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# *In Planta* Sporulation of *Frankia* spp. as a Determinant of Alder-Symbiont Interactions

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**ABSTRACT** The *Alnus* genus forms symbiosis with the actinobacteria *Frankia* spp. and ectomycorrhizal fungi. Two types of *Frankia* lineages can be distinguished based on their ability to sporulate *in planta*. Spore-positive (Sp+) strains are predominant on *Alnus incana* and *Alnus viridis* in highlands, while spore-negative (Sp−) strains are mainly associated with *Alnus glutinosa* in lowlands. Here, we investigated whether the Sp+ predominance in nodules is due to host selection of certain *Frankia* genotypes from soil communities or the result of the ecological history of the alder stand soil, as well as the effect of the sporulation genotype on the ectomycorrhizal (ECM) communities. Trapping experiments were conducted using *A. glutinosa*, *A. incana*, and *A. viridis* plantlets on 6 soils, differing in the alder species and the frequency of Sp+ nodules in the field. Higher diversity of *Frankia* spp. and variation in Sp+ frequencies were observed in the trapping than in the fields. Both indigenous and trapping species shape *Frankia* community structure in trapped nodules. Nodulation impediments were observed under several trapping conditions in Sp+ soils, supporting a narrower host range of Sp+ *Frankia* species. *A. incana* and *A. viridis* were able to associate equally with compatible Sp+ and Sp− strains in the greenhouse. Additionally, no host shift was observed for *Alnus*-specific ECM, and the sporulation genotype of *Frankia* spp. defined the ECM communities on the host roots. The symbiotic association is likely determined by the host range, the soil history, and the type of *in planta Frankia* species. These results provide an insight into the biogeographical drivers of alder symbionts in the Holarctic region.

**IMPORTANCE** Most *Frankia*-actinorhiza plant symbioses are capable of high rates of nitrogen fixation comparable to those found on legumes. Yet, our understanding of the ecology and distribution of *Frankia* spp. is still very limited. Several studies have focused on the distribution patterns of *Frankia* spp., demonstrating a combination of host and pedoclimatic parameters in their biogeography. However, very few have considered the *in planta* sporulation form of the strain, although it is a unique feature among all symbiotic plant-associated microbes. Compared with Sp− *Frankia* strains, Sp+ strains would be obligate symbionts that are highly dependent on the presence of a compatible host species and with lower efficiency in nitrogen fixation. Understanding the biogeographical drivers of Sp+ *Frankia* strains might help elucidate the ecological role of *in planta* sporulation and the extent to which this trait mediates host-partner interactions in the alder-*Frankia*-ECM fungal symbiosis.

**KEYWORDS** *Frankia*, actinorhizal symbiosis, *in planta* sporulation, *Alnus*, host specificity, ectomycorrhizae

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The role of the host species in the biogeography of symbiotic microorganisms has been intensively studied and has often confirmed codispersal patterns (1, 2), particularly for associations with a high level of specialization between the host and its symbionts. The impact of the symbiont's life history traits has been investigated far less, except for species of mycorrhizal fungi with different mechanisms of spore dispersion (3). Alders (*Alnus* spp., Betulaceae family) are involved in tripartite symbioses with ectomycorrhizal (ECM) fungi and nitrogen-fixing actinobacteria belonging to the *Frankia* genus. According to their subgenus or species, alders are associated with specific *Frankia* subgroups (4–6) and a few specific ECM fungi (7–10). This specificity has been confirmed on a worldwide scale, as the biogeography of ECM fungi and *Frankia* is mainly explained by their host biogeography and by abiotic factors, such as elevation or organic matter content (11–13). The life history traits of *Frankia* strains could also explain their high specificity and biogeography. Similar to what is seen with ECM fungi, spore dispersion probably differs among *Frankia* strains. On the one hand, some saprophytic strains only sporulate when cultured *in vitro* and probably in soils but never in the nodules (Sp− strains). On the other hand, uncultured strains are supposedly obligate symbionts and sporulate profusely within the host root nodules (Sp+ strains). These two sporulation phenotypes are, with few exceptions, genetically and phylogenetically distinct, yet they can occur at the same site or colonize the same host species (6, 14). The production of abundant asexual spores, which have high potential for propagation and for resistance to unfavorable conditions, may play an important role in the microbe's fitness and biogeography. Thus, the huge number of spores produced by Sp+ *Frankia* strains may enhance both their survival and dispersion capacity and could therefore explain their high infectivity and competitiveness on host plant roots compared to Sp− strains (15). In addition, spore production may compete with nitrogen fixation by using part of the plant's investment (photosynthesis products and energy) in the nodules and by occupying the infected cells with spores instead of diazovesicles (the specialized cells where nitrogen fixation occurs), thus limiting the benefits for the plant (14). Overall, these studies have led to the assumption that the ability to sporulate *in planta* is an important life trait in *Frankia* spp. (15, 16).

The abundance of Sp+ strains varies among *Alnus* species and stands. Indeed, some sites are exclusively nodulated by Sp+ or Sp− strains, while at other sites, both types cooccur in various proportions (17). Recent studies on European alder species demonstrated that Sp+ strains are dominant on *Alnus viridis* (or *Alnus alnobetula*) and *Alnus incana* strains and are far less abundant on *Alnus glutinosa* (13). Moreover, Sp+ strains from high-altitude alder stands were genetically very close to strains from boreal stands (high latitude) for a given *Alnus* taxon, suggesting that climate could be a driving factor in their distribution. However, since these three *Alnus* species are staggered according to altitude in subalpine, montane, and lowland areas (for *A. viridis*, *A. incana*, and *A. glutinosa*, respectively), the question remains whether the distribution of Sp+ strains reflects high dependency on the host (specificity) or selection by the environmental conditions prevalent at the different elevations of its host.

The phylogenetic clustering of Sp+ strains isolated from different host species (13) and their limited host range deduced from cross-inoculation experiments (15–18) suggest a stronger specificity of Sp+ strains to their host plant than that of Sp− strains. Compatible interactions between *Alnus* species and their specific *Frankia* strains may also affect their interaction with ECM fungi. Indeed, root nodule formation often precedes specific ECM association, and several studies have demonstrated that nodulation efficiency affects ECM diversity (10, 19, 20). As a consequence, and given that Sp+ and Sp− *Frankia* strains differ in their infectivities (15), nitrogen fixation activities (16), and secondary metabolite profiles (21), *in planta* sporulation of *Frankia* spp. may lead to marked differences in the ECM community structures of alders.

To clarify the ecological role of the *Frankia in planta* sporulation phenotype and to explore its consequences on host-symbiont interactions, we decided to study (i) whether the dominance of the Sp+ phenotype in *A. incana* and *A. viridis* stands in highlands can be explained by a preferential selection by the host (*A. incana* and

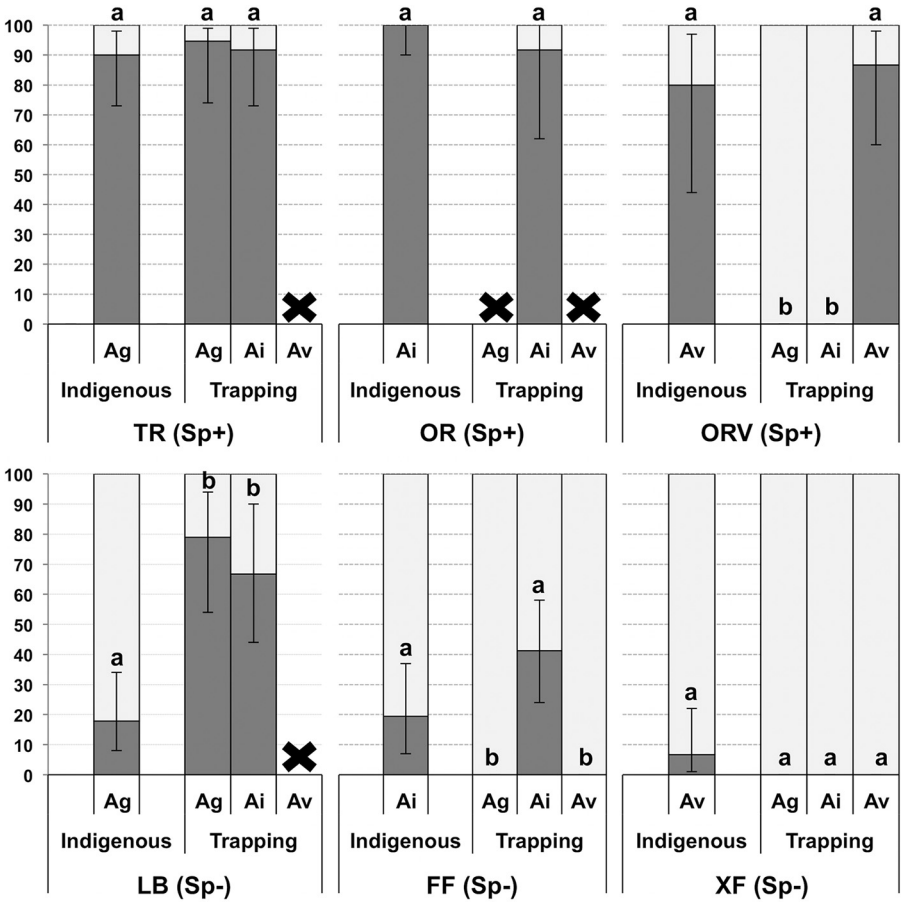


**FIG 1** Histograms of spore-positive percentages among indigenous host species nodules compared to trapping plant species nodules. Percentages were calculated using the estimation of the binomial law. Error bars correspond to 95% confidence intervals. Black crosses indicate an absence of nodules on the plantlets. Panels correspond to the combinations of indigenous host species (Ag, *A. glutinosa*; Ai, *A. incana*; Av, *A. viridis*) and of spore-positive (Sp+) or spore-negative (Sp−) soils. Lowercase letters indicate results obtained by the binomial law test.

*A. viridis* specificity) or by the environmental conditions, (ii) whether soil ecological history (*Frankia* genotype and presence over time of a given *Alnus* species) shapes soil symbiont communities, and (iii) whether the *Frankia* sporulation phenotype could affect ECM communities associated with alder roots. For this purpose, a full factorial plant-trapping experiment was performed with three *Alnus* species, used as traps, on six soils that differed in regards the alder species present (*A. glutinosa*, *A. incana*, or *A. viridis*) and the frequency of the Sp+ *Frankia* phenotype (high versus low) in the field. *Frankia* spp. and ECM fungi from seedling roots were identified, and community assemblages were compared under the different conditions. Furthermore, trapped *Frankia* spp. and ECM fungi were compared with the pool of *Frankia* spp. and ECM fungi sequenced from each native soil.

## RESULTS

**Trapping plant nodulation and *Frankia* Sp+ frequencies.** The number of nodules obtained on the different treatments ranged from 0 (no nodule trapped, e.g., *A. viridis* on Le Blanchet [LB] soil) to 405 nodules (i.e., *A. viridis* on Croix-de-Fer [XF] soil). A total of 168 field nodules and 236 trapping nodules were successfully phenotyped. The three Sp+ sites (Le Tremblay [TR], Ornon site 1 [OR], and Ornon site 2 [ORV]) and the three Sp− sites (LB, Fond-de-France [FF], and XF) harbored more than 80% and less than 20% of the Sp+ field nodules, respectively (Fig. 1). In trapping assays, the three Sp+ sites produced high Sp+ nodule frequencies when the same species present in the field was

**TABLE 1** Nodules per trapping plant for each plant-trapping assay

| Predominant phenotype | Alder stand (indigenous species) | Trapping species (avg ± SD) | | |
|---|---|---|---|---|
| | | *A. glutinosa* | *A. incana* | *A. viridis* |
| Sp+ | TR (*A. glutinosa*) | 3.2 ± 0.4 | 3.0 ± 0.5 | 0 |
| | OR (*A. incana*) | 0 | 3.7 ± 1.2 | 0 |
| | ORV (*A. viridis*) | 2.1 ± 0.3 | 2.1 ± 0.3 | 3.5 ± 0.9 |
| Sp− | LB (*A. glutinosa*) | 10.9 ± 1.0 | 10.8 ± 2.4 | 0 |
| | FF (*A. incana*) | 8.3 ± 0.8 | 9.4 ± 1.8 | 6.2 ± 1.4 |
| | XF (*A. viridis*) | 5.9 ± 0.8 | 5.3 ± 0.6 | 11.3 ± 1.4 |

[a]Spore-positive (Sp$^+$) and spore-negative (Sp$^-$) are the predominant types of *Frankia* strains observed in the field nodules from the 6 alder stand soils used for the trapping. Average values and standard deviations of the nodule numbers per plant are reported for each of the three trapping species (*Alnus glutinosa*, *Alnus incana*, and *Alnus viridis*).

used as a trapping species, with TR, OR, and ORV harboring 94.7, 91.7, and 86.7% of the Sp+ nodules on *A. glutinosa*, *A. incana*, and *A. viridis* plantlets, respectively (Fig. 1). The three Sp− sites harbored different Sp+ frequencies in the trapping experiment from those in the field (Fig. 1). Higher Sp+ frequencies were observed on LB soil when trapped with *A. glutinosa* and on FF soil when trapped with *A. incana* than those in the field (78.9% and 41.2% compared with 17.9% and 19.4%, respectively) (Fig. 1). For XF soil, the rare Sp+ nodules present in the field were not recovered from any *A. viridis* plantlets (6.7% versus 0% of Sp+ nodules) (Fig. 1).

When an alder species other than the indigenous one was used for trapping assays, contrasting results were obtained. *A. glutinosa* plantlets trapped only Sp− strains from FF (*A. incana* Sp− soil), XF (*A. viridis* Sp− soil), and ORV (*A. viridis* Sp+ soil), with average nodule numbers of 8.3, 5.9, and 2.1 per plant, respectively (Fig. 1 and Table 1). On OR (*A. incana* Sp+ soil), *A. glutinosa* plantlets formed only prenodules whose small size and early stage of development precluded any phenotype diagnostics. *A. incana* plantlets produced an average of 3.0 and 10.8 nodules per plant, of which 91.7 and 66.7% were Sp+ nodules, respectively, on TR and LB soils both coming from *A. glutinosa* alder stands in the field. Exclusively Sp− strains were trapped on ORV and XF soils, both corresponding to *A. viridis* alder stands in the field, forming 3.5 and 11.3 nodules per plant, respectively (Table 1). *A. viridis* plantlets formed an average of 6.2 nodules per plant on FF soil, all of them being Sp−. On the three other non-*A. viridis* soils (TR, LB, and OR), *A. viridis* plantlets did not form nodules, and most individuals died after a few weeks.

***Frankia* genetic diversity in nodules.** DNA extractions and *pgk* gene fragment (695 bp) amplifications were successfully conducted for 99 and 207 nodules from the field and the greenhouse trapping plants, respectively (i.e., about 16 per field site and 15 per trapping condition). None of the rarefaction curves obtained from *pgk* sequences of field and trapped nodules reached saturation (Fig. 2).

The richness of *Frankia* strains was higher in the trapped nodules than in field nodules (85.9 against 68 operational taxonomic units [OTUs], respectively; data not shown), regardless of the Sp+ or Sp− phenotype. *Frankia* Sp+ strains always harbored a lower level of richness than *Frankia* Sp− strains in both field nodules and trapped nodules (28 versus 42 OTU in field nodules, respectively, and 39.8 versus 45.9 OTU in trapped nodules, respectively).

The phylogenetic analysis revealed that all *pgk* sequences of the trapped *Frankia* strains could be grouped into three previously described clades (1, 4, and 5) (Fig. 3). Three subclades contained narrow-range strains (1a, 1b, and 5c trapped only by *A. viridis*, *A. incana*, and *A. glutinosa*, respectively), and two subclades contained large-range strains (4a, 4b, and 4c trapped by the three alder species used). Subclade 5a contained middle-range strains (trapped by *A. glutinosa* and *A. incana* but not *A. viridis*). All Sp+ strains trapped on *A. viridis* Sp+ soil (ORV) belonged to clade 1a, while all Sp+ strains trapped on both *A. incana* soils OR and FF were grouped into clade 1b. Clade 4 contained all Sp− *Frankia* strains trapped with the three trapping species on *A. incana*

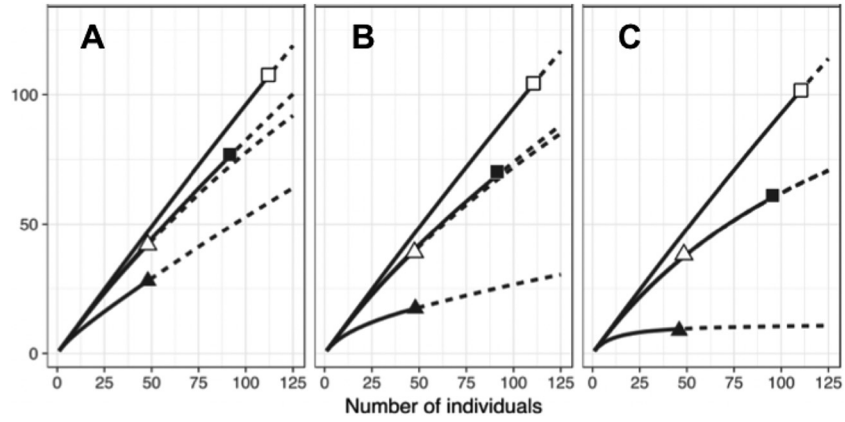


**FIG 2** Accumulation curves of *Frankia* richness (A), diversity (Shannon index) (B), and equitability (Simpson index) (C) estimated from *pgk* sequences at unique sequence threshold. Spore-positive (Sp+; filled symbols) and spore-negative (Sp−; open symbols) *Frankia* strains from indigenous host plant (squares) or trapping plants (triangles). Continuous line, interpolation; dashed line, extrapolation.

and *A. viridis* soils (FF, XF, and ORV), creating three subclades (labeled a, b, and d). Subclades 4d and 4b contained all Sp− strains trapped on ORV soil, subclades 4b and 4a contained all Sp− strains trapped on XF soil, and subclade 4a contained all Sp− strains trapped on FF soil. Sp+ and Sp− strains trapped with *A. glutinosa* and *A. incana* host species from both *A. glutinosa* soils, TR and LB, were grouped in clade 5. No strain of this clade 5 was trapped by the *A. viridis* host species.

***Frankia* community structure in soils and nodules resulting from trapping.** From the 1,310,346 reads obtained in the *nifH* run, 222,678 soil reads and 336,655 nodule reads were considered to be of good quality, with an average of 12,371 and 11,222 reads per sample, respectively. Sequences clustered at the 97% threshold into 163,020 soil reads and 199,432 nodule reads. For each sample, the first 250 *Frankia* OTUs at a 0.03 dissimilarity threshold, each containing a minimum of 200 sequences, were delineated. On average, 10,720 and 266 *Frankia* sequences per sample were obtained for the nodules and the soils, respectively. *Frankia* OTU compositions were compared between soils and trapped nodules. For all the sites studied, the most highly represented *Frankia* OTU in trapping nodules was not the most abundant in the soil (Data S1 in the supplemental material). The dominant OTUs in nodules represent between 3.9 and 18.5% relative abundance, depending on the soil. The predominant OTUs in each soil grouped together in a single clade, while predominant OTUs in nodules formed two distinct clades (data not shown).

To compare *Frankia* OTU compositions in the different soils and in trapped nodules, nonmetric multidimensional scaling (NMDS) was used. *Frankia* OTU compositions in soils were always significantly different from those in trapped nodules (Fig. 4A and B, $F = 14.1$, $R^2 = 0.23$, $P = 0.001$). There was no difference between indigenous soil communities, but the indigenous host species had a strong effect on OTU composition in trapped nodules (Fig. 4A, $F = 9.72$, $R^2 = 0.41$, $P = 0.001$). The trap plant species alone had no statistically significant structuring effect (Fig. 4B, $F = 1.61$, $R^2 = 0.10$, $P = 0.091$). However, there was an interaction between the indigenous species and the trap plant species that explained the *Frankia* OTU compositions in trapped nodules ($F = 7.88$, $R^2 = 0.71$, $P = 0.001$).

**Fungal and ectomycorrhizal diversity in soils and on plantlet roots.** After removing singletons and putative chimeras, 302,972 sequences could be assigned to fungi, of which 30,503 sequences (circa 10%) could be attributed to ECM taxa and 17,114 sequences could be assigned to 10 specific ECM species (Data S2). Species accumulation curves revealed that plantlets always associated with fewer fungi than did the soil samples. Considering ECM fungi, all trapping conditions led to a successful growth of ECM fungi on alder roots, and each alder species trapped more fungal and

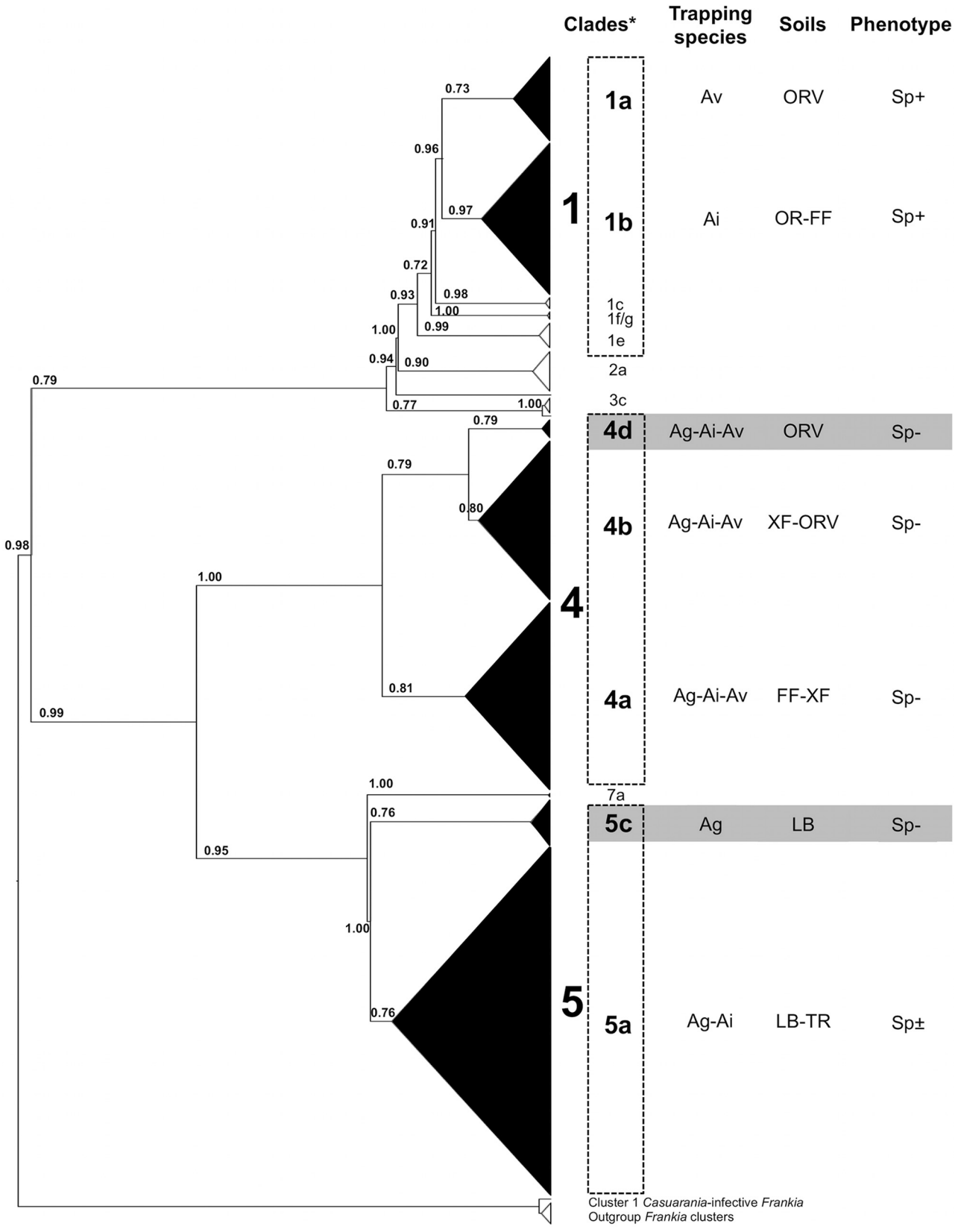


**FIG 3** Positions of trapped *Frankia* strains in the *pgk* phylogenetic tree of *Alnus*-infective (cluster 1) *Frankia* species. The phylogeny was estimated by maximum likelihood (PhyML). Statistical support of the nodes was estimated by aLRT SH-like method. Clades were annotated according to Pozzi et al. (6), and gray zones correspond to the 2 new subclades identified in the present study. Subclades in black are those that contain *Frankia* strains from our trapping assays. Clades 1, 4, and 5 correspond to OTUs 1, 4, and 5 (at the 0.05 threshold) in the present study.

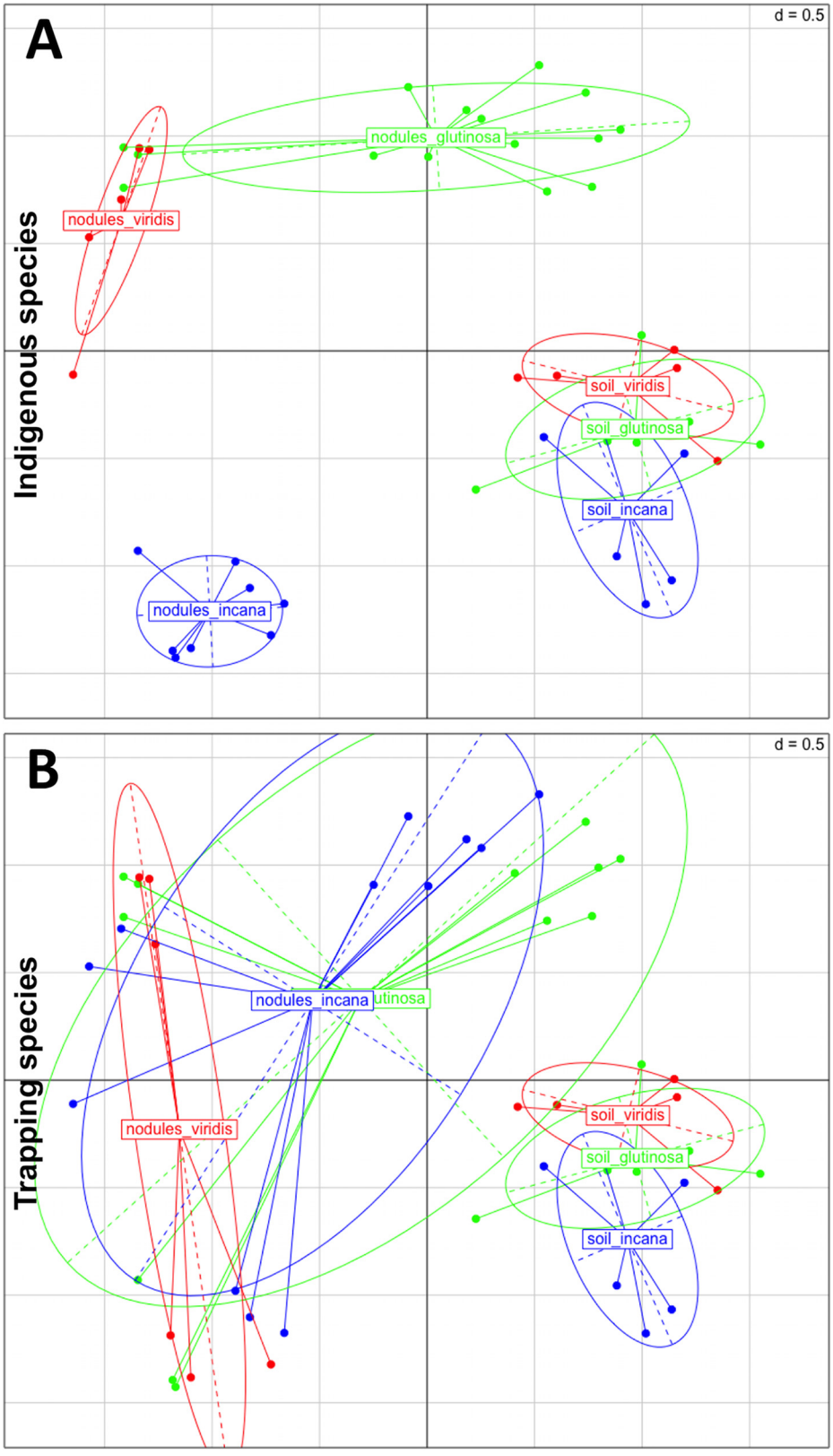


**FIG 4** Effect of the indigenous species (A) and the trapping species (B) on *Frankia* community structure in soils and in the trapped nodules, illustrated by nonmetric multidimensional scaling (NMDS). NMDS were performed on *Frankia* OTU matrices computed from *nifH* sequences. Colors refer to the alder species, both on the field and in the trapping experiment. *P* values of permutational multivariate analysis of variance (adonis function) are given for both factors tested (A and B). Ellipses are graphical overviews.

ECM OTUs than on its own soil (Data S3). The six specific ECM OTUs detected were associated with host species according to their known specificity pattern. No host shifts were detected. However, the identification of three *Alnicola* OTUs and 65 *Tomentella* spp. was not precise enough to investigate their specificity patterns and detect possible host shifts (Data S2).

In the soil, fungal and ECM species richness was not shaped by the same parameters. The sporulation phenotype present in the field had an effect on the number of different fungal species but not on that of ECM (Data S4). Instead, the variations in ECM species richness could be explained by the ecoregion (Data S4). The species richness measured on plantlets followed a different pattern, as the number of both fungal and ECM species was determined first by the indigenous host and, to a lesser extent for the ECM community only, by the trapping host and the ecoregion (Data S4). The soil community structure of the fungal and ECM community was shaped primarily by the indigenous host and then by the ecoregion and the sporulation phenotype present in the field. On plantlets, the fungal and ECM communities were shaped first by the indigenous alder species and then by the ecoregion, the trapping sporulation phenotype, the field sporulation phenotype and, to a lesser extent, the trapping host (Data S4). To summarize, species richness and community structures of all fungi and ECM fungi were mostly shaped by the indigenous host but also partly by the sporulation phenotype and its interaction with the indigenous host (ecoregion).

## DISCUSSION

***Frankia* diversity and distribution.** All trapped strain sequences were grouped into the previously described clades, sometimes constituting new subclades (4d and 5c) that had never been sampled during previous field studies at the same soil collection sites (6, 13). The seven subclades identified differed in their host specificities (i.e., narrow, middle, or large range) and the sporulation phenotypes of the strains. No other clear indication of phylogenetic clustering (trapping species or ecoregion) emerged from our results. The predominance of Sp+ *Frankia* strains in high-altitude zones, associated with *A. incana* and *A. viridis*, was thought to be a result of host species selection and/or climatic factors associated with the habitat (13). Indeed, in the field, very few *A. viridis* stands had a low Sp+ frequency (16, 22), suggesting that alpine habitats may promote Sp+ strains over Sp− strains. Conversely, in the trapping experience, we showed that all the strains trapped from alpine soils belonged to either clade 1 (all Sp+ strains) or 4 (all Sp− strains) and none to clade 5, no matter the trapping species that was used. Under greenhouse conditions, *A. incana* and *A. viridis* form nodules with the strains present, whatever their sporulation phenotype, but with the sole condition that they belong to clade 1 or 4. Thus, they did not actively select Sp+ strains over Sp− strains.

The trapping assays revealed a greater richness of *Frankia* species than previously described in the field, suggesting that greenhouse conditions could affect biomass and function of microbial populations. Both soil preparation (i.e., sieving and mixing) (23–25) and plant age and phenology (26) could influence the recruitment of soil microbial communities in the rhizosphere (27, 28). Young plants often exude substantially higher quantities of organic substances than do mature individuals, in particular phenolic compounds known to play a defensive role in plant-microorganism interactions (29, 30). For instance, age-related resistance (ARR) has been linked with the production of defense compounds (31, 32) that might affect alder symbiotic interactions.

Soils collected from *A. glutinosa* and *A. incana* Sp− alder stands (LB and FF, respectively) revealed higher Sp+ frequencies in trapping trials than with the field when their original alder species was used for the trapping. This result could be explained either by the underestimation of the proportion of Sp+ strains in the field or by the experimental conditions. Although the Sp+ strain proportions in the field have been confirmed by various research studies (data not shown), none of the available tools permit the identification of *Frankia* Sp+ and Sp− strains directly from soil

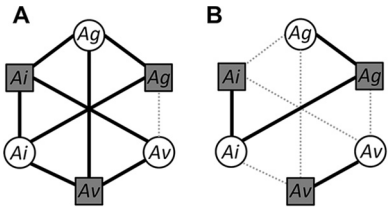


**FIG 5** Compatibility patterns between the plant-trapping species and the spore-negative (A) and spore-positive (B) soils used in the assays. The compatibility between the 3 trapping species (white circles) *Alnus glutinosa* (*Ag*), *Alnus incana* (*Ai*), and *Alnus viridis* (*Av*), and the 6 alder stand soils (gray squares) differing in the indigenous host species (*A. glutinosa*, *A. incana*, or *A. viridis*) is symbolized by a continuous line (presence of compatible strains and formation of mature nodules). The incompatibility is symbolized by a dotted gray line (impeded prenodules and no compatible strain trapped).

communities. Thus, the proportion of infective Sp+ propagules in the soil sampling spots is not known and might be higher than the proportion determined from the nodules. There are currently no available data to explain a difference in the sensitivities of the young plantlets toward Sp+ symbionts, although recent studies on the production of defensin-like peptides by *Alnus* spp. (33) and the detection of plant defense compounds differentially produced in Sp+ and Sp− nodules (21) are both promising lines of research.

This increased proportion of Sp+ strains on trapping plants compared to those under field conditions was not observed in *A. viridis* Sp− soil (XF). Based on crushed-nodule inoculations, Sp+ *Frankia* strains have been described as being about 100 to 2,000 times more infective than Sp− strains (13, 34–36). As discussed above, the low Sp+ frequency observed on nodules (XF soil) would be due to a low abundance in soil rather than host filtering. Therefore, the low probability of an encounter between the roots and Sp+ propagules might explain the finding that Sp− rather than Sp+ nodules are trapped in the greenhouse.

It is worth noting that whatever the trapping species used, Sp− soils always induce a higher number of nodules than do Sp+ soils, independently of the proportion of Sp+/Sp− strains obtained. The most likely hypotheses could be differences in rhizospheric *Frankia* abundance (discussed below) or differences in plant development between Sp+ and Sp− soils. This last hypothesis is supported by significant differences of plantlet root lengths when grown on Sp+ and Sp− soils (15.8 and 17.8 cm, respectively, $P = 0.01$; data not shown).

**Compatibility patterns between soils and trapping species.** Different patterns of compatibility were found between soils and trapping plants depending on the alder species and the proportion of Sp+ nodules present in the field (Fig. 5). *Frankia* strains isolated from alders were long thought to belong to a unique host specificity group, i.e., a group of strains sharing the same compatible hosts, in this case plant species that belong to the *Alnus* genus and Myricaceae (37–40). While this *Alnus*-Myricaceae specificity group concept was confirmed for most *Alnus*-cultured strains, cross-inoculation experiments, using crushed nodules as inocula, suggested that Sp+ strains had a narrower host range than that of Sp− strains (15–18, 41). However, the use of crushed nodules may have three major side effects. First, the presence of plant secondary metabolites could prevent root-*Frankia* recognition and association (5). Second, the inoculum concentration used is generally much higher than the natural *Frankia* concentration in the field (42, 43). Finally, due to the dominance of one strain in the nodules, only a few strains were tested, and this was not representative of the diversity of *Frankia* communities in soils (44), thus giving a simplistic version of the *Alnus-Frankia* compatibility patterns compared with the trapping experiments.

Our results mirrored the narrower host range of Sp+ strains previously described, since most incompatibility patterns concerned Sp+ soils (Fig. 5). Indeed, Sp+ *A. incana* soils and Sp+ *A. viridis* soils never led to compatible associations when alder species other than the indigenous field species (*A. incana* and *A. viridis*, respectively) were used

for trapping (Fig. 5B), since nodules were either absent or very small (impeded prenodules) and not functional (absence of diazovesicles). Moreover, our results corroborate the lack of compatibility between *A. incana* Sp+ strains and *A. glutinosa* hosts, which were previously described using crushed-nodule inocula (15, 16). This incompatibility is even more marked in our soil trapping experiment (no nodules formed) compared to the crushed-nodule inoculations (less infectivity). The high *Frankia* density in crushed-nodule inoculations could force the occurrence of some symbiotic associations never observed in the field, thus indicating an artificially broad host range (15). This result suggests that although *A. incana* Sp+ strains are genetically capable (symbiotic signalization is not lacking) of infecting *A. glutinosa* roots, they actually display low infectivity on this host species.

Equally, several results suggest very narrow specificity patterns between the *A. viridis* host and its associated *Frankia* strains, and especially for the Sp+ strains, as follows: (i) the absence of nodulation of *A. viridis* plantlets on *A. glutinosa* soils, suggesting a total incompatibility between *A. viridis* and clade 5 strains, whatever their sporulation phenotype (Fig. 3); (ii) the absence of nodulation of *A. viridis* plantlets on *A. incana* Sp+ soil, although strains from subclade 1b (all *A. incana* Sp+ strains) and subclade 1a (*A. viridis* Sp+ strains) are genetically close (Fig. 3); and (iii) the strict specificity of *A. viridis* Sp+ strains from subclade 1a that were exclusively trapped by *A. viridis* plantlets. This result is consistent with findings from previous studies (6, 13) where Sp+ strains from clade 1a were described as a monophyletic group considered to be highly specific to their host.

The colonization of a new environment by nonindigenous nitrogen-fixing plants (as shown with a cross-trap plant experiment) often leads to the establishment of novel associations through the recruitment of cosmopolitan strains, implying the presence of compatible low-specificity strains in the soil (45–47). In light of our results, the cosmopolitan status of *Frankia* strains in alder stand soils mainly depends on their Sp+ or Sp− identity. In contrast to earlier hypotheses (48, 49), our findings suggest that specificity within a particular set of mutualists may result in a failure of some alder species to colonize new habitats.

**Impact of alder stand soil history and *Frankia* sporulation phenotype on symbiont diversity.** The next-generation sequencing (NGS) approach, targeting both bacterial and fungal symbionts, was used to determine the extent to which soil communities are shaped by the host species in the field, to explain the symbiotic associations observed on the trapping plants, and to test the potential impact of the *in planta* sporulation of *Frankia* spp. on root ECM fungi.

Although soil *Frankia* communities were the same among the three alder species in the field, differences in *Frankia* communities in trapped nodules were observed according to the field host species. These data indicate that, in agreement with findings from a previous study (50), host species in the field determined the nodule-forming populations of *Frankia* species. Nodule formation has been shown not to be a function of *Frankia* population abundance (51). Similarly, in this study, the sequences of trapped *Frankia* strains harbored relatively low abundances compared to those of soil-dwelling *Frankia* strains, possibly indicating that our metabarcoding approach did not allow for the detection of differences among soil *Frankia* communities beneath the different alder species.

The use of NGS on alder roots revealed mostly ECM fungi; however, sequences were too short to allow a deeper investigation of fungal specificity. By searching for single nucleotide polymorphisms, we did identify some of the *Alnus* symbionts, and, interestingly, all the associations observed in the experiment were congruent with the current information about *Alnus* specificity (8). Here, more ECM fungi were observed on plantlets than in their own soil, probably because, in addition to specialist ECM fungi, general ones were also recruited. This pattern is often observed on *Alnus* spp. in new environments, as recently shown in the case of invasion in New Zealand (48) and in an isolation case in Corsica (52). In these studies, generalist fungi never dominate *Alnus*

**TABLE 2** Factorial plan and conditions

| | Soils (indigenous host)[a] | | |
|---|---|---|---|
| **Sporulation phenotype** | ***A. glutinosa*** | ***A. incana*** | ***A. viridis*** |
| Sp+ | TR | OR | ORV |
| Sp− | LB | FF | XF |

[a]For each condition, these 3 same trapping plant species were used.

roots, and we observed the same pattern in our study, as *Tomentella* and *Alnicola* represented the most abundant genera for the three hosts.

In the context of the numerous studies and reviews on *Alnus*-ECM specificity, our results highlight the importance of the indigenous host and, therefore, the soil history on specificity, and we have also shown that the three hosts can grow and develop ECM on other soils. Interestingly, *A. incana* grew well on both its own soil and on *A. glutinosa* ones, a characteristic that might be correlated with its ability to grow in a wide altitudinal range. As for *Frankia* spp., the indigenous host always had a stronger effect than the trapping host on the fungal and ECM community and diversity. This effect is probably due to the following two major factors: the soil chemistry, when it is particularly distinct between the sites and the hosts (9), and the spore bank.

We investigated the influence of the *Frankia* sporulation phenotype, both predicted from the ecoregion (the soil and the indigenous host present) and as observed on the trapping plantlets. We detected a significant effect of the sporulation phenotype (both predicted and observed) on the ECM community structure but not on ECM species diversity, suggesting that the abundance may change but the identity of the fungi does not. This effect of the sporulation phenotype was often stronger than the trapping host effect, suggesting that the specificity could result from a strong interaction between the host and its diverse symbionts. Several clues suggest an intimate relationship between *Frankia* spp. and ECM fungi (10), and plant investment to nodules and mycorrhizae might depend on their cooccurrence on the host. Here, we did not measure plant investment, but we observed that ECM communities are partly determined by *Frankia* spp. and their phenotype of *in planta* sporulation, even on young plantlets. The underlying mechanism still needs to be deciphered. As the profuse sporulation of *Frankia* spp. within the nodules would require sizable amounts of carbon (spore coat layers), nitrogen (DNA bases for genome copy), and possibly phosphorus (DNA bases and replication enzymes) from the host plant, we hypothesize that the sporulation phenotype of *Frankia* spp. may modify the levels of these elements in the host plant to such an extent that it might mediate host-partner interactions, and even partner-partner interactions, through the host, particularly when these partners are also involved in the C:N:P economy of the symbiotic system.

## MATERIALS AND METHODS

**Sites and sampling.** Six well-developed alder stands about 100 years old were selected in the French alpine region to include three *Alnus* species (*A. glutinosa*, *A. incana*, and *A. viridis*) and the two *Frankia* sporulation phenotypes (Sp+ and Sp−), resulting in 6 distinct ecoregions. These sites included two lowland *A. glutinosa* stands, Le Blanchet (LB) and Le Tremblay (TR); two montane *A. incana* stands, Fond-de-France (FF) and Ornon site 1 (OR); and two subalpine *A. viridis* stands, Croix-de-Fer (XF) and Ornon site 2 (ORV). LB, FF, and XF alder stands had been previously identified as Sp− sites (sites harboring predominantly Sp− nodules), while TR, OR, and ORV were identified as Sp+ sites (13) (Table 2). For each site, soil samples were collected in mid-autumn at 3 different points (0 to 10 cm depth without the upper organic layer), 50 m apart, sieved at 4 mm, and mixed to form a composite soil sample. Three subsamples of 0.5 g from each composite sample were frozen for subsequent genomic analyses. About 25 root nodules were also collected per site from at least 5 alders.

**Plant growth and trapping experiments.** Seeds from the three *Alnus* species (*A. glutinosa* from Grand Lemps, Rhône-Alpes, and *A. incana* and *A. viridis* from Vanoise National Park, France) were sterilized for 30 s in absolute ethanol and then rehydrated, for 48 h at 4°C, under magnetic stirring in sterile water. On each of the six soils, seed germination, plantlet precultivation for 3 weeks, and trapping experiences were conducted. The greenhouse conditions were 16 h of daylight at 10,000 lx, 22°C, and 60% humidity and 8 h of dark at 18°C and 75% humidity. For each soil, 36 plantlets were used for each of the 3 host species (*A. glutinosa*, *A. incana*, and *A. viridis*). Plants were watered twice a week with sterile deionized water, and plant positions within the climatic chamber were randomized weekly. After 4 to 6

months of growth, depending on the species growth rate, plants were harvested. Nodules collected from roots were counted and used for *Frankia* phenotypic and genotypic analyses. Circa 50 root tips per seedling were sampled randomly and used for ECM fungal genotypic characterization.

From each field and trapping nodule, two adjacent lobes were sampled, with one lobe to determine the sporulation phenotype and the other one to genotype the *Frankia* strains. About of 25 field nodules per site and 15 nodules per trapping condition were phenotyped and genotyped.

**Sporulation phenotype determination of nodules.** The sporulation phenotype was determined by microscopic observation of hand-cut sections of nodule lobes stained with lactophenol blue (Réactifs RAL, Martillac, France), as previously described (13). The lactophenol blue stain discriminates between spores from hyphae (refringent and nonstained) and those from diazovesicles (stained deep blue). Nodules were considered to have the Sp+ phenotype when more than one sporangium was observed out of 50 infected plant cells, and the others were Sp−. The proportion of Sp+ strains was estimated for each trapping condition.

**DNA extraction from nodules and *pgk* gene sequencing.** Genotyping consisted of targeting a partial sequence (695 bp) of the housekeeping gene *pgk* coding for the phosphoglycerate kinase. Total nodular DNA was extracted from each nodule lobe individually, using the method previously described (13). For each lobe, amplification was performed using the specific primers and PCR protocol previously described (13). PCR products were single-strand sequenced by Biofidal-DTAMB (Villeurbanne, France) using the Sanger method with the same set of primers. *pgk* sequences were checked, trimmed, and manually corrected using 4Peaks version 1.7.2 (53). Sequences were confirmed to belong to *Frankia* using BLASTN searches against the National Center for Biotechnology Information (NCBI) databases (54) and aligned using the Muscle version 3.8.31 package (55) in SeaView version 4.4.2. The distance matrices were calculated using the DNADIST program. OTU matrices were computed from the alignment in mothur version 1.31.2 (56), with the furthest neighbor clustering and using 0.0049 as the maximum pairwise distance between sequences from an identical OTU. Accumulation curves of *Frankia* richness were computed from the matrices using the iNEXT package in R version 3.0.1 (57), at a 95% confidence interval.

Phylogenetic analyses were performed from our aligned nucleotide sequences and pooled with sequences previously submitted (13), using the maximum likelihood method in the software PhyML (53) with a general time reversible (GTR) + G4 model and the NNI + SPR option for topology exploration. Topologies were rooted with *Frankia pgk* sequences that did not belong to the *Alnus* infective cluster 1 (outgroup). The branch robustness of maximum likelihood (ML) trees was estimated by the approximate likelihood ratio test (aLRT) using the nonparametric Shimodaira-Hasegawa-like branch test (58), implemented in SeaView version 4.4.2. Clades were delineated on the basis of the strain habitat (host plant and/or site) and the *in planta* sporulation phenotype, as previously proposed (13), and using the same numbering (6).

***nifH* metabarcoding analyses from soils and trapped nodules.** A metabarcoding approach was used to assess the *nifH* bacterial community structure from soils and nodules. Soil DNA was extracted from defrosted soil samples using the PowerSoil DNA kit (Qiagen), according to the manufacturer's instructions. Three plants of each trapping condition were randomly selected, and about 8 nodules per plant were sampled for the same DNA extraction protocol previously described (59). Soil and nodule nucleic acid solutions were amplified using IGK3/DVV primers (60), and the PCR conditions were the same as previously described (42). Soil and nodule barcoded amplicons were pooled and used as the template for a single run of Illumina MiSeq sequencing, using the paired-end sequencing technology (2 × 250 bp) at the Genotoul GIS facility, Toulouse, France.

*nifH* reads were processed using the open-source software mothur (version 1.38.0) (56), following the method previously described (42). Once the reads had been processed and the OTUs had been delimited at a 97% dissimilarity threshold, the first 250 most abundant OTUs were screened against the nucleotide database of the National Center for Biotechnology Information (NCBI), using the BLASTn tool to identify *Frankia* OTUs.

**Global and ectomycorrhizal fungal diversities in soils and on plantlet roots.** Root apices were harvested with sterile tweezers and stored in cetyltrimethylammonium bromide (CTAB) buffer until DNA extraction. DNA extraction was performed as previously described (9). The internal transcribed spacer 1 (ITS1) was chosen as a barcode and amplified, both on root apex extracts and on soil DNA, as previously described (42). All ITS1 amplicons were pooled and sequenced on a single run of Illumina MiSeq sequencing. Sequence analysis, according to Schwob et al. (42), enabled us to attribute sequences to seedlings or to soil samples, to group sequences in OTUs at a 97% threshold, and to assign sequences to fungal taxa based on a comparison with GenBank sequences using ecotag in the OBITools package (61). To determine the specificity of the ECM symbionts, sequences belonging to *Alnicola*, *Alpova*, *Lactarius*, *Melanogaster*, *Paxillus*, and *Russula* spp. were aligned with the previously published sequences of specific species associated with alders (8, 9). The alignments, handled with MAFFT (62), were restricted to the ITS1 region. These included reference sequences and all sequences attributed to a given genus. These alignments allowed sequences to be grouped based on their shared single nucleotide polymorphism, and these groups could be identified to the species level. Sequence assignation enabled us to subset ECM OTUs for further analysis. Accumulation curves of ECM OTU richness were computed from the matrices using an iNEXT package in R version 3.0.1 at a 95% confidence interval.

**Community and statistical analyses.** Ninety-five percent confidence intervals of Sp+ frequencies were estimated using the normal approximation of the binomial distribution with the function *binom.test* implemented in the R software (63). Comparisons of Sp+ frequencies on trapping plants, versus those in the field, were performed using the same function. Fungal and ECM OTU richness was computed for each seedling and soil sample using the ade4 package (64). Differences between ecoregions (indigenous

host × dominant *Frankia* phenotype) and the effect of trapping host or indigenous host were tested on plantlet samples using analysis of variance (ANOVA). The Bray-Curtis distance matrices between communities, sampled on seedlings or in the soil, were computed for each gene using the vegan package (65). Bray-Curtis matrices were used to perform nonmetric multidimensional scaling (NMDS) with the *metaMDS* function available in the vegan package (65). The effect of the indigenous host species on fungal, ectomycorrhizal, and *Frankia* communities in soils was tested through a permutational multivariate analysis of variance (PERMANOVA), using the *adonis* function in the vegan package. Likewise, the effect of the trapping host species, the dominant spore phenotype, and the indigenous host species were tested on fungal, ECM, and *Frankia* communities on trapping plantlets. The order in the models was permuted to determine which factor could best explain the variations, and, finally, the model with the least residuals was chosen.

**Data availability.** All *Frankia pgk* sequences were previously deposited with EMBL (https://www.ebi.ac.uk/ena) under accession numbers LT599837 to LT600328. Fastq files were deposited with EMBL under BioProject PRJEB26577 and accession number ERS2462111 for bacteria and under BioProject PRJEB18608 and accession number ERS1473494 for fungi.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01737-18.

**SUPPLEMENTAL FILE 1,** PDF file, 1.1 MB.

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