Symbiosis (2022) 86:273–279
https://doi.org/10.1007/s13199-022-00845-0

Alnus glutinosa seedlings grown following co-inoculation with *Frankia torreyi* strain CpI1 and *Frankia asymbiotica* strain NRRL B-16386

Faten Ghodhbane-Gtari[1] · Mouadh Saadaoui[1] · Issam Ben Mohamed[1] · Moussa Louati[1] · Hatem Chaar[2] · Maher Gtari[1]

Received: 8 December 2021 / Accepted: 16 May 2022 / Published online: 31 May 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract

The potential interference of *Frankia asymbiotica* NRRL B-16386 (nitrogen-fixing but non-infective) during nodule establishment on *Alnus glutinosa* seedlings by *Frankia torreyi* CpI1 (infective and effective) was investigated. Basic plant growth characteristics (stem and root elongation, dry weight production and total chlorophyll) and nodulation were assessed. The analysis showed that NRRL B-16386 did not greatly affect strain CpI1 during root nodule establishment and seedling growth of *A. glutinosa* despite the increase in its quantity and decrease in the symbiotic strain in the inocula. An increase in plant growth was noted even when the level of the effective *Frankia* strain decreased. The asymbiotic strain appeared to have an inhibitory effect on seedling growth and to thrive on the surface of root nodules well established by the symbiotic strain.

Keywords *Alnus glutinosa* · Competition · *Frankia* · Nodulation · Growth

1 Introduction

Alnus glutinosa Gaertn., widely called black alder is an actinorhizal plant that forms symbiosis with members of the genus *Frankia* (McEwan et al. 2017; Roy et al. 2017). This actinobacterial genus encompasses endophytic actinobacteria that have been isolated primarily from nitrogen-fixing nodules in the roots of dicotyledonous host plant designated as actinorhizal plants (Gtari et al. 2013). Based on Koch's postulates of infectivity (nodulation) and effectivity (nitrogen fixation), *Frankia* strains were assigned into symbiotic and asymbiotic endophytes (Gtari et al. 2019). Symbiotic strains are phylogenetically affiliated within cluster 1 (microsymbionts of *Betulaceae*, *Casuarina, Allocasuarina, Comptonia, and Myrica* species), cluster 2 (microsymbionts of *Coriaria, Ceanothus, Datisca, Cercocarpus, Chamaebatia,* and *Purshia species* and of *Dryas drummondii*), and cluster 3 (microsymbionts of *Colletieae, Elaeagnaceae, Gymnostoma* and *Myricaceae* species). Cluster4 contains asymbiotic strains which lack one or two symbiotic characteristics i.e., infectivity and/or effectivity (Gtari et al. 2019). With respect to symbiotic strains, it has been shown that cooperation and competition can occur between various *Frankia* and non-*Frankia* microorganisms during the nodulation process and thus drive nodule occupancy in actinorhizal plants (Kurdali et al. 1990; Hahn et al. 1990; Russo et al. 1993; Sempavalan et al. 1995). A role for host plants has also been reported through selectivity for individual *Frankia* strains or populations (Kurdali et al. 1988; Huss-Danell and Myrold 1994; Mirza et al. 2009; Pokharel et al. 2011; Pozzi et al. 2015). However, the situation remains unclear between asymbiotic and symbiotic *Frankia* strains. Although Ramírez-Saad et al. (1998) reported the co-occurrence of symbiotic and asymbiotic *Frankia* strains in the same *Ceanothus* root nodule samples, the questions of whether asymbiotic strains co-occur as "opportunistic" or "synergistic" and whether they are "parasite" or "beneficial" endophytes remain unanswered.

The ultimate goal of studying nitrogen-fixing symbioses is to maximize their potential to improve plant productivity by accessing an inexhaustible source of nitrogen from the atmosphere (Herridge et al. 2008; Peoples and Craswell 1992). Fundamental, yet challenging, biotechnology practices for nitrogen-fixing symbioses aim to select the most appropriate bacterial inocula (Dommergues 1995;

✉ Maher Gtari
maher.gtari@insat.rnu.tn

[1] USCR Bactériologie Moléculaire et Génomique, Institut National Des Sciences Appliquées et de Technologie, Université de Carthage, Tunis, Tunisia

[2] LR Grandes Cultures, Institut National Agronomique de Tunisie, Université de Carthage, Tunis, Tunisia

Olivares et al. 2013; Sayed 2011; Zhong et al. 2019). The main selection criteria for field inocula are (i) symbiotic performance (ii) ability to accommodate pedoclimatic conditions and (iii) outperformance in relation to less effective indigenous strains.

The aim of the present study was to evaluate the nodulation capabilities of the host plant species *A. glutinosa* with symbiotic *Frankia torreyi* strain CpI1, in the presence of the asymbiotic *Frankia asymbiotica* NRRL B-16386. Basic plant growth characteristics of *A. glutinosa* seedlings, including stem length, root elongation, total chlorophyll, and dry weight, were assessed upon dual inoculation with both *Frankia* strains.

2 Materials and methods

2.1 Plant growth

Alnus glutinosa seeds were collected locally (alder stand in Tamra, Tunisia; 37° 3′ N, 9° 7′ E, 150 m elevation) disinfected by stirring in H_2O_2 (40%) for 30 min, washed several times with sterile distilled water, and germinated in a sterile mix of sand/vermiculite (v/v). The three-week-old seedlings were then grown under hydroponic conditions in a one litter bottle containing Broughton and Dilworth's solution (Broughton and Dilworth 1971) supplied with a nitrogen source, i.e., 5 mM KNO_3. The plant culture in this study was carried out at 20 °C and under a light/dark photoperiod of 16/8 h.

2.2 *Frankia* strains

Strain CpI1 was isolated from root nodules of *Comptonia peregrina* (Callaham et al. 1978) and proposed as a type strain of the species *F. torreyi* by Nouioui et al. (2019). Strain NRRL B-16386 was isolated from *Morella californica* (Lechevalier 1986) and proposed as a type strain of the species *F. asymbiotica* by Nouioui et al. (2017). Both strains were grown and maintained in BAP medium (Murry et al. 1984) supplemented with 2.5 mM propionate as a carbon source at 28 °C without shaking.

2.3 Inoculation experiment

The first set of *A. glutinosa* seedlings was depleted of nitrogen by omitting 5 mM KNO_3 from the DB nutrient solution (BD-N) while the other set was maintained in DB nutrient solution supplemented with 5 mM KNO_3 (BD + N). Four-week-old cultures of each strain were washed with sterile distilled water and syringed several times with sterile 21G needles. Equal stocks of inocula of each strain were prepared on the basis INT (2-(p-iodophenyl-3-(p-nitrophenyl)0.5-phenyl tetrazolium chloride) reduction activity (IR) as described by Prin et al. (1990) and total cellular protein estimated by the BCA (bicinchoninic acid) method (Smith et al. 1985). Briefly 100 µl of 0.2% INT was added to 1 ml of homogenized *Frankia* cells and incubated for one hour at 28 °C in the dark. After methanol extraction for 2 h at 70 °C, the intracellular INTFormazan (INTF) was spectrophotometrically determined at 490 nm. Homogenized *Frankia* cells were solubilized by heating for 15 min at 90 °C in 1.0 N NaOH and total proteins were measured using BCA Protein Assay Kit (Thermo Scientific). All inocula were prepared as an equivalent of 10 µg of protein. To achieve this quantity of inocula, varying amounts of each strain were combined; 0%/100%, 25%/75%, 50%/50%, 75%/25%, and 100%/0% of CpI1 and NRRL B-16386, respectively. Six-week-old seedlings were inoculated and 10 replicates were considered for each inoculum. As a negative control, 10 seedlings were not inoculated.

2.4 Nodulation assessment

After two months, the nodules were harvested from the plants, previously infected with the different combinations of inoculum, numbered and weighed.

2.5 Plant growth assessment

Plant growth was assessed using basic growth parameters. At the end of the experiment, the lengths and dry weights of the stem part and roots were measured.

2.6 Total chlorophyll

Chlorophyll was measured by the method of Arnon (1949) using 200 mg of fresh material in 80% acetone in test tubes for 72 h in the dark and at + 4 °C. After 72 h, the suspension was filtered and the optical density was measured by a spectrophotometer at three wavelengths: 663, 652 and 645 nm and finally the concentrations of chlorophyll ‘a’ and ‘b’ were determined from the following formulas:

$$\text{Chlorophyll a (mg/l)} = (12.7 * \text{OD } 663) - (2.69 * \text{OD } 645).$$

$$\text{Chlorophyll b (mg/l)} = (22.9 * \text{OD } 645) - (4.68 * \text{OD } 663).$$

$$\text{Total chlorophyll (mg/l)} = \text{Chlorophyll a} + \text{Chlorophyll b} = \text{OD } 652 * 1000/34.5$$

2.7 DNA extraction, 16S-23S rDNA ITS-PCR amplification and capillary electrophoresis

To check for the potential co-occurrence of NRRL strain B-16386 with CpI1 in the induced nodules, the automated ribosomal intergenic spacer analysis (ARISA) method was used (Gtari et al. 2007). Nodule lobes were disinfected by

immersion in 30% v/v hydrogen peroxide for 15–20 min, rinsed several times with sterile distilled water containing 2% w/v polyvinylpyrrolidone (PVPP). Peeled and unpeeled lobes from each nodule were crushed separately in 500 µL of extraction buffer (100 mM Tris–HCl pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8. 2, 1.4 M NaCl, 2% (w/v) cetyl trimethyl ammonium bromide (CTAB), 3% w/v PVPP and incubated for 1 h at 65 °C. Afterwards chloroform: isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged at 12 000 9 g for 15 min at room temperatue. A 2/3 volume isopropanol was added to aqueous phase and DNA was precipitated by centrifuging at 12 000 g for 15 min at 4 °C. The resulting DNA pellet was then washed twice in ice-cold 75% ethanol and resuspended in 100 lL of 5 mM Tris–HCl buffer (pH = 8.5). The DNA pellet was then dissolved in 10 ml TE (10 mM Tris–HCl pH 8 20 mM EDTA pH 8.2) (Gtari et al. 2007). Amplification of the ITS 16S-23S rDNA was performed using the forward primer S-d-Bact-1494-a-S-20 (5'-GTCGTAACAAGGTAGCCGTA-3'), labeled at the 5' end with the phosphoramidite dye 6-carboxyfluorescein, and the reverse primer L-D-Bact-0035-a-15 (5'-CAAGGCATC CACCGT-3'). Capillary electrophoresis was performed in an ABI Prism 3710 capillary sequencer as described previously (Gtari et al. 2007; Ghodhbane-Gtari et al. 2010). Aliquots (1–5 µl) of the PCR products were mixed with 1 µl of the 1000-bp internal size standard (Applied Biosystems) labeled with the phosphoramidite dye 6-carboxyrhodamine and 20 µl of deionized formamide. The mixture was denatured at 95 °C for 5 min and cooled in an ice bath. PCR products were then run-on the ABI Prism 3710 genetic analyzer (Applied Biosystems) through a 47 cm/50 µm capillary filled with 4% performance-optimized polymer (Applied Biosystems).

2.8 Statistical analysis

Basic growth parameters, including stem and root length and dry weight, total chlorophyll, and nodule number and weight, were analyzed using a simple linear model with fixed effects (Inoculum, Medium and their interaction). For each variable, residual normality and homogeneity of variances were assessed with Shapiro–Wilk and Levene tests, respectively. Adjusted means (LS-means) were calculated and compared with the Tukey–Kramer (for an equal-variance model) or Dunnett T3 test (for an unequal-variance model) of PROC MIXED of SAS, version 9.4. Table S1 summarizes the main statistical analysis performed in this study.

3 Results and discussion

It is well established that inoculation with compatible *Frankia* strains increases nodulation and growth performance of actinorhizal host plant species even in the presence of indigenous *Frankia* strains (Weber et al. 1989; Sanginga et al. 1989; Nickel et al. 2001). In this study, it was proposed to investigate nodulation and growth of *A. glutinosa* seedlings upon co-inoculation with two *Frankia* strains, one symbiotic *F. torreyi* CpI1 and the other asymbiotic *F. asymbiotica* NRRL B-16386. The potential interaction between the two strains was then analyzed.

Root nodules were first observed after 3 weeks of inoculation in all tested *A. glutinosa* seedlings, except those inoculated with NRRL B-16386 strain only and the negative control. This result is concordant with previous studies reporting that root nodules appeared 2–3 weeks after inoculation in members of *Alnus* species (Huss-Danell 1978; Kohl and Baker 1989; Yamanaka et al. 2016). The assessed time course for nodule appearance was not affected by the presence or absence of the asymbiotic strain NRRL B-16386. The nodulation results obtained in the present study also confirm previous reports on the ability of the CpI1 strain (from phylogenetic cluster 1) to efficiently nodulate *A. glutinosa* (Lalonde 1979; Nouioui et al. 2019) and the complete inability of strain NRRL B-16386 (phylogenetic cluster 4) to nodulate any of the actinorhizal species tested, including its original host of isolation, *Morella californica* and *Alnus* species (Lechevalier 1986; Huguet et al. 2005; Nouioui et al. 2017). ARISA performed on selected root nodules confirmed the omnipresence of the CpI1 strain in all induced root nodules, whereas the strain NRRL B-16386 was detected in only 10% of unpeeled lobes and 3% of peeled lobes (Fig. S1). Thus, strain NRRL B-16386 is more likely to be a contaminant of the surface of root nodules of *Morella californica* (Lechevalier 1986). Similarly, incompatible R43 strain (cluster 3) does co-infect the root nodules formed by compatible *F. casuarinae* CcI3 strain (cluster 1) in *Casuarina equisetifolia* (Vemulapally et al. 2019) and is likely being a surface contaminant (Zhang et al. 1984).

The establishment of optimal symbiosis between *F. torreyi* strain CpI1 and *A. glutinosa* seedlings was significantly influenced ($p < 0.05$) by the availability of mineral nitrogen in the medium (Table S1). The presence of nitrogen in the plant growth medium significantly affected nodulation and the number of nodules induced (Fig. 1A). Thus, the number of nodules in plants grown in BD + N and inoculated only with the symbiotic strain CpI1 was significantly lower compared with the number of nodules found in BD-N. Yamanaka et al. (2016) reported that the number of nodules formed on *A. sieboldiana* seedlings inoculated with *Frankia* was reduced when sufficiently supplied with mineral nitrogen. The seedlings appear to have no need to form nodules in a nitrogen-rich environment since nodule formation is energetically costly. This result is similar to those reported previously (Griffiths and McCormick 1984; Knowlton and Dawson 1983; MacConnell and Bond 1957; Smolander and Sundman 1987; Stewart and Bond 1961).

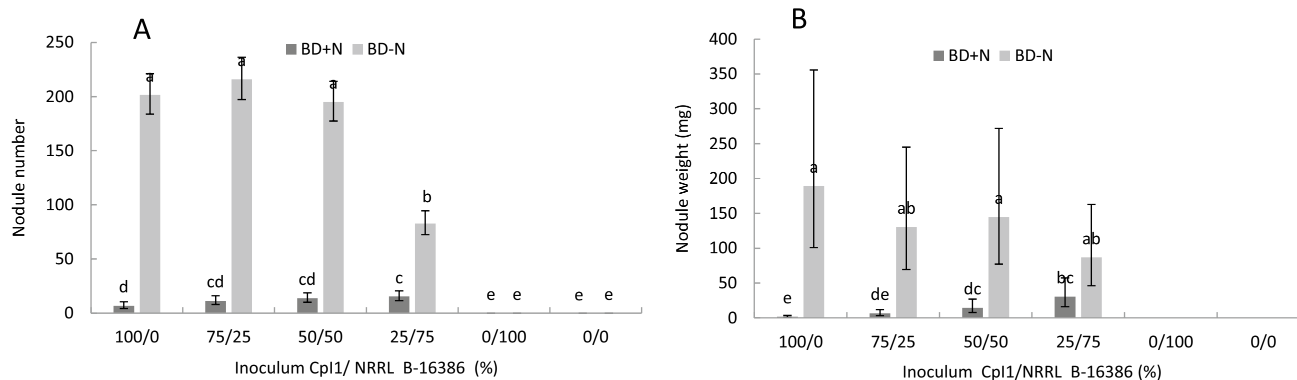

Fig. 1 Average nodule number (**A**) and dry weight (**B**) as function of CpI1/ NRRL B-16386 inoculum (in %). Bars represent the confidence interval. Values with different letters are significantly different at 0.05 (Tukey–Kramer test)

The number of nodules formed following co-inoculation with symbiotic strain CpI1 and the asymbiotic strain NRRL B-16386 was significantly reduced relative to inoculation only with strain CpI1 (Fig. 1A). These results seem to be in agreement with those obtained for *A. incana* plants (Kurdali et al. 1990) where the double inoculation showed that the spore positive strain reduced the number of nodules induced by the spore negative strain. Martin et al. (2003) reported that co-inoculation with two symbiotic *Frankia* strains, AvcI1 and ArI5, increases nodulation in *A. rubra* compared to simple inoculation with each strain.

Although the number of nodules induced in BD-N (Fig. 1A) was significantly different, the total nodule weight for each plant was not significantly affected (Fig. 1B, Fig. S2). Wheeler et al. (1981) and Hooker and Wheeler (1987) reported that, to some extent, increased nodule weight may be a compensatory effect for low specific activity in nitrogen fixation. For the rhizobia-Legume symbiosis, this compensatory mechanism has also been reported when inoculation of legume species was done by effective strains and in the presence of ineffective indigenous strains (Singleton and Stockinger 1983; Singleton and Tavares 1986).

Growth characteristics of seedlings assessed by stem length and dry weight (Fig. 2A, Figs. S3-S4) and roots (Fig. 2B, Figs. S5-S6), showed that nodulated seedlings grown in BD + N or in BD-N had the same growth performance than those grown. In BD-N, the growth of seedlings inoculated with *Franki*a strain CpI1 and seedlings co-inoculated with both strains at the same time did not differ significantly (Fig. 2, Figs. S3-S6). In fact, seedlings infected only with *Frankia* NRRL B-16386 showed no significant difference in growth compared with the negative control (non inoculated seedlings). This suggests that seedlings are not able to profit from the nitrogen fixed by asymbiotic strain NRRL B-16386 (Nouioui et al. 2017). Kurdali et al. (1990) show that co-inoculation with spore-positive (Sp +) and spore-negative (Sp-) strains did not influence the growth of the host plant. Growth parameters (length and dry weight) of the aerial part as well as root dry weight showed no significant difference compared to simple inoculation

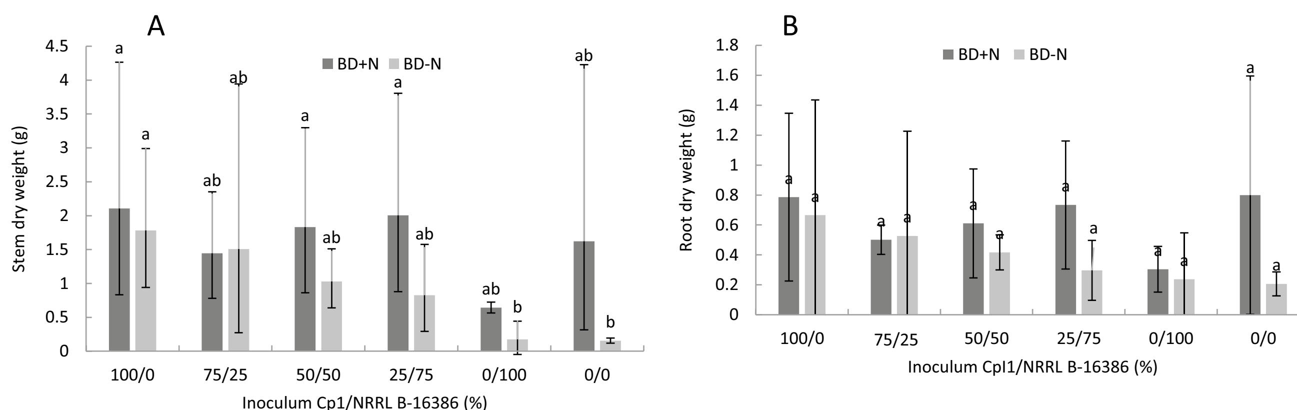

Fig. 2 Average dry weight of stem (**A**) and root (**B**) as function of CpI1/ NRRL B-16386 inoculum (in %). Bars represent the confidence interval. Values with different letters are significantly different at 0.05 (Tukey–Kramer test)

(Kurdali et al. 1990). Upon an analysis of eight *Alnus* species co-inoculated with an equal mixture of three symbiotic strains of *Frankia*: ACN1^{AG}, ACoN24d, and AG10^{AI}, Prat (1989) showed that co-inoculation generally provided better seedling growth than the best individual strain. Similarly, co-inoculation with AvcI1 and ArI5, two symbiotic *Frankia* strains, resulted in increased *A. rubra* seedling growth and nodulation compared to inoculation with each strain alone (Martin et al. 2003). Using PCR–RFLP analysis of the *gln*II gene, the authors reported that the ArI1-like pattern was most prevalent in the co-inoculation experiment. Another study showed that double infection of *A. glutinosa* plants with two strains of *Frankia*, one symbiotic and the other asymbiotic, caused an increase in plant growth but the number of nodules was not affected (Hahn et al. 1990). This suggests the various asymbiotic strains have diverse effect on the establishment and functioning of actinorhizal symbioses ranging from inhibition to stimulation.

Stem/root weight and length ratios showed that in the presence of nitrogen (BD + N), the seedlings tended to significantly expand the aerial part in terms of weight and length more than the root part (Fig. S7-S8). In BD-N and in the presence of the symbiotic CpI1 strain or both strains at the same time, the root part was significantly more developed than the aerial part compared to the negative control or to the seedlings inoculated only with the strain NRRL B-6386 (Fig. S7-S8). This root extension may be attributed to an expanding nutrients uptake afterward active nodule induction or to vitamins or phytohormones like auxins or cytokinine produced by the microsymbiont *Frankia* (Hahn et al. 1990).

The assay showed that the amount of total chlorophyll was significantly higher in the BD-N than in the BD + N medium (Fig. S9). In BD-N, seedlings inoculated with either strain CpI1 or both strains showed higher amounts of total chlorophyll than seedlings inoculated only with strain NRRL B-16386, which showed the lowest amount of total chlorophyll of all treated seedlings. However, this difference was only significant for seedlings inoculated with a 75%/25% mix (CpI1/NRLL B-16386) (Fig. S9). Although increased photosynthetic activity is expected to improve vital plant activities, it also appears essential to support the additional metabolic expense due to the intracellular growth of microsymbionts. Alder plants inoculated with *Frankia* strains have higher productivity as assessed by shoot and root length, dry matter production, and chlorophyll content (Vendan et al. 1999). These results illustrate that the symbiotic strain CpI1 promotes plant growth in a nitrogen-poor medium, whereas the nitrogen-fixing and asymbiotic NRRL B-16386 strain alone inhibits seedling growth and/or induces defensive plant immune response. It appears that this effect is delayed in the presence of the symbiotic strain CpI1. This may be due to evasion or suppression of host immune responses by symbiotic CpI1 effectors as has been suggested in the legume-rhizobia symbiosis (Yasuda et al. 2016). The symbiosis between *F. torreyi* CpI1 strain and *A. glutinosa* promotes plant growth even in a nitrogen-replete environment, while the presence of the CpI1 strain and the formation of nodules cover the nitrogen needs of the plant. Black alder co-infection did not influence plant development, but it did influence nodule number.

In conclusion, our current analysis suggests that the asymbiotic strain NRRL B-16386 did not greatly affect the symbiotic strain CpI1 during root nodule establishment and growth of *A. glutinosa* seedlings despite the increase in its amount along with the decrease in the amount of the symbiotic strain in the inocula. A compensatory mechanism for the nodule number may be achieved by increasing the nodule weight in order to sustain nitrogen-fixation rate and satisfy plant demand. The NRRL B-16386 strain did not penetrate inside root nodules, regardless of its amount in the inocula, but may occur as a contaminant of the nodule surface. The ability these asymbiotic strains to fix nitrogen in the rhizosphere should be assessed. Assessments of basic growth characteristics and nodulation of *A. glutinosa* seedlings upon dual inocula suggest that the competition result depends on the symbiotic capacity of strains used for inoculation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-022-00845-0.

References

Arnon D (1949) Copper enzymes in isolated chaloroplasts. Poly-phenoloxidase in beta vulgaris. Plant Physiol 24:1–15

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J 125:1075–1080

Callaham C, Deltredici P, Torrey JG (1978) Isolation and cultivation in vitro of the actinomycete causing root nodulation in *comptonia*. Science 199:899–902

Dommergues YR (1995) Nitrogen fixation by trees in relation to soil nitrogen economy. Fertilizer Res 42(1):215–230

Ghodhbane-Gtari F, Nouioui I, Boudabous A, Gtari M (2010) 16S–23S rRNA intergenic spacer region variability in the genus *Frankia*. Microb Ecol 60(3):487–495

Griffiths AP, McCormick LH (1984) Effects of soil acidity on nodulation of *Alnus glutinosa* and viability of *Frankia*. Plant Soil 79(3):429–434

Gtari M, Brusetti L, Cherif A, Boudabous A, Daffonchio D (2007) Heteroduplex structures in 16S–23S rRNA intergenic transcribed spacer PCR products reveal ribosomal interoperonic polymorphisms within single *Frankia* strains. J Appl Microbiol 103(4):1031–1040

Gtari M, Nouioui I, Sarkar I, Ghodhbane-Gtari F, Tisa LS, Sen A, Klenk HP (2019) An update on the taxonomy of the genus *Frankia* Brunchorst, 1886, 174 AL. Anton Leeuw 112(1):5–21

Gtari M, Tisa LS, Normand P (2013) Diversity of *Frankia* strains, actinobacterial symbionts of actinorhizal plants. In Symbiotic endophytes. Springer, Berlin, Heidelberg, pp. 123–148

Hahn D, Starrenburg MJC, Akkermans ADL (1990) Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains. Plant Soil 122(1):121–127

Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311(1):1–18

Hooker JE, Wheeler CT (1987) The effectivity of Frankia for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. Physiol Plantarum 70(2):333–341

Huguet V, Gouy M, Normand P, Zimpfer JF, Fernandez MP (2005) Molecular phylogeny of *Myricaceae*: a reexamination of host–symbiont specificity. Mol Phylogenet Evol 34(3):557–568

Huss-Danell K (1978) Nitrogenase activity measurements in intact plants of *Alnus incana*. Physiol Plantarum 43:372–376

Huss-Danell K, Myrold DD (1994) Intragenic variation in nodulation of *Alnus*: Consequences for quantifying *Frankia* nodulation units in soil. Soil Biol Biochem 26:525–531

Knowlton S, Dawson JO (1983) Effects of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia*. Can J Bot 61(11):2877–2882

Kohl SJ, Baker DD (1989) Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant Soil 118:171–179

Kurdali F, Domenach AM, Bardin R (1990) Alder-poplar associations: determination of plant nitrogen sources by isotope techniques. Biol Fertil Soils 9(4):321–329

Kurdali F, Domenach AM, Fernandez M, Capellano A, Moiroud A, De la Paz-Fernandez M (1988) Compatibility of *Frankia* spore positive and spore negative inocula with *Alnus glutinosa* and *Alnus incana*. Soil Sci Plant Nutr 34:451–459

Lalonde M (1979) Immunological and ultrastructural demonstration of nodulation of the European *Alnus glutinosa* (L.) Gaertn. host plant by an actinomycetal isolate from the North American *Comptonia peregrina* (L.) Coult. root nodule. Bot Gaz 140:S35–S43

Lechevalier MP (1986) Nitrogen-fixing actinomycetes of the genus Frankia. In: Megusar F, Gantar M (eds) Perspectives in Microbial Ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia, pp 253–256

MacConnell JT, Bond G (1957) A comparison of the effect of combined nitrogen on nodulation in non-legumes and legumes. Plant Soil 8(4):378–388

Martin KJ, Tanaka Y, Myrold DD (2003) Dual inoculation increases plant growth with *Frankia* on red alder (*Alnus rubra* Bong.) in fumigated nursery beds. Symbiosis 34:253–260

McEwan NR, Wilkinson T, Girdwood SE, Snelling TJ, Collins T, Dougal K et al (2017) Evaluation of the microbiome of decaying alder nodules by next generation sequencing. Endocyt Cell Res 28:14–19

Mirza BS, Welsh A, Rasul G, Rieder JP, Paschke MW, Hahn D (2009) Variation in *Frankia* populations of the *Elaeagnus* host infection group in nodules of six host plant species after inoculation with soil. Microb Ecol 58:384–393

Murry M, Fontaine M, Torrey J (1984) Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. Plant Soil 78:61–78

Nickel A, Pelz O, Hahn D, Saurer M, Siegwolf R, Zeyer J (2001) Effect of inoculation and leaf litter amendment on establishment of nodule-forming *Frankia* populations in soil. Appl Environ Microbiol 67:2603–2609

Nouioui I, Ghodhbane-Gtari F, Jando M, Tisa LS, Klenk HP, Gtari M (2019) *Frankia torreyi* sp. nov., the first actinobacterium of the genus Frankia Brunchorst 1886, 174 AL isolated in axenic culture. Anton Leeuw 112(1):57–65

Nouioui I, Gueddou A, Ghodhbane-Gtari F, Rhode M, Gtari M, Klenk HP (2017) *Frankia asymbiotica* sp. nov., a non-infective actinobacterium isolated from *Morella californica* root nodule. Int J Syst EvolMicrobiol 67(12):l4897-4901

Olivares J, Bedmar EJ, Sanjuán J (2013) Biological nitrogen fixation in the context of global change. Mol Plant Microbe Interact 26(5):486–494

Peoples MB, Craswell ET (1992) Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. Plant Soil 141(1):13–39

Pokharel A, Mirza BS, Dawson JO, Hahn D (2011) *Frankia* populations in soil and root nodules of sympatrically grown *Alnus* taxa. Microb Ecol 61:92–100

Pozzi AC, Bautista-Guerrero HH, Nouioui I, Cotin-Galvan L, Pepin R, Fournier P, Menu F, Fernandez MP, Herrera-Belaroussi A (2015) In-planta sporulation phenotype: a major life history trait to understand the evolution of *Alnus*-infective *Frankia* strains. Environ Microbiol 17:3125–3138

Prat D (1989) Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. Plant Soil 113(1):31–38

Prin Y, Neyra M, Diem HG (1990) Estimation of Frankia growth using Bradford protein and INT reduction activity estimations: application to inoculum standardization. FEMS microbiology letters 69(1–2):91–95

Ramírez-Saad H, Janse JD, Akkermans AD (1998) Root nodules of *Ceanothus caeruleus* contain both the N2-fixing Frankia endophyte and a phylogetically related Nod-/Fix-actinomycete. Can J Microbiol 44(2):140–148

Russo RO, Gordon JC, Berlyn GP (1993) Evaluating Alder-Endophyte (*Alnus acuminata-Frankia*-Mycorrhizae) Interactions: Growth Response of *Alnus acuminata* Seedlings to Inoculation with *Frankia* Strain Ar13 and Glomus intra-radices, Under Three Phosphorus Levels. J Sustain for 1(1):93–110

Roy M, Pozzi AC, Gareil R, Nagati M, Manzi S, Nouioui I et al (2017) Alder and the Golden Fleece: high diversity of Frankia and ectomycorrhizal fungi revealed from Alnus glutinosa subsp. barbata roots close to a Tertiary and glacial refugium. PeerJ 18:e3479. https://doi.org/10.7717/peerj.3479

Sanginga N, Danso SKA, Bowen GD (1989) Nodulation and growth response of *Allocasuarina* and *Casuarina* species to phosphorus fertilization. Plant Soil 118:125–132

Sayed WF (2011) Improving *Casuarina* growth and symbiosis with *Frankia* under different soil and environmental conditions. Folia Microbiol 56(1):1–9

Sempavalan J, Wheeler CT, Hooker JE (1995) Lack of competition between *Frankia* and *Glomus* for infection and colonization of roots of *Casuarina equisetifolia* (L.). New Phytol 130(3):429–436

Smith PK, Krohn RI, Hermanson GT et al (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85

Smolander A, Sundman V (1987) *Frankia* in acid soils of forests devoid of actinorhizal plants. Physiol Plant 70(2):297–303

Stewart WDP, Bond G (1961) The effect of ammonium nitrogen on fixation of elemental nitrogen in *Alnus* and *Myrica*. Plant Soil 14(4):347–359

Singleton PW, Stockinger KR (1983) Compensation against ineffective nodulation in soybean 1. Crop Sci 23(1):69–72

Singleton PW, Tavares JW (1986) Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. Appl Environ Microbiol 51(5):1013–1018

Vendan RT, Rajeshwari T, Thamizh R, Narayanan R (1999) Studies on strain specificity of *Frankia* in *Alnus nepalensis*. Trop Agric Res Ext 2:124–125

Vemulapally S, Guerra T, Hahn D (2019) Localization of typical and atypical *Frankia* isolates from *Casuarina* sp. in nodules formed on *Casuarina equisetifolia*. Plant Soil 435(1):385–393

Weber A, Sarsa ML, Sundman V (1989) *Frankia-Alnus incana* symbiosis: effect of endophyte on nitrogen fixation and biomass production. Plant Soil 120:291–297

Wheeler CT, McLmighlin ME, Steele P (1981) A comparison of symbiotic nitrogen fixation in Scotland in *Alnus giutinosa* and *Alnus rubra*. Plant Soil 61:169–188

Yamanaka T, Okabe H, Kawai S (2016) Growth and nodulation in *Alnus sieboldiana* in response to *Frankia* inoculation and nitrogen treatments. Trees 30(2):539–544

Yasuda M, Miwa H, Masuda S, Takebayashi Y, Sakakibara H, Okazaki S (2016) Effector-triggered immunity determines host genotype-specific incompatibility in legume–Rhizobium symbiosis. Plant Cell Physiol 57(8):1791–1800

Zhang Z, Lopez MF, Torrey JG (1984) A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of Casuarina species. Plant Soil 78:79–90

Zhong C, Zhang Y, Wei Y, Meng J, Chen Y, Bush D, Bogusz D, Franche C (2019) The role of *Frankia* inoculation in casuarinas plantations in China. Anton Leeuw 112:47–56

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.