is surrounded by a winged fruiting perianth. *F. x bohémica* produces a large number of fertile achenes each year (Lamberti-Raverot *et al.*, 2019). Achenes of *F. x bohémica* were harvested from an unique *F. japonica* individual at the Chambon-Feugerolles site (Loire (42), France - 45°23'49"N ; 4°18'33"E) in winter 2021 and were stored at 4°C until the experimentation.

The soil was harvested at Saint Maurice de Gourdans (Ain (01), France - 45°48'15"N ; 5°10'29"E) five days before the start of the experiment, sieved to 4 mm and stored at 4°C, in order to limit any changes in mineral nitrogen levels and microbial communities. This soil was chosen (1) for its physicochemical characteristics (Table 1); low mineral nitrogen (N) contents allowing the creation of a N gradient and (2) because *Fallopia* spp. has never been detected at this site.

A gradient was carried out with six N amendment treatments (Table 2), respecting the initial ammonium/nitrate coefficient of soil (0.36; see Table 1) with respectively adding 0, 4.1, 6.83, 17.08, 27.33 and 68.33 of N ($\mu\text{gN-NH}_4^+ + \text{NO}_3^- \cdot \text{g dry soil}^{-1}$) in soil, hereafter named no add, Add-N1, Add-N2, Add-N3, Add-N4 and Add-N5, respectively. Amendments were prepared by adding KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ powder to 150 ml of distilled water as in Table 2. Each N amendment was added to two kilograms of soil with a sprayer, in order to moisten the soil, add the solution evenly and blend the soil.

Experimental design and harvests

The six treatments (one treatment without and five with N amendments) were each distributed in fourteen pots (7 x 7 cm) with 140 g of soil (+/- 1 g) per pot. In total, the experiment had 84 pots. On each fourteen pots by treatment, twelve were sown with an achene of *F. x bohemica* and two remain unplanted (control without plant). After sowing, all pots were randomly placed in a phytotron (Sanyo MLR-351 - Versatile Environmental Test Chamber) at the “Serre et Chambres Climatiques” platform (FR BioEEnVis UCBLyon1) regulated at 22°C with 16 h light/8 h dark. Every two days, pots were watered with 20 mL of water, their places were randomly changed in the phytotron to avoid position effects and weeds removed.

The plant and soil samples were collected at early vegetative phenological stages following 20 and 40 days of plant growth after emergence (Fig. 1A), corresponding to seedlings at the 12th and 13th stages respectively on the BBCH scale (Hack *et al.*, 1992). The *F. x bohemica* emergence (i.e., 09 on the BBCH scale) occurs eight days after sowing. Thus, 20 days after this emergence, plants reach the 12th stage with 2 expanded leaves and 40 days after, the 13th stage with 3 expanded leaves. Four planted pots and one unplanted pot were harvested by treatment and harvest time for a total of 30 samples by harvest date. The T20 pots were randomly selected for the T20 harvest. The T20 harvest had no effect on the T40 pot cohort, since each plant being in an individual pot. The four replicates of planted soil by treatment were used (1) for plants, to measure the plant functional traits and (2) to collect rhizosphere soil (the soil under root influence), to analyse the microbial activities and the microbial functional gene abundances. The unplanted soils were only used to test the plant presence effect. Plant presence effect compared to unplanted soil (%) is measured according to the following formula:

$$Plant\ effect\ (\%) = 100 \times \frac{(X_i - X_{UN})}{(X_{UN})}$$

where X is the tested variable for a planted treatment, i is the individual sample and X_{UN} is the value for the tested variable for the unplanted treatment.

For plants, individual aboveground and root parts were separately collected from each pot to measure biomass and root traits. Denitrification activities as well as the soil nitrate concentration were measured on fresh soil stored to 4°C until analysis, and a subsample was immediately frozen at -20°C for the DNA extractions and gene abundance analysis.

Plant traits

After sowing, seedling emergence analysis were conducted every two days during the experiment. For each treatment, total survival (%) was calculated utilizing this equation:

$$\text{Total survival (\%)} = 100 \times \frac{(\text{total sown plants} - \text{total dead plants})}{(\text{total sown plants})}$$

Plant growth was evaluated as the mean relative growth rate in height of aboveground system (RGRh; Hunt, 1982) of individual seedlings was calculated as:

$$\text{RGRh (cm. j}^{-1}\text{)} = \frac{(\ln(h_2) - \ln(h_1))}{(t_2/t_1)}$$

where initial height (h₁) measurements were taken 10 days after emergence, final height (h₂) measurement was recorded at the time of harvests (i.e. after 20 and 40 days).

Immediately following the two harvests, the fresh aboveground and root system was weighed and root system stored in distilled water for a few days at 4 °C until determination of the fresh root morphology by digital scanning. Each seedling root systems were suspended in 1 cm of water in a 20 cm×25 cm clear acrylic tray and scanned at 600 dpi with a scanner (Epson V850 Pro Perfection). Images were analyzed with WinRHIZO™ software (Regent Instruments Inc., Canada) to determine the root length and average root diameter (ARD). Following the root morphology analysis, the samples (aboveground and root parts) were dried for 72 hours at 65 °C and weighed. Specific root length (SRL, m.g⁻¹) was computed as the ratio of root length to dry matter (DM). Aboveground and root dry matter content, respectively ADMC and RDMC (mg.g⁻¹), were computed as aboveground or root DM divided by aboveground or root fresh mass. Aboveground and root DM are used to calculate the plant shoot-to-root ratio (SRR). Root mass ratio (RMR, %) was calculated as the ratio of whole root system dry mass to total plant dry mass, multiplied by 100 (Roumet *et al.*, 2008).

Physico-chemical soil measurements

Fresh soil samples (2.5 g equivalent of dry soil) were placed in plasma vials (150 ml). A volume of 0.01M of CaCl₂ solution (Houba *et al.*, 2000) was added after consideration of the soil moisture. Soil suspensions were sealed with M[®] parafilm, incubated at 20 °C and shaken at 140 rpm for 2 h at 10 °C. The suspensions were filtered through a pore size of 0.22 µm. The NO₃⁻ concentration was quantified using a Smartchem 200 photometer (KPM Analytics - AMS Alliance, Villeneuve-la-Garenne, France).

Soil humidity was measured according to the ISO 11465 protocol. Two grams of fresh soil samples were placed in aluminium cups (previously weighed and annotated) and weighed. The samples were then placed in an oven at 105 °C for 24 h, weighed again and moisture was calculated as the mass of water per 100 g of dry soil according to the following calculation:

$$\text{Soil humidity (\%)} = 100 \times \frac{(\text{fresh soil mass}) - (\text{dry soil mass})}{(\text{dry soil mass})}$$

Fresh and dry soil masses are expressed in grams.

Soil microbial measurements

Denitrification enzyme activity (DEA) was measured as N₂O production using a method adapted from Dassonville *et al.* (2011). Five grams (equivalent dry weight) of fresh soil were placed in a flask hermetically sealed with a rubber stopper. In each flask, air was removed and replaced with a He/C₂H₂ mixture (90:10, v/v) to create anoxic conditions and inhibit N₂O-reductase. A nutritive solution containing glucose (0.5 mg of C-glucose.g⁻¹ of dry soil), glutamic acid (0.5 mg of C-glutamic acid.g⁻¹ of dried soil), potassium nitrate (50 µg of N-KNO₃.g⁻¹ of dry soil) and distilled water was added through the rubber stopper using a syringe to ensure non-limiting amounts of carbon and NO₃⁻ for denitrification activity. The amount of N₂O during incubation at 28°C was measured each hour for 6h. The slope of the linear regression was used to estimate anaerobic respiration (denitrification) as the N₂O produced (µg N-N₂O.g⁻¹ dry soil.h⁻¹). Gases (CO₂ and N₂O) were measured with a gas chromatograph coupled to a micro-catharometer detector (IGC-R990; SRA instruments, Marcy L'Etoile, France). To highlight BDI strategy developed by *F. x bohemica*, plant effect on DEA is used (see plant effect calculation in. §*Experimental design and harvests*). BDI is observed when denitrification under the soil with *F. x bohemica* is significantly decreased compared to the soil without *F. x bohemica* (i.e. with a significant negative plant effect percentage).

Microbial DNA was extracted from each soil sample using the NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co, Düren, Germany), following the manufacturer's protocol. DNA concentrations were determined with the QuBit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted DNA was stored at -20° C until use. The abundance of the nitrite reductase genes *nirS* and *nirK*, as proxies for the abundance of denitrifying bacteria, were quantified based on the gene copy numbers present per gram of dry soil. Quantification was performed by quantitative real-time PCR (qPCR), using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) as the

fluorescent dye on a CFX Connect Real-Time PCR Detection System (Bio-Rad). The standard curves for *nirK* and *nirS* qPCR were generated by amplifying 10-fold dilutions (107–102) of a linearised plasmid. Melting curve analysis confirmed the specificity of amplification and gene amplification efficiencies were in the range 85-99%. R-squared (R^2) values were always above 0.95. Possible inhibition of PCR was tested in advance and appropriate dilutions were chosen.

Statistical analyses

The data were subjected to JUMP PRO 17.0 (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA) and were analysed using ANOVA two ways (harvested dates, N amendment treatments and the interaction), Student's t-test and Pearson correlation analysis, as appropriate. The normality of residuals and homogeneity of variance were tested by Shapiro and Levene's tests, respectively. When necessary, data for microbial activities were transformed. Significance levels and other data formats are as illustrated in the figure and table legends.

RESULTS

Effects of plant presence on denitrification and microbial denitrifying community

On the no nitrogen (N) add treatment (named no add hereafter), we observed significant effects of plant presence on denitrification (DEA, Fig. 2) depending on the plant growth time with a negative effect of plant presence on DEA after 20 days of plant growth (T20 : -9.16 % in average) and a positive effect after 40 days (T40 : 15% in average). For microbial denitrifying community in the no add treatment, we observed contrasted effects of plant presence depending on the considered microbial population (i.e., *nirK* or *nirS* gene abundance). A negative effect of plant presence was found at each growth time for *nirK* abundance (-16.77% and -22.9% respectively for T20 and T40, Fig. 3A) and for *nirS* abundance only at T40 (average -20.57%, no effect at T20, Fig. 3B).

By comparing unplanted and planted pots, we observed that plant presence had contrasted effects on DEA depending on the N amendment treatment and plant growth time (significant N amendment treatment and time interaction; $F_{1,5} = 6.62$, $P < 0.001$, Fig. 2). Throughout all N amendments, the plant effect on DEA increased with plant growth time with an average plant effect of -9.98 % at T20 and +6.36 % at T40 on DEA ($F_{1,1} = 16.81$, $P < 0.001$). For T20, a negative effect of plant presence on DEA was found for the three strongest N add (average -19.47%, -9.92% and -18.75%; respectively for Add-N3, Add-N4 and Add-N5). This negative effect was negated by the addition of the first and second amendments of N

(average -2 % for Add-N1 and -0.47 %, respectively with no difference between unplanted and planted soil; Fig. 2). For T40, the stimulation of DEA associated with the plant presence was reduced twofold when the soil was amended to the strongest N add (average -10% in Add-N5; Fig. 2) and increased with the third N add (average +28% in Add-N3). At the T40, the Add-N1, Add-N2 and Add-N3 showed no difference on DEA between planted and unplanted soil.

For the plant presence effect on *nirK* abundance, we observed significant changes only with the N amendment treatments ($F_{1,5} = 12.49$, $P < 0.0001$). The plant effect on *nirK* abundance was reduced in the Add-N4 for T20 (-27.32%) and increased in the Add-N5 (48.55% and 21.7% respectively for T20 and T40, Fig. 3A). As for the plant presence effect on *nirS* abundance, we did not observe significant differences between plant growth times for the plant presence effect on *nirS* abundance. We observed that plant presence had contrasted effects on *nirS* abundance depending on the N amendment treatment and plant growth time (significant N amendment treatment and time interaction; $F_{1,5} = 13.41$, $P < 0.0001$, Fig. 3B). For T20, a negative effect of plant presence on *nirS* abundance was found for the Add-N4 and Add-N5 treatments (average -18.07% and -43.87%; respectively) and a positive effect on the Add-N2 treatment (17.54%, Fig. 3B). For T40, a negative effect of plant presence on *nirS* abundance was found for the Add-N2 treatments (average -20.57% and -24.83%; respectively) and a positive effect on the Add-N5 treatment (18.05%, Fig. 3B).

Plants also influenced the relationships between DEA and microbial communities, such as *nirS* abundances or the concentration of nitrate in soil in this experiment (Figure 4). When considering all N treatments together, DEA was positively correlated to plant presence effect on *nirS* abundance after 20 days of plant growth but not after 40 days ($R^2 = 0.39$; $F_{1,22} = 14.03$, $P = 0.0011$, Fig. 4A). For T20 and T40, plant presence effect on DEA was negatively correlated to plant presence effect on soil nitrate concentration ($R^2 = 0.48$; $F_{1,22} = 20.05$, $P = 0.0002$, and $R^2 = 0.23$; $F_{1,22} = 6.79$, $P = 0.016$, respectively for T20 and T40, Fig. 4B).

In conclusion, a reduction of DEA was observed after T20 and a stimulation after T40 in the no add treatment. The increase of soil N content with an ammo-nitrate gradient shown few and contrasted plant effects on activity and abundance of soil denitrifying community compared to the no add treatment.

Comparison between plant growth and N amendment treatments for aboveground and root traits

Total survival of *F. x bohemica* seeds were variable depending on the N amendment treatments with 76.9% for no add, 100 % for Add-N1 and Add-N2, 92.3% for Add-N3 and Add-N4, and 84.6% for Add-N5 (data not shown).

For aboveground traits, we observed significant changes with plant growth time ($F_{1,1} = 121.6$, $P < 0.0001$ for aboveground biomass; [supplementary figure 1A]) with an increase of 228.7% for aboveground biomass at T40 compared to T20. No effect of N amendment treatment was found. No effect of plant growth time and N amendment were found on relative growth rate (RGR_h , $F_{1,11} = 1.31$, $P = 0.26$, [supplementary figure 2]). For aboveground dry matter content (ADMC), we observed that changes depending on the N amendment treatment and plant growth time (significant N amendment treatment and time interaction; $F_{1,5} = 3.40$, $P = 0.0128$, Fig. 5A). Plant growth time shown a significant positive effect on ADCM with increased ADCM in T40 compared in T20 for each N amendment treatment ($F_{1,1} = 196.3$, $P < 0.0001$) except for Add-N1 which shown no difference between T20 and T40 (Fig. 5A).

For root traits, time have significant effect on root biomass ($F_{1,1} = 133.9$, $P < 0.0001$) with an increase of 405.6% in average after 40 days of growth compared to 20 days ([supplementary figure 1B]). Same results were found in the shoot:root ratio (SRR) with a decrease between T20 and T40 ($F_{1,1} = 44.33$, $P < 0.0001$; ([supplementary figure 1C])). For root mass ratio (RMR), the same results were found, no effect of N treatment and an increase of 10 % in average of the biomass allocation towards root biomass than in aboveground biomass after 40 days ($F_{1,1} = 62.15$, $P < 0.0001$; Fig. 6).

Root dry matter content (RDMC) had contrasted responses depending on the N amendment treatment and plant growth time (significant N amendment treatment and time interaction; $F_{1,5} = 10.47$ $p < 0.0001$, Fig. 4B). An increase of RDMC were found between T20 and T40 for no add, Add-N3, Add-N4 and Add-N5. At the T40, Add-N3 and Add-N4 have significant higher values of RDMC compared to Add-N1 and Add-N2 (Fig. 4B). None N amendment or plant growth effects was found in specific root length (SRL, ([supplementary figure 3])). The average root diameter (ARD) was significantly higher at T20 than at T40 ($F_{1,1} = 20.93$, $P < 0.0001$, [supplementary figure 3])). RDMC gain between T20 and T40 (measured as RDMC gain (mg.g⁻¹) = values of RDMC at T40 – values of RDMC at T20) was significantly higher in treatment where plants develop a BDI strategy at T20 ($\chi^2 = 11.76$, $P = 0.0006$; Fig. 7). BDI treatment were grouped thanks to the Fig. 2 results at T20 with BDI in no add, Add-N3, Add-N4 and Add-N5 treatments and no BDI in Add-N1 and Add-N2 treatments.

The key results are that plant traits ADMC, RDMC, RMR, SRL, ARD and RGR_h of *F. x bohemica* showed no change when comparing the N amended soils to the soil without N add after 20 and 40 days of plant growth. The root biomass was found to be higher than aboveground biomass at T40, see RMR and SRR.

Correlations between plant traits and the denitrifying microbial community after 20 and 40 days of plant growth

Regardless the plant growth time, plant traits correlations were found for SRL that was negatively correlated to ARD and to RDMC (for SRL/RDMC : $r = -0.56$ and $P < 0.01$ both for T20 and T40; and for SRL/ARD : $r = -0.66$ and $P < 0.001$ for T20 and $r = -0.47$ and $P < 0.05$ for T40; Table 3A).

For the T20, another correlation between plants traits was found, with positive links between SRR and SRL (Table 3A). Moreover, correlations between plants and microbial traits were found with negative relationships between ADMC and two microbial traits (% DEA : $r = -0.42$, $P < 0.05$ and *nirS* abundance : $r = -0.49$, $P < 0.05$) and between RDMC and *nirK* abundances ($r = -0.49$, $P < 0.05$, Table 3B). Plant traits were also correlated to some soil parameters as RGR_h with soil nitrate content and ARD with soil humidity ($r = -0.40$ and $P < 0.05$ and $r = -0.41$ and $P < 0.05$, respectively, Table 3C).

For the T40, another correlation between plant traits was found between ADMC and SRL ($r = -0.66$, $P < 0.001$, Table 3A). One correlation between plant and microbial traits with ARD negatively correlated to *nirS* abundances ($r = -0.48$, $P < 0.05$, Table 3B) and one correlation between plant traits and soil parameters with ADMC negatively correlated to soil humidity ($r = -0.43$, $P < 0.05$, Table 3C).

In conclusion, a negative correlation between RDMC and SRL and between SRL and ARD were found, as commonly observed in plant ecology. Other correlations were found between plant traits and microbial parameters, but preferentially found at the younger stage T20.

DISCUSSION

Recently, Bardon and co-workers identified a novel plant strategy to improve soil nitrate (NO_3^-) acquisition by plants (Bardon *et al.*, 2014; Bardon *et al.*, 2018). This strategy, called biological denitrification inhibition (BDI) (Bardon *et al.*, 2014), was able to inhibit bacterial denitrification by procyanidin root exudation (Bardon *et al.*, 2016a; Bardon *et al.*, 2016b). Subsequent studies demonstrated the potential for the *Fallopia* spp complex. to inhibit denitrification on a significant scale of environmental conditions (i.e., in field conditions: Dassonville *et al.*, 2011 or in mesocosm conditions:

Cantarel *et al.*, 2020), but only on plants regenerated from vegetative propagules (rhizome fragments). For the first time, we focused on (1) the BDI establishment at early growth stages (20 and 40 days after emergence), (2) the impacts of the soil nitrogen conditions (i.e., by N amendments) and (3) the influence on plant growth.

On the soil without nitrogen add, a reduction of soil denitrification activity (DEA) was preferentially found on soil with *Fallopia x bohemica* presence compared to unplanted soil after twenty days of growth (T20), whereas a stimulation of the DEA was found after forty days of plant growth (T40, Fig. 2). Many studies have reported that the denitrifying activity and the structure of soil heterotrophic denitrifying communities can be affected by plant presence (Philippot *et al.*, 2007). Generally, studies found a stimulatory effect of plants on DEA (Henry *et al.*, 2008; Guyonnet *et al.*, 2017) induced by root-released carbon exudates called the rhizosphere effect (Bais *et al.*, 2006). Exudation is a cost for the plant and young seedlings exude about 30 to 40% of the fixed carbon by photosynthesis (Whipps, 1990). Root exudation is influenced by the root morphology, with an increase in exudation for the fine roots (Meier *et al.*, 2020). In our study, the DEA reduction found after 20 days of *F. x bohemica* growth is suggesting an early implementation of BDI that is later counteracted after 40 days of growth by the potential increase of a rhizosphere effect. In the case of another inhibition of nitrogen cycle by plants (i.e., biological nitrification inhibition or BNI), the nitrification inhibitor production was reported for *Sorghum* varieties at early seedling growth stages (i.e., 7 days; Czarnota *et al.*, 2003), suggesting that inhibitor plant metabolites could to be produced very early in plant growth. Using the DMAC (p-Dimethylaminocinnamaldehyde) method (i.e., analytical colorimetric method that specifically quantifies the total content of soluble proanthocyanidins (Prior *et al.*, 2010), the 20-day-old and 40-days-old *F. x bohemica* roots revealed a strong blue coloration, synonymous with the presence of significant amounts of proanthocyanidins (red arrows, Fig. 1B). These findings suggest that seedlings produce and exudate this complex molecule even after 40 days of growth, suggesting that BDI persist after 20 days. In the early life stage of *F. x bohemica*, BDI strategy appears to be very dynamic with an early establishment after the 12th stage of BBCH scale, but also depending on implemented photosynthesis rate and the linked root exudation.

The *nirS*-type denitrifier community known to be more closely associated with plants was found to be more impacted by the rhizosphere effect than the *nirK*-type community (Guo *et al.*, 2013; Kastl *et al.*, 2015; Hou *et al.*, 2018). In our study, *nirK* and *nirS* abundances were generally reduced under *F. x bohemica* presence in the no add treatment for all dates (Figure 3). Only for *nirS*-type community at T20,

no change was found between planted and unplanted soil (Fig. 2B). The BDI implementation highlighted at T20 by the DEA reduction seems to be more linked to the *nirK*-type community than the *nirS*-community in our sandy clay loam soil. Galland *et al.* (2019; 2020) have previously found that procyanidins, condensed tannin exudated by roots permitting BDI, have similar effects on *nirK*-type and *nirS*-type communities on sandy clay loam or loamy sand soils. Our results suggest more complex interactions between denitrifying communities and root exudation (Philippot *et al.*, 2007) than procyanidins add alone. The reduction of *nirK* and *nirS* abundances at T40 highlights a potential persistence of BDI after 40 days of plant growth, perhaps linked to a dampened BDI than at T20, and/or a BDI concealed by an increase of root-released carbon exudates (i.e., rhizosphere effect). Several studies indicated that *nirK*-type community colonized habitats with high contents of easily degradable carbon (Ollivier *et al.*, 2011; Kastl *et al.*, 2015), this could be taken as an indication of changes in root exudation of the *F. x bohemica* during its seedling growth with simple carbohydrates majorly exudated at T20 and more complex carbohydrates exudated at T40.

Generally, amendment of soil with nitrate-based fertilizers are known to promote the microbial denitrification activity, with an increase of the abundance of denitrifier communities, that is especially true for *nirK*-type community (Hallin *et al.*, 2009; Ye *et al.*, 2022). However, in our study, the increase of soil N content with an ammo-nitrate gradient (Table 2) shown few and contrasted plant effects on activity and abundance of soil denitrifying community compared to the no add treatment. The contrasting effects observed between N amendments and the abundance of denitrification genes (*nirK* and *nirS*) suggest complex interactions between the N-added dose and the denitrifying communities. For instance, the N level in soil could impact different microbial populations harboring the *nirK* or *nirS* gene leading to these effects (Yang *et al.* 2017). After 20 days of plant growth, BDI was found only for the highest N amendments (Fig. 2 - Add-N3, Add-N4 and Add-N5). Cantarel *et al.* (2020) have shown similar findings with the strongest BDI on a nitrate rich-soil compared to a nitrate poor-soil. Contrary to the no add treatment, DEA stimulation was not found after 40 days of plant growth, except for the Add-N3 treatment. In our study, carbon exudation of *F. x bohemica* roots would not stimulate enough the denitrifying microbial community to see a rhizosphere effect. These results suggested either this rhizosphere effect is countered by an active BDI at T40 or either by the persistence of the procyanidin effects of the BDI developed at T20, leading to the plant effect on the DEA close to zero (Fig. 2). For the nitrification inhibition, plant inhibitor metabolite effects are known to be stable in soils, often lasting up

to 30 days (Tesfamariam *et al.*, 2014), it may also be the case for procyanidin effects. Recent results shown that the procyanidin effects on denitrifying activity can persist during 50 days with same intensity and decrease only after 76 days (Galland *et al.*, 2020). Moreover, negative correlations found between plant effect on DEA and soil nitrate concentration after 20 and 40 days of plant growth (Fig. 4B) with an increase of soil nitrate content when plants developing a BDI strategy reducing DEA. As the negative correlation is also significant after 40 days of plant growth, this result advocates for a persistence of BDI strategy implemented at T20, concealed by a rhizosphere effect at T40.

For denitrifying communities, N amendments and plant effects seem to be difficult to disentangle. The N amendment has different effects on *nirS*-type and *nirK*-type communities depending on the N-add and the plant growth (Fig. 3). The application of procyanidins are known to reduce the abundance of *nirS*-type and *nirK*-type communities (Galland *et al.*, 2019; Ye *et al.*, 2022) and their inhibitory efficacy seems better with ammo-nitrate type fertilizers (Ye *et al.*, 2022). Our results reflect not an alone procyanidin effects but the whole mechanisms through which plants shape rhizosphere denitrifying communities. The concentrations of key factors regulating denitrifying communities in rhizosphere, such as available labile carbon, nitrate and oxygen availability are drastically different among plant growth stages (Philippot *et al.*, 2007). However, these changes in structure of microbial communities under plant effects remain unclear. Interestingly, for Add-N5 treatment at T20, where BDI is strong (Fig. 2), the plants seem also to inhibit the *nirS*-type community (Fig. 3A) consistent with the responsiveness of the *nirS*-type community to the rhizosphere effect found in previous studies (Guo *et al.*, 2013; Kastl *et al.*, 2015; Hou *et al.*, 2018).

Soil N is particularly important in plant growth, development and nutrition by regulating biochemical and physiological functions of plants, as photosynthesis rate for example (Vitousek & Farrington, 1997). Surprisingly, the aboveground and root dry biomasses, root mass ratio (RMR) and the relative growth rates (RGR_h) of *F. x bohemica* showed no changes when comparing the N amended soils to the soil without N add after 20 and 40 days of plant growth. Usually, studies conclude that plants, more particular plants with high nutrient uptake and high growth as knotweeds, are favoured by increased N availability and responded rapidly to this N availability (Lowe *et al.*, 2003). When plants are exposed to increased N availability, they will benefit from an accelerated nutrient uptake (James & Richards, 2006). Here, our plant growth results seem reflect no changes in nitrogen uptake status of *F. x bohemica* seedlings, suggesting a unique ability for *F. x bohemica* to grow regardless soil N status. In this study, we

found that aboveground dry matter content (ADMC) and root dry matter content (RDMC) at T20 are similar regardless of nitrogen levels (Fig. 5). Dry matter content is a key functional trait commonly associated with plant growth strategies (Westoby, 1998). Higher values indicate higher lignin content and higher proportion of xylem for roots and stems (Hummel *et al.*, 2007) and are often associated with a resource conservation strategy in response to resource limitation (Roumet *et al.*, 2008). We can assume that there are no N-limitations at this stage of seedling development and that these changes reflect change in plant storage status. After 40 days of growth, we found that ADCM and RDMC decreased with the lower add of nitrogen (Add-N1, Fig. 4) compared to the no add treatment and the other N amendments (Add-N2, N5 for ADCM and Add-N3, N4 for RDMC). Our results on the amended treatments do not agree because the highest values of RDMC and ADCM are concomitant for the lowest N-level (no add) and the highest (Add-N5), suggesting, here, that the dry matter content do not vary according to N-resource limitation. Once again, *F. x bohemica* seedlings have an exceptional growth ability regardless soil N status. However, ADCM and RDMC values generally increase between T20 and T40 (except for Add-N1 for ADCM and Add-N1 and Add-N2 for RDMC). This could mean that seedlings respond at T40 with a shift in resource use strategy towards an increased resource conservation in their organs. Indeed, *F. x bohemica* is known for its high resource storage capacity in belowground system (Price *et al.*, 2001), with a rhizome establishment after five months of growth in average. It is possible that this belowground storage starts as early as 40 days (Fig. 6). A monitoring of these resource allocation changes in plant organs with total carbon and nitrogen measurements would be particularly interesting to confirm our hypothesis.

Plant economics spectrum postulates that aboveground and root traits lead to the identification of plant trait syndromes for the understanding of resource acquisition and/or conservation by plants (Wright *et al.*, 2004; Reich, 2014). Only few studies have focused on plant seedlings when analysing these trait syndromes (Kramer-Walter *et al.*, 2016; Shen *et al.*, 2022). We examined the relationships between plant and root traits of *F. x bohemica* seedlings, N-associated microbial traits and soil parameters. Two known and consistent trait syndromes were found for all harvested dates in *F. x bohemica* seedlings (Table 3). As in previous studies on fine roots (Prieto *et al.*, 2015; Roumet *et al.*, 2016; Valverde-Barrantes *et al.*, 2017), we reported a negative correlation between RDMC and the specific root length (SRL), and between SRL and the average root diameter (ARD). These results confirm the already known trait syndrome where plants (here *F. x bohemica*) invest either in long fine roots (high SRL) associated to

higher nutrient uptake, exudation and greater resource acquisition, or either in thick and dense roots associated to longer root life spans and greater resource conservation (Eissenstat *et al.*, 2000). In an experiment with *F. x bohemica* growing with procyanidins, Bardon *et al.* (2017) have observed a significantly increase of the lateral root proliferation associated with an increase in SRL and a decrease in RDMC, known to explain an increase in lateral root meristematic activity (Malamy, 2005). These results may be related to the BDI activity observed in our study at T20 in *F. x bohemica* seedlings. Few relationships were found between seedling growth (RGR_h or biomass) and root traits. These results are coherent with others studies. Shen *et al.* (2022) found that the variation in seedling growth could not be totally captured by root traits. Recent studies explained this lack of correlation that could be due to the more multidimensional aspect of root traits developed in a more complex environment (coupling soil, water, nutrient, microbial parameters), so a single acquisition-conservation axis cannot adequately capture all trait syndromes and the correlation with plant performance traits (Kramer-Walter *et al.*, 2016; Shen *et al.*, 2022). In our study, we found only a negative correlation between SRL and ADCMC at T40. This result could be explained by the shift found between T20 and T40 in plant resource allocation in favour to root biomass (Fig. 6, with RMR values and [supplementary figure 1], with shoot/root ratio (SRR)), suggesting an increase of root foraging capacity by an increase of fine roots (high SRL) concomitantly to a reduction in resource allocation to the aboveground part (low ADCMC).

Interestingly, relationships between plants and microbial parameters are preferentially found for young *F. x bohemica* stage (i.e., at T20, Table 3). ADCMC are negatively correlated with the plant effect on DEA, suggesting either BDI is implemented (i.e., for the lowest plant effect values) and the resource storage in aboveground part increases (i.e., the highest values of ADCMC), or the seedlings with higher storage capability have enough energy to implement the BDI. This correlation is supported by the negative correlation between RGR_h and nitrate soil level (Table 3). If BDI is implemented at T20, this represented a cost for seedlings reducing their growth ability, but in the same time this BDI increases of available soil nitrate (as already shown by Dassonville *et al.*, 2011; Bardon *et al.*, 2014). Still in line with the BDI establishment, the dry matter contents (ADMC and RDMC) are shown to be negatively correlated to *nirS*-type or *nirK*-type communities respectively. These correlations observed between plant and microbial traits at T20 seem reflect early and strong chemical interactions between plants and the denitrifying community, which decreased with plant growth and the increase of the rhizosphere effect.

Conclusion

To conclude, the biological denitrification inhibition (BDI) strategy seems to be a very dynamic plant process and specific to the soil conditions (i.e., nitrogen level, microbial communities, etc.). All these results advocate for (1) an early implementation of the BDI, particularly after 20 days of growth for no Add and the highest N treatments, and (2) a unique plant growth capability for *F. x bohemica*, regardless of soil N levels. This early BDI lead to an efficient acquisition of N resources, with a latent period between the BDI establishment (T20) and the effective storage in plant organs (T40). Moreover, *F. x bohemica* appeared to change their allocation strategy to a root conservative strategy after 40 days, involving resource storage (higher root and aboveground dry matter). We found an alteration of allocation to aboveground and belowground parts (shift to root allocation apparent in root mass ratio), an investment in fine roots (decrease of the average root diameter), and all, regardless of the N treatment. In perspectives, we must study the evolution of this root dry matter gain, linked to the early BDI implementation, for the plant performance. Monitoring root morphology in the first life stages of *F. x bohemica* could confirm this root storage hypothesis with the development of particular root morphology (i.e., strong coarse root system) or the rhizome establishment. *F. x bohemica* is a rhizomatous species and the development of its seedlings must involve, after the formation of the root system, the formation of the rhizome (generally after five months of growth). It now seems clear that this storage strategy starts very young with an early BDI establishment, allowing this clonal plant exceptional storage, multiplication and competitive capacities. As *Fallopia* spp. genotypes are known to be highly variable, it would be interesting to validate these conclusions on a large panel of *Fallopia x bohemica* genotypes.

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Author contributions:

A.A.M. Cantarel, M. Simonin and F. Piola contributed to the study conception, design and funding acquisition. Material preparation, experiment monitoring, and data collection were performed by A. Signoret, J. Gervais, P. Defour, A. Delort, C. Creuzé des Châtelliers, C. Beligon, C. Béraud, C. Boisselet, C. Marais, C. Lobreau, E. Louvez and E. Lacroix. Data analysis were performed by A. Signoret, A.A.M. Cantarel, M. Simonin and F. Piola. The first draft of the manuscript was written by A.A.M. Cantarel and F. Piola and all authors commented on previous versions of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability:

All data that support the findings of this study are available from the corresponding author, Amélie A.M. Cantarel, upon reasonable request.

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APPENDIX

Abbreviations

ADMC: aboveground dry matter content

ARD: average root diameter

BDI: biological inhibition of denitrification

DEA: denitrification enzyme activity

DM: dry matter

N: nitrogen

NO_3^- : nitrate

NH_4^+ : ammonium

RDMC: root dry matter content

RGRh: relative growth rate in height

RMR: root mass ratio

SRL: specific root length

SRR: shoot-to-root ratio

Tables

Table 1. Saint Maurice de Gourdans (Ain (01), France - 45°48'15"N ; 5°10'29"E) soil characteristics at the start of the experiment (named hereafter initial soil conditions) : soil nitrate concentration (NO_3^-), soil ammonium concentration (NH_4^+), organic matter(OM), total carbon (C), total nitrogen (N), soil texture (Clays/Silts/Sands), total carbonates (Cbt) and cation exchange capacity (CEC).

pH	Moisture	NH_4^+	NO_3^-	Ratio	Soil Texture	Clays	Silts	Sands	OM	C	N	Cbt	CEC
	% for 100g of dry soil	$\mu\text{g N-NH}_4^+ \cdot \text{g dry soil}^{-1}$	$\mu\text{g NNO}_3^- \cdot \text{g dry soil}^{-1}$	$\text{NH}_4^+ / \text{NO}_3^-$	Jamagne diagram (1967)	g.Kg^{-1}	g.Kg^{-1}	g.Kg^{-1}	g.Kg^{-1}	g.Kg^{-1}	g.Kg^{-1}	g.Kg^{-1}	mol.Kg^{-1}
8.01	16.49	0.279	0.761	0.366	Sandy-clay loam	245	304	451	59.93	34.84	3.88	479	120.89

Table 2. The studied nitrogen gradient with six nitrogen amendments with ascending values of nitrate and ammonium respecting the initial ammonium/nitrate coefficient in the initial soil (0.36 – see initial values of soil ammonium and nitrate concentration in Table 1).

Nitrogen treatment	Nitrate add ($\mu\text{gN-NO}_3^- \cdot \text{g dry soil}^{-1}$)	Ammonium add ($\mu\text{gN-NH}_4^+ \cdot \text{g dry soil}^{-1}$)	Total N add ($\mu\text{gN-NH}_4^+ \text{NO}_3^- \cdot \text{g dry soil}^{-1}$)
no add	0	0	0
Add-N1	3	1.1	4.1
Add-N2	5	1.83	6.83
Add-N3	12.5	4.58	17.08
Add-N4	20	7.33	27.33
Add-N5	50	18.33	68.33

Table 3. Principal pairwise comparisons of Pearson's correlation among plant traits, microbial traits and soil parameters after 20 (T20) and 40 (T40) days growth of *Fallopia x bohemica*. Abbreviations are given as ADMC: aboveground dry matter content; ARD: average root diameter; NO_3^- : nitrate; RDMC: root dry matter content ; RGR_h : relative growth rate in length.

T20				T40			
a) Plant trait correlations				r			
RDMC	-	SRL	-0.56 **	RDMC	-	SRL	-0.56 **
ARD	-	SRL	-0.66 ***	ARD	-	SRL	-0.47 *
ADMC	-	SRL	-0.09	ADMC	-	SRL	-0.66 ***
b) Plant & microbial trait correlations				r			
ADMC	-	%DEA	-0.42 *	ADMC	-	%DEA	0.28
ADMC	-	nirS	-0.49 *	ARD	-	nirS	-0.48 *
RDMC	-	nirK	-0.41 *	RDMC	-	nirK	0.17
c) Plant trait & soil parameter correlations				r			
RGR_l	-	NO_3^-	-0.40 *	RGR_h	-	NO_3^-	-0.01
ARD	-	Humidity	-0.41 *	ADMC	-	Humidity	-0.43 *

Pearson correlation coefficient (r). Significance levels ($P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

In **gray**: no significant correlations

Figures

Figure 1. *Fallopia x bohemica* seedlings after 20 and 40 days of growth after emergence. Plant general morphology (A) and (B) focus on root system after using DMAC method (p-Dimethylaminocinnamaldehyde; Prior et al., 2010) to highlight soluble proanthocyanidins production at root system (red arrows shown blue color conferred by DMAC method when proanthocyanidins are produced).

Figure 2. Effects of *Fallopia x bohemica* on denitrifying enzyme activity (DEA) after 20 (T20 – white circles) and 40 (T40 – gray circles) days of growth after emergence, as function of nitrogen amendments (Add-N1<Add-N2<Add-N3<Add-N4<Add-N5), expressed as percent change from unplanted soil. Means and standard errors are presented per treatment and date ($n = 4$). Negative values indicate a negative effect of plant presence on DEA (plant microbial activity values are lower than unplanted soil values), and vice versa for positive values. Multiple one-sample t -tests were conducted to discover differences between plants and unplanted soil ($\mu=0$) and the level of significance is indicated by asterisks such as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3. Effects of *Fallopia x bohemica* on (A) *nirK* and (B) *nirS* gene abundances after 20 (T20 – white circles) and 40 (T40 – gray circles) days of growth after emergence, as function of nitrogen amendments (Add-N1<Add-N2<Add-N3<Add-N4<Add-N5), expressed as percent change from unplanted soil. Means and standard errors are presented per treatment and date ($n = 4$). Negative values indicate a negative effect of plant presence on denitrifying gene abundances (gene abundances values under plants are lower than unplanted soil values), and vice versa for positive values. Multiple one-sample t -tests were conducted to discover differences between plants and unplanted soil ($\mu=0$) and the level of significance is indicated by asterisks such as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Correlation between the effects of *Fallopia x bohemica* compared to unplanted soil after 20 (T20 – white) and 40 (T40 – gray) days of growth after emergence for all N treatment values. (A) plant effect on denitrification (DEA) vs plant effect on *nirS* gene abundance and (B) plant effect on

DEA vs plant effect on concentration of soil nitrate. When a correlation is significant, the R^2 and P -value are indicated, otherwise NS for Non-Significant is displayed.

Figure 5. Effects of nitrogen amendment treatments and time on plant dry matter content (C: aboveground - ADMC and D: root - RDMC) after 20 (T20 – white) and 40 (T40 – gray) days of growth after emergence. Means and standard errors are presented ($n = 4$), different letters indicate significant differences between the interaction of nitrogen amendment treatments and time ($P < 0.05$) based on HSD Tukey tests.

Figure 6. Effects of nitrogen amendment treatments and time on root mass ratio (RMR, %) after 20 (T20 – white) and 40 (T40 – gray) days of growth after germination. Means and standard errors are presented ($n = 4$), different letters indicate significant differences between the interaction of nitrogen amendment treatments and time ($P < 0.05$) based on HSD Tukey tests.

Figure 7. Comparison between BDI and no BDI treatment for the root dry matter content gain between 20 and 40 days of growth. The BDI and no BDI treatments are defined by Figure 2 for T20. BDI treatment is defined as treatments with significant DEA decreases (no add, Add-N3, Add-N4 and Add-N5 treatments) and the no BDI treatment include the Add-N1 and Add-N2 treatments. All values are presented following their N-treatment (no add, Add-N1, Add-N2, Add-N3, Add-N4 and Add-N5), each symbol is explain in the graphic legend. Means and standard errors are presented (dark dots with error bars on the BDI treatment $n = 16$ and the no BDI treatment, $n = 8$).

Figure 1

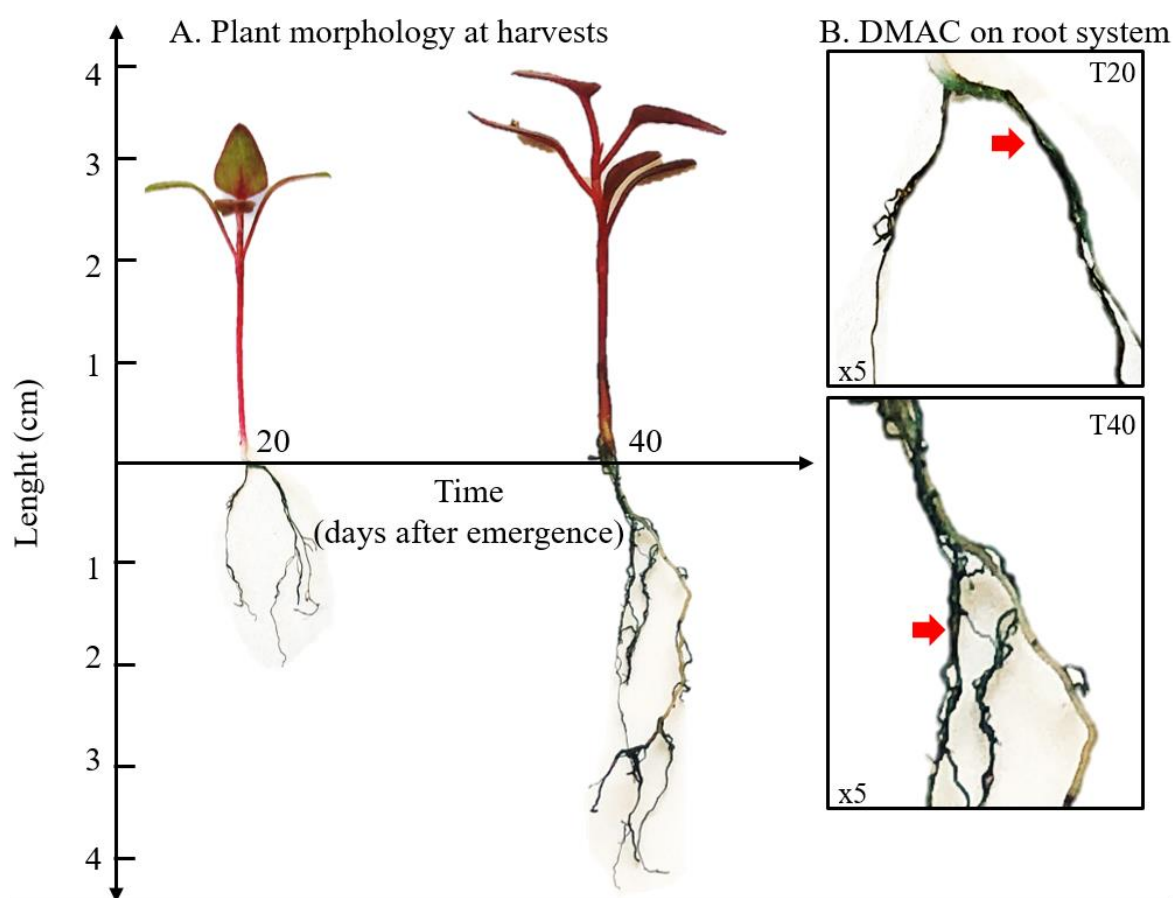
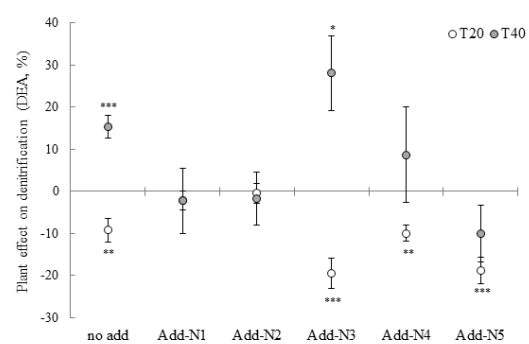


Figure 2



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Figure 3

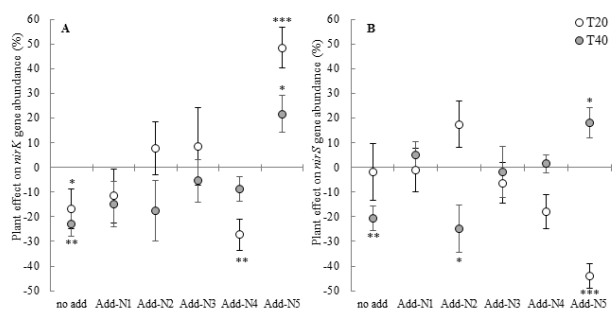


Figure 4

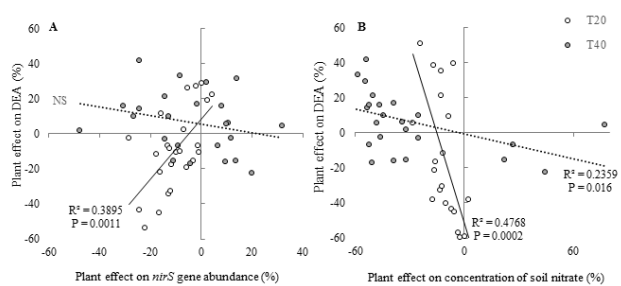


Figure 5

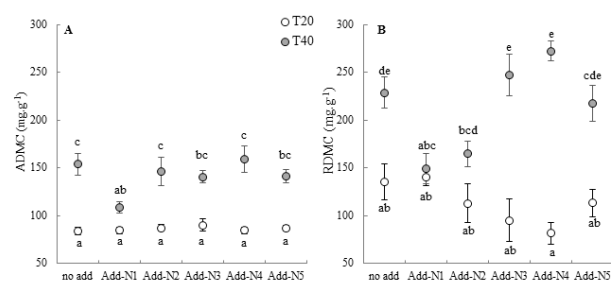


Figure 6

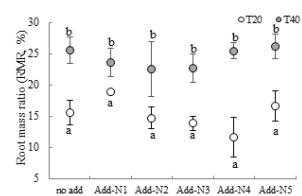


Figure 7

