Control of Endophytic *Frankia* Sporulation by *Alnus* Nodule Metabolites

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A unique case of microbial symbiont capable of dormancy within its living host cells has been reported in actinorhizal symbioses. Some *Frankia* strains, named Sp+, are able to sporulate inside plant cells, contrarily to Sp– strains. The presence of metabolically slowed-down bacterial structures in host cells alters our understanding of symbiosis based on reciprocal benefits between both partners, and its impact on the symbiotic processes remains unknown. The present work reports a metabolomic study of Sp+ and Sp– nodules (from *Alnus glutinosa*), in order to highlight variabilities associated with in-plant sporulation. A total of 21 amino acids, 44 sugars and organic acids, and 213 secondary metabolites were detected using UV and mass spectrometric-based profiling. Little change was observed in primary metabolites, suggesting that in-plant sporulation would not strongly affect the primary functionalities of the symbiosis. One secondary metabolite (M27) was detected only in Sp+ nodules. It was identified as gentisic acid 5-*O*-β-D-xylopyranoside, previously reported as involved in plant defenses against microbial pathogens. This metabolite significantly increased *Frankia* in-vitro sporulation, unlike another metabolite significantly more abundant in Sp– nodules [M168 = (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-glucopyranoside]. All these results suggest that the plant could play an important role in the *Frankia* ability to sporulate in planta and allow us to discuss a possible sanction emitted by the host against less cooperative Sp+ symbionts.

Bacterial sporulation is a complex process resulting in the production of metabolically dormant cells—or spores—generally established to escape from nutritionally local conditions unfavorable to growth (Dworkin and Shah 2010). In soil, many bacterial populations spend part of their lives as spores to deal with environmental assaults. Among the most studied, *Bacillus subtilis* forms remarkably resistant endospores (e.g., to heat, desiccation, radiation, and chemical insult) in response to nutrient starvation, *Streptomyces* spp. differentiate, in the latter stages of their life cycle, chains of conidiospores from aerial hyphae. In addition to temporal escape, spores can also be spatially relocated (via wind, water, living hosts) to favorable environments for

germination and vegetative growth (Nicholson et al. 2000). Bacterial sporulation, therefore, represents a highly successful strategy for survival and spatial dispersion of bacterial life. However, a unique case of endophytic symbionts able to sporulate inside host plant cells has been reported in some *Frankia* strains. Though endosymbionts, which benefit from a highly favorable habitat for their development and trophic interactions with their hosts (Torrey 1987; Schwintzer 1990), these strains produce sporangiospores with respiration rates 10-fold lower than those for vegetative hyphae (Krumholz et al. 2003). Observing such sporulation directly within host cells is, thus, quite unexpected in a symbiotic context.

The actinobacteria *Frankia* are able to establish symbiotic associations with actinorhizal plants (e.g., members of genera *Alnus* and *Casuarina*), resulting in root nodules in which carbohydrates provided by the host plant are exchanged for nitrogen supplied by the bacteria. In this model, clear differences in microbial partner functional traits have been found, manifested as different types of nodules (van Dijk 1978; van Dijk and Merkus 1976). All available cultured *Frankia* strains form both vesicles (nitrogen-fixation site) and sporangiospores within sporangia under some cultural conditions. However, in the nodule, two types of strains have been described: Sp+ strains (spore-positive), forming numerous spores within host plant cells, contrarily to Sp– strains, incapable of in-plant sporulation (Pozzi et al. 2015; Schwintzer 1990; Simonet et al. 1994; Torrey 1987). In Sp+ nodules, bacterial spore production inside plant cells is often massive (up to 10⁸ spores per gram of nodules) and occurs very early in nodule differentiation (Cotin-Galvan et al. 2016). Given that spore formation was generally described as an energy-intensive and time-consuming process, it raises the question of the impact of in-plant bacterial sporulation on both partner interactions and the host influence on this microbial trait. Previous studies suggest that Sp+ strains are less efficient in nitrogen fixation than Sp– strains (Houwens and Akkermans 1981; van Dijk 1984; Vandenbosch and Torrey 1984; Wheeler et al. 1986), which could affect carbon and nitrogen exchanges between plant and bacteria in Sp+ nodules. Sp+ strains may trade off their own multiplication by producing considerable sporangia in host cells at the expense of mutualistic behavior (Pozzi et al. 2015). This trade-off could result in sanctions from the host (Denison 2000; Kiers et al. 2003), leading to increased plant defense mechanisms in Sp+ nodules compared with Sp– nodules. Important modifications in nodule primary and secondary metabolites can, thus, be hypothesized due to *Frankia* in-plant sporulation.

The purpose of the present study is to compare Sp+ and Sp– nodule metabolome, in order to highlight modifications associated with in-plant sporulation, using UV and mass spectrometric-based metabolite profiling. This metabolomic approach aims to identify all metabolites in a cellular system and to monitor their

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quantities. In recent years, it has become a powerful tool to understand the complexity of plant systems, used for chemotaxonomy or metabolic change characterizations after biotic and abiotic stresses (Abdel-Farid et al. 2014; Allwood et al. 2010; Ott et al. 2003; Pedras and Zheng 2010), among available applications. Here, we compared the metabolomic diversity of a large number of Sp+ and Sp− nodules from *Alnus glutinosa* (48 samples, including associated-root samples). These nodules were all collected from the field, in order to take into account the diversity of Sp+ and Sp− *Frankia* strains associated with the *A. glutinosa* host (Pozzi et al. 2015). They came from six different alder stands previously described as “Sp+ sites” or “Sp− sites” (Pozzi et al. 2015). Chemical extractions were performed for all samples and resulting extracts were analyzed by ultra-high pressure liquid chromatography (UHPLC)/UV/ESI-MS (electrospray ionization-mass spectrometry) for secondary metabolite profiling. Analyses of amino acids were achieved through HPLC/UV, after online derivatization, and analyses of sugars and organic acids (SOA) through gas chromatographic (GC)/EI-QQQ (triple quadrupole). Discriminant metabolites between Sp+ and Sp− nodules were characterized and tested for their effects on *Frankia* respiration and in-vitro sporulation.

RESULTS

A total of 48 samples (15 Sp+ nodule samples, 14 Sp− nodule samples, and 19 associated-root samples) were successively extracted in methanol/water 50:50 (vol/vol) and pure methanol. The average dried extract quantity obtained from all samples was $29.61 \text{ mg} \pm 3.13 \text{ mg}$.

Characterization of discriminant primary metabolites between Sp+ and Sp− nodules: SOA and amino acids.

The quality control (QC), made from 12 standard sugars and 5 μl of each sample (as described below), and samples were analyzed by GC/EI-QQQ. A total of 44 SOA were detected from the total ion current (TIC) chromatogram (SOA 1 to 44 [Supplementary Data S1]). The principal component analysis (PCA) of sample metabolic profiles showed a slight discrimination between Sp+ and Sp− nodules, with PC1 and PC2 scores of 12.19 and 7.62%, respectively (Fig. 1A).

Sixteen SOA showed significant differences in their relative abundances (i.e., relative peak area calculated over the total area of the sample chromatogram, expressed as a percentage) between both types of nodules (Wilcoxon rank sum test, P value < 0.05). SOA 16 (glucose) was the most abundant metabolite in all nodule samples, with a relative abundance significantly higher in Sp+ nodules. Among these 16 SOA, three were at least two times more abundant in Sp+ nodules: SOA 9 (fumarate), SOA 23 (annotated as D-galactose), and SOA 33 (annotated as melezitose). Five SOA were at least two times more abundant in Sp− nodules: SOA 5 (annotated as xylopyranoside), SOA 12 (annotated as D-mannitol), SOA 32 (annotated as sucrose), SOA 38 (annotated as melibiose), and SOA 40 (unidentified) (Table 1). The most different metabolites between Sp+ and Sp− nodules were metabolites annotated as D-galactose, 12-fold more abundant in Sp+ nodules, and D-mannitol and sucrose, sevenfold more abundant in Sp− nodules. In addition, the nodule mean relative abundance of each of the nine previously mentioned metabolites was computed from all nodule samples, including Sp+ and Sp−. Their root mean relative abundance was computed

Fig. 1. Principal component analysis of Sp+ (including *Frankia* spores in plant cells) and Sp− (without *Frankia* spores) nodule metabolic profiles for **A**, sugars and organic acids, **B**, amino acids, and **C**, secondary metabolites. Graphic representations were obtained with factors one and two.

in a similar way for all associated-root samples (Table 1). Fumarate and metabolites annotated as D-galactose, melezitose, and melibiose showed a significantly higher abundance in nodules compared with roots (Wilcoxon rank sum test, P value < 0.05), with melibiose being up to 25-fold more abundant. Glucose, in contrast, was significantly more abundant in roots.

A total of 21 amino acids were detected using LC/UV-DAD (diode array detection) analysis and were identified by comparison with the standard mixture (QC). Although the PCA did not show clear discrimination in nodule metabolic profiles according to their in-plant sporulation phenotype (Fig. 1B), three amino acids highlighted significantly different abundances between Sp+ and Sp- nodules, namely, citrulline, arginine, and valine (Wilcoxon rank sum test, P value < 0.05). Citrulline and arginine were more abundant in Sp- nodules, while valine was more abundant in Sp+ nodules (Table 1). In addition, citrulline and arginine also showed a significantly reduced relative abundance in nodules compared with roots (Wilcoxon rank sum test, P value < 0.05).

Characterization and identification of discriminant secondary metabolites between Sp+ and Sp- nodules.

The LC/UV/ESI-Q-TOF (quadrupole-time-of-flight) chromatograms of all 48 samples led to the detection of 213 secondary metabolites (M1 to M213). The PCA of these metabolites showed a strong discrimination between Sp+ and Sp- nodules, with PC1 and PC2 scores of 57.94 and 25.23%, respectively (Fig. 1C). A total of 114 secondary metabolites significantly varied in their relative abundances between both types of nodules (Wilcoxon rank sum test, P value < 0.05). In addition to the 114, 57 metabolites were at least two times more abundant in Sp+ nodules compared with Sp- nodules and vice versa for 12 metabolites (the remaining 45 metabolites showed ratios lower than 2 between their mean relative abundances in Sp+ and Sp- nodules and were considered of less interest in this study).

Only secondary metabolites with mean relative abundances higher than 1% were considered abundant enough to attempt purification and identification. A total of six metabolites were selected, four among 57 for Sp+ nodules (M27, M92, M137, and M171) and two among 12 for Sp- nodules (M168 and M180) (Table 2). Only metabolites M27 and M168 were successfully purified from Sp+ and Sp- nodules, respectively. M27

was specific to Sp+ nodules (meaning totally absent from roots and Sp- nodules). Its nuclear magnetic resonance (NMR) and MS spectral data analyses and results comparison with database led to the identification of gentisic acid 5-*O*- β -D-xylopyranoside (Nassar et al. 2003) (white amorphous powder, calculated $C_{12}H_{14}O_8$ 286.068870 uma) (Fig. 2A). M168 was significantly more abundant in nodules compared with roots (Wilcoxon rank sum test, P value = $3.38e^{-4}$) (Table 2) and, following NMR and MS, was identified as (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*- β -D-glucopyranoside (Lee et al. 2000) (white amorphous powder, calculated $C_{25}H_{34}O_{10}$ 494.215200) (Fig. 2B).

It is noticeable that the metabolite M132 was largely UV-dominant (at 280 nm) in all extract metabolite profiles (roots and nodules), with more than 62.72% of mean relative abundance. Although this metabolite was not discriminant between Sp+ and Sp- nodules, it was successfully purified and identified as oregonin [(5S)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*- β -D-xylopyranoside] (Kuroyanagi et al. 2005) (white amorphous powder, calculated $C_{24}H_{30}O_{10}$ 478.183900 uma) by NMR and MS spectral data analyses (Fig. 2C).

Effects of nodule extracts and metabolites on *Frankia* ACN14a in-vitro respiration and sporulation.

Sp+ and Sp- nodule total extracts were tested on *Frankia* ACN14a INT (iodo-nitro-tetrazolium) respiration at three different concentrations (0.3, 3, and 30 μ g/ml). Three days after inoculation, both types of extracts showed a significantly positive effect on respiration at the highest concentration (30 μ g/ml) compared with the control without nodule extract (Tukey's honest significant difference [HSD] test, P value < 0.05) (Fig. 3A and B). Bacterial respiration was also significantly higher in the presence of Sp+ nodule extract at a concentration of 3 μ g/ml (Tukey's HSD test, P value = $9.042e^{-3}$) compared with the control (Fig. 3A). Ten days after inoculation, both Sp+ and Sp- nodule extracts significantly decreased bacterial lethality at the highest concentration (optical density of 0.25 and 0.33, respectively, compared with 0.09 for the control; Tukey's HSD test, P value < 0.05). A significant (Tukey's HSD test, P value = $3.574e^{-3}$) and similar effect was only observed for Sp- nodule extract 7 days after inoculation.

Purified secondary metabolites M27 and M168 were also tested on *Frankia* ACN14a INT respiration at 0.1, 1, and 10 μ M.

Table 1. Discriminant primary metabolites (sugars, organic acids, and amino acids) between Sp+ (with *Frankia* spores in plant cells) and Sp- (without *Frankia* spores in plant cells) nodules and roots

Compound	Rt ^a	Nodule relative mean abundance (%) ^b		Sp+/Sp- ratio (P value) ^c	N/R ratio (P value) ^d	Database annotation (%) ^e
		Sp+	Sp-			
SOA9	11.93	1.20	0.28	4.29 ($1.7e^{-5}$)	15.75 ($1.13e^{-6}$)	Fumarate (100%)
SOA16	17.10	18.28	14.13	1.29 ($9.41e^{-3}$)	0.70 ($9.35e^{-6}$)	Glucose (100%)
SOA23	21.75	2.07	0.17	12.18 ($1.405e^{-5}$)	4.81 ($6.38e^{-4}$)	D-galactose (72% NIST)
SOA33	25.71	6.42	2.21	2.90 ($5.097e^{-6}$)	1.52 ($3.48e^{-3}$)	Melezitose (86% AMDIS)
SOA5	7.04	0.42	1.57	0.27 ($1.514e^{-3}$)	2.67 (>0.05)	Xylopyranoside (93% NIST)
SOA12	14.55	0.40	2.95	0.14 ($5.211e^{-4}$)	0.67 (>0.05)	D-mannitol (98% AMDIS)
SOA32	24.98	0.54	3.93	0.14 ($1.955e^{-2}$)	0.93 (>0.05)	Sucrose (91% AMDIS)
SOA38	30.87	4.95	12.13	0.41 ($7.273e^{-3}$)	25.62 ($1.06e^{-8}$)	Melibiose (68% NIST)
SOA40	31.49	0.57	2.39	0.24 ($4.551e^{-3}$)	2.53 (>0.05)	Non-identified
Citrulline	8.06	60.62	72.90	0.83 ($7.903e^{-3}$)	0.90 ($4.00e^{-2}$)	–
Arginine	8.52	0.50	1.84	0.27 ($4.281e^{-4}$)	0.49 ($3.35e^{-3}$)	–
Valine	11.76	4.52	2.69	1.68 ($6.805e^{-3}$)	1.57 ($5.04e^{-3}$)	–

^a Rt = retention time in minutes.

^b Relative peak areas reported to the total area of chromatograms.

^c Ratios between relative abundances in Sp+ and Sp- nodules. P values were obtained by comparing mean relative abundances in both nodule types with Wilcoxon rank sum statistical tests.

^d Ratios between relative abundances in nodules (N) and roots (R) (including all Sp+ and Sp- nodule samples for N and all associated-root samples for R). P values were obtained by comparing N and R mean relative abundances with Wilcoxon rank sum statistical tests.

^e For metabolite annotation, we used the National Institute of Standards and Technology (NIST) database and AMDIS (automated mass spectral deconvolution and identification system) software. The percent identification is given in parentheses (100% indicates when identification was confirmed by comparison with standards).

Total nodule extracts (0.3, 3, and 30 $\mu\text{g/ml}$) and purified metabolites (0.1, 1, and 10 μM) were also tested on *Frankia* ACN14a sporulation 10 days after inoculation (Fig. 4). While Sp+ nodule extract significantly inhibited sporangia production only at the highest concentration (30 $\mu\text{g/ml}$), Sp- nodule extract induced a significantly lower sporulation at all concentrations, as compared with the control (Tukey's HSD test, P value < 0.05) (Fig. 4A). Regarding purified secondary metabolites, an opposite effect was observed between M27 and M168 at the highest concentration (10 μM) (Fig. 4B). M27 significantly induced bacterial sporulation (Tukey's HSD test, P value = 8.860e^{-3}), compared with the control, while M168 significantly inhibited sporangia production (P value = 1.949e^{-2}). The commercial gentisic acid significantly increased sporulation at 0.1 μM compared with the control (Tukey's HSD test, P value = 6.972e^{-4}) and tended to inhibit at the highest concentration (Tukey's HSD test, P value = 7.186e^{-2}) (Fig. 4B).

In addition to metabolite profiling, total carbon and nitrogen nodule contents were also compared between Sp+ and Sp- nodules. Sp+ nodules showed a significantly lower carbon content compared with Sp- nodules (47.80 against 50.50% in Sp- nodules, analysis of variance [ANOVA], P value = $2.297e^{-2}$). No significant difference was observed in nitrogen content between both types of nodules.

Plants and microorganisms are capable of symbiotic sustainable associations often based on trophic complementarities. These interactions lead to reciprocal enhancement in the fitness of both partners involving a reciprocal cost. To our knowledge, Sp+ *Frankia* strains are the only case of microbial symbiont

Sp+ and Sp- nodules shared more than 70% of detected primary metabolites (amino acids, OAS), suggesting that the bacterial in-planta sporulation would not strongly affect the primary functionalities in *Frankia-Alnus* symbiosis. No major difference could be detected in organic acids involved in nitrogen fixation or assimilation (e.g., glutamic acid, 2-oxoglutarate, and derivatives

Table 2. Discriminant secondary metabolites between Sp+ nodules, Sp- nodules, and roots^a

Compound	Rt ^b	Relative mean abundance (%) ^c		Sp+/Sp- ratio (<i>P</i> value) ^d	N/R (<i>P</i> value) ^e	ESI-MS (negative mode) <i>m/z</i> ^f	UV maxima absorption (nm)
		Sp+	Sp-				
Sp+							
M27	4.93	2.27	0	Sp+ nodule-specific ^g		285.0678 ([M-H] ⁻); 217.0084; 141.0190	200sh, 210, 230, 312
M92	11.40	1.16	0.30	3.87 (2.195e ⁻²)	6.51 (1.24e ⁻⁴)	493.1782; 420.0988	216, 242, 300sh, 326
M137	16.04	2.66	0.92	2.89 (1.408e ⁻⁵)	0.82 (>0.05)	475.1699 ; 327.1248	196, 220, 250, 342
M171	19.58	1.42	0.65	2.18 (7.696e ⁻⁶)	1.05 (>0.05)	891.4081; 445.2019; 295.1433	194, 220, 276
Sp-							
M168	19.08	2.68	5.38	0.49 (1.903e ⁻⁴)	1.89 (3.38e ⁻⁴)	493.2374 ([M-H] ⁻);	198, 218sh, 280
M180	20.85	0.68	2.08	0.33 (1.093e ⁻³)	2.26 (>0.05)	359.1667; 217.0086	198, 220sh, 280

^g Undetected in Sp- nodules and roots.

[Schultz and Benson 1990]). However, significant differences between Sp+ and Sp- nodules were observed for two amino acids, citrulline and arginine. In the nodule, fixed nitrogen can be stored as an amide (i.e., asparagine) or as urea-cycle amino acids (i.e., citrulline or arginine) (Berry et al. 2011). In the present study, citrulline was the most abundant amino acid in nodule and root extracts (more than 60% of the total amino acids content). This observation is strongly consistent with previous studies suggesting that, in *Alnus*, the arginine pathway including

citrulline predominates and that citrulline is the major soluble nitrogenous compound in nodules and other plant tissues (Berry et al. 2011; Bollard 1957; Brooks and Benson 2016; Leaf et al. 1958; McClure et al. 1983; Miettinen and Virtanen 1952; Schubert et al. 1981). Additionally, both citrulline and arginine were significantly more abundant in roots as compared with nodules, suggesting their role in nitrogen compound export from nodules.

When considering both amino acid amounts in Sp+ and Sp- nodules, a significantly lower concentration was observed in

Fig. 3. Effect of **A**, Sp+ (with *Frankia* spores in plant cells) and **B**, Sp- (without *Frankia* spores in plant cells) and **C**, pure compounds M27, **D**, M168, and **E**, gentisic acid on *Frankia* ACN14a respiration rates. *Frankia* respiration was measured using tetrazolium/formazan iodo-nitro-tetrazolium, as described by Carro et al. (2016). Controls were made in the presence of absolute ethanol/water 75:25 (% vol/vol) (A and B) or absolute ethanol/water 50:50 (% vol/vol) (C, D and E), used to dilute nodule extracts or pure compounds, respectively. Error bars indicate standard deviations. An asterisk (*) indicates significant differences compared with the control condition (Tukey's honest significant difference test, P value < 0.05).

Sp+ nodules compared with Sp−, suggesting either a higher nitrogen export from Sp+ nodules, less storage of nitrogen amino acids, or both. This hypothesis could be explained by either i) a lower ability of Sp+ strains to fix nitrogen, as proposed in the literature (Houwers and Akkermans 1981; van Dijk 1984; Vandenbosch and Torrey 1984; Wheeler et al. 1986) or ii) a higher arginine and citrulline uptake by Sp+ strains (e.g., to produce sporangia), or both. In the other well-known sporulating bacteria model *Clostridium*, arginine is metabolized to citrulline, required to produce spores (Perkins and Tsuji 1962).

In contrast to primary metabolites, 50% of secondary metabolites were found significantly different between Sp+ and Sp− nodules. Both plant and bacteria are able to produce secondary metabolites, generally known to be involved in diverse functions including, but not limited to, defense against biotic or abiotic stresses and communication with other organisms (Demain and Fang 2001; Mazid et al. 2011). The origin of these metabolites is, therefore, hard to elucidate, since they were present in nodules, resulting from plant and bacteria interactions. A bacterial origin of discriminant secondary metabolites between Sp+ and Sp− nodules could not be excluded. Indeed, bioinformatics analyses of *Frankia* genomes revealed an unexpected number of secondary metabolic biosynthesis gene clusters (Udway et al. 2011). However, in our study, metabolite purification and NMR analyses led to the identification of compounds usually found in plants. The secondary metabolite M132, largely dominant in all nodule extracts (more than 60%), was, indeed, identified as oregonin [(5S)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside], previously reported from different tissues of *Alnus* species, such as *Alnus rubra* bark (Sati et al. 2011). Another diarylheptanoid was found at significantly higher levels in Sp− nodules, the metabolite M168, identified as (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-glucopyranoside (Lee et al. 2000). Diarylheptanoids are very common in several organs of *Alnus* spp., such as bark, leaves, or fruits (Choi 2013; Gonzalez-Laredo and Karchesy 1997; Kang et al. 2004; Yadav and Gupta 2014), although, as far as we know, they have never been described in nodules. These types of compounds were previously observed to be responsible for different activities, such as antibacterial and antioxidant (Ilic-Tomic et al. 2016; Ponomarenko et al. 2014; Saxena et al. 1995). They could be produced by the host plant as a defense-like response to control bacteria in the rhizosphere, including pathogen and symbiotic microorganisms. The diarylheptanoid M168 is greatly interesting

because of its abundance in nodules compared with roots, and particularly, in Sp− nodules. The host plant could control the invading symbiotic bacteria by producing this metabolite as a defense molecule. Plant defenses would also be required in the very close symbiotic rhizobia-legume system for optimal nodule functioning (Gourion et al. 2015). In *Alnus* nodules, the diarylheptanoid M168 metabolite could be produced in the nodule periderm (since several diarylheptanoids were isolated from bark, the metabolites could therefore be present in this tissue), although to date, there is no relevant indication on diarylheptanoids localization in Sp+ and Sp− nodules.

Interestingly, we observed here that the diarylheptanoid M168 did not impact *Frankia* growth but inhibited *Frankia* in-vitro sporulation as well as total Sp− nodule extract (unlike Sp+ nodule extract). This observation suggests that, in Sp− nodule extracts, some secondary metabolites emitted by the host plant could prevent the bacterial symbiont to sporulate, and this metabolic cocktail would be finely regulated to ensure that the bacteria remain metabolically active for plant benefit (in vegetative form to fix nitrogen).

The most important finding that emerges from this work is the secondary metabolite M27, the only metabolite among 213 detected, specific to Sp+ nodules (not detected in roots and Sp− nodules). This metabolite was identified as gentisic acid 5-*O*-β-D-xylopyranoside (Nassar et al. 2003). It was previously described as a pathogen-inducible signal for activation of plant defenses in tomato and cucumber and was associated with systemic infections in compatible plant-pathogen interactions (Bellés et al. 1999; Fayos et al. 2006).

The “cheater” behavior of Sp+ *Frankia* strains (i.e., less-beneficial endophyte that mimics the “genuine mutualists” without totally fulfilling its symbiotic obligations) was recently discussed by Pozzi et al. (2015). Sp+ strains would be less efficient in nitrogen fixing and less cooperative than Sp− strains by diverting part of the plant energy resources for the production of many sporangia (Pozzi et al. 2015; Schwintzer 1990; Torrey 1987). In *Rhizobia*-legume symbioses, such behavior induces host plant defense mechanisms to sanction the cheater bacterial symbionts (Kiers et al. 2003). Under this hypothesis, gentisic acid 5-*O*-β-D-xylopyranoside M27 could be produced in the nodule by the host plant as a sanction against Sp+ *Frankia* strains. However, the involvement of Sp+ strains in M27 production can actually not be excluded, since the high ability of *Frankia* spp. to synthesize many different secondary

Fig. 4. Effect of **A**, nodule extracts and **B**, pure compounds on *Frankia* ACN14a sporulation. In **A**, white bars = Sp+ (able to sporulate inside plant cells), black bars = Sp− (unable to sporulate inside plant cells). Controls were made in the presence of absolute ethanol/water 75:25 (vol/vol), used to dilute nodule extracts. In **B**, white bars = M27, black bars = M168, and hatched bars = gentisic acid. Controls were made in presence of absolute ethanol/water 50:50 (vol/vol), used to dilute pure compounds. Error bars indicate standard deviations. An asterisk (*) indicates significant differences compared with the control condition (Tukey's honest significant difference test, *P* value < 0.05).

metabolites, as previously discussed. A plant-bacteria coproduction could also be possible. Furthermore, at this stage, we still ignore where this metabolite is actually produced in nodule tissues. Host infected cells full of Sp+ *Frankia* spores could be a likely location, which might be confirmed using matrix-assisted laser desorption–imaging mass spectrometry (MALDI-IMS), recently used to investigate metabolites in *Medicago truncatula* nodules and roots (Gemperline et al. 2015; Ye et al. 2013).

Our study also demonstrated a stimulating effect of this metabolite on *Frankia* spp. in-vitro sporulation, without any negative effect on bacterial respiration rate (the bacterial respiration rate was even better in the presence of M27 under some conditions). Interestingly, the gentisic acid, for which xyloside substituent is lacking, compared with M27, showed a different effect on *Frankia* sporulation. These results suggest the specific stimulation effect of gentisic acid 5-*O*- β -D-xylopyranoside M27, at high concentrations, on bacterial respiration and sporulation. This effect raises the question of a possible adaptation of *Frankia* Sp+ to bypass the host plant sanction by producing more sporangia. In the current state of knowledge, it remains difficult to understand whether *Frankia* in-plant sporulation could be a cause or a consequence of specific plant defense metabolites in Sp+ nodules.

Under the cheater hypothesis, another form of sanctions against Sp+ strains could also be a limited supply of carbohydrates to the nodule, as suggested by the significantly lower carbon content in Sp+ nodules compared with Sp– nodules. It is also worth noting that, despite the small difference in primary metabolite content between both nodule types, one major change was observed for the metabolite SOA 32, sevenfold less abundant in Sp+ nodules, and chemically close to sucrose. The latter is the form of photo-assimilate most commonly used in long-distance transport in the plant phloem (Lemoine 2000). Its lower abundance in Sp+ nodules could mean that they are less supplied with carbon sources by the host plant than Sp– nodules or, simply, reflect the higher ability of Sp+ strains to rapidly metabolize these carbohydrates. In addition, more complex polymers were found in Sp+ nodules, like fumarate (organic acid) and melezitose (trisaccharide), while a disaccharide, melibiose, was significantly more abundant in Sp– nodules. A recent study illustrated that fumarate induced longer and lower *Frankia* respiration dynamics, compared with *Frankia* spp. preferred carbon sources (Carro et al. 2016). The higher presence of complex compounds in Sp+ nodules could suggest a possible way for the host plant to slow down the dissemination of sporulating—and potentially less cooperative—symbionts.

Conclusion.

In the present study, we conducted, for the first time, to our knowledge, the metabolite profiling of Sp+ and Sp– nodules, in order to highlight differences associated with *Frankia* in-plant sporulation in SOA, amino acids, and secondary metabolites. More changes between both nodule types were globally observed in secondary metabolites, as compared with primary metabolites. Two secondary metabolites analyzed as discriminant were isolated and characterized, i.e., the gentisic acid 5-*O*- β -D-xylopyranoside (M27), specifically produced in Sp+ nodules, and the (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*- β -D-glucopyranoside (M168), more abundant in Sp– nodules. Both metabolites remind defense molecules emitted by the host plant but their respective effects on *Frankia* sporulation were totally opposite. While the metabolite isolated from Sp– nodules (M168) inhibited *Frankia* in-vitro sporulation (as well as Sp– total extract), a significantly higher production of spores was observed in the presence of M27. These results suggested that the host plant could play an important role in the *Frankia* ability to sporulate in planta. It could exert control over potentially less cooperative Sp+ symbionts through specific secondary metabolites and, to a lesser extent, by regulating the flow of photosynthates provided to the

nodule. Promising approaches such as MALDI-IMS could be tested to investigate the nodule localization and to quantify the different metabolites associated with in-plant sporulation. Finally, further genomic and transcriptomic analyses of both plant and bacteria might be helpful to decipher genes involved in metabolic pathways of metabolites of interest and their expression in Sp+ versus Sp– nodules and roots.

MATERIALS AND METHODS

Alnus glutinosa Sp+ and Sp– field nodule and associated-root sampling.

All nodules and roots were collected between September and October 2012 from six *Alnus glutinosa* stands, previously described as “Sp+ sites” (three sites) or “Sp– sites” (three sites) in Pozzi et al. (2015) (Table 3). Three different trees were randomly selected per site and, for each of them, a minimum of three nodules were collected with associated roots. All nodules were phenotypically and genetically analyzed to confirm Sp+ or Sp– phenotype, as described by Pozzi et al. (2015). All collected material was stored at –80°C before being freeze-dried (Christ Alpha 1-4 freeze dryer).

In our experiments, a nodule sample is defined as a pool of three nodules collected on three different trees (one nodule per tree) coming from the same site, which represents a minimum of three nodule samples per site. Similar sampling was done for associated roots (at least three pooled root samples were also made per site). Each sample, whether nodule or associated root, was made from 150 mg of freeze-dried material, was crushed to very fine dry powder (Retsch TissueLyser II), and was kept in liquid nitrogen, until the first extraction. A total of 48 samples (15 Sp+ nodule samples, 14 Sp– nodule samples, and 19 associated-root samples [Table 3]) were extracted.

Metabolic extraction.

Each nodule and associated-root sample was processed as described as follows. A first metabolic extraction was performed in 1 ml of methanol/water 50:50 (vol/vol). Dissolution was expedited by vortexing (Heidolph Top-mix 94323) and sonication (Branson Ultrasonic cleaner 2510E-DTH), followed by 15 min of centrifugation at 15,870 $\times$ g. The resulting supernatant was kept as a first extract (E1). The remaining pellet was used for the second metabolic extraction, performed in 1 ml of methanol (100%) and was followed by a dissolution similarly expedited as for E1, to get the second extract (E2). Both extracts E1 and E2 were mixed and dried (CentriVap concentrator LABCONCO) before lyophilization. They were stored at –20°C, prior to chromatographic analyses.

Chromatographic analyses.

Sugars and organic acids analysis by GC/EL-QQQ. Dried extracts underwent two derivatization reactions with Hydroxylamine

Table 3. Origins of Sp+ and Sp– nodule (with and without *Frankia* spores in plant cells, respectively) samples

Nodule phenotype	Site reference	Location ^a	Number of N and R ^b
Sp+	S1+	Le Tremblay, Bourget-du-Lac (#28)	4 N, 3 R
	S2+	Thury (pop. V, #3)	4 N, 3 R
	S3+	Miribel (#17)	7 N, 3 R
Sp–	S1–	Arandon (#21)	4 N, 4 R
	S2–	Le Grand-Lemps (#22)	3 N, 3 R
	S3–	Le Blanchet, Bourget-en-Huile (#27)	7 N, 3 R

^a Site numbers as referenced by Pozzi et al. (2015) are in parentheses.

^b Numbers of nodules (N) or roots (R) pooled and collected from three different trees per site.

hydrochloride and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) in order to transform all SOA into a more volatile form that is suitable with the GC analysis. Extracts were first dissolved at 10 mg/ml in a 30-mg/ml hydroxylamine HCl solution and were incubated 30 min at 80°C. BSTFA was then added at 300 µl/ml, before incubating 10 min at 80°C.

GC analyses were performed for derivatized samples within 24 h after derivatization, using Agilent Technologies 7000A EI-Triple Quad, coupled with the 7890A GC system and Agilent 190915-433 column (HP5-MS, 0.25mm × 30m × 0.25 µm) with helium as vector gas at a constant flow of 1 ml/min. The inlet temperature was 290°C with a 20:1 split, and the temperature gradient (35 min) was as follows: 165°C for 3 min; 165°C to 195°C at 2°C per min; 195°C to 290°C at 15°C per min, and 290°C to 300°C at 15°C per min. For mass analysis, electrons were accelerated to 70 eV. The gradient rinsing solvent was pyridine (solvent A) and isopropanol (solvent B). A QC containing 5 µl of each derivatized sample and 12 standard sugars (Supplementary Data S2) was similarly analyzed.

Finally, TIC chromatograms were obtained with Agilent Mass Hunter qualitative analysis software.

Amino acid analysis by HPLC/DAD. Amino acid analyses were performed on the HPLC Agilent 1100 coupled with photodiode array detector, using C18 Eclipse AAA Agilent Zorbax column (3.5 µm, 4.6 × 150 mm). Two on-line derivatizations with o-phthalaldehyde (OPA) and fluorenylmethoxy chloroformate were applied. Samples were dissolved at 10 mg/ml in methanol/water 50:50 (vol/vol), along with a QC sample (mixture of 5 µl of each sample and 24 standard amino acids [Supplementary Data S3]). The eluting solvents were: solvent A, 40 mM NaH₂PO₄·2H₂O buffer, pH 7.8, solvent B, acetonitrile/methanol/water 45:45:10 (vol/vol/vol). The B solvent gradient was as follows: 0% for 1.9 min; 0 to 57% from 1.9 to 18.1 min; 57 to 100% from 18.1 to 18.6 min; and 100% up to 26 min (2 ml/min constant flow) (Henderson et al. 2000). Chromatograms were analyzed at 338 nm for most amino acids, except for proline, which was detected at 262 nm, with the Agilent LC 1100 offline ChemStation for LC and LC-MS analysis software.

Secondary metabolites analysis by HPLC/DAD/ESI-Q-TOF. Secondary metabolites were analyzed using Agilent Technologies accurate-mass Q-TOF LC-MS 6530, with the LC 1290 Infinity system and Poroshell 120 EC-C18 column (3 × 100 mm, 2.7 µm). Dried extracts were dissolved at 10 mg/ml in methanol/water 50:50 (vol/vol), were sonicated and were centrifuged to discard insoluble material. HPLC analyses were performed at 25°C with a flow-rate of 0.7 ml/min, using acetic acid 0.4% in ultrapure water (solvent A) and acetonitrile (solvent B). The gradient was 98:2 A/B (0 to 3 min), 70:30 A/B (3 to 25 min), and 0:100 A/B (25 to 33 min), 98:2 A/B (33 min to 38 min) (Table 4). Mass analyses were obtained in negative mode, with the nebulization gas (nitrogen) at a flow of 12 liters per minute and 40 psi pressure. The capillary tension was 3,000 V and gave ionization energy of 100 eV. A QC sample containing 5 µl of each sample was initially

analyzed and was replicated after every 10 samples. Chromatograms were explored at 280 nm with Mass Hunter qualitative analysis B.05.00 software.

Metabolite profiling data treatment and statistical analyses.

For each of the three chromatographic analyses (SOA, amino acids, and secondary metabolites), a preliminary work consisted in peak alignment between all samples, based on peak retention time. This work resulted in three metabolite profiling matrices, with samples in rows and absolute area (A_{abs}) of detected metabolites within SOA, amino acids, or secondary metabolites chromatograms in columns. For each metabolite within a given sample, its relative peak area (i.e., relative abundance in the sample = A_{rel}) was calculated over the total area of the sample chromatogram ($\sum A_{abs}$) as follows: $\%A_{rel} = A_{abs} / \sum A_{abs} \cdot 100$.

PCA were made from these matrices in order to visualize the effect of the nodule sporulating phenotype on nodule metabolite profiles. The effect of nodule phenotype on metabolite relative abundances was also statistically tested, in order to highlight significantly different metabolites between Sp+ and Sp- nodules. Statistical analyses were performed using R software version 2.15.3. Since relative abundance data were not normally distributed, Wilcoxon rank sum statistical tests were used. Among significantly different metabolites between both types of nodules, we applied a minimal fold-change threshold of 2, below which differential relative abundances between Sp+ and Sp- nodules is unlikely to be of interest for any metabolite. We finally selected the most abundant metabolites (mean relative abundance in nodule samples >1%) to attempt purification.

Identification of discriminating SOA.

Discriminating SOA were annotated using the Fiehn database (AMDIS software) (Fiehn 2008) and National Institute of Standards and Technology (NIST02) database (MassHunter qualitative analysis B.05.00, Agilent Technologies software). When possible, comparisons with standards also allowed confirming metabolite identity.

Purification and identification of discriminating secondary metabolites.

Purification of M27 by semipreparative HPLC. A total of 208.2 mg of Sp+ nodule extract was submitted to semipreparative HPLC on a HP1100 system with a photodiode array detector (Agilent Technologies). The column used was Macherey-Nagel VP Nucleodur 100-5 C18ec (C18; 250 × 10 mm; 5 µm). HPLC analyses were performed at 40°C with a flow rate of 3.5 ml/min, using acetic acid 0.4% in ultrapure water (solvent A) and acetonitrile (solvent B) (Carlo Erba reagents). Ultrapure water, acetic acid, and acetonitrile were obtained from Direct-Q 5 UV (Merck Millipore), Carlo Erba Reagents, and VWR (Prolabo Hipersolv Chromanorm), respectively. The gradient was 95:5 A/B (0 to 3 min), 75:25 A/B (3 to 23 min), and 0:100 A/B (23 to 29 min). Injected volume was 100 µl of a 40-mg/ml solution of Sp+ total extract in methanol. Detection of M27 was done at 280 and 320 nm. Retention time for the M27 was 14.22 min and collection was done between 14 and 14.5 min. The purification resulted in 2.0 mg (dried weight) of M27 (6.9×10^{-3} mol; yield 1%). NMR analyses were then done in CD₃OD.

Purification of M132 and M168 by semipreparative HPLC. Both metabolites were purified from 170.0 mg of total Sp- nodule extracts. A liquid-liquid extraction was performed twice in 10 ml of ultrapure water and 10 ml of CH₂Cl₂, resulting in the organic phase EA (12.8 mg, dried weight). The aqueous layer was similarly partitioned in 10 ml of ethyl acetate (Hipersolv Chromanorm, VWR Prolabo), resulting in the organic phase EB

Table 4. Gradient used for UPHLC/DAD/ESI-Q-TOF^a analyses of secondary metabolites

Time (min)	Solvent A (%)	Solvent B (%)
0	98	2
3	98	2
25	70	30
29	0	100
33	0	100
34	98	2
38	98	2

^a Ultra-high pressure liquid chromatography diode array detection electrospray ionization-quadrupole-time-of-flight.

(27.3 mg, dried weight), and, finally, in 10 ml of n-butanol (Hipersolv Chromanorm, VWR Prolabo), resulting in the organic phase EC (39.1 mg, dried weight). The three organic phases were pooled to obtain ED (70.7 mg, dried weight) and were analyzed by semipreparative HPLC, using a HPLC 1100 system with a photodiode array detector. The column used was Macherey Nagel VP Nucleodur 100-5 C18ec (C18; 250 × 10 mm; 5 µm). Analyses were performed at 40°C with a flow rate of 3.5 ml/min, using acetic acid 0.4% in ultrapure water (solvent A) and acetonitrile (solvent B) (Carlo Erba Reagents). The gradient was 80:20 A/B (0 to 3 min), 61:39 A/B (3 to 21 min), and 0:100 A/B (21 to 23 min). Injected volume was 100 µl of 20- mg/ml ED in methanol/water 50:50 (vol/vol). Retention time for M132 was 10.72 min and collection was done at 280 nm between 10.45 and 10.85 min. Retention time for M168 was 13.67 min and collection was done at 320 nm between 13.5 and 13.7 min. The purification resulted in 2.0 mg of M168 and 18.9 mg of M132 (dried weights), yielding in 1.1 and 11.1%, respectively (4.04×10^{-6} and 3.95×10^{-5} mol, respectively).

Identification of M27, M132, and M168 by semipreparative HPLC. NMR spectra were recorded on a Bruker DRX 500 spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C) in CD_3OD as solvent (internal reference, tetramethylsilane). D4-methanol used for NMR analysis was from Magnisolv Merck (Darmstadt, Germany).

Biological material, growth condition, and in-vitro tests of purified metabolites.

The ACN14a *Frankia* strain (Normand and Lalonde 1982) was grown in liquid BAP (Murry et al. 1984) medium without ammonium (BAP-) under the same conditions described by Carro et al. (2016). Seven-day-old cultures with two induction steps in BAP- medium were made before in-vitro tests. M27 and M168 nodule purified metabolites and commercial gentisic acid (Sigma Aldrich) were solubilized in ethanol/water 50:50 (vol/vol) and were supplemented in *Frankia* ACN14a BAP- cultures at final concentrations of 0.1, 1, and 10 µM. Nodule extracts were solubilized in ethanol/water 75:50 and were supplemented in *Frankia* cultures at 0.3, 3, and 30 µg/ml at final concentration. Five replicates per condition were performed. Positive controls were also included with the same volume of solvent ethanol/water, 50:50 or 75:50 (vol/vol), without purified metabolites or nodule extracts. The effects of M27, M168, M132, and Sp+/ Sp- nodule extracts were also tested on INT reduction, without *Frankia* cells in the reaction, in order to verify their redox properties. None of them reduced INT, confirming they did not affect *Frankia* respiration rates (data not shown). After 3, 7, and 10 days of growth, measures of respiration (using tetrazolium/formazan INT) and fixation (based on acetylene reduction) were performed as described by Carro et al. (2016). Sporangia production was also assessed after 10 days of culturing, using light microscopy (Nikon Eclipse E400). For this purpose, sporangia were enumerated for each tested condition, from at least 23 hyphae clusters—named colonies—randomly observed under the microscope. The mean number of sporangia per colony was computed. Since data were normally distributed, ANOVA and Tukey's HSD were used to compare effects of purified metabolites or nodule extracts on respiration, acetylene fixation, and sporulation (using R software version 2.15.3).

Total carbon and nitrogen nodule content measurement.

For each sampled tree, total carbon and nitrogen content of nodules were measured using a Flash EA1112 elemental analyzer (Thermo Fischer Scientific Inc.), following the procedure previously described by Grigulis et al. (2013) and Saccone et al. (2013), from 3 to 4 mg of lyophilized and ground material. A total of eight Sp+ and nine Sp- nodule samples were analyzed.

Since data were normally distributed, one-criterion ANOVA was made to test the effect of Sp+/Sp- nodule phenotype on carbon and nitrogen contents (using R software version 2.15.3).

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