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Regulation of metabolite production during the encounter between the actinobacterium Frankia and its host Alnus glutinosa

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Abstract

Our study focuses on the metabolomic changes observed during the early stages of *Alnus glutinosa* and *Frankia alni* symbiosis. Amino acid metabolic profiling by HPLC-DAD-FLD and untargeted metabolomic analysis of secondary metabolites by UHPLC-QTOF were performed on the shoots and roots of *A. glutinosa* as well as on bacterial pellets of *F. alni*. Two culture conditions were compared: a single culture condition (where *A. glutinosa* or *Frankia* was grown alone) and a co-culture condition (where *A. glutinosa* and *Frankia* were grown together) at different culture times (D1, D2 and D3). Our results reveal a change in metabolism (primary and secondary) in both partners in the co-culture condition. For amino acids, this change was more important in the shoots than in the roots and in *Frankia*. A total of 16 amino acids (Asp, Asn, Ser, Gln, Gly, Cit, GABA, Ala, Arg, Tyr, Trp Val, Phe, Ile, Lys and Pro) were overproduced in the presence of *Frankia* in the shoots on the different sampling days. We hypothesised that the plant would modify its amino acid content in its shoots in anticipation of a transfer to *Frankia* for growth. At the same time, a drastic change in secondary metabolites occurs in the shoots, roots and *Frankia* at the three time points considered between the control condition and the co-culture condition. Statistical analyses enabled us to highlight the ions characterising the co-culture condition in the different biological compartments (i.e. shoots, roots and *Frankia*). The biomarkers identified in the shoots and *Frankia* varied greatly depending on the sampling day (i.e. D1, D2 and D3), revealing strong dynamics. The root biomarkers appear to be more stable over time, as several of them are common to all three sampling days.

Introduction

Mutualistic symbiosis is a striking example of co-evolution that promotes plant growth by facilitating access to limiting nutrients, particularly nitrogen (Hay et al., 2020; Kucho et al., 2010). Certain plants develop strategies for interacting with diazotrophic bacteria that are capable of reducing atmospheric nitrogen (N_2) to ammonia (NH_3) using a specific metalloenzyme called nitrogenase. This symbiosis between plants and diazotrophic bacteria is found in very few plant species with two types of bacteria: legume/*Rhizobium* symbioses and actinorhizal plant/*Frankia* symbioses. In both cases, the symbiotic association takes place in several stages: chemotaxis, adhesion/infection, colonisation/proliferation and formation of mature nodules where trophic exchanges take place (Liu et al., 2019). The bacteria provide ammonium to the plant in exchange for carbon compounds, energy and a protective niche (Hay et al., 2017; Tjepkema et al., 1986).

In the legume/*Rhizobium* system, all stages of symbiosis are well characterised. The plant secretes flavonoids that attract compatible *Rhizobia* and regulate the expression of their nodulation genes (Nod genes). In turn, these genes are involved in the synthesis and excretion of NOD factors (LipoChitoOligosaccharides) that are specifically recognised by the host plant, leading to the deformation of root hairs (Poupot et al., 1995). The bacteria then penetrate the root tissues, colonise the cortical cells and ultimately lead to the formation of a new specialised organ called a nodule. A molecular dialogue is therefore established between the plant and the bacteria for the establishment of

this symbiosis. Unlike the legume/*Rhizobium* system, the actinorhizal plant/*Frankia* system is poorly characterised, mainly due to the slow growth of *Frankia* and the lack of genetic tools for its bacterial transformation (Popovici et al., 2010; Pujic et al., 2019). Actinorhizal plant/*Frankia* symbiosis is found in more than 260 plant species, mainly trees and shrubs belonging to eight different families: Betulaceae, Coriariaceae, Elaeagnaceae, Rhamnaceae, Rosaceae, Myricaceae, Datiscaceae, and Casuarinaceae (Benson and Silvester, 1993). It also plays an important role in the functioning of ecosystems, as it is responsible for approximately 15% of biologically fixed nitrogen inputs on Earth (Kucho et al., 2010). Alders are the main nitrogen-fixing trees found in the northern hemisphere (Normand et al., 2007), thriving in temperate, cool, and alpine ecosystems. They also provide a wide range of ecosystem services, resulting directly from the fixation of atmospheric nitrogen by *Frankia* in root nodules (i.e. increase in soil fertility via high N and MO inputs in soil) or indirectly through *Frankia* impact on tree fitness (i.e. maintain of biodiversity, soil and water quality regulation and greenhouse gas (GHG) regulation) (Guigard et al., 2025).

Currently, the *Alnus glutinosa*/*Frankia alni* pair appears to be the model for studying the molecular mechanisms involved in the establishment of this actinorhizal symbiosis. *Alnus glutinosa* is one of the most emblematic species found in riparian zones and is naturally widespread across all of Europe, from mid-Scandinavia to the Mediterranean countries, including northern Morocco and Algeria (Claessens et al., 2010). It belongs to the Betulaceae family and the Fagales order. It grows in moist, sunny soils. Its leaves are petiolate, glabrous and shiny. *Frankia alni* is a Gram+ actinobacterium that is both symbiotic and saprophytic, with a high C + G base ratio in its DNA (Ventura et al., 2007). It is filamentous and capable of fixing atmospheric nitrogen symbiotically by forming nodules and in a free state in the form of vesicles. Genomic approaches have made it possible to explore the determinants of host specificity for "*Alnus*-infective strains" (i.e., *Frankia* strains belonging to Cluster Ia, Normand et al., 1996). For example, several genes were specifically found in these strains, including an agmatine deiminase which could possibly be involved in various functions as access to nitrogen sources, nodule organogenesis or plant defence (Kim Tiam et al., 2023). The establishment of symbiosis between *A. glutinosa* and *Frankia alni* is characterised by several major stages (Pujic et al., 2022). After two and a half days of contact between the two partners, deformation of the root hairs is observed; this marks the beginning of the infection process. The formation of a pre-nodule appears after seven days, allowing *Frankia* to colonise the interior of the root tissues. At 21 days, nodule formation allows trophic exchanges between the partners to take place. However, the early changes induced on the metabolome of the two symbiotic partners are still poorly characterised. To do this, a targeted (amino acids) and untargeted (secondary metabolites) metabolomics approach was used to better understand the metabolome changes induced on the two symbiotic partners during early stage of symbiosis.

Material and Methods

Biological materials

The seeds of *Alnus glutinosa* were collected from a tree located on the left bank of the Rhône in Lyon in December 2020. They were germinated in aluminium trays for 6 weeks, then the seedlings were transferred to opaque pots containing modified Fahraeus culture medium (Fåhraeus, 1957) with 0.5 g/L KNO_3 to allow for optimal plant growth (Supplementary Material 1). Four seedlings were transferred to each pot for a total of 35 pots. After three weeks of growth in Fahraeus medium, nitrogen withdrawal was carried out by transferring the seedlings to Fahraeus 0 medium (nitrogen-free). The purpose of this withdrawal is to place the plant in conditions favourable to the establishment of actinorhizal symbiosis. A volume of 8 L of *Frankia alni* ACN14a (Normand and Lalonde, 1982) culture was prepared in FBM medium (Supplementary Material 2). The cells were collected by sedimentation and rinsed in sterilised ultrapure water, then transferred to Fahraeus 0. The suspension was homogenised by passing the cells through a syringe. Each dialysis tube (Float-A-Lyzer G2, MWCO = 100 kD) was filled with 8 mL of *Frankia* suspension. Using this biological material, three experiments were then carried out (Fig. 1): "Alder": alder seedlings placed in opaque pots containing 500 mL of Fahraeus 0 medium (4 seedlings/pot), "*Frankia*": 8 mL of *Frankia* suspension per dialysis tube placed in opaque pots containing Fahraeus 0 medium (5 dialysis tubes/pot) and 'Alder + *Frankia*': alder seedlings and *Frankia* cells in dialysis tubes (8 mL of *Frankia* suspension/dialysis tube) were placed in opaque pots containing Fahraeus 0 medium (5 dialysis tubes and 4 seedlings/pot). Five replicates were performed for each condition. One replicate corresponds to one pot. The shoots, roots and bacterial cells were sampled at different times (D1, D2, and D3) for metabolomic analyses. For the '*Frankia*' and 'Alder + *Frankia*' conditions, the contents of the 5 tubes from the same pot were combined in a 50 mL Falcon tube and centrifuged (5100 rpm, 20°C, 15 min). The supernatant was then transferred to a new Falcon tube and stored at -20°C. The cell pellet is transferred to a 1.5 mL microtube and centrifuged (13,500 rpm, 20°C, 5 min), the supernatant is removed, and the tube is immersed in liquid nitrogen and stored at -20°C. For the 'Alder' and 'Alder + *Frankia*' conditions, the shoots of the four seedlings from the same pot are grouped together, then coarsely ground in liquid nitrogen using a mortar and pestle and stored at -20°C. Similarly, the roots of the four seedlings from the same pot were grouped together, coarsely ground in liquid nitrogen using a mortar and pestle, and stored at -20°C.

Freeze-drying and extraction

After freeze-drying at -55°C for 48 hours (Christ Alpha 1–4, Fisher Scientific), the samples were ground in a TissueLyser II (Qiagen) at a frequency of 15.4 revolutions per second for 3 times 2 minutes. Norvaline (Agilent Technologies), a synthetic amino acid, was chosen as an internal standard. Two successive extractions with 60% EtOH (VWR) were performed, followed by an extraction with ultra-pure water (Direct-Q 5-UV Millipore). The solvent (ethanol or water) was added to the sample using a ratio of 1:20 (1 g of dry matter to 20 mL of solvent). The sample was then vortexed for 2 min, and sonicated for 15 minutes in an ultrasonic bath (Bransonic Ultrasonic cleaner 2510E-DTH). After centrifugation (12,000 rpm, 10 min) the supernatant was collected. The supernatants from the three extractions were pooled, dried with a Speedvac® for 2 hours (Centrivap Coldtrap Concentrator LABCONCO) and lyophilised. All dry extracts were then weighted and resuspended at 10 mg/mL in 60% EtOH, followed by 15 minutes of

sonication and 5 minutes of centrifugation at 1500 rpm. Finally, a volume of 400 µL of supernatant was transferred to vials for metabolomic analysis. A quality control (QC) sample was also prepared by taking 5 µL from all extracts under both conditions.

Metabolic profiling of amino acids

Identification and quantification of amino acids was performed using high-performance liquid chromatography coupled with a diode array detector and a fluorescence detector (HPLC-DAD-FLD, Agilent 1100, Agilent Technologies). The column used contains a C18 reversed-phase grafted phase conditioned at 40°C (Zorbax Eclipse AAA Agilent Technologies 150×4.6 mm, 3.5 µm). Standard mixtures of 24 amino acids (primary and secondary) were prepared at two different concentrations (100, 250 µM). These mixtures were composed of a standard solution of 17 amino acids: aspartate (Asp), glutamate (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), citrulline (Cit), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys) and proline (Pro) and six additional amino acids solubilised in H_2O: asparagine (Asn), glutamine (Gln), tryptophan (Trp), norvaline (Nor), γ-abscisic acid (GABA), α-abscisic acid (AABA) and ornithine (Orn), all supplied by Agilent Technologies. The method used is described in Henderson et al., (2000). The mobile phase consists of solvent A: 40 mM Na_2HPO_4 at pH 7.8 and solvent B: acetonitrile (CH_3CN)/MeOH/H_2O (45:45:10). The gradient used is described in (Supplementary Material 3). The injection volume is set at 18 µL and the flow rate at 2 mL/min. However, for the soots and roots, this volume was multiplied by 9 in order to obtain a more intense signal. Thus, prior to injection, the samples were derivatised with a reaction mixture composed of OPA (O-phthalaldehyde) to visualise primary amino acids and FMOC (9-fluorenylmethyl chloroformate) to visualise secondary amino acids.

Secondary metabolites analysis

The analysis of secondary metabolites was carried out at the Bio-organic Mass Spectrometry Technical Platform of the National Museum of Natural History (MNHN) in Paris. The analyses are carried out on an ultra-high performance liquid chromatography system (UHPLC, ELUTE, Bruker) coupled with a high-resolution hybrid quadrupole time-of-flight mass spectrometer (Compact, Bruker) equipped with an electrospray ionisation source (ESI-Qq-TOF). The extracts are injected at a volume of 3 µL for the stems and 4 µL for the shoots and roots onto a C18 column (Polar Advances II 2.5 pores 2.1X100mm -Thermo), then eluted at a flow rate of 300 µL.min-1 using a mobile phase composed of H2O + 0.4% HCOOH (solvent A) and CH3CN + 0.1% HCOOH (solvent B). The elution gradient was: 95:5 (0 to 2 min), 50:50 (2 to 9 min), 10:90 (9 to 17 min) and 95:5 from 19 to 21 min (Supplementary Material 4). Regarding the mass spectrometer parameters, the different ions were analysed in positive auto MS/MS mode at 2–4 Hz on m/z between 50-1500. The nebulisation gas was dinitrogen heated to 250°C, at a flow rate of 300 nL/min. The capillary voltage was 3500 volts, providing an ionisation energy of 2 eV. The collision energy used to select the daughter ions was 8 eV. In addition, an internal formate (Na) calibration solution was injected at the start of each sample analysis to check the system.

Data analysis

Amino acids. Using Agilent Technologies Chemstation software (B.04.03 SP2), the chromatograms of our samples are overlayed with that of the mixture of known amino acid standards using the FLD detector. If primary amino acids such as Glu, Gln and Arg are saturated, the chromatograms are visualised at a wavelength of 368 nm. If it is the secondary amino acid, proline, a wavelength of 262 nm is used. For each chromatogram, the peak areas are integrated manually. This provides a matrix containing the area of each amino acid for each sample. To deduce the concentration of each amino acid in each sample, an average response coefficient (RC) is calculated for each AA in the standard mixture. RC is calculated by dividing the area of the amino acid (Ai) by the area of norvaline (Anor) in the standard mixture used. From this RC, the absolute concentration is calculated for each amino acid in our samples by applying this formula:

$$Ci = \frac{Ai\ (sample)}{\varvecA\varvecn\varveco\varvecr\ (sample)} \varvec* \frac{1}{\varvecK} * \frac{\varvecA\ \varvecn\varveco\varvecr\ (sample)}{\varvecA\varvecn\varveco\varvecr\ (\varvecs\varvect\varvecd)} * [std]$$

Where Ci = Concentration of the amino acid in the sample, Ai = Area of the amino acid in the sample, A(nor) = Area of norvaline, K= response coefficient calculated in the standard, [Std] = 100µM for shoots and roots, and 250 µM for *Frankia* pellets.

Concentrations were expressed in nmol/mg of dry biomass of the weighed sample, taking into account the extraction yield.

Secondary metabolites. For secondary metabolites, raw data from MS/MS analyses were processed using MetaboScape 4.0 software (Bruker). The list of peaks was generated from recalibrated MS spectra (< 0.5 ppm) in a window of 1 to 15 minutes of the LC gradient. Ions with a minimum intensity of 5,000 counts (*Frankia* pellets and shoots) and 4,000 counts (roots) for at least 10% of all samples were detected and realigned by combining all charge states and isotopic forms.

Statistical analyses

Using the open source software MetaboAnalyst (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml) (Pang et al., 2024), one-factor statistical analyses of the metabolic profiles of amino acids and secondary metabolites are performed. PCA (Principal Component Analysis) and PLS-DA (Partial Least Square Discriminant Analysis) are generated in order to compare the two conditions (culture alone vs. co-culture) for each compartment on

D1, D2 and D3. Data relating to secondary metabolites are normalised (centred and reduced data). Then, given the small size of the replicates (n = 5), non-parametric Kruskal-Wallis mean comparison tests (t-tests) are performed on the amino acids and secondary metabolites between conditions, setting a maximum p-value of 0.05.

Results

Effect of co-culture on the amino acid composition of A. glutinosa and Frankia

Amino acids composition variation in alders' shoots and roots. In order to see the influence of molecules secreted by *Frankia* on amino acid content during the establishment of actinorhizal symbiosis, shoots and roots content was analysed. A total of 22 AAs was detected, methionine, was not detected in these extracts. Figure 2 shows the PCA following metabolic profiling of the 22 AAs in samples of shoots (Fig. 2A) and roots (Fig. 2B) of alder grown alone (purple triangle) or with *Frankia* (pink circle) on D1, D2 and D3. The variability explained by axes 1 and 2 was greater than 60% for all PCAs. In terms of separating the two growing conditions, there appears to be more difference in the shoots than in the roots. The presence of *Frankia* seems to affect the AAs of the shoots (particularly on D1 and D3, low overlap of ellipses), unlike the roots, where we didn't note a clear separation.

This was confirmed by statistical analyses since the presence of *Frankia* impacted significantly the concentration of AAs for all sampling times in the shoots (Fig. 3A, B, C). On the contrary, amino acid production was only impacted at D2 in the roots, with an increase of Ala and Phe production when alder was co-cultivated with *Frankia* (Fig. 3E). In the shoots, eight amino acids were significantly different between the single culture (pink) and the co-culture (purple) growing conditions on D1 (Fig. 3A). Asn, Gln, Ala, GABA, Val, Phe, Ile and Pro were overproduced in the presence of *Frankia* with a factor ranging from 1.9 for Gln to 3.08 for GABA. On D2, Ala and Tyr were overproduced in the presence of *Frankia*, while AABA was underproduced (Fig. 3B). On D3, the production of 14 amino acids (Asp, Asn, Ser, Gln, Gly, Cit, Arg, GABA, Tyr, Val, Trp, Phe, Lys and Pro) increased in the co-culture condition with a factor ranging from 1.97 for Tyr to 5.06 for Cit (Fig. 3C).

Amino acids composition variation in Frankia. Comparing the AA content in bacterial pellets between the two culture conditions (single culture and co-culture) reveals the influence of molecules secreted by alder on the intracellular metabolome of *Frankia*. Figure 2C shows the PCA obtained from the amino acids matrices prepared following analysis of the pellets of *Frankia* grown alone (purple triangle) or in the presence of *A. glutinosa* (pink circle) on D1, D2 and D3. Axes 1 and 2 represent 93.1%, 93.3% and 96.4% of the overall variation on D1, D2 and D3. PCAs show no or low separation of culture condition (overlap of ellipses). Non-parametric t-tests comparing means reveal differential amino acids on D1 and D2 (data not shown). These are glutamine (Gln), which increases in co-culture conditions by a factor of 1.31 and 1.95 on D1 and D2 respectively, and Cit, which decreases in the presence of alder by a factor of 0.78 on D2.

Effect of co-culture on the secondary metabolites composition of A. glutinosa and Frankia

Secondary metabolites composition variation in alders' shoots and roots. Non-targeted analysis of secondary metabolites in extracts from shoots and roots reveals changes in the metabolome of alder in the presence of *Frankia*. Shoots and roots appear to have very distinct metabolic compositions. In the shoots, non-polar compounds were detected at the end of the chromatogram with higher intensity (Supplementary Material 5). This difference is also visible in the total number of ions detected by LC/MS. A total of 3,493 ions are detected in the shoots compared to 2,643 ions in the roots.

Figure 4 shows the PLS-DA performed on shoots (Fig. 4A) and roots (Fig. 4B) between the single culture condition (dark green triangles) and co-culture with *Frankia* (light green circles) on D1, D2 and D3. The co-culture and single culture samples are separated along both axes for each of the days considered and for both the shoots and the roots. Secondary metabolite profiles varied greatly in co-culture compared to single culture for both shoots and roots. Of the 3,493 ions detected in the shoots, 677, 286 and 407 were significantly impacted at D1, D2 and D3 respectively (representing 19.4, 8.2 and 11.6% of the detected ions) while of the 2,643 ions detected in roots, 71, 269 and 177 were significantly impacted at D1, D2 and D3 respectively (2.7, 10.2 and 6.7% of the detected ions) (Fig. 5A). The majority of compounds significantly impacted in shoots and roots were overproduced with the strongest effect observed in shoots at D1 with a total of 643 metabolites overproduced when alders were co-cultivated with *Frankia* (Fig. 5A). Of this significantly impacted ions, only few were common to all three days, with 5 marker ions in the shoots and 24 in the roots (Fig. 5B). On the contrary, the concentration of most ions is significantly affected at only one of the three sampling times (Fig. 5B). Altogether these results indicate a rapid evolution of the metabolome in the plant.

Secondary metabolites biomarkers of Frankia discriminating between single culture and co-culture conditions. A total number of 3,549 ions are detected in *Frankia* pellets (all conditions combined). The metabolic profile of the QC shows a diversity of metabolites produced that are polar in nature (Supplementary Material 6). The PLS-DA analyses (Fig. 4C) highlight a difference in the metabolic composition of cultures grown alone (dark green triangles) or in co-culture with the plant (light green circles). Indeed, the conditions of culture alone are clearly separated from the conditions of co-culture on D1, D2 and D3, suggesting the presence of very discriminating peaks between the two conditions. The number of biomarker ions discriminating between the two culture conditions is presented in Fig. 5A. A total of 1023, 174 and 345 ions are significantly different on D1, D2 and D3 respectively ($p\text{-value} < 0.05$). At D1, a large number of ions are downproduced when *Frankia* is co-cultivated with the plant (927/1023), then the trend reverses at D3 since there are more overproduced (254) than underproduced (91) ions. Very few ions (2) were common to all three days in the *Frankia* pellet (Fig. 5B) reflecting, like in the plant, the rapid evolution of metabolome.

Discussion

Role of discriminating amino acids observed during this early stage of actinorhizal symbiosis

Studies of primary metabolism in the *Frankia/Alnus* symbiotic complex are mainly carried out after nodule formation (Carro et al., 2015; Hay et al., 2020; Lundberg and Lundquist, 2004). Our study focuses on the stage preceding nodule formation. In particular, we have attempted to characterise the content of AAs involved in the establishment of this symbiosis by comparing different culture conditions (culturing *Frankia* or alder alone and co-culturing both partners). These analyses enable us to highlight the metabolic changes induced by the molecules secreted in both partners.

Under co-culture conditions, the change in AA content is more pronounced in the shoots than in the roots and in *Frankia* (during the experiment the concentrations of 17 AAs are altered under co-culture conditions in the shoots, compared to 2 in the roots and 2 in *Frankia*). A total of 16 AAs (Asp, Asn, Ser, Gln, Gly, Cit, GABA, Ala, Arg, Tyr, Trp Val, Phe, Ill, Lys and Pro) are overproduced in the presence of the bacterium in the shoots over the different sampling days, compared to only one, AABA, which decreased on D2. This increase in AA has already been noted in *Alnus glutinosa* nodules compared to uninfected roots (Brooks and Benson, 2016). The authors measured the amount of AA in nodules (roots infected with the *Frankia* CpI1 strain) and uninfected roots and detected higher concentrations of certain AAs in nodules, such as Asn, Glu, Gln, Thr, Cit, Tyr and Ala. Five of these AAs (Asn, Gln, Cit, Tyr and Ala) were found in higher concentrations in the plant under co-culture conditions (compared to single-culture conditions) in our study. These results are also consistent with the work of Hay et al. (2020). The authors identified marker AAs in the *Alnus-Frankia* ACN 14a interaction by comparing nodules (from the field and greenhouse) with non-nodulated roots. Four AAs (Glu, Arg, Cit and Asp) were significantly different between greenhouse nodules and non-nodulated roots. In field samples, certain AAs such as Arg, Glu, Val, His, Ser, Trp, Lys, Ill, Phe, Tyr and Pro were found in higher concentrations in nodules than in roots. Eleven of these AAs were also found in our study (Cit, Asp, Arg, Val, Ser, Trp, Lys, Ill, Phe, Tyr and Pro). Our results are consistent with the two studies cited above, despite the difference in the symbiotic stage studied. Thus, we find an increase in the same AAs in the shoots in response to the presence of the bacteria. In addition, Hay et al. (2020) studied the effect of seven amino acids (including Asp, Asn, Gln, Cit, Ala, GABA and Val) on the growth, respiration and nitrogen fixation of *Frankia* ACN 14a. The authors were able to show that Asp and Gln stimulated the respiration and growth of the bacterium, while Asn, Cit, GABA, Ala and Val had a positive effect on nitrogen fixation and slightly stimulated the growth and respiration of the bacterium. We therefore hypothesised that in the early stages of symbiosis, the plant would modify its primary metabolism (i.e. Asp, Asn, Gln, Cit, GABA, Ala and Val) in its leaves in anticipation of a transfer to the bacteria to stimulate its growth and respiration.

Secondary metabolism

The mechanisms involved in establishing symbiosis remain a complex interactive process that has been well understood in the *Rhizobium*-legume system (Oldroyd et al., 2011) and is partially understood in actinorhizal symbiosis. The main limitations are the lack of genetic transformation tools for *Frankia* and the slow growth rate of bacterial isolates (Popovici et al., 2010). Recent developments in metabolomics (particularly untargeted metabolomics and corresponding statistical analyses) now offer an interesting

approach to deciphering the modification of the metabolomes of both symbionts induced by secreted molecules.

Our study on the profiling of secondary metabolites in the preliminary stage of alder/*Frankia* symbiosis shows a drastic change in intracellular metabolism for both partners on all days considered. This change occurs at all levels (shoots, roots and *Frankia*), unlike the results for amino acids, where changes were almost exclusively visible in the shoots. Thus, a significant number of biomarkers are identified using the PLS-DA approach. For example, at the start of the interaction (D1), a total of 1023, 677 and 71 biomarker ions are counted on *Frankia*, shoots and roots, respectively. This shows that metabolic changes precede the deformation of root hairs (observed at 2.5 days in the symbiosis timeline). Furthermore, Pujic et al. (2019) conducted a transcriptomic and proteomic study of the molecular dialogue between *Alnus/Frankia* at the early stage (2.5 days). The authors identified 1,071 genes (611 overexpressed versus 460 underexpressed) and 443 proteins (283 overexpressed versus 160 underexpressed) involved in the *Alder/Frankia* interaction. These results illustrate the very rapid transcriptomic modification of *Frankia* during its transition from a free state to a symbiotic state. This strong dynamic is also reflected in our results on secondary metabolites (i.e. effects of the co-culture condition on the *Frankia* metabolome observable from D1 to D3, Fig. 4C).

Brooks & Benson (2016) studied changes in the metabolome of *Alnus glutinosa* nodules compared to uninfected roots. They found 192 differential metabolites (54 known and 138 unknown) between nodules and roots. Most of these were phenolic compounds (caffeic acid, isonicotinic acid, valeric acid, etc.) and were overproduced in the nodules. At the same time, Popovici et al. (2010) highlighted the role of eight flavonoids, five of which are dihydrochalcones present in *Myrica gale* (*Myricaceae*) during the early stage of *the Frankia-M. gale* interaction. Two of these (Myrigalone B and P) strongly stimulated the growth of *Frankia* ACN 14a. These studies strongly suggest the involvement of plant phenolic compounds, particularly flavonoids, in the early stages of actinorhizal symbiosis. Ion annotation was attempted in our study using databases accessible from the MetaboAnalyst platform, but this approach was unsuccessful due to few matches in the databases with too little confidence. Future studied should focused on annotation using complementatry approaches such as molecular networking.

Conclusion

The molecular mechanisms involved in the *Alnus glutinosa/Fran*kia interaction are poorly understood. Our targeted (amino acids) and untargeted (secondary metabolites) metabolomic study revealed changes in the metabolomes of both partners at the preliminary stage. We were able to demonstrate that this symbiosis requires an increase in amino acid content in the plant's shoots from the early stage of the interaction. Some of these amino acids (e.g. Gln, Val, etc.) appear to stimulate the growth and respiration of the bacteria, enabling them to transition from a free state to a symbiotic state. In addition, the encounter between the two partners modifies their production of secondary metabolites, which are either overproduced or underproduced in the different compartments studied (shoots, roots and *Frankia*).

For the future, the study of its results must be further developed by annotating the biomarkers detected. Finally, it would also be relevant to analyse sugars and organic acids by GC/MS in order to understand all the mechanisms involved in the *Alnus/Frankia* symbiosis.

Declarations

Competing interests policy

The authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Author Contribution

SKT designed the experiment. PF, PJ and SKT performed the *Alnus-Frankia* experiment. EF performed the analysis of primary and secondary metabolites. GM and MR supervised EF in the analysis of primary metabolites performed at LEM. BM, AM and RP supervised the analysis of secondary metabolites performed at MNHN. EF, SKT and AE performed the analysis and interpretation of data and wrote the main manuscript text and prepared figures and tables.

Data Availability

our data is newly generated and are available upon request

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Figures

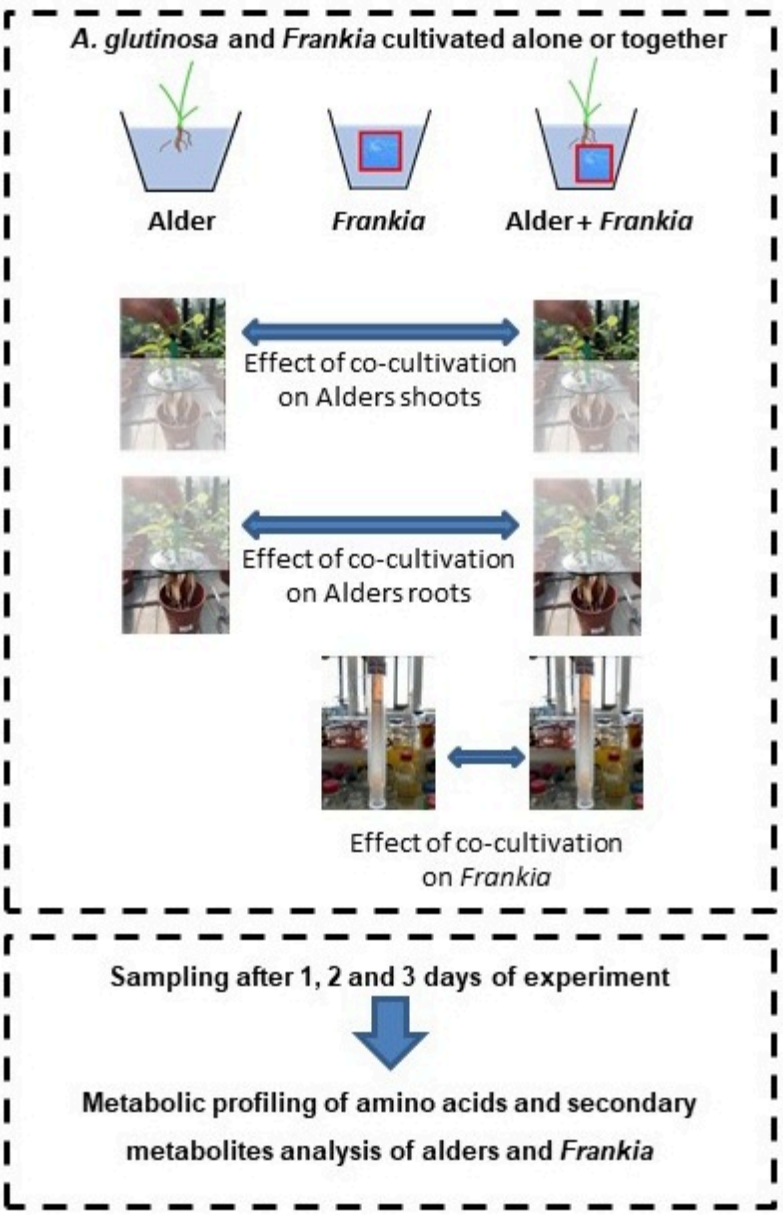


Figure 1

Illustration of the experimental setup designed to determine the regulation of metabolite production during the encounter between the actinobacterium *Frankia* and its host *Alnus glutinosa*.

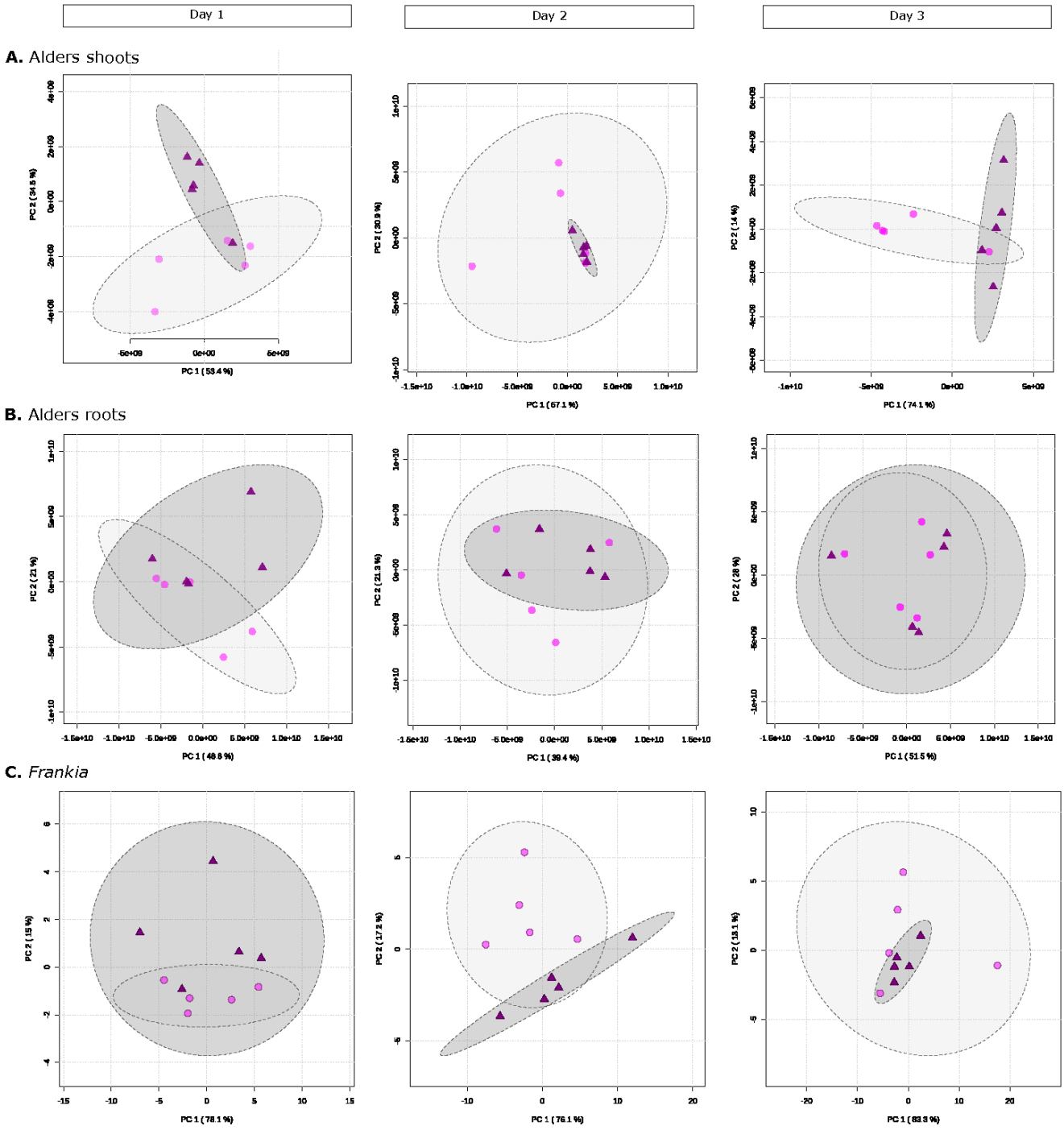


Figure 2

Principal Component Analyses following metabolic profiling of the 22 amino acids in alder's shoots (A), alder's roots (B) and *Frankia* (C) of alder or *Frankia* grown alone (purple triangle) or together (pink circle) on day 1, day 2 and day 3 of the experiment.

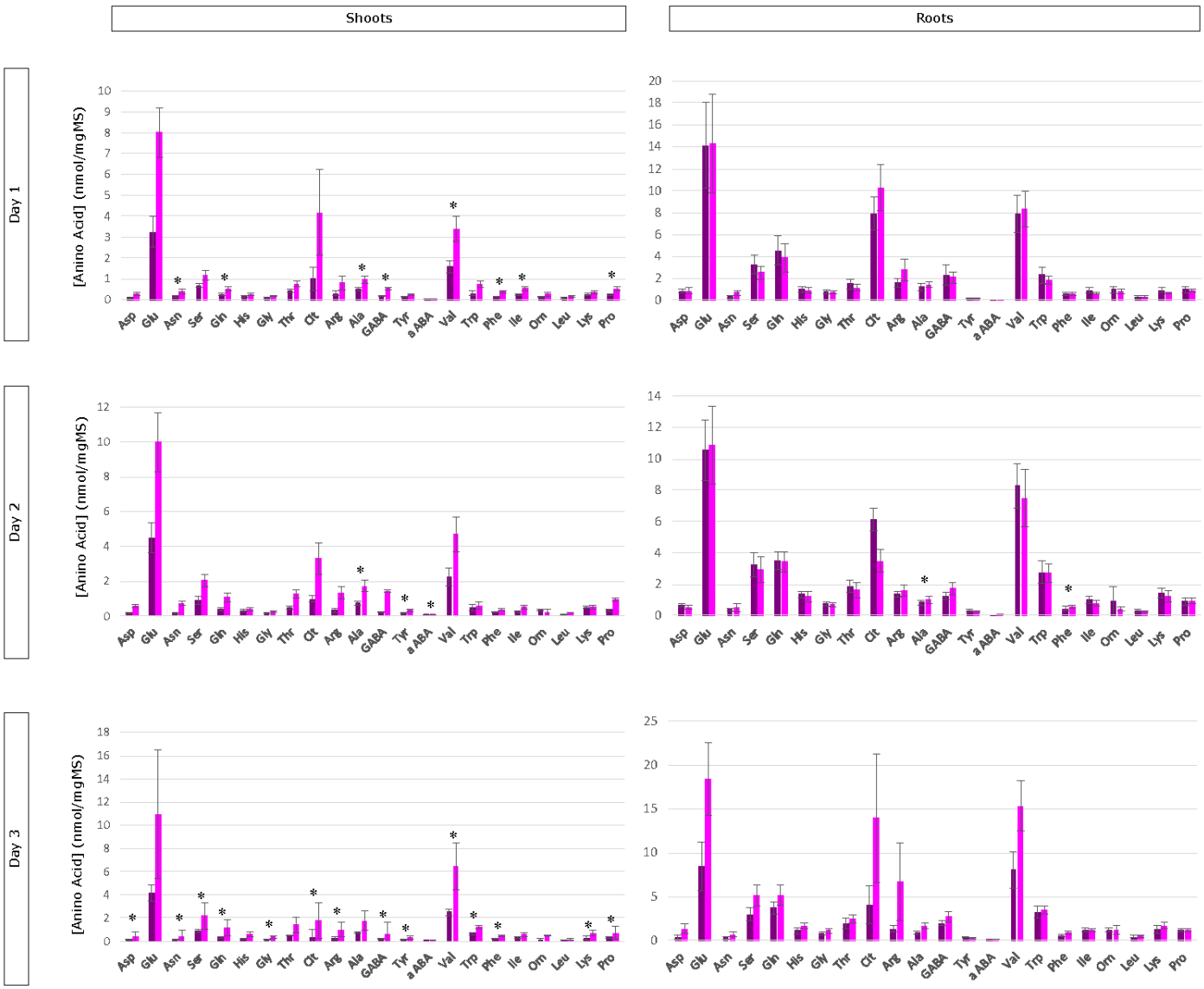


Figure 3

Concentration (expressed as mean value ± standard error) the 22 amino acids in shoots (ABC) and roots (DEF) of alder grown alone (purple) or together with *Frankia*(pink) on day 1, day 2 and day 3 of the experiment. Stars indicate statistical difference between alder grown alone or together with *Frankia* (n=5, *p<0.05).

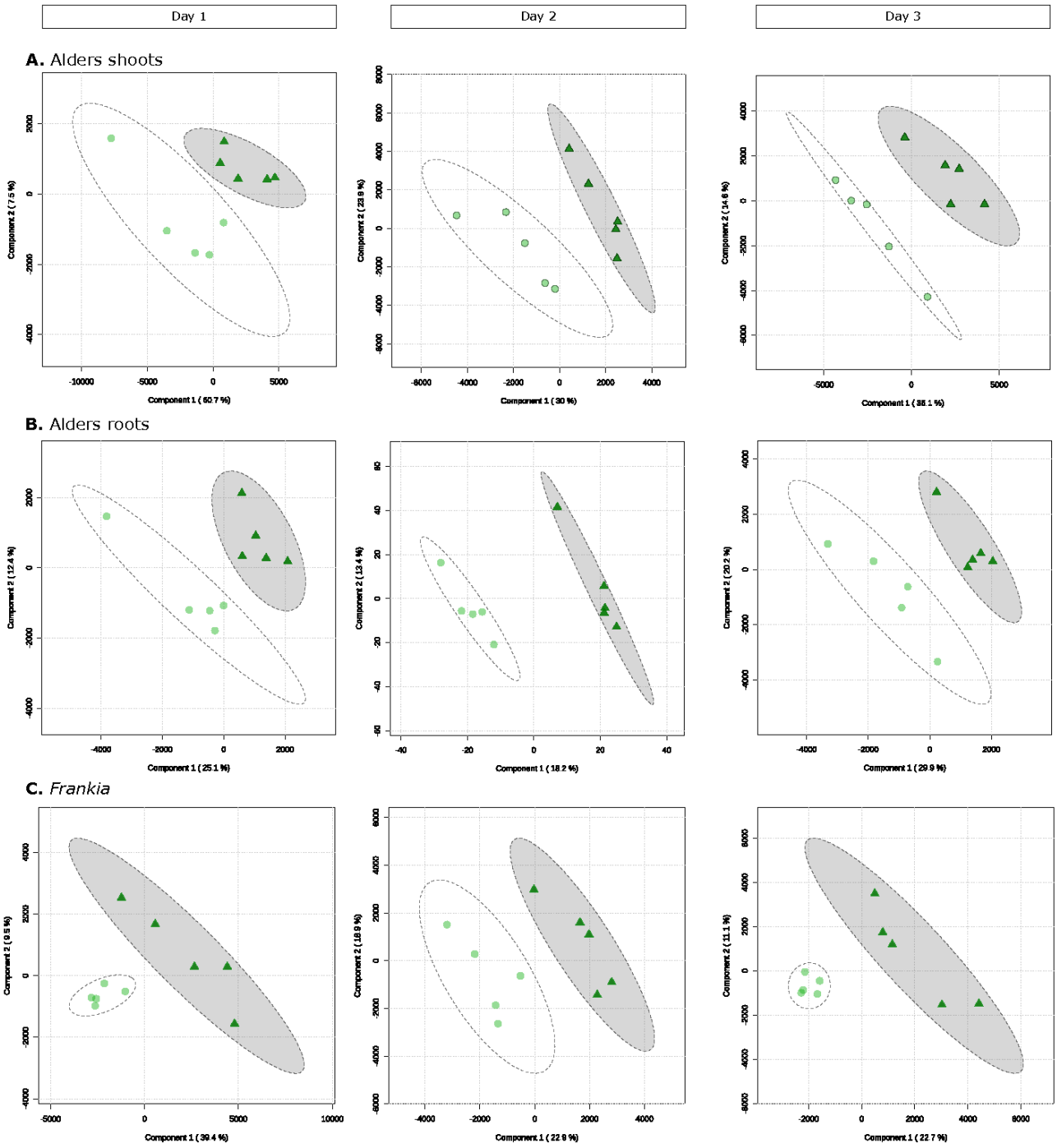


Figure 4

Partial Least Squares Discriminant Analysis (PLS-DA) performed on alder's shoots (A), alder's roots (B) and *Frankia* (C) samples on D1, D2 and D3. Dark green triangles represent the single culture condition and light green circles represent the co-culture condition.

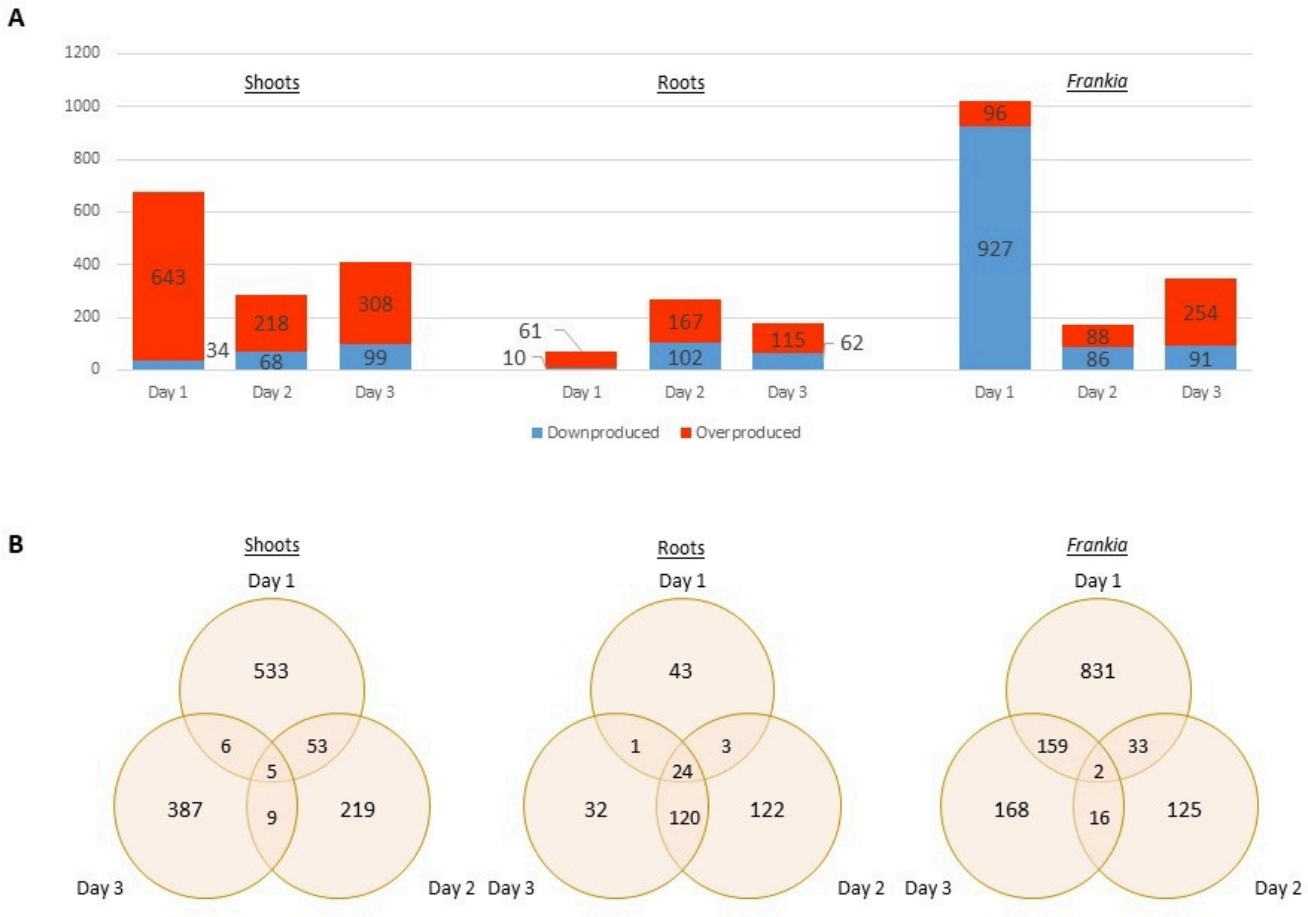


Figure 5

Number of compounds significantly impacted in shoots, roots and *Frankia* when alders or *Frankia* were co-cultivated (n=5, p<0.05) (A). Venn diagrams showing the number of compounds common to 1, 2 or 3 days of experimentation among those significantly impacted by the co-culture condition in shoots, roots and *Frankia* (B). Compounds downproduced are presented in blue and compounds overproduced are presented in red.

Supplementary Files

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