EXPERIMENTAL TEST OF THE HYPOTHESIS THAT NITROGEN INPUT INHIBITS GROWTH AND NODULATION OF *ALNUS GLUTINOSA* GROWING ON SOILS IN MINING AREAS

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ABSTRACT

Alder (*Alnus glutinosa*) is a nitrogen-fixing species commonly used in the reclamation of mining sites. Nitrogen supply is assumed to be beneficial for soil development, soil carbon storage and overall ecosystem recovery. However, earlier studies show that in old alder plantations, growth is slow. The experiment reported here was done to test the hypothesis that a surplus of nitrogen can suppress the nitrogen fixing activity of *Frankia*, which is the nitrogen-fixing symbiont of alder, resulting in a reduction in the growth of alder. Potted alder seedlings were grown in a greenhouse into two types of non-sterilised spoil heap soils from the Sokolov brown coal mining district in Czechia. Two different concentrations of ammonium nitrate were tested, which mimic the addition of 25 and 50 kg $N.ha^{-1}$, together with a control in which no nitrogen was added. The addition of nitrogen to the soil significantly reduced both alder growth and the formation of *Frankia* tubers. This indicates that once there is sufficient nitrogen in soil, alder growth will be suppressed even in the absence of competition from other plants, which has implications for using alder in reclamation.

Keywords: alder; ecosystem; *Frankia*; reclamation; symbiosis

Introduction

Post-mining sites are often poor in available nitrogen (N) (Bradshaw 1996). Planting N-fixing trees is a common approach to improving soils and increasing the N pool in soil organic matter (Batterman et al. 2013; Brookshire et al. 2019; Levy-Baron et al. 2019; Kou-Giesbrecht and Menge 2021). Alders are a genus of actinorhizal plants, which are often used for afforestation of post-mining sites in central Europe. This is due to their pioneer status (Pietrzykowski et al. 2018; Perakis and Pett-Ridge 2019) and nitrogen-fixing ability of their bacterial symbionts of genus *Frankia,* which fix atmospheric nitrogen. *Frankia* are Gram-positive bacteria belonging to the *Actinobacteria phylum*. Among them, *Frankia alni* is the only formally identified species known to form a symbiotic relationship with actinorhizal plants of the genus *Alnus*. In associations with members of the Betulaceae family, *Frankia* typically initiate intracellular infections (Santi et al. 2013). These bacteria possess the ability to fix atmospheric nitrogen (N_2), both while residing within plant root tubers and as free-living organisms in the soil (Sellstedt and Richau 2013). Nitrogen fixation is facilitated by nitrogenase, an enzyme complex that is highly sensitive to oxygen (Jang et al. 2000). The process of nitrogen fixation in root nodules can demand up to 12% of the host plant's total photosynthetic input, making the symbiotic relationship particularly beneficial in environments where nitrogen availability islow.

Earlier studies at the Sokolov post-mining sites (Frouz et al. 2015) show that the growth of 30-year old alder, *A. glutinosa*, is stunted. A plausible explanation is that there is already suffcient nitrogen in the soil and this reduces *Frankia* performance, consequently negatively affecting the growth of alder. This assumption is supported by several laboratory experiments, as well as field observations on other alder species.

A greenhouse experiment using hydroponic culture, Bélanger et al. 2011, reports that if exogenous nitrogen (potassium nitrate) concentrations are increased they gradually inhibit the symbiosis between *Alnus glutinosa*, *A. viridis*, *A. incana* and *Frankia*. In another greenhouse study, different concentrations of ammonium nitrate reduced nodulation and nitrogen fixation in *Alnus sieboldiana* (Yamanaka et al. 2016). Tiffany Laws and Graves (2005) report that ammonium nitrate negatively affects nodule (tuber) activity in *Alnus maritima* and *Frankia*, and growth of alder.

In addition, field studies with various species of alder, namely *Alnus rubra, A. incana* and *A. alnobetula* spp. *crispa*, report that the addition of N in the form of organic or inorganic fertilizer reduces *Frankia* infestation and nitrogenase activity, and consequently tree growth (Hurd et al. 2005; Gaulke et al. 2006; Markham and Anderson 2021).

The aim of this study was to test the hypothesis that nitrogen addition will reduce *Frankia* infestation and alder growth at post-mining sites. It is also expected that this

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effect will be more pronounced in old spoil soils where the natural background of nitrogen is already high.

Materials and Methods

Materials

Alder [*Alnus glutinosa* (L.) Gaertn.] seeds were obtained from Lesy České republiky Company (Czech Republic).

Soils used in this study came from the Sokolov mining district (Czech Republic), specifically sites that are located at 450–550 m above sea level, where average annual rainfall is 650 mm and average annual temperature is 6.8 °C. Soils used in this experiment originated from clay-rich tertiary mudstone deposits in post-mining spoil heaps after brown coal mining. The material consists mainly of clays (kaolinite, illite and montmorillonite), quartz and calcium carbonate. Two soils were used in this study, both coming from a chrono sequence of alder plantations (Frouz et al. 2001). The first soil, hereafter called young, came from an 18-year old alder plantation, while the other called old, came from a 70 years old plantation. Carbon content in the young soil was 4% and 10% in the old soil. N content was 0.2 and 0.6%, and pH was 8.5 and 6.5 in young and old soil, respectively (Bartuška and Frouz 2015).

Pot experiment

Seeds of *Alnus glutinosa* (L.) Gaertn. were washed in distilled water and then allowed to germinate for one week. Once germinated, similarly developed seedlings were chosen and planted individually in 150 ml pots filled with post-mining spoil heap soils from the Sokolov mining district in Czechia.

The pots were then treated with the equivalent 0 kg/ha, 25 kg/ha and 50 kg/ha ammonium nitrate and each treatment was replicated 7 times.

In addition to irrigation and providing fertilizer, which was provided by top-watering, plants were checked every other day and watered from the bottom with pure water as needed.

The experimental plants were then placed in a greenhouse kept at an average temperature of 20 °C and under a light regime of 14/10 LD, 600 $W.m^{-2}$ for four months.

After this period, the plants were carefully extracted from the pots, dried at room temperature and weighed.

Data analysis

Tree biomass and *Frankia* infestation were tested using two-way ANOVA using soil age and N fertilization level as factors. In addition, one-way ANOVA was used to test the effect of the N fertilizer on both soils separately. If the ANOVA showed significant results, LSD post hoc test was used. Computation was done using Statistica 13.0.

Results

Over the course of the experiment the effect of the fertilizer on plant growth was obvious. Dry aboveground biomass of alder seedlings was significantly affected by both soil age and application of nitrogen fertilizer (Table 1). The growth of seedlings was strongly negatively affected by the nitrogen fertilizer, with seedlings growing in the nonfertilizer treatment having a significantly higher dry mass than those growing in both fertilizer treatments, with no significant difference between the latter two (Fig. 1). Seedlings in the old soil grew slightly and significantly better than in young soil (Fig. 1, Table 1).

Production of *Frankia* tubers was significantly (Table 1) affected by nitrogen fertilizer; no tubers were formed in any of the fertilizer treatments, thus tuber production in non-fertilizer treatments were significantly higher than in those treated with fertilizer (Fig. 2). In terms of

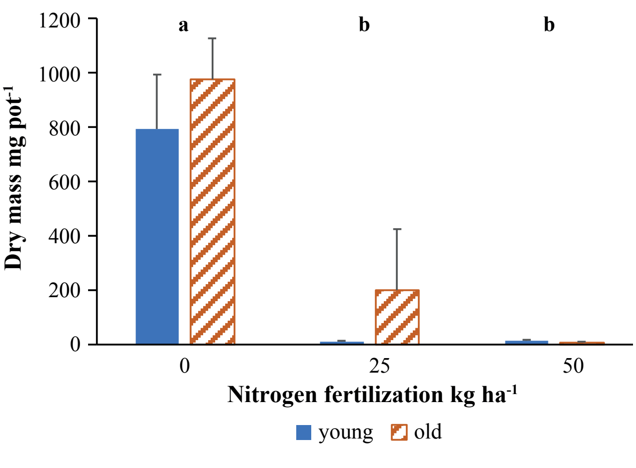

Fig. 1 Aboveground dry mass of alder in young and old soil exposed to various levels of N fertilization (mean and SD). Statistically homogeneous groups of fertilization treatments are marked by the same letter (LSD post hoc test $p < 0.05$).

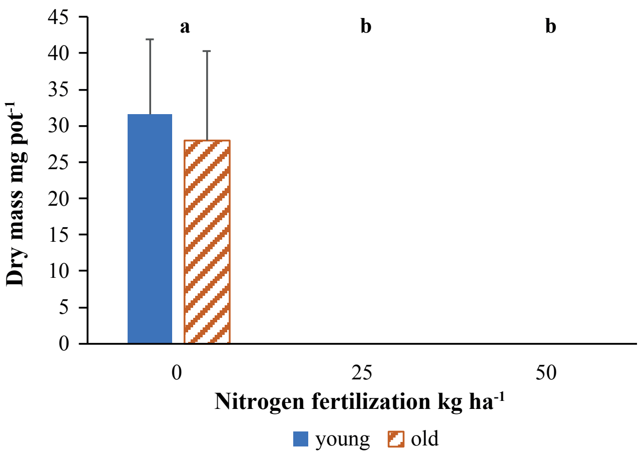

Fig. 2 Mass of *Frankia* tubers on alder roots in young and old soils (mean and SD) exposed to various levels of N fertilization. Statistically homogeneous groups of fertilization treatments are marked by the same letter (LSD post hoc test $p < 0.05$).

Table 1 Output of two-way ANOVA (p values) exploring effect of soil age and nitrogen fertilization and their interaction on aboveground biomass of alders and mass of *Frankia* tubers.

Parameter	Soil age	N fertilization	Interaction
aboveground biomass	0.0123	> 0.0001	ns
mass of tubers	ns	> 0.0001	ns

tuber production, there was no significant difference between the two soils (Fig. 2, Table 1).

Discussion

In agreement with the hypothesis and previous research (Hurd et al. 2005; Gaulke et al. 2006; Bélanger et al. 2011; Yamanaka et al. 2016; Markham and Anderson 2021), the addition of nitrogen affects the symbiotic relationship between alders and *Frankia*. This indicates that the poor growth of alder trees in old soils (Frouz et al. 2015) may be at least partly associated with surplus nitrogen.

Contrary to the hypothesis, alder seedlings grew better in old than young soil, this however, was not recorded for the *Frankia* tubers. In the present experiment, soils from alder plantations which were already inoculated with *Frankia* strains that had time to establish on the local spoil heap soils, so it is unlikely that there were any problems with colonization or mismatch between alder and *Frankia* strains (Balkan et al. 2019; Wolfe et al. 2022; Yuan et al. 2023). Colonization of trees by *Frankia* in young soil can be negatively affected by the availability of phosphorus and micronutrients as the soil was alkaline (Buchbauerová et al. 2024). Slightly better performance of alder seedlings in old soil can be due to better availability of nutrients in these soils. Also, the symbiotic relationship is further affected by the presence and activity of other symbionts, like mycorrhizal fungi and rhizobacteria (Yamanaka et al. 2005), which may be better developed in old soil.

Conclusion

Results clearly show that surplus of nitrogen suppresses colonization of alder roots by the N-fixing symbiont *Frankia* and have a negative effect on alder growth. This indicates that accumulation of nitrogen in soil due to the activity of alders and their symbionts may have negative effect on these symbiotic interactions and eventually impair performance of alder trees. These findings have a clear implication for the restoration of spoil heap soils. While planting nitrogen-fixing trees can speed up soil development, their long- term persistence at a site is unlikely.

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REFERENCES

Balkan MA, Stewart NU, Kauffman ES, Wolfe ER, Ballhorn DJ (2019) Genotypic diversity and host-specificity of *Frankia* bacteria associated with sympatric populations of *Alnus rubra* and *Alnus rhombifolia* in Oregon. Northwest Sci 93: 244–252.

Bartuška M, Frouz J (2015) Carbon accumulation and changes in soil chemistry in reclaimed open-cast coal mining heaps near Sokolov using repeated measurement of chronosequence sites. Eur J Soil Sci 66: 104–111.

Batterman SA, Hedin LO, van Breugel M, Ransijn J, Craven DJ, Hall JS (2013) Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. Nature 502: 224–227.

Bélanger P-A, Bissonnette C, Bernèche-D’Amours A, Bellenger J-P, Roy S (2011) Assessing the adaptability of the actinorhizal symbiosis in the face of environmental change. Environ Exp Bot 74: 98–105.

Bradshaw AD (1996) Underlying principles of restoration. Can J Fish Aquat Sci 53: 3–9.

Brookshire ENJ, Wurzburger N, Currey B, Menge DNL, Oatham MP, Roberts C (2019) Symbiotic N fixation is sufficient to support net aboveground biomass accumulation in a humid tropical. Sci Rep 9: 7571.

Buchbauerová L, Ardestani MM, Rydlová J, Veselá H, Frouz J (2024) Establishment of nitrogen-fixing *Frankia*, arbuscular mycorrhizal fungi, and their effects on alder (*Alnus glutinosa* L.) growth in post-mining heap soils. Soil Systems 8: 98.

Frouz, J, Dvorščík P, Vávrová A, Doušová O, Kadochová Š, Matějíček L (2015) Development of canopy cover and woody vegetation biomass on reclaimed and unreclaimed post-mining sites. Ecol Eng 84: 233–239.

Frouz J, Keplin B, Pižl V, Tajovský K, Starý J, Lukešová A, Nováková A, Balík V, Háněl L, Materna J, Düker C, Chalupský J, Rusek J, Heinkele T (2001) Soil biota and upper soil layer development in two contrasting post-mining chronosequences. Ecol Eng 17: 275–284.

Gaulke LS, Henry CL, Brown SL (2006) Nitrogen fixation and growth response of *Alnus rubra* following fertilization with urea or biosolids. Sci Agric (Piracicaba, Braz.) 63: 361–369.

Hurd TM, Gökkaya K, Kiernan BD, Raynal DJ (2005) Nitrogen sources in Adirondack wetlands dominated by nitrogen-fixing shrubs. Wetlands 25: 192–199.

Jang SB, Seefeldt LC, Peters JW (2000) Insights into nucleotide signal transduction in nitrogenase: Structure of an iron protein with MgADP bound. Biochemistry 39: 14745–14752.

Kou-Giesbrecht S, Menge DNL (2021) Nitrogen-fixing trees increase soil nitrous oxide emissions: A meta-analysis. Ecology 102: e03415.

Levy-Varon JH, Batterman SA, Medvigy D, Xu X, Hall JS, van Breugel M, Hedin LO (2019) Tropical carbon sink accelerated by symbiotic dinitrogen fixation. Nat Commun 10: 5637.

Markham J, Anderson P (2021) Soil moisture, N, P, and forest cover effects on N fixation in alders in the southern boreal forest. Ecosphere 12: e03708.

Perakis SS, Pett-Ridge JC (2019) Nitrogen-fixing red alder trees tap rock-derived nutrients. Proc Natl Acad Sci USA 116: 5009–5014.

Pietrzykowski M, Woś B, Pająk M, Wanic T, Krzaklewski W, Chodak M (2018) The impact of alders (*Alnus* spp.) on the physico-chemical properties of technosols on a lignite combustion waste disposal site. Ecol Eng 120: 180–186.

Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. Ann Bot 111: 743–767.

Sellstedt A, Richau KH (2013) Aspects of nitrogen-fixing Actinobacteria, in particular free-living and symbiotic Frankia. FEMS Microbiol Lett 342: 179–186.

Tiffany Laws M, Graves WR (2005) Nitrogen inhibits nodulation and reversibly suppresses nitrogen fixation in nodules of *Alnus maritima*. J Amer Soc Hort Sci 130: 496–499.

Wolfe ER, Singleton S, Stewart NU, Balkan MA, Ballhorn DJ (2022) *Frankia* diversity in sympatrically occurring red alder (*Alnus rubra*) and Sitka alder (*Alnus viridis*) trees in an early successional environment. Trees 36: 1665–1675.

Yamanaka T, Akama A, Li C-Y, Okabe H (2005) Growth, nitrogen fixation and mineral acquisition of *Alnus sieboldiana* after inoculation of *Frankia* together with *Gigaspora margarita* and *Pseudomonas putida*. J For Res 10: 21–26.

Yamanaka T, Okabe H, Kawai S (2016) Growth and nodulation in *Alnus sieboldiana* in response to *Frankia* inoculation and nitrogen treatments. Trees 30: 539–544.

Yuan Y, Chen Z, Huang X, Wang F, Guo H, Huang Z, Yang H (2005) Comparative analysis of nitrogen content and its influence on actinorhizal nodule and rhizospheric microorganism diversity in three *Alnus* species. Front Microbiol 14: 1230170.