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Assessing the adaptability of the actinorhizal symbiosis in the face of environmental change

Pier-Anne Bélanger[a], Cyntia Bissonnette[a], Audrey Bernèche-D'Amours[a], Jean-Philippe Bellenger[b,c], Sébastien Roy[a,*]

[a] Centre d'étude et de valorisation de la diversité microbienne, Département de Biologie, Université de Sherbrooke, Sherbrooke, Qc, J1K 2R1, Canada
[b] Department of Geosciences, Princeton Environmental Institute, Guyot Hall, Princeton University, Princeton, NJ 08544, USA
[c] Centre d'étude et de valorisation de la diversité microbienne, Département de Chimie, Université de Sherbrooke, Sherbrooke, Qc, J1K 2R1, Canada

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ABSTRACT

Human activity, and in particular industrial activity, has altered natural environments. Here we present an experimental approach adapted to study the actinorhizal symbiosis in alder trees and shrubs submitted to abiotic stress. We measured the impact of exogenous nitrogen on the establishment of the alder symbiosis with *Frankia* sp., and its primary function; nitrogen fixation. Results showed our version of the growth pouch method was functional, and corroborated the gradual inhibition of symbiosis in the presence of increasing exogenous nitrogen concentrations. In mountain alder (*Alnus viridis* ssp. *crispa*) there was a gradual and suppressive effect of nitrogen on the relative number or root nodules, while in black alder (*Alnus glutinosa*) results suggested a threshold effect at 45 ppm N. Shoot to root biomass ratios were increased in the presence of the microsymbiont, and this effect was generally maintained even in the presence of heavy metals (As, Se or V). Alders and the actinorhizal symbiosis were not heavily affected by the presence of heavy metals, confirming potential applications in soil rehabilitation, however the distribution of metals in plant tissues sometimes changed when high levels of metals were present. *A. glutinosa* plants exposed to high levels of As significantly increased the allocation of As to roots (≈90%), while those exposed to high levels of Se rose their aerial tissue Se allocation to roughly 86%. *A. glutinosa* plants exposed to high V levels did not change behavior: V was in all cases preferentially accumulated in underground tissues (≥90%). Our results detail the use of a high-throughput approach to study the plasticity of the actinorhizal symbiosis in the presence of fluctuating nitrogen and metal conditions. These methods are transposable to numerous actinorhizal studies in both fundamental and applied research.

1. Introduction

Human activity and natural phenomena such as the use of activated sludge amendments, volcanic eruptions and landslides are examples of perturbations that can increase concentrations of trace elements in soil. In the case of anthropogenic soil contamination, heavy metals, salts and hydrocarbons are commonly encountered. Obviously, these perturbations affect soil microorganisms but also plants which are closely associated with them. One plant–microbe association that may be affected by such environmental change is the actinorhizal symbiosis.

The microbial symbionts in this association are actinobacteria of genus *Frankia*. Frankiae are ubiquitous bacteria able to fix nitrogen as soil-dwelling saprophytes or as plant symbionts (inside root nodules). Frankiae can infect over 200 host plant species of 24 genera found in a large variety of ecosystems worldwide (Gualtieri and Bisseling, 2000; Schwencke and Caru, 2001; Normand et al., 2007; Franche et al., 2009). Within this root nodule-forming association, *Frankia* can supply 70–90% of the nitrogen needed by its host plant, giving the host a significant advantage to grow in nutrient poor soils (Nickel et al., 2001). The nitrogen input of this symbiosis is important in forests, wetlands and disturbed sites of tropical and temperate regions (Zahran, 1999). The actinorhizal symbiosis is comparable to the *Rhizobium*–legume symbiosis on a number of levels, notably in nitrogen fixation rates (Schwencke and Caru, 2001; Franche et al., 2009). While actinorhizal host plants are biologically very diverse, one genus of particular interest is that of alders (*Alnus* spp.), in which all 47 species are symbiotic (Pawlowski and Newton, 2008). Alders are pioneer trees and shrubs known for their robustness and adaptability to a large range of ecosystems. They enrich and ameliorate soil structure through leaf litter and root exudates (Roy et al., 2007). Alder utilizations in forestry, agroforestry, phytotechnologies and revegetation of perturbed lands is very well documented (Nickel et al., 2001; Schwencke and Caru, 2001; Normand, 2006; Roy et al., 2007; Franche et al., 2009). In addition to the bacterial symbiosis, alders can also be mycorhized

* Corresponding author. Tel.: +1 819 8218000x62505; fax: +1 819 8218049.
E-mail address: Sebastien.Roy@USherbrooke.ca (S. Roy).

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both by endo- and ectomycorrhizal fungi which will improve their capacity to extract macronutrients such as phosphorus from soil (Godbout and Fortin, 1983; Schwencke and Caru, 2001; Markham, 2005; Oliveira et al., 2005; Franche et al., 2009). Because of their capacity to form tri- and tetrapartite symbiosis, alders are of interest for the revegetation of contaminated soils (Roy et al., 2007).

Although both *Frankia* and alders are known to survive in naturally harsh environments, their capability to adapt to environmental stress is little understood and this could be overcome with more research focusing on their tolerance for practical applications. In the last years, much work has been done to study the actinorhizal symbiosis in controlled conditions (Valverde and Wall, 1999; Gabbarini and Wall, 2008). These studies were mostly centered on essential elements (N, P, O_2) that could influence symbiosis development and efficiency (Tjepkema and Murry, 1989; Valverde et al., 2002; Valverde and Wall, 2003, 2005; Peret et al., 2007). None of these studies, however, determined the possible impact of a stress (biotic or abiotic) on nodulation. Some studies looked at the tolerance of actinorhizal symbiosis towards heavy metals, but unfortunately, those studies were done on different plant species, different *Frankia* strains and various growth conditions making comparisons between them difficult (Wheeler et al., 2001; Markham, 2005; Gaulke et al., 2006; Cusato et al., 2007). Another problem leading to the lack of knowledge about actinorhizal symbiosis stress tolerance is the absence of efficient techniques that allow the monitoring of symbiosis establishment and development through a gradient of stressful conditions. Being able to test a gradient of contaminants is essential to measure phenomena such as threshold effects that could give us a clearer understanding of how organisms and their symbiosis adapt to environmental changes (Vandenhove et al., 2006). Such studies could focus on the mechanisms through which compounds affect organisms, or the adaptation mechanisms induced by their presence. In all cases, it is also necessary to use a controlled culture method where most abiotic factors would be absent in order to solely focus on the impact of contaminants on these organisms. To eliminate such interferences, a sequence of axenic and gnotobiotic alder culture methods was developed (Echbab et al., 2007).

Aiming to perform controlled studies on the actinorhizal symbiosis, we first defined: 1 – an effective method to improve alder seed germination rate by getting rid of germ-less seeds (Jobidon and Thibault, 1981), 2 – an efficient seed sterilization method for different alder species, and 3 – since nitrogen is crucial in symbiosis establishment, we performed a "nitrogen calibration trial" using *Frankia alni* ACN14a and three species of alder to define the optimal conditions for our subsequent hydroponic experiments. Hence, the method presented here is gnotobiotic (elimination of biotic interferences) and hydroponic (to override abiotic interferences). Finally, to validate the reliability of the suite of methods we present here, we exposed the *Frankia*–alder symbiosis to heavy metals, and subsequently evaluated symbiosis establishment, development and function.

2. Material and methods

2.1. Bacterial strain and culture methods

The *Frankia* strain used throughout these experiments was *F. alni* ACN14a (ULQ010201401) isolated by Normand and Lalonde in Tadoussac, Canada in 1982 (Normand and Lalonde, 1982). *F. alni* ACN14a was kindly provided by Prof. D. Khasa, *Centre d'étude de la forêt*, Laval University, Québec, Canada. *F. alni* ACN14a was grown at 30 °C (obscurity, static) in BAP culture medium as described by Igual and Dawson (1999). BAP medium was supplemented with sodium succinate at 5 g/L.

2.2. Alder seeds provenance, preparation and use

Seeds of *Alnus glutinosa* (L.) Gaertn. (seed lot 8180890.0), *Alnus viridis* (Chaix) DC. ssp. *crispa* (seed lot 8360545.0), *Alnus incana* (L.) Moench ssp. *rugosa* (seed lot 8431680.0), *A. viridis* (Chaix) DC. ssp. *sinuata* (seed lot 20017120.0) and *Alnus rubra* Bong. (seed lot 8470601.0) were provided by the National Tree Seed Centre of Canada (Fredericton, NB), and stored at −20 °C. Viable seeds were separated from non-viable seeds using hexanes. Seeds destined for hydroponic experiments were surface-sterilized using 30% hydrogen peroxide for 20 min, and then rinsed three times with sterile distilled water for 10 min. After 24 h of stratification at 4 °C in the dark, the sterilization procedure was repeated. Seeds were placed to germinate on Petri dishes with germination medium (for 1 L: 25 g sucrose, 4.3 g Murashige and Skoog basal salt mixture (Sigma, M5524), 0.15 g $CaCl_2{\cdot}2H_2O$, 0.05 g $MgSO_4{\cdot}7H_2O$, 3 g Gelzan™ CM (Sigma, G1910), 1 mL Gamborg's Vitamin Solution 1000× (Sigma, G1019), pH 5.7). The Petri dishes were sealed with Parafilm to prevent drying, and positioned on a 45° slant for 3–4 weeks, under a 16 h light at 22 °C/8 h dark at 18 °C regime, 500–600 μmol photons m^{-2} s^{-1}. To assess the efficiency of the hexane separation procedure, germination rates were assessed for both seeds that floated and those that sanked in hexane.

2.3. Hydroponic culture method

After 3 or 4 weeks of germination on Petri dishes, alder seedlings were transferred to sterile germination pouches (CYG germination pouches) (Mega International, St-Paul, MN). Three alder seedlings were placed into each pouch and the pouches were held upright in autoclaved custom-made wire racks placed in an autoclaved polycarbonate container. These polycarbonate containers had two 2-inch holes that were sealed with 0.22 μm MilliWrap membranes (Millipore Corporation, Bedford, MA) to allow gas exchanges while maintaining sterility. Pouches were watered weekly with 15 mL of Hoagland's solution 0.1× (Xiong et al., 2006). A nitrogen calibration assay was performed for *A. glutinosa* (L.) Gaertn., *A. viridis* (Chaix) DC. ssp. *crispa* and *A. incana* (L.) Moench ssp. *rugosa*. In this experiment, the Hoagland's solution was supplemented with 0, 5, 15, or 45 ppm nitrogen (as KNO_3). Incubation of these plants was performed in the conditions described above for seed germination. Two weeks following seedlings transfer in pouches, homogenized *F. alni* ACN14a was added at the collar of half of the seedlings as 15 mL of a 1 μL PCV (packed cell volume) per milliliter Hoagland's solution. Inoculum viability had been previously confirmed with a tetrazolium salt reduction method as described recently (Bélanger et al., 2011). Pouches were watered weekly for 8 weeks. Nodulation was monitored qualitatively 4 weeks post-inoculation (results not shown) and alder harvest and dissection took place 8 weeks post-inoculation. Evaluation of the following parameters was done during the dissection: plant tissue weight (roots and aerial parts), number of nodules, nodule weight and nitrogen fixation activity (nitrogenase acetylene reduction assay).

2.4. Nitrogenase assay

Nodules from each growth pouch (i.e. 3 seedlings) were combined and placed in 10 mL vials (Chromatographic Specialties Inc, Rockwood, TN) in order to perform the acetylene reduction assay (ARA). Vials were sealed with 20 mm septa (BellCo Glass Inc, Vineland, NJ) and aluminum seals (Chromatographic Specialties Inc, Rockwood, TN). An air volume of 1 mL was removed using a syringe and replaced by purified acetylene to obtain a final acetylene concentration of 10% (v/v). Vials were incubated 24 h in the dark in the same conditions as seedlings. Following incubation, 5 mL of gas volume was removed from each vial and stored in