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NMR and LC-MS-Based Metabolomics to Study the Effect of Surfactin on the Metabolome of Flax

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Abstract: Flax (*Linum usitatissimum)* is a versatile plant used in a range of applications, from textiles to nutrition. Surfactin, a cyclic lipopeptide biosurfactant produced by bacteria such as *Bacillus subtilis*, has potential as a biocontrol agent or as a plant defense inducer in agriculture. This work aims to determine the effects of surfactin treatment at two kinetic points on the metabolism of flax hydroponic cultures, using advanced metabolomic techniques, including ^{1}H NMR and LC-MS analyses. Surfactin, detected in the roots, has a significant local impact on the metabolic profiles of flax roots, leading mainly to a higher content of cyanogenic compounds and amino acids and a lower content of carbohydrates. Surfactin, which is not detected in the aerial parts, also induces contrasted changes in amino acids, sugars, and secondary metabolite accumulation between stems and leaves. Surfactin treatment of flax leads to both a local and systemic effect on flax metabolism. These changes suggest that plant response to surfactin treatment could induce an enhanced plant defense. This could suggest potential applications of surfactin in the agricultural field as a biostimulant or biocontrol agent, to limit the use of chemical compounds in culture, and to limit their negative impact on both health and the environment.

Keywords: *Linum usitatissimum*; surfactin; metabolomics; ^{1}H NMR; LC-MS; systemic effects; local effects

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1. Introduction

Flax, also known as *Linum usitatissimum* (Linaceae), is a multipurpose plant used for its fiber and nutritious seeds. It grows until 0.9 to 1.2 m tall, with thin stalks, short lance-shaped leaves, and blooms with five petals in a variety of hues. The fruits are little capsules with glossy brown seeds accumulating high contents of omega-3 fatty acids and lignans. Flax has been domesticated for almost 8000 years, beginning in the Fertile Crescent region [1].

Flax fiber, one of the earliest textile fibers known to humans, is produced by processes such as retting, drying, crushing, and beating. Its strength, durability, and moisture absorption make it ideal for a wide range of items, including delicate linen textiles and industrial materials such as canvas, twine, and fire hoses. Linen clothing is popular because it keeps the user cool and is resistant to microbes [2]. Linen culture for fiber production is mainly found in northwestern Europe [1,3].

Flaxseed contains omega-3 fatty acids, lignans, dietary fiber, protein, and minerals. It can be eaten raw or roasted; mixed into salads or baked items; or extracted to make linseed

oil for paints, varnishes, and supplements. Flax culture for seed production is mainly found in nations such as Russia, Kazakhstan, Canada, and China [4].

Genetic research, combined with advances in genomics and metabolomics, allows for improvement in flax culture and applications [5]. Because flax culture can be damaged by several biotic or abiotic stresses, it is interesting to develop new practices to limit the use of chemical products that potentially damage the environment and health.

The use of biostimulants and biocontrol agents has economic potential in agriculture because they can increase production, reduce input costs, and promote sustainable agricultural practices [6]. They can improve yields, reduce dependency on chemical pesticides, and promote global food security. Importantly, they can be economically competitive with traditional ways, promoting their inclusion into contemporary agricultural systems. These products may be derived from natural microbes or plants. As a result, they can decrease or eliminate damage caused by certain pests and diseases while also improving plant performance [7–9].

Among these agents, *Bacillus* strains are available in different commercial products. These bacteria produce several metabolites, belonging to the lipopeptide family, which can induce plant resistance to different pathogens. A study on surfactin produced by *Bacillus subtilis SF1* against the fungal agent *Fusarium foetens*, which causes potato wilt, has shown that surfactin and other lipopeptides produced by *Bacillus* may have biocontrol properties against plant diseases [10]. Surfactant molecules have also been recognized as powerful biostimulants in agricultural and horticultural systems due to their ability to solubilize large organic molecules and increase their availability for uptake by plants [11]. Surfactin is a cyclic lipopeptide biosurfactant. Its structure consists of a circular heptapeptide containing specific amino acids such as L-leucine, D-leucine, L-valine, L-aspartate, and L-glutamate, along with a long-chain fatty acid [11,12].

Surfactin has a variety of uses in plant protection and has been proven to trigger induced systemic resistance (ISR) in plants, thus increasing their pathogen defense systems [13]. For example, surfactin has been shown to activate ISR in plants such as tobacco and tomato. Furthermore, surfactin has been shown to improve resistance to diseases such as cucurbit powdery mildew in melon plants. These findings emphasize surfactin's potential as a biocontrol agent in agriculture, promoting plant health and disease management [13,14]. Surfactin has also been shown to boost the host's defensive capabilities [15]. Additionally, surfactin has been found to increase the uptake of certain minerals and nutrients, which in turn may influence plant metabolism. Surfactin can potentially influence the activity of enzymes engaged in metabolic processes. All of these impacts may interact to influence a plant metabolome. This systemic impact is a series of molecular processes that spread beyond the treated region, influencing other areas of the plant [16–18].

Therefore, this work aims to study how these metabolic changes manifest in flax plants when exposed to surfactin treatment.

Metabolomics research on flax has been proven to be a powerful approach to elucidate its metabolic functions [19]. Flax is known to contain numerous metabolites in its aerial parts and roots, often serving as biomarkers of plant health [19,20]. Employing a combination of analytical methods is interesting for studying flax metabolism, with gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC—MS), and nuclear magnetic resonance (NMR) allowing for the detection of differently accumulated metabolites in the plant [21,22].

In our investigation into the systemic and local effects of surfactin on flax, advanced metabolomics analyses using both ^{1}H NMR and LC-MS were employed on different extracts of the three vegetative parts of flax. The aim of this study was to evaluate the changes observed in terms of primary and secondary (semi-)polar metabolites, which can be affected by surfactin treatment and are involved in plant resistance mechanisms.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

Flaxseeds of the Progress variety were supplied by a French company (LINEA Semences de Lin, Martainneville, France). Plants were grown in a hydroponic system in Hoagland solution [23,24] in a growth chamber at 21 °C, 70% relative humidity, and 16 h of photoperiod. After 30 days of culture, surfactin was added to the medium of half the cultivated plants. A total of 25 plants were used for this study, with 13 plants allocated to the control group and 12 plants allocated to the surfactin-treated group. Then, the control and treated plants were harvested at two different kinetic points: 56 h (56 h), with 6 control plants and 5 surfactin-treated plants, and 10 days (10 D), with 7 control plants and 7 surfactin-treated plants, after applying treatment (Figure 1). The aerial and root parts of each plant were separated, immersed in liquid nitrogen, stored at −80 °C, and freeze-dried. The dry weight of each freeze-dried plant sample was precisely determined using a Sartorius ME235P balance, and the average dry weight of each organ in each condition of culture was determined to evaluate the growth of the plant (Section 3.1).

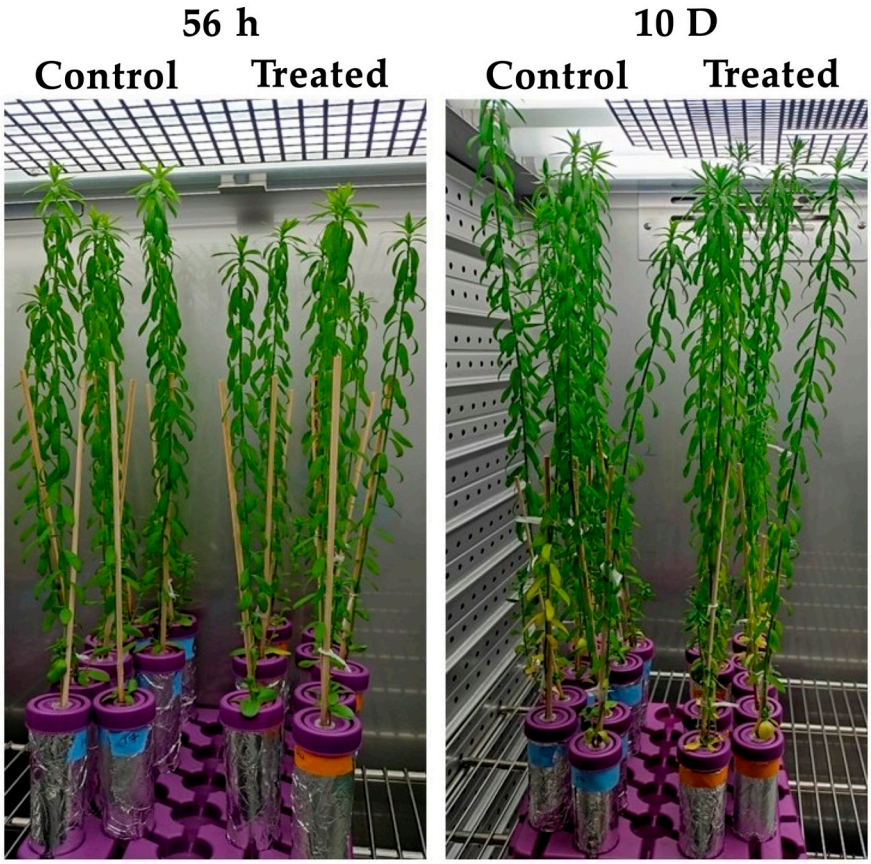


Figure 1. Flax plants cultivated in hydroponic devices. Photos taken before the 56 h harvest (**left**) and the 10 D harvest (**right**); for each kinetic point, the left rack shows the control plants, and the right rack shows the surfactin-treated plants. Wooden stick height: 30 cm.

Surfactin was obtained through the purification of an extract of *Bacillus velezensis* (purity 90%) [25]. Briefly, 30 mg of surfactin was dissolved in 50 mL of MilliQ water, and 1 mL of this solution was added to 49 mL of the plant culture medium directly in Falcon® tubes for a final concentration of 12 µM (CMC value ~15 µM). The treatment with surfactin was performed 30 days after the transfer of plants in a hydroponic system, and the control condition resulted in the addition of 1 mL of MilliQ water instead of surfactin stock solution.

2.2. Metabolite Extraction

The extraction was performed with an Eppendorf® epMotion 5073 robot to extract (semi-)polar metabolites; this device is an automated pipetting system that allows for enhanced reproducibility. Briefly, 20 ± 0.02 mg of root powder and 60 ± 0.02 mg of leaf and stem powder were weighed and used for solid/liquid extraction. A water/methanol (1:1) solvent was used for extraction. Additionally, 400 µL was added for roots, and 500 µL was added for leaves and stems. The samples were mixed at 60 °C for 10 min using a ThermoMixer® (Eppendorf AG, Hamburg, Germany) at 2000 rpm, followed by sonication at 60 °C for 30 min using a 35 kHz ultrasonic bath. The centrifugation of the samples was performed at 12,000 rpm for 10 min at 4 °C to separate extract and plant powder. This extraction procedure was performed three times. For the second and third extractions, the same volume of MeOH/H_2O solvent as that of the collected supernatants was added to plant powder pelleted in the microtube at the beginning of each new extraction. This corresponded to 300 µL for roots or 400 µL for leaves and stems. Final volumes of 900 µL for roots and 1200 µL for leaves and stems were then collected, of which aliquots of 700 µL for roots and 800 µL for leaves and stems were used for NMR analysis; the remaining 200 µL for roots or 400 µL for leaves and stems were used for LC-MS analysis. The described extraction was adapted from [19,20,26].

2.3. Metabolite Analysis by NMR

This part of the experiment was adapted from [19].

2.3.1. Sample Preparation

For each sample prepared for NMR analysis, the pH was precisely adjusted to 6.00 ± 0.02. A nitrogen flux was used to remove the hydroalcoholic solvent. The dry extract was then dissolved in 700 µL of MeOD/D_2O solvent (1:1), where KH_2PO_4 was added at a concentration of 0.1 M for buffer properties. TMSP was added at a concentration of 0.125 g/L for external standardization, and sodium azide was added at a concentration of 0.6 mg/mL as a microbial inhibitor. pH was checked and again adjusted to 6.00 ± 0.02 with deuterated solutions of DCl or NaOD. After vortexing, sonication, and centrifugation, the supernatant was then placed in a 5 mm NMR tube prior to NMR analysis.

2.3.2. NMR Data Acquisition

A Bruker Avance III 600 MHz spectrometer (Bruker, Wissembourg, France) operating at 600.13 MHz for ^1H and 150.91 MHz for ^{13}C was used to record all the NMR spectra with a 5 mm BBO probe (Bruker, Wissembourg, France), at 300 K. CD_3OD was used as an internal lock. The 1D ^1H NMR spectra were acquired using a water-suppressed pulse sequence, with the following acquisition parameters: number of scans of 32 scans, number of data points of 131 K, relaxation delay of 13 s, and spectral width of 8417 Hz. The Bruker software (Topspin v3.5) was used to automatically phase the spectra to perform a baseline correction and a calibration to TMSP at 0.0 ppm.

2.3.3. NMR Data Treatment

This step was exactly performed in the same way as that published in the paper cited at the beginning of Section 2.3 and required the use of the airPLS 2.0 algorithm [27,28], the icoshift algorithm (v 1.2.1), and the DAB technique [29,30].

2.4. Metabolite Analysis by LC-MS

This part of the experiment was adapted from [19].

2.4.1. Sample Preparation

A dilution with MeOH/H_2O solvent (1:1) was performed at 1/10 for root extracts and at 1/60 for stem and leaf extracts. The filtration of the diluted extracts was performed through 0.22 µm PTFE membrane filters before placing them in glass vials. For each root,

stem, or leaf sample, a QC was prepared by thoroughly mixing 10 µL from each diluted extract. For each plant part, LC-MS analysis was run in a randomized order.

2.4.2. LC-MS Analysis and Data Treatment

The LC-MS analysis was performed in the same conditions (column, elution, and MS parameters) as those published in the paper cited at the beginning of Section 2.4, but a VION Q-TOF mass spectrometer (Waters Micromass, Manchester, UK) controlled by UNIFI software (v 1.9.4) was used with a m/z range of 50–2000. For untargeted metabolomics, UNIFI processing was used to generate a data matrix of variables with a retention time chosen from 0.5 to 6 min. This matrix was then treated with the XCMS package (R software, v 4.2.2) to allow for multivariate analysis.

2.5. Statistical Treatment of Data

Statistical analysis was performed with SIMCA-P software (version 17.0; Umetrics, Umea, Sweden) and R Statistical Software (v 4.4.2; R Core Team 2024). Dry weights of plant organs were compared using a Kruskal–Wallis test. From the obtained datasets from NMR and LC-MS analyses, an OPLS-DA was performed. The OPLS-DA model resulted in a predictive component that was interpreted and represented by the *X*-axis, indicating the difference between the groups, and a second internal component indicated the intergroup differences. The Pareto scaling method was used in multivariate analysis. The VIP values > 1 were used to highlight important variables in the separation model [31].

3. Results and Discussion

3.1. Effect of Surfactin on Plant Growth

A comparison of dry weight was made for each organ between the treated and control plants at the two kinetic points (Figure 2). The growth of the plant in both control and surfactin treatment conditions was observed through an increase in dry weight, which was significant for roots and stems from 56 h to 10 days, whereas a significant increase in dry weight was observed for leaves only in the surfactin treatment condition. A significant decrease in the dry weight of roots and leaves in the treated plants compared to control plants was observed at 56 h after treatment with surfactin, whereas no significant change was observed in the dry weight of roots, stems, and leaves in the treated plants compared to control plants at 10 days. These observations, as shown in Figure 2 for the aerial parts, confirm the observations from the photo of flax plants grown in both conditions (surfactin-treated and control ones) presented in Figure 1, where no difference in size in the plants grown could be observed between the different culture conditions, suggesting that surfactin treatment did not alter flax growth after 56 h nor after 10 days of surfactin treatment.

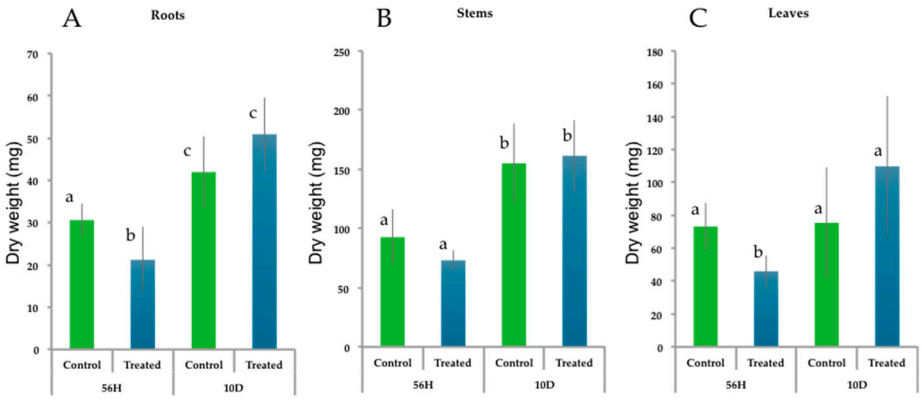


Figure 2. Comparison of the dry weight (mg) of each organ in both surfactin-treated (blue) and control (green) plants for both kinetic points harvesting: (**A**) roots, (**B**) stems, and (**C**) leaves, harvested (56 h, 10 D). For each organ, different letters indicate significant differences determined with the Kruskal–Wallis test.

3.2. Distribution of Surfactin in Flax Tissues

Surfactin treatment occurs by adding surfactin solution in the culture medium of hydroponic cultures of flax. At first, the local effect of surfactin was studied by analyzing root extracts, given that roots are the first organ in contact with surfactin.

3.2.1. Detection of Surfactin in Root Extracts

By comparing the data with the NMR spectrum of surfactin standard, a signal corresponding to surfactin was detected in surfactin-treated root extracts, analyzed by 1D ^{1}H NMR, which was a doublet at 0.85 ppm (J = 6.7 Hz). It was not present in the spectra of control root extracts (Figure 3A). Considering the 2D NMR spectrum, a correlation signal of 0.85 ppm/25 ppm was also observed on the ^{1}H/^{13}C HSQC spectrum of the surfactin-treated root extracts but was absent on the ^{1}H/^{13}C HSQC spectrum of the control root extracts.

The comparison of LC-MS chromatograms of root extracts obtained from the control or treated flax plants reveals three additional peaks with a retention time of around 6.3 min in the treated conditions. By comparing with the surfactin standard, these three peaks corresponding to $[M\text{-}H]^{-}$ ions could be assigned to surfactin A (6.27 min, 1006.6414 m/z), surfactin B (6.35 min, 1020.6557 m/z), and surfactin C (6.43 min, 1034.6714 m/z) (Figure 3B,C). The relative area of these three peaks showed that surfactin B was the main form of the surfactin standard (more than 40%), followed by surfactin C (less than 40%) and surfactin A (less than 20%). The structure of these molecules of surfactin is presented in Table 1. The three surfactin forms had a similar amino acid basic structure, whereas the fatty acid chain length of surfactin A, B, and C was 13, 14, and 15 carbon atoms, respectively. These signals were removed from the dataset to perform multivariate data analysis. The quantitative analysis of surfactin in root extracts revealed that around 60% and 80% of the surfactin added in the culture medium was found in the root extracts 56 h and 10 days after the treatment, respectively.

Table 1. Different types of surfactin detected in negative mode ionization on treated root extracts analyzed by LC-MS.

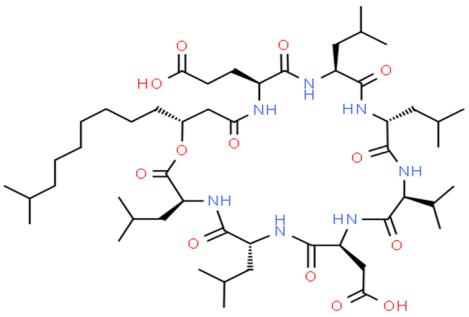
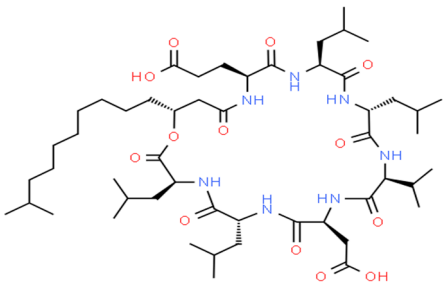
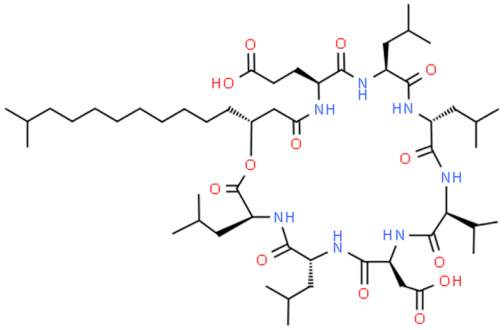
Surfactin Type Molecular Formula Monoisotopic Mass	Chemical Structure	ID Reference ChemSpider
Surfactin A $C_{51}H_{89}N_7O_{13}$ 1007.6518		28533730
Surfactin B $C_{52}H_{91}N_7O_{13}$ 1021.6674		28533731

Table 1. *Cont.*

Surfactin Type Molecular Formula Monoisotopic Mass	Chemical Structure	ID Reference ChemSpider
Surfactin C $C_{53}H_{93}N_7O_{13}$ 1035.6831		391754

In parallel to the effect of surfactin at the point of application, i.e., in the roots, the effect of surfactin far from the point of application, i.e., in the aerial parts of the flax, was studied.

3.2.2. No Detection of Surfactin in Aerial Part Extracts

The same analyses carried out on the root extracts were carried out on the leaf and stem extracts and revealed no peak corresponding to the three forms of surfactin. In LC-MS chromatograms, there was no additional peak in the area at the retention time range of 6.2–6.5 min when comparing the surfactin-treated samples with the control samples. These results were also confirmed by the absence of a doublet signal at 0.85 ppm in ^{1}H NMR spectra. This reveals that surfactin stays at the root level and does not transfer to stems and leaves.

This finding is consistent with previous research showing a general response to exogenous substances, attacks by pathogens and pests, and interaction with microbiota, reporting that systemic effects induced by local effects can alter the way plants defend themselves and respond to stress, with all of these elements being considered as part of the complex interactions between plants and their environment that can activate molecular signaling [32,33].

3.3. Metabolite Changes in Root

The OPLS-DA score plot (Figure 4) showed a good separation between the two groups. Symbols in green represent the control plant samples, which are grouped in the left part, whereas symbols in blue represent the surfactin-treated plant samples, which are grouped in the right part. The values for PC1 were 44% and 30% for the NMR dataset and LC-MS dataset, respectively, suggesting the highest contribution of more accumulated metabolites in the separation between the control and surfactin-treated samples because NMR analysis is less sensitive than LC-MS analysis. The values of PC2 were 9% for the NMR dataset and 17% for the LC-MS dataset. This component can explain the effect of kinetics on the separation. For the OPLS-DA score plot corresponding to the NMR dataset, most of the 56 h samples were in the upper part, whereas most of the 10 D samples were in the lower part, especially for the surfactin-treated samples.

Among the annotated variables from the corresponding VIP list, the discriminant metabolites belong to several biochemical families such as amino acids, carbohydrates, organic acids, or secondary metabolites (Table S1).

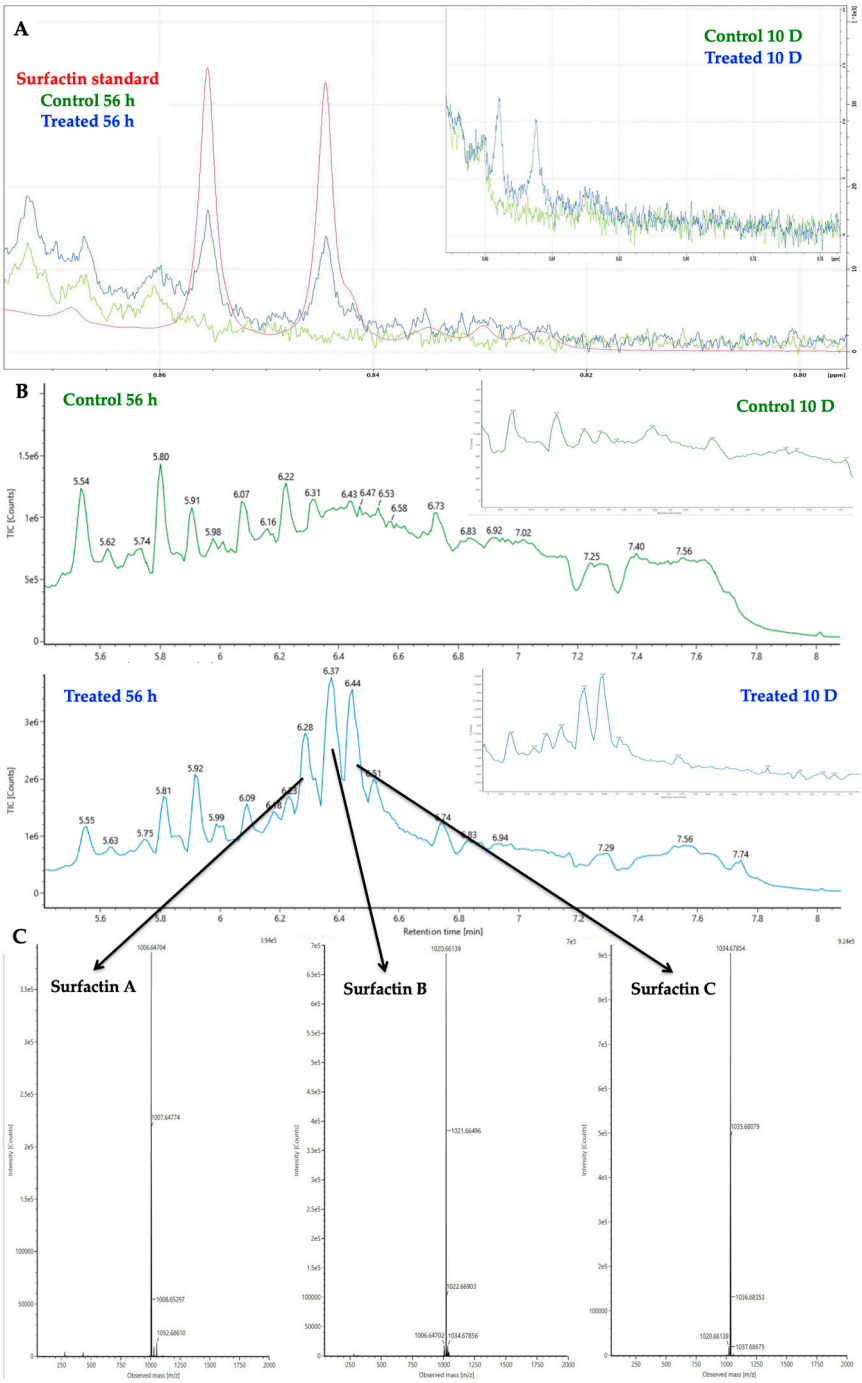


Figure 3. Signals of surfactin detected (**A**) by NMR analysis; doublet (δ 0.85 ppm, J = 6.7 Hz), present in standard (red) and 56 h surfactin-treated flax root extract (blue) or absent in 56 h control flax root extract (green); (**B**) by LC-MS analysis; peaks at 6.27, 6.35, and 6.43 min present in 56 h surfactin-treated flax root extract (blue) or absent in 56 h control flax root extract (green), (**C**) with the corresponding m/z of $[M\text{-}H]^-$ below.

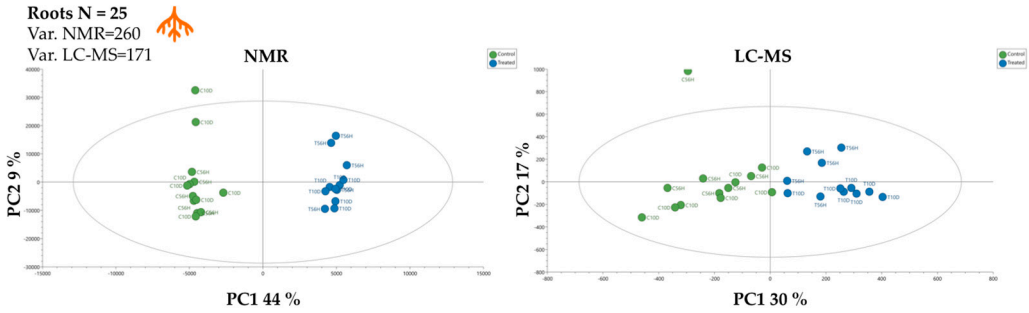


Figure 4. OPLS-DA score plot derived from ^{1}H NMR (**left**) and LC-MS (**right**) datasets: control flax roots (green) versus surfactin-treated flax roots.

Amino acids and organic acids:

Seven discriminant amino acids were annotated in the NMR dataset from root extracts, namely GABA, alanine, proline, valine, leucine, isoleucine, and glutamic acid. As can be seen in Figure 5, most of these amino acids were more accumulated in surfactin-treated flax roots compared to control flax roots, while in surfactin-treated flax, only one amino acid presented a decrease in its content for both kinetic points, namely glutamic acid. This amino acid is a precursor of proline and GABA, which were both accumulated at 56 h and 10 D in surfactin-treated conditions. The importance of this branchpoint has been recently described in plant defense and, in this work, could be associated with surfactin treatment [34]. In this review, they also mentioned the implication of malic acid in this biosynthesis regulation; changes in malic acid content were also observed in our study, with a decrease at 56 h and an increase at 10 D in the surfactin-treated flax roots. The different accumulation of amino acids has already been described in metabolomic studies on flax samples subjected to osmotic stress, suggesting that treatment with surfactin induces metabolic changes to prepare the plant for potential stress [16]. Two other amino acids, valine and isoleucine, were differentially accumulated in flax roots between surfactin treatment and control conditions. Their content highly increased at 10 D, similar to the content of cyanogenic compounds because valine is a precursor of linamarin biosynthesis, and isoleucine is a precursor of lotaustralin.

Cyanogenic compounds:

Both NMR and LC-MS datasets showed increases in linamarin and lotaustralin in surfactin-treated flax roots (Figure 5). These increases were more important at 56 h than at 10 D, whereas the two precursors were less accumulated at 56 h than at 10 D, suggesting that an increase in cyanogenic compounds led to precursor consumption at the beginning of surfactin treatment and that the flax plant responded by increasing the pool of precursors later after the beginning of surfactin treatment. The implication of cyanogenic glycoside levels in plants has already been described in plant stress resistance mechanisms, especially when submitted to herbivore attack [35,36], and in flax when submitted to abiotic stress [19].

Carbohydrates:

From the NMR dataset (Figure 5), we found that the level of sucrose, glucose, and fructose decreased at 10 D after the beginning of surfactin treatment, whereas the treatment did not alter sucrose and fructose accumulation early in the kinetics and induced an increase in glucose at 56 h in the treated roots. The implication of carbohydrates has been already reported in relation to plant stress response [37].

Phenolic compounds:

From the LC-MS dataset (Figure 5), we found that surfactin treatment induced a slight decrease in the lignan SMG and the neolignane DCG content in roots at 56 h and an increase at 10 D. A metabolomic study of osmotic stress on flax showed also an increase in SMG from 5 days after stress application but not 1 day after stress application, suggesting a

relatively important delay to induce metabolic change in the content of this lignan [19]. Another phenolic compound had a lower content in the surfactin-treated roots at the two kinetic points, namely 4-caffeoylquinic acid.

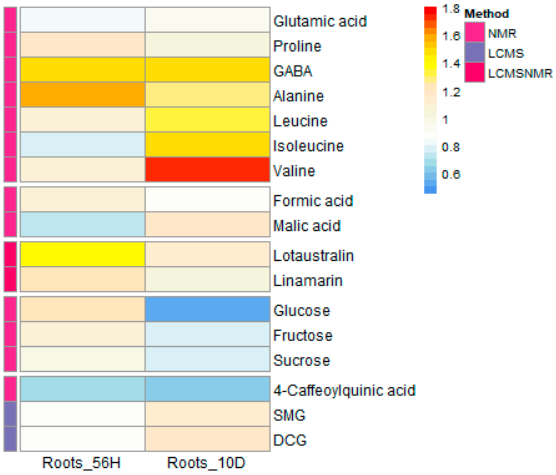


Figure 5. Heatmap of the discriminant metabolites differently accumulated in flax root extracts between surfactin-treated and control conditions. The scale bar (from red to blue) on the right represents the ratio of metabolite content in surfactin-treated flax roots to that in control flax roots. The column on the left indicates the kinetic point at 56 h and the column on the right indicates the kinetic point at 10 D. The analytical methods used to determine discriminant metabolites are indicated on the left of the graph; the red color corresponds to the combination of NMR and LC-MS, the blue color corresponds to LC-MS, and the purple color corresponds to NMR.

3.4. Metabolite Changes in Aerial Parts

The metabolic changes were studied in the aerial organs. These implicated the same biochemical families as those observed in roots, such as amino acids, carbohydrates, organic acids, and cyanogenic compounds, as well as phenolic compounds.

The OPLS-DA score plot (Figure 6) also showed a good separation between the two groups. Symbols in green represent the control plant samples, which are grouped on the left side, whereas symbols in blue represent the surfactin-treated plant samples, which are grouped on the right side.

Stems:

The values for PC1 were 34% and 36% for the NMR dataset and the LC-MS dataset, respectively, suggesting the similar contribution of more and less accumulated metabolites in the separation between the control and surfactin-treated samples. The values of PC2 were 3% for the NMR dataset and 17% for the LC-MS dataset. This component can explain the kinetic effect. For the OPLS-DA score plot corresponding to the NMR dataset, most of the 56 h samples were in the upper part, whereas most of the 10 D samples were in the lower part, especially for the surfactin-treated samples. Equivalent clustering on the OPLS-DA score plot corresponding to the LC-MS dataset was observed, but most of the 56 h samples were in the lower part, whereas most of the 10 D samples were in the upper part.

Leaves:

The values for PC1 were 33% and 44% for the NMR dataset and the LC-MS dataset, respectively, suggesting the highest contribution of less accumulated metabolites in the separation between the control and surfactin-treated samples. The values of PC2 were 23% for the NMR dataset and 10% for the LC-MS dataset. This component can explain the kinetic effect. The clustering of samples from a kinetic point of view was also observed for both the NMR and LC-MS datasets, with most of the 56 h samples in the upper part, while most of the 10 D samples were in the lower part.

Among the annotated variables in the VIP list corresponding to all the NMR and LC-MS data from the leaf and stem extracts, the discriminating metabolites belonged to the same biochemical families as the ones of root extracts (Table S1).

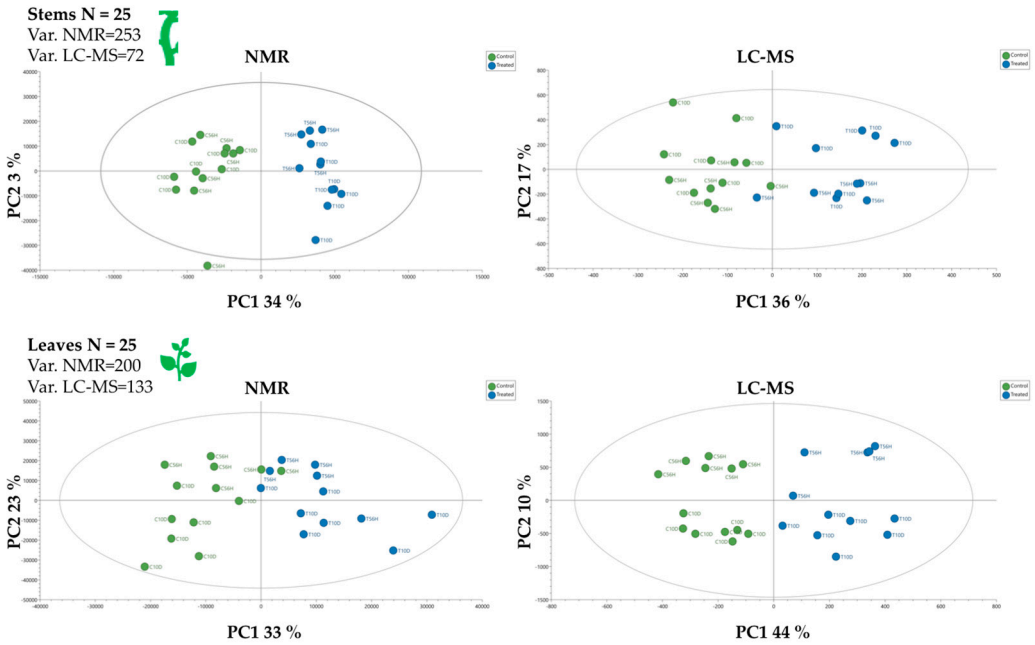


Figure 6. The OPLS-DA score plot derived from ^{1}H NMR (**left**) and LC-MS (**right**) datasets obtained from stem extracts (**upper part**) or leaf extracts (**lower part**); control flax plants (green) versus surfactin-treated flax plants (blue).

Amino acids and organic acids:

In the stem and leaf extract, the discriminant amino acids due to surfactin treatment were the same as those observed in the root extract, with the observation of threonine for stems and leaves and without the observation of proline in leaves (Figure 7).

In the stems, the accumulation of all amino acids decreased at 56 h, except for threonine, and increased at 10 D, except for glutamic acid and GABA. The decrease in amino acid content observed at 56 h could be due to the remobilization of the amino acids from stems to roots. At 10 D, the plants could activate the synthesis of these primary metabolites to correct the lack of them at 56 h. Glutamic acid was still less accumulated. The same hypothesis regarding a deviation occurring mainly through proline and less through GABA was established as previously for the roots. Malic acid, as for the roots, was less accumulated at 56 h and more at 10 D, whereas formic acid was more accumulated at the two kinetic points and fumaric acid content decreased at 56 h and at 10 D. The organic acids represent a way to store fixed carbon and can be allocated to form amino acids or carbohydrates [38].

In the leaves, the content of all the discriminant amino acids, except glutamic acid, increased at both kinetic points, suggesting that the plant counterbalanced the consumption of these amino acids in the other organs and biosynthetic pathways. The change in the organic acid content was in the same order of range as the one observed for stems.

Compounds:

As already observed in the roots, linamarin and lotaustralin concentrations were modified in the aerial parts by surfactin treatment (Figure 7). In the stems, their content decreased at 56 h; thus, their pool can be used to allow for an increased content in the roots, whereas they increased at 10 D; thus, the plant could improve their synthesis de novo. The content of cyanogenic compounds varied in the same way as valine and isoleucine content, their precursors. In the leaves, at both kinetic points, the content of cyanogenic compounds decreased, whereas the content of their precursors increased. The cyanogenic

compounds can be redistributed from the aerial parts to the roots, where surfactin treatment was applied [39].

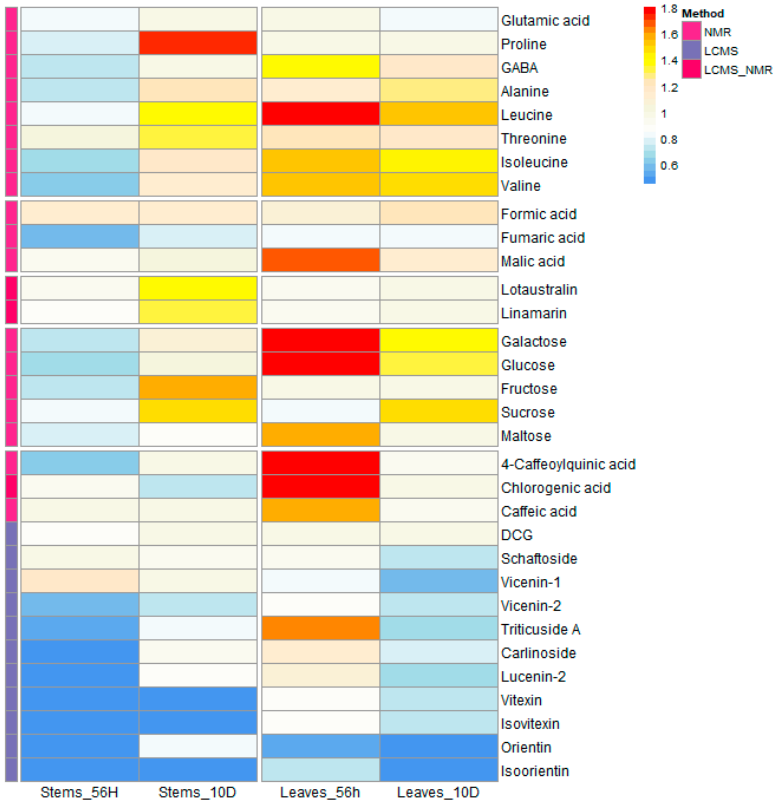


Figure 7. Heatmap of the discriminant metabolites differently accumulated in the flax stem (**left**) and leave (**right**) extracts between the surfactin-treated and control conditions. The scale bar (from red to blue) on the right represents the ratio of metabolite content in surfactin-treated flax aerial parts to that in control flax aerial parts. For each organ, the column on the left indicates the kinetic point at 56 h, and the column on the right indicates the kinetic point at 10 D. The analytical methods used to determine discriminant metabolites are indicated on the left of the graph; the red color corresponds to the combination of NMR and LC-MS, the blue color corresponds to LC-MS, and the purple color corresponds to NMR.

Carbohydrates:

Two monosaccharides (glucose and fructose) and the resulting disaccharide (sucrose) were the discriminant metabolites in the roots; they were also discriminant in the aerial parts—except fructose in the leaves—and another disaccharide (maltose) and monosaccharide (galactose) were discriminant for both the stems and leaves.

In the stems, the content of carbohydrates decreased at 56 h and increased at 10 D (except for maltose), and in the leaves, their content increased at both kinetic points (except for sucrose at 56 h and maltose at 10 D). The stems seemed to exhibit opposite behavior compared to the roots at 56 h and 10 D, and the transport of molecules seemed to occur between these two organs. Carbohydrate content variations between the three organs were in accordance with the intermediate position of stems between the roots and leaves.

Phenolic compounds:

In the aerial parts, several phenolic compounds were identified by NMR when enough accumulated or by LC-MS when accumulated in small amounts, using standards and/or by matching the MSe spectra with the molecules cited in other studies involving flax extracts. From both NMR and LC-MS datasets (Figure 7), we found that almost all the phenolic compounds exhibited changes in concentration in the same way.

In the stems, it is noticeable that the content of isovitexin, lucenin-2, isoorientin, vitexin, orientin, carlinoside, triticuside A, and vicenin-2 strongly decreased at 56 h after surfactin treatment. All these compounds belong to the flavonoid family with one or two glycoside unit(s) linked to the flavone core via C bound. The content of chlorogenic acid isomers, DCG, shaftoside and vicenin-1 decreased slightly or increased at 56 h. At 10 D, the content of all these phenolic compounds decreased with surfactin treatment but less strongly than at 56 h. The influence of surfactin on phenolic content has already been studied in other models, showing, for example, that surfactin treatment did not modify phenolic content in peanut in control conditions but induced phenolic increase when exposed to a pathogen. The phenolic content was evaluated in the stems 10 D after surfactin treatment, and the results agreed with our results, showing that surfactin affects phenolic content early after surfactin treatment, but this effect is attenuated late after surfactin treatment [40].

In the leaves, some of the phenolic compounds increased with surfactin treatment at 56 h, namely caffeic acid, chlorogenic acid isomers, carlinoside, triticuside, and lucenin-2, whereas the other ones slightly decreased. At 10 D, in the leaves, all the phenolic compound content decreased. The ratios of surfactin treatment conditions vs. control conditions were lower at 10 D than at 56 h for all the phenolic compounds. In another study, the phenolic content in the sprouts of sesame seeds treated by *Bacillus clausii* increased, which is in accordance with our results obtained at 56 h regarding the leaves treated with surfactin, produced by *Bacillus velezensis* [41].

This suggests that the stems and leaves exhibit opposite behavior during the kinetic response of the plant after surfactin treatment. Stems react more like roots than leaves.

4. Conclusions

In this research, we used advanced metabolomic methods, including ^{1}H NMR and LC-MS, to assess the effects of surfactin on the metabolic composition of flax (*Linum usitatissimum*) roots, stems, and leaves. Our results indicate significant changes to both primary and secondary metabolic pathways.

The capacity of flax roots to take up surfactin was demonstrated. It was found that surfactin treatment induced a local effect in this organ with an increase in amino acids and cyanogenic glycosides and a decrease in carbohydrate content.

Surfactin treatment also induced a systemic effect demonstrated by the metabolic changes in the aerial parts of the plant. The leaves and stems presented an increase in several amino acids and carbohydrates. Additionally, we observed a decrease in the metabolites associated with the phenylpropanoid pathway, including flavonoids.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app142411999/s1, Table S1: NMR chemical shift and/or retention time and m/z value of the signal used for semi-quantitation of the different discriminant metabolites present in the different organs of flax grown in control conditions or with surfactin-treatment.

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