Federal University of São Carlos
Exact Science and Technology Centre
Department of Chemistry

DOCTORAL THESIS

Bioactivity, chemical and microbial diversity in *Alternanthera brasiliana*: the influence of endophytes on antibiotic production

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This work is dedicated to all those who have encouraged,
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"Love the life you Live,
Live the life you Love"
Bob Marley

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Abbreviations

AA – Arachidonic acid
ABA – Abscisic acid
ANOVA- -Analysis of Variance
AOS – Allene Oxide Synthase
ATCC –
BABA – b-aminobutyric acid
bp – base pairs
CC – Calibration Curve
COX - Cycloxygenase
CYP – Cytochrome p450
DGLA – dihomo-g-linolenic acid
DHA – Docosahexaenoic acid
dNTP – Deoxynucletide
EET – Epoxyeicosatrienoic acid
EMA – European Medicines Agency
EPA – Eicosapentanoic acid
ESI – Electrospray Ionization
FDA – Food and Drugs Agency
GC – gas chromatography
HODE – Hydroxy-octadecadienoic acid
HPLC – High Performance Liquid Chromatography
HPLC-ESI-MS - High Performance Liquid Chromatography coupled with Mass Spectrometry using Electrospray Ionization
HPLC-MS – High Performance Liquid Chromatography coupled with Mass Spectrometry
HQC – High Quality Control
HRMS/MS – High Resolution Mass Spectrometry
IAA – Indole Acetic Acid
INA – 2,6-dichloroisonicotinic acid
IS – Internal Standard
JA – Jasmonic Acid
JA-Ile – Jasmonic Acid Isoleucine conjugate
kb – kilo base pairs
KODE – Keto-octadecadienoic acid
LC-MS/MS - Liquid Chromatography hyphenated with Tandem Mass Spectrometry
LC-PDA – Liquid Chromatography hyphenated with Photodiode Array
LC-PDA-MS/MS – Liquid Chromatography hyphenated with Photodiode Array and Tandem Mass Spectrometry
LOD – Limit of Detection
LOQ – Limit of Quantification
LOX – Lipoxygenase
LQC – Low Quality Control

MALDI – Matrix Assisted Laser Desorption Ionization
MQC – Medium Quality Control
MRM – Multiple Reaction Monitoring
MS – Mass Spectrometry
MS/MS – Tandem Mass Spectrometry
NMR – Nuclear Magnetic Resonance
OPDA – 12-Oxophytodienoic Acid
PCR – Polimerase Chain Reaction
PDA – Photodiode Array
PDA – Potato Dextrose Agar
PGE2 – Prostaglandin E2
PXG – Peroxigenases
QC – Quality Control
qPCR – quantitative Polimerase Chain Reaction
ROS – Reactive Oxygen Species
RSD – Relative Standard Deviation
SA – Salicylic Acid
SAR – Systemic Acquired Resistance
SIR – Systemic Induced Resistance

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Alternanthera brasiliana is a plant widely used by Brazilian people as a folk medicine for the treatment of numerous diseases, especially due to its antibiotic properties. Using dereplication strategies, we identified antibiotic compounds as modified fatty acids, which are closely related to oxylipins. The biosynthesis of these compounds could be related with the presence of endophytic bacteria. Thus, we established axenic cultures of *A. brasiliana* and developed methods for detection and quantification of bacterial cells in plant tissues. We also performed initial re-inoculation experiments with endophytic bacteria isolated from *A. brasiliana*. In addition, method for quantification of phytohormones and oxylipins in plant tissues were developed. The results reinforced the correlation between both the amount of bacteria and oxylipins in *A. brasiliana* tissues.

Doctor of Science

"Bioactivity, chemical and microbial diversity in *Alternanthera brasiliana*: the influence of endophytes on antibiotic production"

by Marilia Almeida Trapp

Universidade Federal de São Carlos

Centro de Ciências Exatas e de Tecnologia
Departamento de Química

Doutor em Ciência

Alternanthera brasiliana é uma planta amplamente utilizada na medicina popular brasileira para o tratamento de diversas doenças, principalmente devido a suas propriedades antibióticas. Durante este trabalho, utilizou-se estratégias de desreplicação a fim de se identificar os compostos antibióticos presentes nesta planta. Esses compostos foram identificados como ácidos graxos oxidados pertencentes a classe das oxilipinas. A biossíntese destes compostos esta comumente relacionada a situações de estresse biótico e abiótico em plantas. Dessa forma, nos estabelecemos métodos para a detecção e quantificação de células bacterianas em tecidos vegetais. Também foram realizados experimentos iniciais de re-inoculação de bactérias endofíticas isoladas de *A. brasiliana*. Além disso, foram desenvolvidos métodos para a quantificação de fitohormônios e oxilipinas em tecidos vegetais. Os resultados obtidos até o momento ressaltam a possibilidade de bactérias endofíticas influenciarem a produção de oxilipinas (compostos antibióticos) na planta *A. brasiliana*..

"Bioatividade, diversidade química e microbiana associadas à *Alternanthera brasiliana*: a influência de micro-organismos endofíticos na produção de compostos antibióticos "

Marilia Almeida Trapp

Summary

1 Background and Motivation

2 Dereplication Studies in *Alternanthera brasiliana*

3 Oxylipins: Methods and Concepts

1

Background and Motivation

The first chapter presents the general aspects of plant-microbes interaction and how chemical ecology can be used for discovering of new bioactive molecules, which can be used in agriculture and medicine.

1.1 General Introduction

Evolution generates a large diversity in chemical structures and selects the most useful ones. Such selection, developed by evolution/time can be used in order to discover new interesting molecules valuable to solve human challenges in agricultural and life science.[1] Therefore, observing the interaction between plants, microorganisms, insects and other organisms as well as identifying the molecules that rise from these interactions is a promising approach to shorten the time consuming path to get hands on active molecules that have been refined by evolution processes.[2–4]

In this context, the improvement of plant growth and resistance, reduction of environmental pollution and production of specialized molecules (including discovering of new drugs) are some of the remarkable points in plant chemical ecology studies.

For example, once the mechanisms used by plants to defend themselves from pathogens and pests are elucidated, we can take advantage of it and develop new methods to combat pests in agriculture.[5,6] Such approach could be, for instance, the activation of Systemic Induced Resistance (SIR) and/or Systemic Acquired Resistance (SAR) using molecules like 2,6-dichloroisonicotinic acid (INA), β-aminobutyric acid (BABA), benzothiadiazole (BTH), and salicylic acid, among others (Figure 1.1). [5,7] In the same way, both plant growth and resistance can be promoted directly by microorganisms. The molecules involved in such plant-microbe interactions can be synthetically produced and used to stimulate plant growth and defence.[5] Such strategies demonstrate how the knowledge of ecological interactions might help us to solve agricultural challenges in an ecological-friendly way. Besides the compounds that improve growth and resistance, during infection processes, plants can also produce bioactive and pharmaceutically interesting compounds that have been widely used in drug research. [8,9]

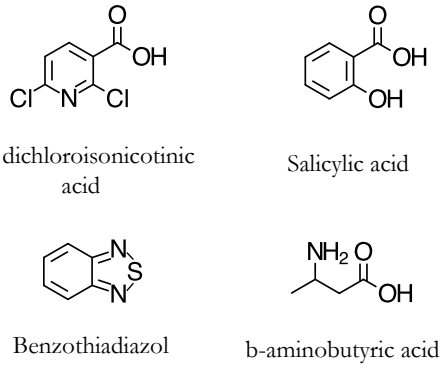

Figure 1.1: Some molecules involved in Systemic Induced Resistance (SIR) and/or Systemic Acquired Resistance (SAR)

1.1.1 Ecological interactions and drug discovery

Plant and microorganisms have been extensively used on the development of new drugs. Medicinal plants, for instance, have been used in folk medicine for at least 60,000 years and they remain as an important source for treatments all over the world. The World Health Organization estimates that such plants currently constitute the main modality of heath care for almost 65% of world's population. [10]

In fact, pharmaceutical companies have used plant secondary metabolites for both crude extract production and as a source of new bioactive molecules.[11,12] However, just few works have been done in order to figure out how and why such compounds are produced in plant tissues and what is their function in a complex ecological system.

Furthermore, biosynthesis of secondary and specialized metabolites present in plant tissues must be seen as result of many biotic and abiotic factors. Such compounds can be produced either exclusively by a plant or they can also arise from ecological interactions between plant and other organisms. Microorganism, perhaps, are able to produce active metabolites inside plant tissues, which can be misleading with respect to their biosynthetic source.[13]

Therefore, understanding the ecological relationship in which active secondary metabolites are involved and synthesized is necessary and might become a

powerful tool for both finding new active molecules and improving metabolites' production. However, the identification of new molecules, which are either involved in ecological interactions or have and biological activity (i.e. antibiotic, analgesic, insecticide) is not a trivial task. These compounds are frequently found in a very low concentration in complex matrices (i.e. plant extracts), and they usually present challenging molecular structures. In order to overcome these challenges we can use high-throughput screening combining spectroscopic analysis, biological assays and database searching to identify active molecules in complex samples. In this way, based on analytical, organic and spectroscopic knowledge, chemists can considerably contribute to reveal and to elucidate either key molecules involved in ecological relationships or bioactive compounds present these complex matrices.

During the past years, we have studied *A. brasiliana* to identify the antibiotic compounds present in this Brazilian medicinal plant.

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2

Dereplication Studies in *Alternanthera brasiliana*

This chapter introduces Alternanthera brasiliana, a Brazilian medicinal plant. It also presents the dereplication studies about the antibiotic compounds found in this plant.

2.1 *Alternanthera brasiliana*

Alternanthera is one of the 65 genera of Amaranthaceae family being composed by approximately 1000 species. It can be found in tropical, subtropical and temperate regions. The *Alternanthera* genus comprehends around 80 species, and 30 of them can be found in Brazil. [14]

Alternanthera brasiliana, a Brazilian medicinal plant, is widely used in Brazilian folk medicine due its antibiotic properties. It is usually called "penicillin" or "terramycin" due to its pharmacological properties. Some of these properties reported by folks have also been proved by pharmacological studies, for instance analgesic, anti-inflammatory and healing properties. [15–23]

Many pharmacological studies have proved that *A. brasiliana* has antibiotic activity against gram positive bacteria.[24,25,16] Strikingly, the compounds that had been so far isolated from *A. brasiliana* did not explain these antimicrobial activities.[26,17] This makes the involvement of additional sources such as endossimbiontes likely, although there were no reports regarding endophytic microorganisms associated with this plant. So we have used dereplication strategies in order to identify the antibiotic compounds present in *A. brasiliana* and developed further studies to understand the biosynthetic origin of such compounds.

Figure 2.1: *Alternanthera brasiliana* in nature

2.2 Dereplication and high-throughput screening

The term dereplication seems to predate 1978, when it was used for the first time in the CRC Handbook of Antibiotics Compounds. The aim of dereplication is to identify natural compounds partial or completely without any or with only partial isolation, avoiding spending too much effort on isolation of known compounds.[27]

Nowadays, dereplication has been extensively used to shorten drug discovery processes.[28] Using biological assays, separation methods (GC, HPLC), spectroscopic techniques (PDA, MS, NMR), and computational support, the high-throughput screening prevents the isolation of known compounds saving efforts to be spent on more promising natural sources. In Brazil, the dereplication studies are becoming are being spread quickly. In 2013, we were the second country in number of publications covering dereplication topics (6 articles), behind only the USA (11 articles).[29] It is probably happening due to the high natural diversity and the evolution of natural products research, which is well established in our country.

During this PhD project, we used dereplication to identify the antibiotic compounds present in *A. brasiliana* tissues (paper published in *Phytochemistry*, item 2.4). It is important to highlight that only the target compounds were isolated and identified. We have isolated 11 compounds (Figure 2.2) and identified other 6 (Figure 2.3) using the fragmentation profile provided by mass spectrometry analysis. The main fragmentation pathways are discussed in the item 2.5.

We have found that the antibiotic compounds present in *A. brasiliana* tissues are modified fatty acids closely related to oxylipins. Some of these compounds have already been reported as anti-inflammatory, anti-cancer, and antibiotic substances, among others.

The biosynthesis of oxylipins seems to be related with plant infection and elicitation.[30–34] Among the isolated compounds from *A. brasiliana* there are

some oxylipins: 9*Z*,12*Z*,14*E*-16-hydroxy-9,12,14-octadecatrienoic acid (**1**) and 9*Z*,13*E*,15*Z*,-12-hydroxy-9,13,15-octadecatrienoic acid (**2**) that have been reported as compounds which rise up during plant defence upon infection by *Pseudomonas aeruginosa*.[35] On the other hand, compounds **3** and **4** (9*Z*,11*E*,15*Z*,-13-hydroxy-9,11,15-octadecatrienoic acid and 9*Z*-octadecenedioic acid) have been widely reported as biotransformation products of *Pseudomonas aerugiona*.[35–46] Similarly, there are some papers reporting the isolation of compounds **5** and **6** (9*Z*, 11*E*-13-hydroxy-9,11-octadecadienoic acid and 10*E*, 12*Z*-9-hydroy-10,12-octadecadienoic acid) from algae. Just three works can be found where such compounds were isolated from plants; however these references also called them unusual plant metabolites.[47–51] In this context we tried to understand whether these oxylipins were involved in some interactions between plants and microorganisms. So, we have isolated and cultured some endophytes isolated from *A. brasiliana* as can be seen in the paper published in Phytochemistry (item 2.4).

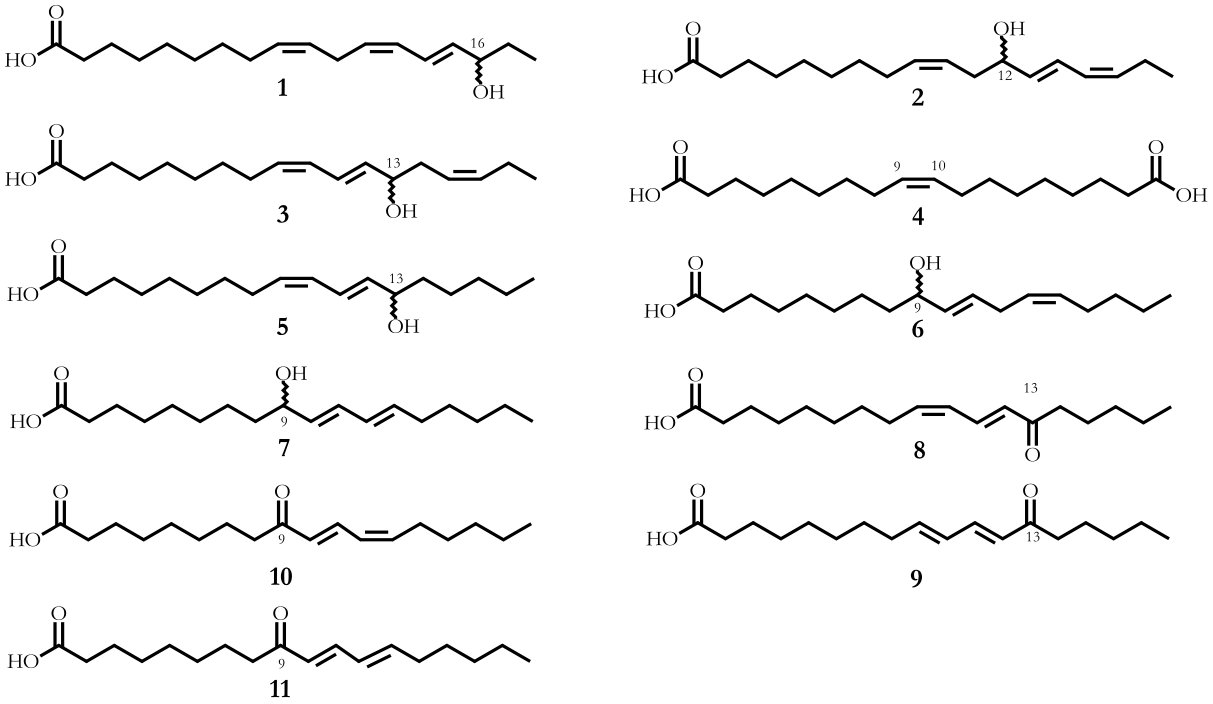

Figure 2.2: Oxylipins with antibiotic properties isolated from *A. brasiliana*

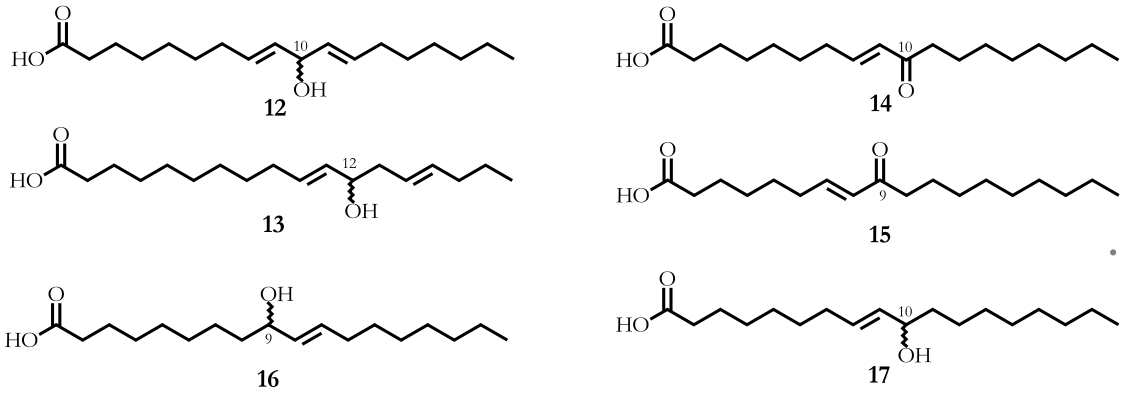

Figure 2.3: Oxylipins identified in *A. brasiliana* during dereplication studies

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2.4 Dereplication studies in *A. brasiliana*

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Antibiotic oxylipins from *Alternanthera brasiliana* and its endophytic bacteria

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ABSTRACT

Bioassay-guided fractionation of *Alternanthera brasiliana* stem extracts resulted in the isolation of an antibiotically active fraction. Five human pathogenic bacteria were used to guide the fractionation process for the isolation of antimicrobial compounds. Finally, 17 linoleate oxylipins were identified by LC–MS/MS and NMR spectroscopy. Five of the isolated compounds present in *A. brasiliana* tissues were also detected to be synthesized by endophytic bacteria of the genus *Bacillus* that were isolated from *A. brasiliana*. It is speculated that the antibiotic oxylipins from *A. brasiliana* might derive from bacteria and be involved in an ecological relationship between this plant and its endophytes.

1. Introduction

Alternanthera brasiliana (Fig. 1), a plant belonging to the family of Amaranthaceae, is an indigenous herb widely used in Brazil as a medicinal plant for treatment of general inflammation, cold, influenza virus, headache, diarrhea, and fever (Agra et al., 2007; Vendruscolo and Mentz, 2006). Pharmacological assays have proven many of these important biological activities for *A. brasiliana* extracts (Brochado et al., 2003; Macedo et al., 2004; Souza et al., 1998). Among them, the antibiotic activity was reported to be similar to that shown by tetracycline (Caetano et al., 2002) and this is why *A. brasiliana* is popularly known as "Tetracycline", "Terramycin" and "Penicillin" in Brazil. Pereira and co-workers have reported the inhibitory activity of *A. brasiliana* crude extracts on Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) at 1250 μg/mL (Pereira et al., 2007). In the same way, Avancini and co-workers showed that the aqueous extract of *A. brasiliana* inhibits the growth of *S. aureus*, one of the causative agents of mastitis in cattle (Avancini et al., 2008; Kumar et al., 2011).

Despite these biological activities, little is known about the chemical composition of this plant and the nature of the bioactive molecules present in its tissues. The only study dealing with antimicrobial molecules present in *A. brasiliana* extracts was developed by Silva et al. (2011). During their studies, these researchers showed that *S. aureus* is inhibited by a methanol soluble fraction of *A. brasiliana* extracts at a Minimum Inhibitory Concentration (MIC) of 780 μg/mL. They also isolated quercetin and sitosterol glycoside and evaluated their activity against such bacteria. However, sitosterol glycoside was not active even at 1000 μg/mL, and quercetin exhibited a MIC of 300 μg/mL. If at all, these compounds are most likely not the only active molecules present in *A. brasiliana*, since they are well known plant metabolites and present a very high MIC.

These inconclusive results motivated conduct of a survey for antibiotic compounds present in *A. brasiliana* tissues, using microdilution antibiotic assays combined with LC–MS screening. A fraction containing seventeen oxidized fatty acid derivatives, termed oxylipins was isolated and found to have antibiotic activity; its chemical components were structurally characterized.

2. Results and discussion

2.1. Bioactivity-guided fractionation

The crude ethanol/ethyl acetate extract obtained from *A. brasiliana* was fractionated in a silica gel column, resulting in five

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0031-9422/

fractions (**F1**–**F5**). Fractions F2–F5 were tested against five pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus luteus* and *Staphylococcus aureus*) using the antibiotic microdilution broth assay performed according to NCCLS standardization (NCCLS, 2003). The final tested concentration for each fraction was 500 μg/mL. This test was performed using resazurin as an indicator of bacterial cell viability. Changes in the dye color (from blue to pink) indicate aerobic respiration confirming the presence of viable bacterial cells. The bioassay results (Fig. 1S, supplement material) indicated the chromatographic fractionation to be an effective procedure to discriminate the active compounds present in the extract of *A. brasiliana*, since only fraction **F3** was able to inhibit the growth of Gram-positive bacteria (*B. subtilis*, *M. luteus*, *S. aureus*) at the tested concentration. Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were not inhibited by any fraction. For confirmation this test was repeated twice with the same result.

TLC and ^{1}H NMR spectroscopic analysis indicated that the inactive fraction **F1** consists of an acyl glycerides mixture. The main difference among fractions **F2**–**F5,** when analyzed by LC–PDA–MS/MS, was the accumulation of some chromatographic peaks with retention time between 20.0 and 29.0 min in **F3** only (Fig. 2S, supplement material). Thus, these molecules could be correlated with the antibiotic activity observed for such fraction. In agreement with this hypothesis, the other three fractions (**F2**, **F4** and **F5**), showing a very different chromatographic profile when compared with **F3**, were inactive (Fig. 2S).

The active fraction **F3** was processed through a C18 SPE cartridge and re-fractionated in a preparative HPLC system, using an optimized chromatographic method (see Section 4). This process yielded 7 sub-fractions (**F3.1**–**F3.7**), which were collected according to the dashed lines shown in Fig. 3S. Fractions F3.1–F3.6 had their antibiotic activity evaluated against Gram-positive bacteria. Among these fractions, **F3.2**, **F3.3** and **F3.4** concentrated the antibacterial activity and again showed growth inhibition of the three Gram-positive bacteria species at a MIC of 50 μg/mL (Fig. 4S). Gram-negative bacteria were not inhibited in any of the tested concentrations (500, 250, 100 and 50 μg/mL).

Accordingly, active fractions **F3.2**–**F3.4** were analyzed by LC–MS. The accumulation of isomers with molecular weight 294, 296 and 298 Da (detected as peaks at *m/z* 293, 295 and 297, respectively, using negative ion ESI mode) in those fractions suggested a correlation between these compounds and the antibiotic activity.

Thus, these three active fractions (**F3.2**–**F3.4**) were subjected to a new chromatographic separation protocol. The HPLC method was optimized for each sample, in order to reach the best chromatographic resolution. The compounds were collected according to highlighted areas showed in Fig. 5S. Such procedure resulted in 9 isolated compounds (**1**–**8** and **11**) and one fraction containing two compounds (**9** and **10**). All of them were further analyzed by LC–PDA–MS, LC–PDA–MS/MS and NMR spectroscopy, except for compounds **1** and **7**, which could not be analyzed by NMR due to the low isolated amount.

2.2. Compound identification

The NMR spectra of isolated compounds show intense signals in the shielded region (δ 1.27) and some signals between 6.0 and 7.5 ppm, indicating that they have a long carbon chain with only few modifications. These NMR spectroscopic data and the fragmentation profiles by mass spectrometry indicated that the isolated compounds are modified fatty acids. As mentioned above, all of them have molecular weights of 294, 296 or 298 Da, suggesting that the difference among them is on the degrees of unsaturation.

Compound **1** and **2** were identified as (9*Z*,12*Z*,14*E*)-16-hydroxy-9,12,14-octadecatrienoic acid and (9*Z*,13*E*,15*Z*)-12-hydroxy-9,13,15-octadecatrienoic acid by comparison of their ^{1}H NMR spectroscopic data (Table 1) with those published by Pagani et al. (2011). These structures are also in agreement with MS data, both compounds **1** and **2** having precursor ions at *m/z* 293. The main fragment in the MS/MS spectra originates by cleavage between the carbinolic and vinyl carbons (Fig. 3), this being the main fragmentation for an unsaturated hydroxy fatty acid. It corresponds to *m/z* 235 for compound **1** and 211 ($[M-H-C_7H_{10}O_2]^-$), *m/z* 183 ($[M-H-C_7H_{10}O]^-$), and *m/z* 109 ($[M-H-C_{11}H_{20}O_2]^-$) for compound **2** (Fig. 2).

Compound **3** was identified as (9*Z*,11*E*,15*Z*)-13-hydroxy-9,11,15-octadecatrienoic acid by comparison of its NMR spectroscopic data (Table 1) with those published by (Bang et al., 2002). This structure was also confirmed by correlations in the COSY spectrum (see Fig. 6S, Supplementary Information) and fragments in the MS/MS spectrum (Fig. 2): *m/z* 275 ($[M-H-H_2O]^-$), 224 ($[M-H-C_5H_9]^-$), *m/z* 195 ($[M-H-C_6H_{10}O]^-$), and *m/z* 113 ($[M-H-C_7H_{16}O_2]^-$).

Compound **4** was identified as (9*Z*)-9-octadecenedioic acid (Fig. 3), since it shows only a few signals in its ^{1}H NMR spectrum (Table 1) with no ^{1}H resonance for a terminal methyl group. The HRMS and MS/MS data confirm the proposed structure with precursor ion at *m/z* 311.22122 ($C_{18}H_{31}O_4$, calcd 311. 22169) and product ions at *m/z* 293 ($[M-H-H_2O]^-$), *m/z* 267 ($[M-H-CO_2]^-$), and *m/z* 249 ($[M-H-H_2O-CO_2]^-$) (Fig. 3).

Compounds **5** and **6** (296 Da) each contain one double-bound less than **2** and **3** (294 Da), which was confirmed by analysis of both ^{1}H NMR and MS/MS spectra. These compounds were assigned as (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid and (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid, respectively (Fig. 3). The structures were confirmed by comparison of ^{1}H NMR spectroscopic data (Table 1) with those published by Li et al. (2009). Moreover, compound **5** has its main peaks at *m/z* 195 ($[M-H-C_6H_{12}O]^-$), and *m/z* 113 ($[M-H-C_{11}H_{18}O_2]^-$) in the MS/MS spectrum (Fig. 2),

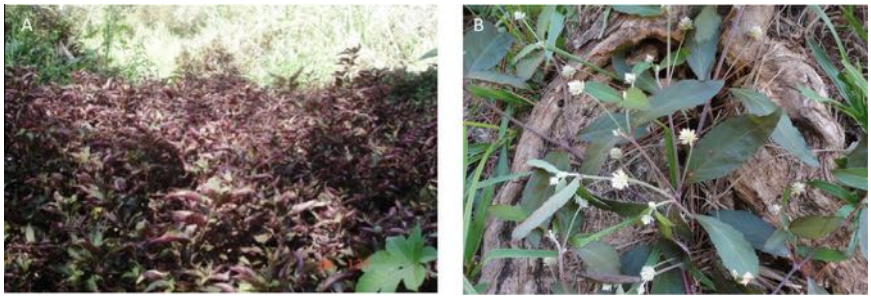
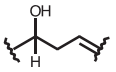
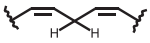
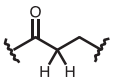
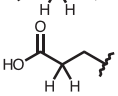
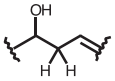
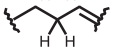
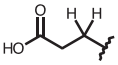
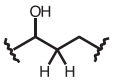
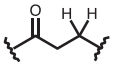


Fig. 1. *Alternanthera brasiliana.*

Table 1
^{1}H NMR spectroscopic data of oxidized fatty acids isolated from *A. brasiliana* tissues.

	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
HC=C	6.53 (1H, ddt, 15/11/1 Hz)	6.55 (1H, ddt, 15/11/1 Hz)	6.55(1H, ddt, 15/11/2 Hz)	–	6.50 (1H, ddt, 15/11/1 Hz),	6.48 (1H, ddt, 15/11/1 Hz),	7.52 (1H, ddd, 15/12/1 Hz)	7.14 (1H, ddd, 15/10/1 Hz)	7.51 (1H, ddd, 15/11/1 Hz)
HC=C	6.00 (1H, t, 11 Hz),	6.00 (1H, t, 11 Hz),	6.00 (1H, t, 11 Hz)	–	5.98 (1H, t, 11 Hz),	5.98 (1H, t, 11 Hz),	6.20 (1H, d, 15 Hz),	6.15–6.20 (1H, m),	6.21 (1H, dd, 11/12 Hz),
HC=C	5.69 (1H, dd, 15/6 Hz)	5.70 (1H, dd, 15/6 Hz)	5.71 (1H, dd, 15/6 Hz)	–	5.68 (1H, dd, 15/7 Hz)	5.66 (1H, dd, 15/7 Hz)	6.18 (1H, dd, 11/12 Hz),	6.15–6.20 (1H, m)	6.19 (1H, d, 15 Hz),
HC=C	5.60 (1H, dtt, 11/7/1 Hz)	5.60 (1H, dtt, 11/7/1 Hz)	5.60 (1H, dtt, 11/8/2 Hz)	–	–	–	5.92 (1H, dt, 8/ 11 Hz	6.11 (1H, d, 11 Hz	5.94 (1H, dt, 8/ 11 Hz)
HC=C	5.48 (1H, m)	5.48 (1H, m)	5.47 (1H, dt, 11/8 Hz),	–	5.47 (1H, dt, 11/ 7 Hz),	5.45 (1H, dt, 11/ 7 Hz),	–	–	–
HC=C	5.36 (1H, m)	5.40 (1H, m)	5.38 (1H, dtt, 11/8/2)	5.35 (2H, t 5 Hz)	–	–	–	–	–
	4.12 (1H, q, 7 Hz)	4.17 (1H, q, 6 Hz)	4.25 (1H, q, 6 Hz)	–	4.18 (1H, q, 7 Hz),	4.18 (1H, q, 7 Hz),	–	–	–
	2.95 (2 H, q, 7 Hz)	–	–	–	–	–	–	–	
	–	–	–		–	–	2.56 (2H, t, 8 Hz)	2.54 (2H, t, 8 Hz)	2.53 (2H, t, 8 Hz)
	2.35 (2H, t, 8 Hz)	2.35 (2H, t, 7 Hz)	2.36 (2H, t, 8 Hz)	2.35 (4H, t, 7 Hz)	2.36 (2H, t, 7 Hz)	2.36 (2H, t, 7 Hz)	2.35 (2H, t, 8 Hz)	2.36 (2H, t, 8 Hz)	2.36 (2H, t, 8 Hz)
	–	2.30–2.38 (2H, m)	2.30–2.40 (2H, m),		–	–	–	–	–
	–	2.20 (2H, q, 8 Hz)	2.19 (2H, q, 8 Hz)	–	2.19 (2H, q, 7 Hz)	2.19 (2H, q, 7 Hz)	2.19 (2H, q, 8 Hz)	2.19 (2H, q, 8 Hz)	2.19 (2H, q, 8 Hz)
	2.05 (2H, quin, 8 Hz)	2.07 (2H, quin, 8 Hz)	2.09 (2H, quin, 8 Hz)	2.00 (4H, m)	–	–	–	–	–
	1.62 (2H, quin, 7 Hz).	1.62 (2H, quin, 7 Hz).	1.62 (2H, quin, 8 Hz).	1.66 (4H, quin, 7 Hz)	1.64 (2 H quin, 7 Hz)	1.64 (2 H quin, 7 Hz)	1.64 (2 H quin, 8 Hz)	1.62 (2 H quin, 8 Hz)	1.64 (2 H quin, 8 Hz)
	1.58 (2H, m)	–	–	1.54 (2H, m)	1.54 (2H, m)				
	–	–	–		–	–	1,58 (2H, t, 8 Hz)	1,57 (2H, t, 8 Hz)	1,58 (2H, t, 8 Hz)
Chain	1.32 (10H, s)	1.32 (10H, s)	1.30 (10H, s)	1.31 (16H, s)	1.30 (10H, s)	1.30 (10H, s)	1.30 (8H, s)	1.30 (8H, s)	1.30 (8H, s)
Methyl	0.98 (3H, t, 8 Hz)	0.96 (3H, t, 8 Hz)	0.97 (3H, t, 8 Hz)	–	0.91 (3H, t, 7 Hz)	0.91 (3H, t, 7 Hz)	0.93 (3H, t, 7 Hz)	0.93 (3H, t, 7 Hz)	0.93 (3H, t, 7 Hz)

while compound 6 shows mainly one fragment at *m*/*z* 171 ($[M-H-C_9H_{16}]^-$ which is generated by the cleavage between the carbinolic and vinylic carbons, as shown in Fig. 3.

As mentioned above, it was not possible to obtain the ^{1}H NMR spectra of compound **7** due to the low isolated amount. Since compounds **7** and **6** have exactly the same MS/MS spectra (Fig. 2) and a very similar chromatographic behavior (Fig. 3S), they should have a very similar structure. Indeed, the difference between them is the geometry of one double bond, being the compound **7** related to the (10*E*,12*E*)-9-hydroxy-10,12-octadecadienoic acid (Fig. 3).

The ^{1}H NMR spectra of compounds **8**–**10** contain signals for ^{1}H in deshielded region. The comparison of these deshielded resonances with those reported by Dufour and Loonis (2005), combined with the MS data interpretation shown below (Figs. 2 and 3), allowed the identification of these three unsaturated fatty acids as (9*Z*,11*E*)-13-oxo-9,11-octadecadienoic acid (**8**), (9*E*,11*E*)-13-oxo-9,11-octadecadienoic acid (**9**) and (10*E*,12*Z*)-9-oxo-10,12-octadecadienoic (**10**).

The two pairs of oxylipins **8**/**9** and **10**/**11** also displayed product ions at *m*/*z* 113 and *m*/*z* 185 in MS/MS spectra (Fig. 2), which are important diagnostic ions for 13-oxo and 9-oxo conjugated fatty acids, respectively (Dufour and Loonis, 2005). Therefore, the MS/MS spectrum obtained for **11** (Fig. 2), which is identical to that produced by **10**, was prompt associated with the (10*E*,12*E*)-9-oxo-10,12-octadecadienoic acid (**11**) (Fig. 3).

Other minor compounds with molecular weight 296 and 298 Da were also identified in the active fraction **F3**. Thus, a new chromatographic method was developed to separate them (Fig. 7S), in order to acquire individual MS/MS spectra. Considering the very similar ionization and chromatographic behavior of these compounds with those discussed above, they all might be linoleic acid-derivatives.

In addition, four of these compounds have the same molecular weight of compounds **5** and **6** (296 Da), showing deprotonated ion at *m*/*z* 295. Such isomers (**12**–**15**) appear in the chromatogram as two pairs of peaks spread in the fatty acids retention window (10.19, 10.50, 15.09 and 15.38 min, Fig. 7S). The first fragmentation step for **12** and **13** is loss of water (*m*/*z* 277), indicating that they are hydroxy-diene fatty acids instead of keto-dienes. On the other hand, the compounds **14** and **15** show loss of CO_2 (44 Da) suggesting they are keto-ene linoleic acid derivatives. The main fragments present in the MS/MS spectrum obtained for the hydroxy-diene **12** (Fig. 4) are *m*/*z* 183 ($[M-H-C_8H_{16}]^-$), and *m*/*z* 155 ($[M-H-C_9H_{16}O]^-$), which coincide with the data reported for the (8*E*,12*Z*)-10-hydroxy 8,12-octadecadienoic acid (Garscha and Oliw, 2007).

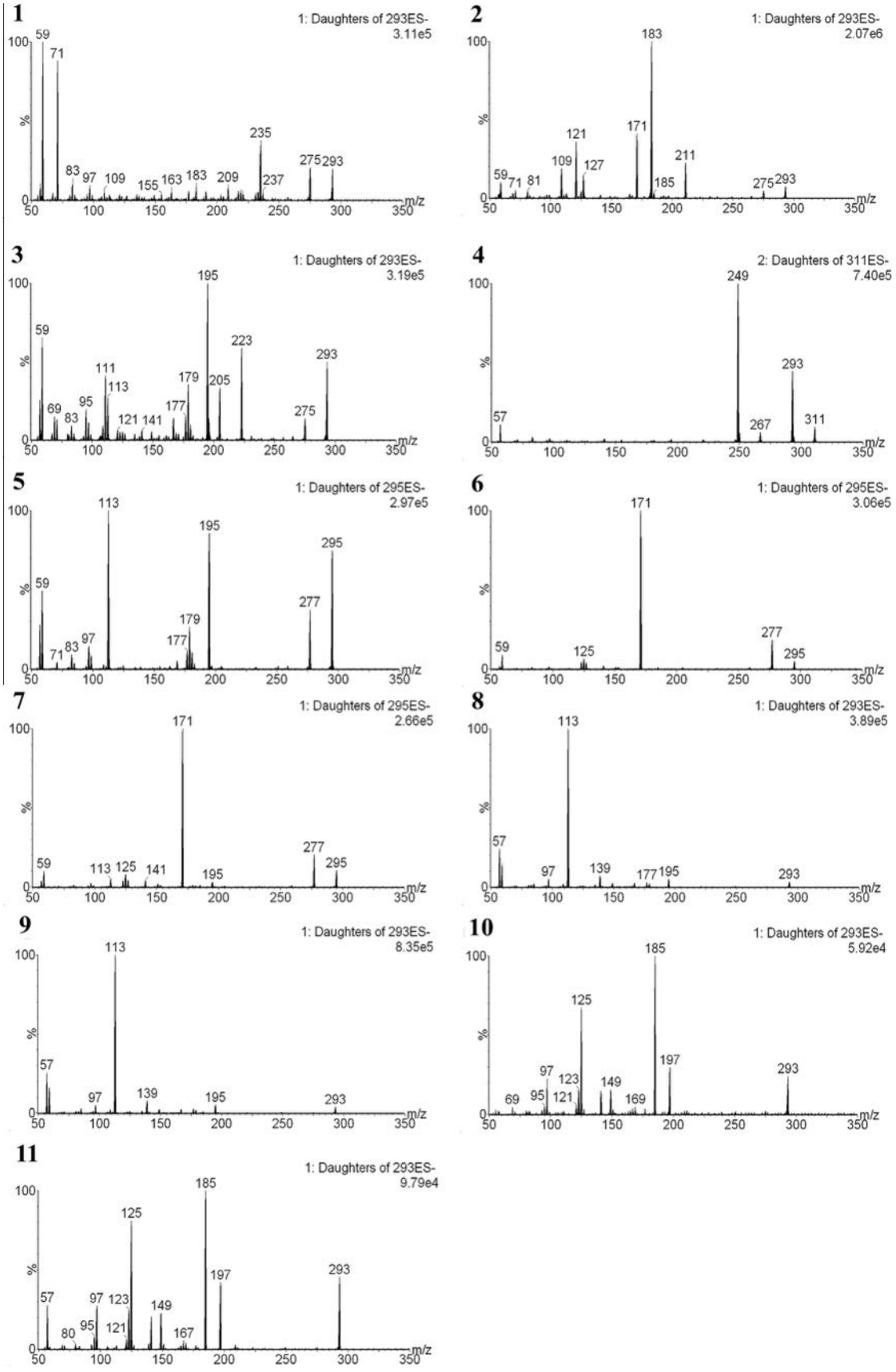


Fig. 2. MS/MS spectra of compounds **2**–**11** identified in *A. brasiliana.*

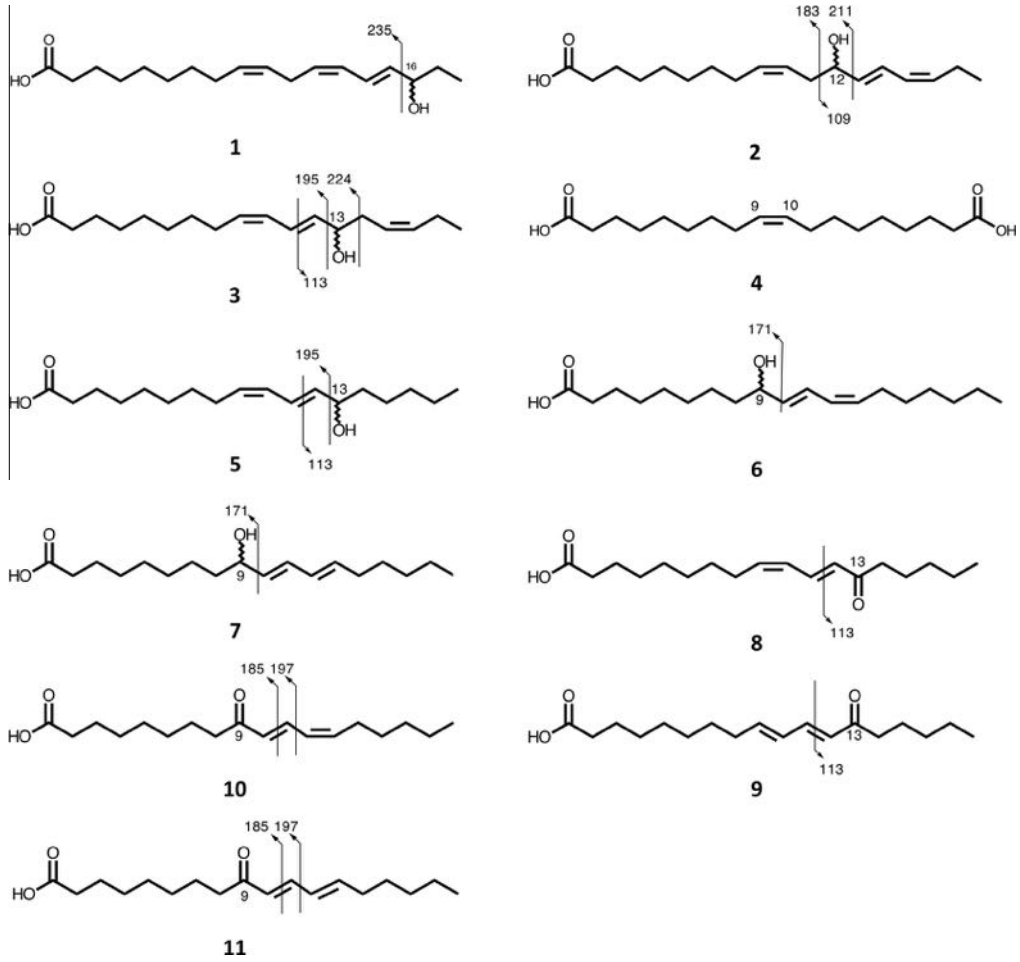


Fig. 3. Molecular structures of compounds **2**–**11** found in *A. brasiliana* with the proposed fragments.

Compound **13** also has a *m*/*z* 183.13845 ($C_{11}H_{19}O_2$, calc. 183.13872) as base–peak in the MS/MS spectrum (Fig. 4), which corresponds to fragment $[M-H-C_7H_{12}O]^-$. Comparison between the fragmentation profiles of **12** and **13** with those published by Yoshida and co-workers, allowed identification of compound **13** as (9*Z*,13*E*)-12-hydroxy-9,13-octadecadienoic acid (Yoshida et al., 2008).

Although many keto-ene linoleic acid derivative isomers are reported in the literature, little information has been provided regarding the ESI-MS/MS spectra for such compounds. Therefore, the other two fatty acids with a deprotonated ion at *m*/*z* 295 were elucidated by comparing their MS/MS spectra (Fig. 4) with those obtained from similar compounds. Hamberg and co-workers reported MS^2 and MS^3 data for both (10S)-hydroperoxy-(8*E*)-octadecenoic acid (10-HPOME) and (9S)-hydroperoxy-(10*E*)-octadecenoic acid (9-HPOME) (Hamberg et al., 2010). During that study, those compounds were analyzed in an ion trap mass spectrometer with the MS^3 data recorded for the ion *m*/*z* 295 generated by fragmentation of precursor ion *m*/*z* 313. The MS^3 spectral profiles of 10-HPOME and 9-HPOME were very similar to those obtained for compounds **14** and **15**, respectively. Since dehydration is the early fragmentation step for hydroperoxides during MS/MS experiments, the ion *m*/*z* 295 mentioned above might correspond to ketones of 10-HPOME and 9-HPOME. The similarity between the product ion spectra of these compounds and those obtained for **14** and **15** (Fig. 4) suggests that these compounds correspond to (8*E*)-10-oxo-8-octadecenoic acid and (7*E*)-9-oxo-7-octadecenoic acid respectively (Fig. 5).

The last pair of linoleates, eluted in an intermediary position in the fatty acid chromatographic window (12.65 and 12.97 min, Fig. 7S), gave *m*/*z* 297 in their ESI-MS spectra. The precursor ion *m*/*z* 297 decomposes by CID and produces abundant *m*/*z* 279 in both MS/MS spectra, corresponding to a loss of water. The MS/MS spectrum for each of the compounds **16** and **17** also contain abundant ions at *m*/*z* 171 ($[M-H-C_9H_{18}]^-$) and 155 ($[M-H-C_9H_{18}O]^-$) respectively, arising from bond cleavage next to the hydroxyl group (Dufour and Loonis, 2005; Garscha and Oliw, 2007; Hou, 1995; Li et al., 2009). Thus, according to literature data (Rodríguez et al., 2001), these hydroxyl-ene linoleates were identified as (10E)-9-hydroxy-10-octadecenoic (**16**) and (8*E*)-10-hydroxy-8-octadecenoic acid (**17**) acids.

2.3. Oxylipins as antibiotics in plants and microorganisms

As previously discussed, despite the many pharmacological studies regarding antimicrobial activity of *A. brasiliana*, only one paper has reported the identification of putative antimicrobial compounds from this plant, namely quercetin and sitosterol glycoside. When tested against *S. aureus*, quercetin exhibited a Minimum Inhibitory Concentration (MIC) of 300 μg/mL, whereas sitosterol glycoside was not active below 1000 μg/mL (Silva et al., 2011). In the present work samples of root, stem and leaves of *A.*

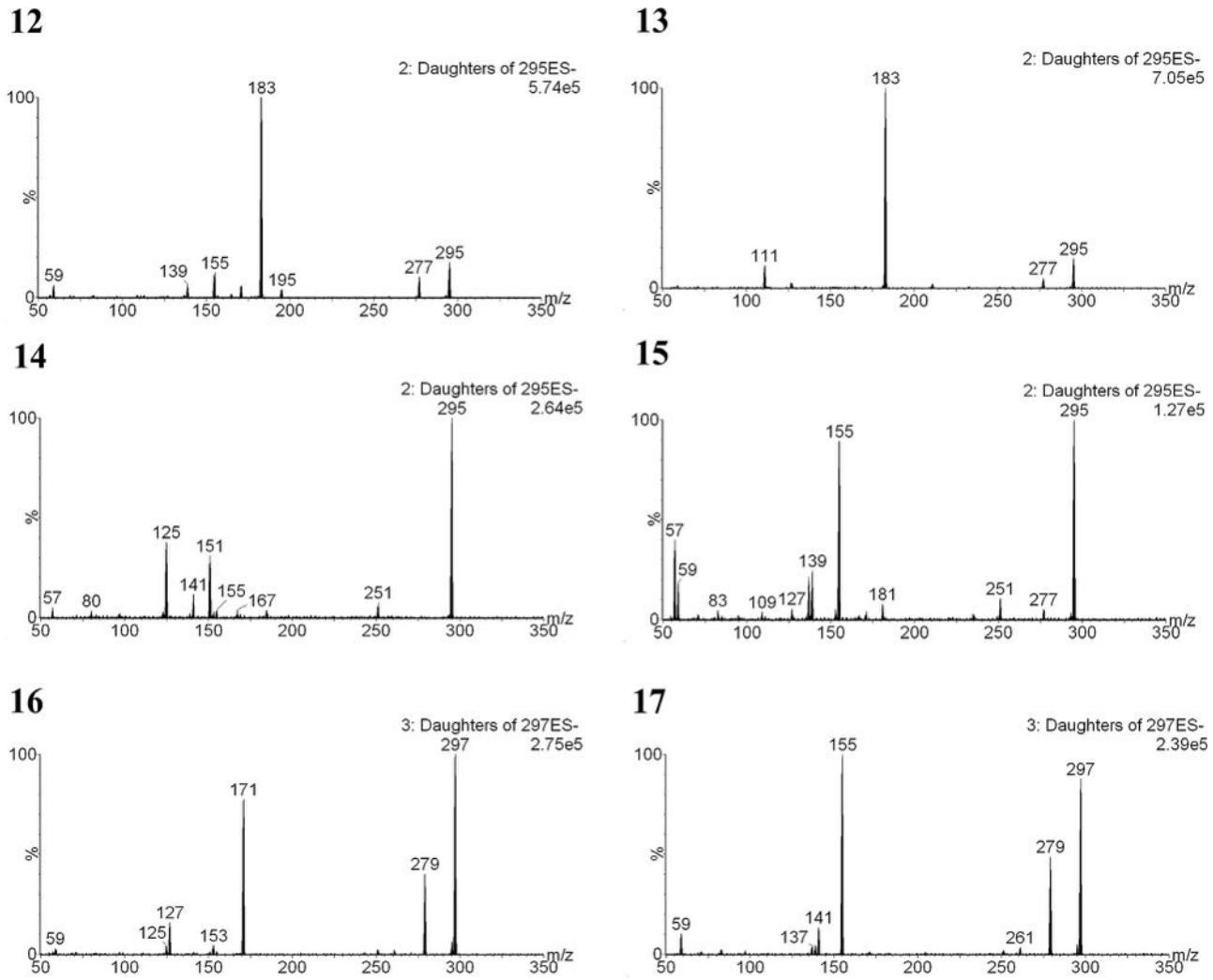


Fig. 4. MS/MS spectra of compounds **12–17** identified in *A. brasiliana*.

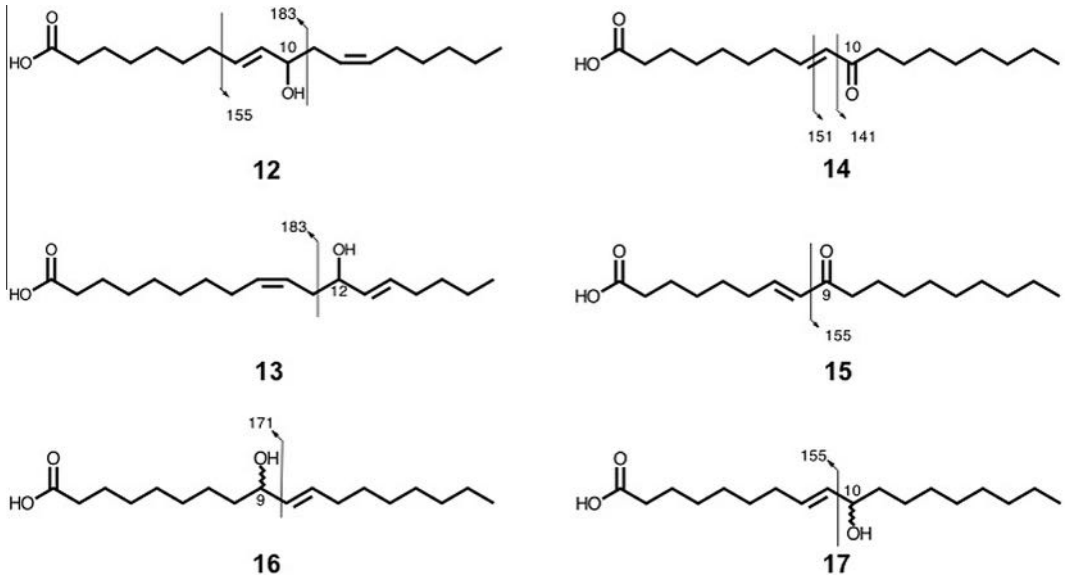


Fig. 5. Molecular structures of compounds **12–17** found in *A. brasiliana* with the proposed fragments.

brasiliana were extracted according to Silva et al. (2011) and analyzed by LC–HRMS, but no masses which could correspond to these compounds, quercetin and sitosterol glycoside, were found. It is possible that in the evaluated plants these compounds were modified or the biosynthetic pathway was diverted to other secondary metabolites.

The oxidized fatty acids reported here are the first compounds detected in *A. brasiliana* that can justify the antibiotic properties associated with this plant. The isolated compounds **1**–**10** (except **7**) were able to inhibit the growth of Gram-positive bacteria (*B. subtilis* (ATCC 6623), *M. luteus* (ATCC 9341) *S. aureus* (ATCC 25923)) at 50 μg/mL. This observation is also supported by previous studies that describe oxidized fatty acids as antibiotics compounds (Dang et al., 2008; Dasgupta and Hogan, 2001; Furuno et al., 2011; Prost et al., 2005; Weber et al., 2006). However, this is the first time that such oxylipins are described for plants from genus *Alternanthera*.

These oxylipins may also explain the anti-inflammatory properties reported for *A. brasiliana*. Perhaps, 9-HODE (compound **5**) has been reported as an anti-inflammatory compound while 13-HODE (compounds **6** and **7**) presents pro-inflammatory properties (Altmann et al., 2007; Belvisi and Mitchell, 2009; Hattori et al., 2008; Obinata and Izumi, 2009; Tam et al., 2013). Compounds **1**, **5** and **8** also display anti-inflammatory activity toward TPA-induced Edema on mouse ear with inhibition effect between 39 and 79% (Dong et al., 2000). Compounds **9**, **10** and **14** reduced the production of LPS induced TNF-α in THP-1 macrophages (Reyes et al., 2014).

On the other hand, plant oxylipins are usually related to stress signaling and stress responses that can be induced by biotic or abiotic factors. The best known oxylipins are the jasmonates that are generated enzymatically and accumulate in due to various stresses, in particular wounding, herbivory, and pathogen infection (Wasternack and Hause, 2013; Wasternack, 2007). Additionally, non-enzymatically formed oxylipins, such as phytoprostanes, also play important roles in plant signaling and stress responses (Shah, 2005; Thoma et al., 2004). Besides a role in signaling, a direct impact of oxylipins on attacking organisms, such as pathogens has been described as well but not very often (Blée, 2002). For example, some oxylipins found in *A. brasiliana* (9-KODE and 13-KODE) were already reported as accumulating metabolites during the infection of *Arabidopsis* leaves by *Pseudomonas syringae*. However, the accumulation of these compounds occurred more prominently when the leaves were infected by non-pathogenic strains (Vollenweider et al., 2000). This can occur due to the activation of 9- and 13-lipoxygenases, and biosynthesis of some oxylipins such as compound **2**, which seems to be related to the recognition of bacterial avirulence protein (AvrRpm1) by *Arabidopsis thaliana* (Andersson et al., 2006).

These modified fatty acids have also been reported as active compounds against *Bacillus brevis*, *B. subtilis* and various *Pseudomonas* strains, at 30 μg/mL (Prost et al., 2005; Weber et al., 2006). For example, compound **10** is active against some strains of plant pathogenic bacteria, such as *P. syringae* pv *syringae*, *P. syringae* pv *maculicola*, *Xanthomonas campestris* pv *campestris*, at 30 μg/mL (Prost et al., 2005). Yet, neither has activity against *P. syringae* pv *tomato* nor *P. syringae* pv *tabaci*.

Some oxylipins, such as compounds **1**, **5**, **7**, **10**, **12**, **13**, **16** and **17**, are also toxic against fungal plant pathogens, i.e., *Cladosporium phlei* and anti-rice blast fungus (Kato et al., 2001; Koshino et al., 1987). *Phleum pratense* (timothy plants) becomes resistant against *C. phlei* when this plant is infected by *Epichloe typhina*. It probably happens due to some oxylipins (compounds **12**, **13**, **16** and **17**) which are produced by *E. typhina* (Koshino et al., 1987), during plant infection. These results suggest that these oxylipins are involved in an ecological relationship between *P. pratense* and its pathogens.

Table 2
Summary of oxylipins production by endophytic bacteria grown in culture (nd = not detected).

Strain	**5**	**6** and **7**	**14** and **15**
Bacillus flexus	✔	✔	✔
Bacillus megaterium	✔	✔	✔
Bacillus safensis	✔	✔	✔
Bacillus atrophaeus	nd	nd	nd
Bacillus amyloliquefaciens	nd	nd	nd

On the other hand, compounds **16** and **17** have been reported as biotransformation products of oleic acid generated by *P. aeruginosa*, as well as 10-hydroxy 6,12-octadecanoic acid, which is similar to **13** (Hamberg et al., 2010; Rodríguez et al., 2001). Additionally **5**, **6**, **15** and **16** were reported as secondary metabolites produced by some microorganisms (Bastida et al., 1999; Cullere et al., 2001; Hamberg et al., 2010; Martin-Arjol et al., 2010). Therefore, this complex scenario concerning the role of oxylipins in plant-microorganisms interaction prompted this investigation for any presence of endophytic bacteria in *A. brasiliana*, as well as their ability to produce oxylipins we found in plant tissues.

2.4. Oxylipin identification in endophytic bacteria isolated from *A. brasiliana* tissues

Twenty-eight strains of endophytic bacteria were isolated from *A. brasiliana* tissues (leaves and stem). Up to now, only some of these bacteria could be clearly identified using 16S rRNA sequencing. Five of these strains (Table 2) were cultivated and the extracts were analyzed using Selected Reaction Monitoring experiment (SRM), and by searching for compounds **5**–**7**, **12** and **13**, as described in 3.9.

Three out of the five chosen *Bacillus* species (*B. megaterium*, *B. mycoides*, and *B. cereus*) isolated as endophytic bacteria were able to produce exactly the same oxylipins (**5**–**7**, **12** and **13**) found in the plant tissues (Fig. 8S; Table 2). Although some studies showed the ability of *Pseudomonas* species to oxidize fatty acids that were provided from outside (Hou, 2006, 2005; Lanser, 1998; Lanser et al., 1992), as well as the association of such microorganisms with plants (Mastretta et al., 2009; Melnick et al., 2011), there is neither information concerning the production of compounds **5**–**7** and **12** by *Bacillus* species nor about a relationship between plants and these microorganisms in oxylipins production.

Although the role of oxylipins in plant-microorganisms interaction is poorly understood, the data presented here and the information reported previously suggest that the antibiotic compounds present in the *A. brasiliana* tissues arise from an intriguing interaction between this plant and its endophytic bacteria. Moreover, it raises the question as to which is the real producer of the oxylipins and the ecological relevance of such compounds in the interaction between *A. brasiliana* and the endophytic bacteria. Both questions will be addressed in the near future by generation of sterile and re-infected *A. brasiliana* plants.

3. Conclusion

Seventeen oxylipins were identified as the antibiotic compounds from *A. brasiliana*. This is the first time that such oxylipins are described for plants from the genus *Alternanthera*. Since three strains of *Bacillus* (isolated as endophytes from *A. brasiliana*) are

able to produce exactly the same oxylipins present in the plant tissues in vitro, a bacterial origin for these compounds is likely. Thus, it is speculated that these oxylipins might be involved in an intriguing relationship between this plant and its endophytic bacteria.

4. Experimental

4.1. General experimental procedures

A. brasiliana extract and fractions were analyzed in a Alliance 2695 HPLC from Waters coupled to Micromass triple-Q Quattro LC mass spectrometer using a Phenomenex Synergy C-18 column (4.6 mm × 250 mm; 5 μm) column. Purification of oxylipins was performed in a Shimadzu HPLC system composed of two pumps LC-20AP, a controller unit CBM-20 A, degasser 20 A5, detector UV–vis SPD 20AV and fraction collector FRC-10A. H^1 and COSY measurements were recorded on Bruker Avance III 600 MHz using $CDCl_3$ as solvent and HRMS analyses were performed in a Ultimate 3000 series RSLC UHPLC (Dionex) combined to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) and Acclaim C18 column (150 × 2.1 mm, 2.2 μm, Dionex) mounted to a C18 3.5 μm guard column (2.1 × 10 mm, Waters).

4.2. Plant material

Parts of *A. brasiliana* were collected at São Carlos area during the end of 2008. Stem was washed with tap H_2O, dried and grinded, yielding a powdered material (534.6 g).

4.3. Extraction and sample preparation

The powdered parts of *A. brasiliana* were extracted with EtOH (200 ml × 3) and EtOAc (200 mL solvent 3) for 48 h for each extraction. The solvents were evaporated and the solid residues were combined to give a solid extract (14.3 g). The fractionation process was performed with 10 g of the solid extract in a silica gel column (7.5 × 35 cm) under reduced pressure. Five fractions were collected using subsequently the following eluent compositions (200 mL each): Hexane:EtOAc (1:1, **F1**), EtOAc:CH_2Cl_2 (4:1, **F2**), EtOAc:CH_2Cl_2 (1:1, **F3**), EtOAc:CH_2Cl_2:MeOH (4:1:0.1, **F4**) and MeOH (**F5**).

4.4. High-performance liquid chromatography and electrospray ionization-mass spectrometry

The fractions of *A. brasiliana* were analyzed by LC–PDA–MS and LC–PDA–MS/MS (at 1 mg/mL). The LC method (Method 1) was developed using a Phenomenex Synergy C-18 column (4.6 mm × 250 mm; 5 μm) mounted on an Alliance 2695 HPLC from Waters, at a flow rate of 1.0 mL/min. A flow splitter (15:1) was used to couple the HPLC with a Micromass triple-Q Quattro LC mass spectrometer equipped with a Z-spray ESI ion source. The chromatographic method employed a linear gradient program with mobile phase composed of H_2O (solvent A) and MeOH (solvent B) from 30% to 100% B in 30 min, then holding 100% of B for 10 min. The mass spectrometer was operated on the ESI negative mode (Capillary at 3.6 kV, Cone at 45 V and Extractor at 4 V). For LC–PDA–MS and LC–PDA–MS/MS analyses, fractions were solubilized in MeOH (1.0 mg/mL) and it was injected 20 μL of this solution into the LC–MS system. A second chromatographic method (Method 2) was developed to achieve the necessary resolution to distinguish isomeric fatty acids. The best separation was achieved using a Supelco-Ascentis Express C-18 column (100 × 4.6 mm; 2.7 μm). The mobile phase was composed of H_2O (solvent A) and CH_3CN (solvent B). During these analyses, it was employed a multistep gradient: 0–10 min 30–60% B, 10–20 min 60% B, 20–25 min 60–100% B and 25–30 min 100% B. The column was equilibrated during 8 min (30% B) before the injections. This method was also used in LC–PDA–MS/MS analyses.

4.5. Isolation of antibiotic compounds

Isolation of oxylipins was carried out in a Shimadzu HPLC system composed of two pumps LC-20AP, a controller unit CBM-20 A, degasser 20 A5, detector UV–vis SPD 20AV and fraction collector FRC-10A. The isolation was performed in two steps. Initially, the active fraction of *A. brasiliana* (**F3**) was processed through a C18 SPE cartridge eluted with MeOH:H_2O and MeOH. The methanolic fraction was re-fractionated in a Phenomenex Luna Phenyl-Hexyl column (21.2 mm × 250 mm; 10 μm) with mobile phase composed of H_2O (solvent A) and CH_3CN (solvent B) and flow of 21.5 mL/min, employing a multistep gradient as following: 0–20 min 45–65% B, 20–27 min 65–80% B, 27–29 min 100% B and 29–36 min 100% B. This procedure yields 3 samples (F3.1–F3.3), which were subjected to a new separation process using the same chromatographic system (column and solvents). The composition of mobile phase was optimized for each sample: Fraction F3.1 (43% of CH_3CN and flow 25 mL/min); Fraction F3.2 (50% of acetonitrile and flow 25 mL/min) and Fraction F3.3 (50% of CH_3CN and flow 29 mL/min). The isolated compounds were analyzed by NMR spectroscopy.

4.6. Compound characterization

4.6.1. (9Z,12Z,14E)-16-Hydroxy-9,12,14-octadecatrienoic acid (**1**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21150 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 1.8 mg.

4.6.2. (9Z,13E,15Z)-12-Hydroxy-9,13,15-octadecatrienoic acid (**2**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21126 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 2.7 mg.

4.6.3. (9Z,11E,15Z)-13-Hydroxy-9,11,15-octadecatrienoic acid (**3**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21187 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 4.0 mg.

4.6.4. (9Z)-9-Octadecenedioic acid (**4**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 311.22122 $[M-H]^-$ (calcd for $C_{18}H_{31}O_4$, 311.22169). HRMS/MS (−) see Table 1S. Isolated amount: 2.7 mg.

4.6.5. (9Z,11E)-13-Hydroxy-9,11-octadecadienoic acid (**5**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 295.22699 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S. Isolated amount: 2.3 mg.

4.6.6. (10E,12Z)-9-Hydroxy-10,12-octadecadienoic acid (**6**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 295.22681 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S. Isolated amount: 2.3 mg.

4.6.7. (10E,12E)-9-Hydroxy-10,12-octadecadienoic acid (**7**)

Colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 295.22661 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S.

*4.6.8. (9Z,11E)-13-Oxo-9,11-octadecadienoic acid (**8**)*

Colorless oil; ^{1}H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21126 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 1.7 mg.

*4.6.9. (9E,11E)-13-Oxo-9,11-octadecadienoic acid (**9**)*

Colorless oil; ^{1}H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21109 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 3.3 mg (fraction composed by 9 and 10 according to Fig. 5S).

*4.6.10. (10E,12Z)-9-Oxo-10,12-octadecadienoic (**10**)*

Colorless oil; ^{1}H NMR ($CDCl_3$, 600 MHz) see Table 1. HRESIMS (−) *m/z* 293.21137 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 3.3 mg (fraction composed by 9 and 10 according to Fig. 5S).

*4.6.11. (10E,12E)-9-Oxo-10,12-octadecadienoic acid (**11**)*

HRESIMS (−) *m/z* 293.21147 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.21220). HRMS/MS (−) see Table 1S. Isolated amount: 1.9 mg.

*4.6.12. (8E,12Z)-10-Hydroxy-8,12-octadecadienoic acid (**12**)*

HRESIMS (−) *m/z* 295.22712 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S.

*4.6.13. (9Z,13E)-12-Hydroxy-9,13-octadecadienoic acid (**13**)*

HRESIMS (−) *m/z* 295.22724 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S.

*4.6.14. (8E)-10-Oxo-8-octadecenoic acid (**14**)*

HRESIMS (−) *m/z* 295.22729 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S.

*4.6.15. (7E)-9-Oxo-7-octadecenoic acid (**15**)*

HRESIMS (−) *m/z* 295.22689 $[M-H]^-$ (calcd for $C_{18}H_{31}O_3$, 295.22787). HRMS/MS (−) see Table 1S.

*4.6.16. (10E)-9-Hydroxy-10-octadecenoic (**16**)*

HRESIMS (−) *m/z* 297.24285 $[M-H]^-$ (calcd for $C_{18}H_{33}O_3$, 297.24265). HRMS/MS (−) see Table 1S.

*4.6.17. (8E)-10-Hydroxy-8-octadecenoic acid (**17**)*

HRESIMS (−) *m/z* 297.24243 $[M-H]^-$ (calcd for $C_{18}H_{33}O_3$, 297.24265). HRMS/MS (−) see Table 1S.

4.7. HRMS measurements

Ultra high-performance liquid chromatography–high resolution tandem mass spectrometry (UHPLC–MS) was accomplished with a UHPLC system of the Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) combined to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Ten microliter of the *A. brasiliana* fraction were separated on an Acclaim C18 column (150 × 2.1 mm, 2.2 μm, Dionex) linked to a C18 3.5 μm guard column (2.1 × 10 mm, Waters, Dublin, Ireland) with a flow rate of 300 μl min^{-1} in a binary solvent system containing H_2O (A) and CH_3CN (B) (hypergrade for LC–MS, Merck, Darmstadt, Germany), both supplemented with 0.1% (v/v) HCO_2H (eluent additive for LC–MS, Sigma Aldrich, Steinheim, Germany). Starting conditions were set to 99.5% A and 0.5% B. Gradient was linearly increased within 15 min to 100% B, which was then held for 5 min, before decreasing to the initial conditions, which were kept for 5 min to re-equilibrate the column for the next injection. Ionization was achieved using electrospray ionization source using parameters of 5 kV for spray voltage, 35 V for transfer capillary voltage at a capillary temperature 275 °C. The samples were measured in negative ion mode in the mass range of *m/z* 150–1200. Fragmentation was achieved using several energies (10, 20, 30, 40, 50 arb. unit) of collision-induced dissociation (CID) in the LTQ and higher energy collision dissociation (HCD) in the HCD cell. Mass spectra were acquired using 7500 m/Δ*m* resolving power in the Orbitrap mass analyzer. Data were interpreted using XCALIBUR software (Thermo Fisher Scientific, Waltham, MA, USA).

4.8. Antibiotic assay

All the antibiotic assays were performed by microdilution broth method according to NCCLS standardization (NCCLS, 2003). For leaf and stem extracts, the stock solutions (17.5 mg/mL in DMSO) were diluted with Müller–Hinton broth up to 500 μg/mL and DMSO at a final concentration of 3%. The oxylipin rich fractions were tested at concentrations varying from 50 to 500 μg/mL, and the isolated compounds were tested at 50 μg/mL. The antibiotics tetracycline and norfloxacin (4.0 μg/mL) were used as a positive control, and DMSO at 3% was used as a negative control. All samples were tested in triplicate and the tests were repeated twice. The bacteria used in these tests, *E. coli* (ATCC 25922), *B. subtilis* (ATCC 6623), *P. aeruginosa* (ATCC 15442), *M. luteus* (ATCC 9341) and *S. aureus* (ATCC 25923) were grown overnight in Müller–Hinton broth and diluted to a final concentration of 5×10^5 CFU/mL per well. The plates were incubated under aerobic conditions at 37 °C for 20 h. The inhibition of bacteria was evaluated after incubating the plates with resazurin solution (15 μL, 100 μg/mL) per well for 30 min.

4.9. Isolation of endophytic bacteria

Isolation of endophytic bacteria was carried out using aerial parts of *A. brasiliana*. Leaves and stem were washed immediately after the collection and endophytic bacteria were isolated according to Petrini et al. (1992) The surface sterilization was performed introducing pieces of *A. brasiliana* (leaves and steam) into a sequence of solvents (sterile H_2O, EtOH:H_2O (70:30, v/v) sterile H_2O, hypochlorite 4–6% and sterile H_2O).

After sterilization, plant fragments were introduced into a Petri dish containing sterile nutrient agar medium. They were maintained at 37 °C until the bacterial growth. The last sterile H_2O was sown in a Petri dish as a negative control.

At the beginning of growth, bacteria were transferred onto a new plate containing nutrient agar medium. In order to purify the isolated strains, a serial dilution was performed. After the bacteria growth, one colony was transferred to a 15 mL tube containing sterile distilled H_2O. Such suspensions were diluted serially and inoculated in new Petri dishes containing nutrient agar media. They were kept for 24 h at 37 °C. Twenty-eight strains were obtained after the purification process. Five bacterial strains were identified through 16S rRNA gene sequencing.

4.10. Microorganism cultivation and analysis

Five of the isolated bacteria were cultivated in 250 mL Erlenmeyer flasks containing liquid media (nutrient broth) under stirring (136 rpm and room temperature) for 10 days. Hereafter, the bacterial metabolites were extracted adding EtOAc (50 mL) into each Erlenmeyer flask. This procedure was repeated three times. The solutions containing bacterial metabolites were combined and the solvent was removed under reduced pressure. These extracts were analyzed in a LC–MS/MS system, using SRM detection mode with transitions associated with oxylipins fragmentation (295 > 195 for **5**; 295 > 171 for **6** and **7**; 295 > 183 for **12** and **13**).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.11.005.

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Antibiotic oxylipins from *Alternanthera brasiliana* and its endophytic bacteria.

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Supplementary Information

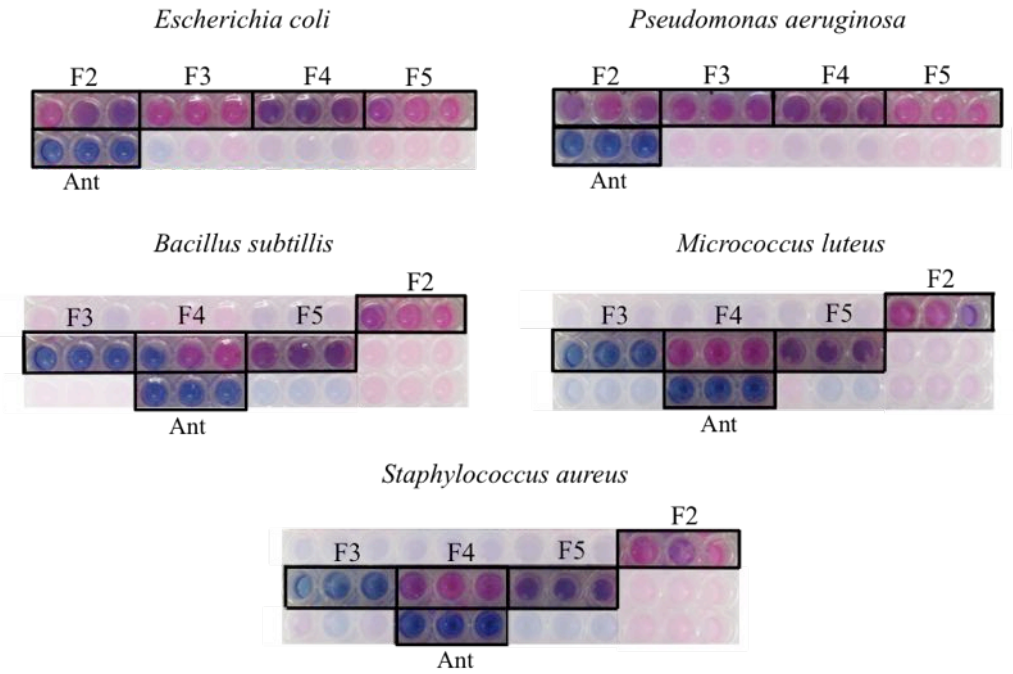


Figure 1S: Antibiotic assay of *A. brasiliana* fractions (at 500ug/mL) against Gram-positive and Gram-negative bacteria.

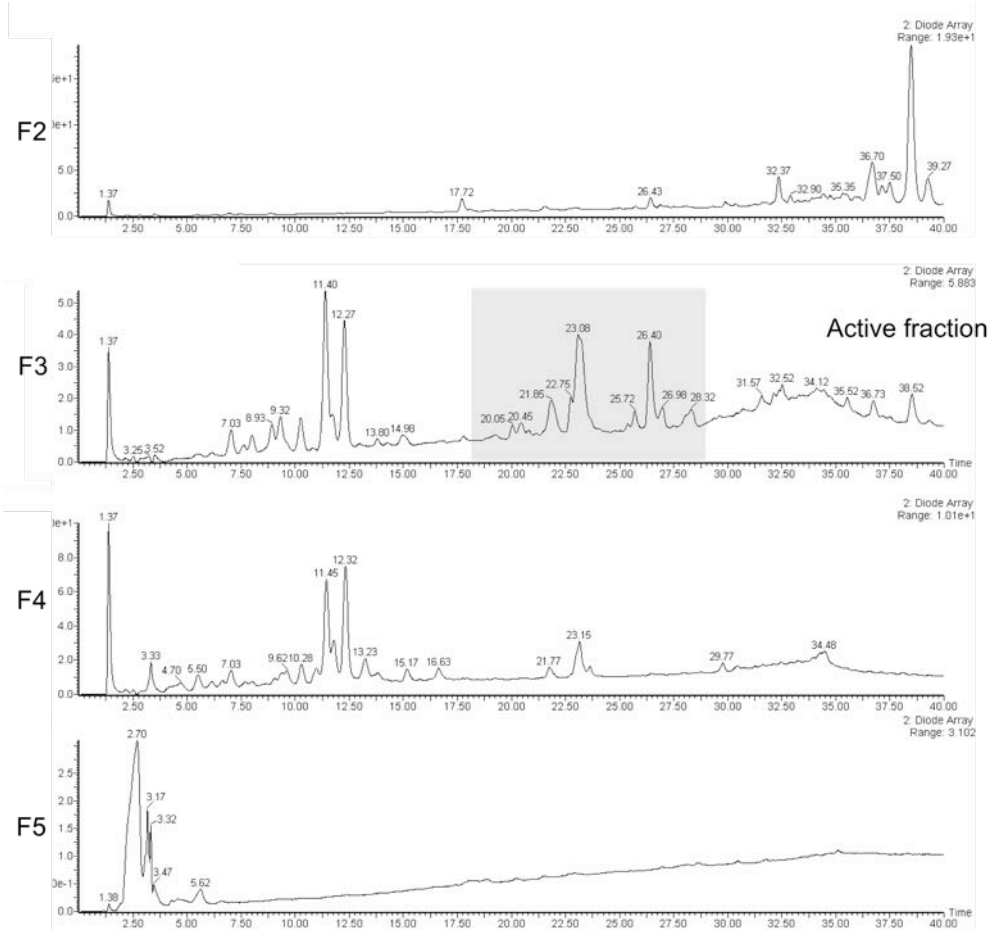


Figure 2S: LC-PDA chromatograms for stem fractions of *Alternanthera brasilian*. The dashed lines in F3 chromatogram represent the collected fractions during the semi-preparative fractionation process given **F3.1** - **F3.5**.

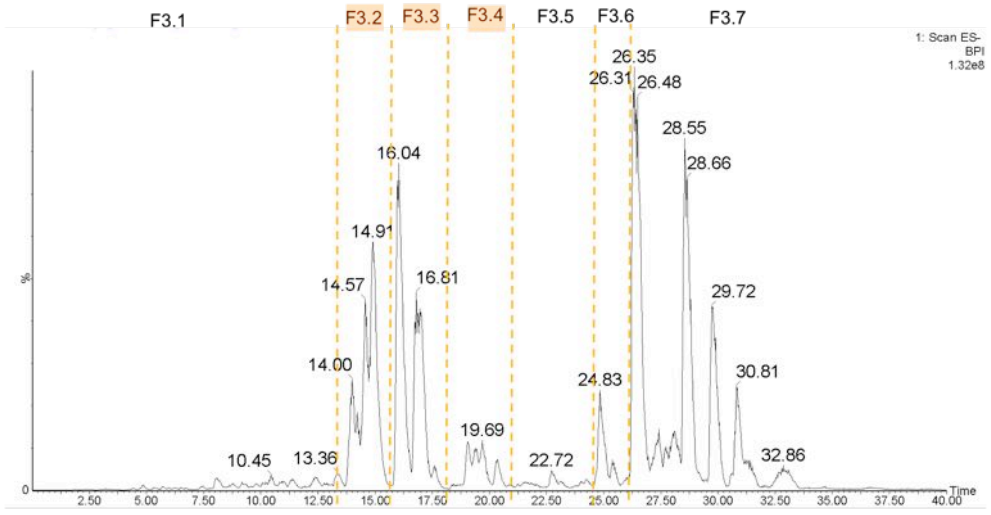


Figure 3S: LC-PDA chromatogram obtained for **F3**. The dashed lines represent the collected fractions during the preparative fractionation process, and the highlighted codes correspond to active fractions.

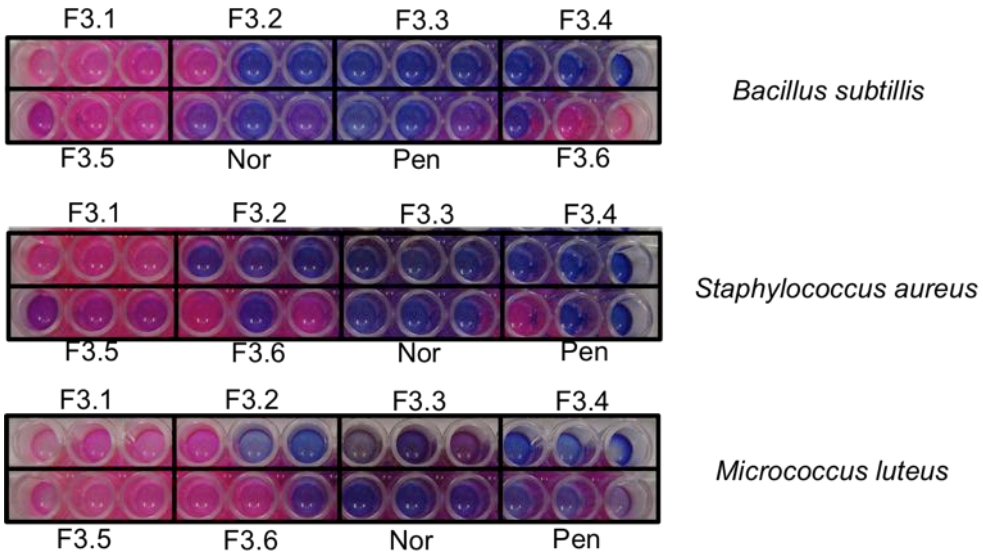


Figure 4S: Antibiotic assay of *A. brasiliana* fractions (at 50ug/mL) against Gram-positive bacteria.

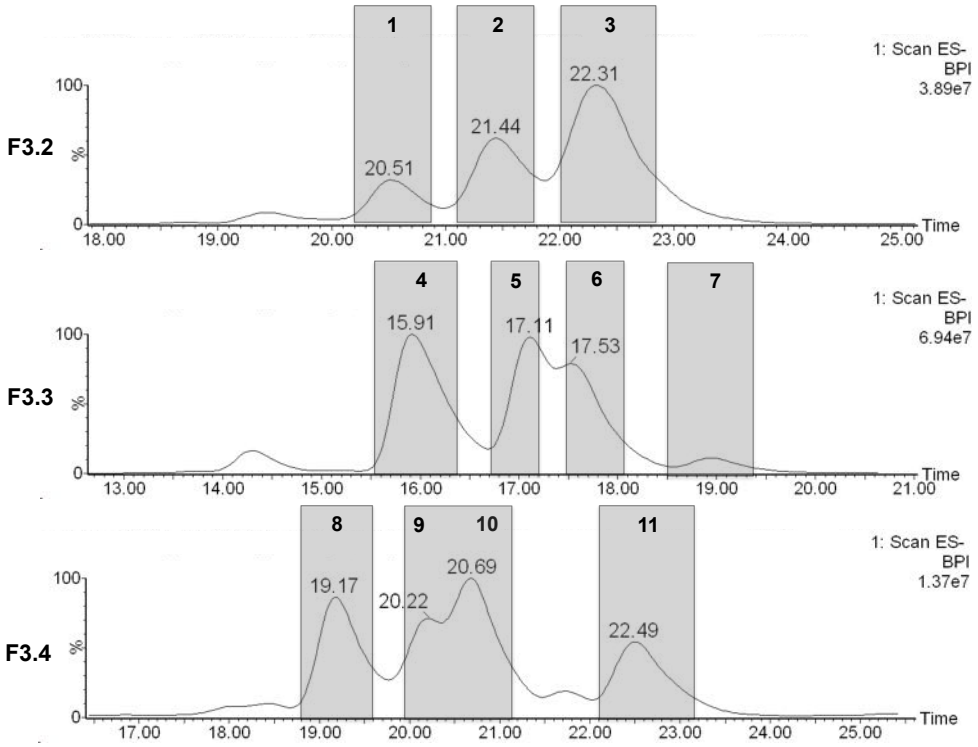
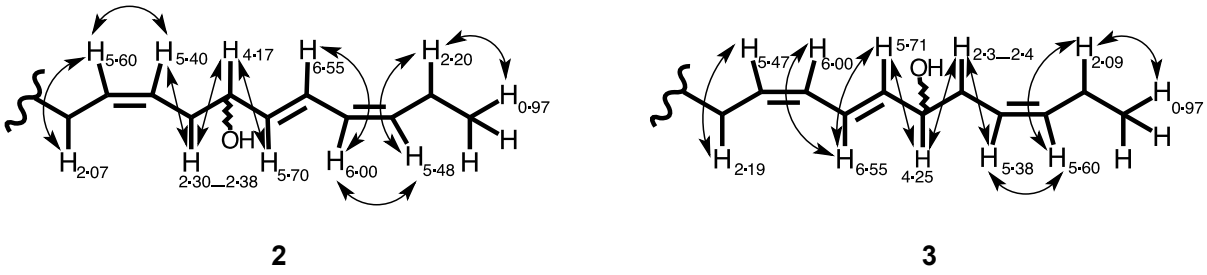


Figure 5S: LC-PDA chromatogram obtained for **F3.2, F3.3** and **F3.4** after chromatographic optimization. The highlighted area corresponds to collected bands in preparative scale.



2 **3**

Figure 6S: COSY correlations for compounds **2** and **3**.

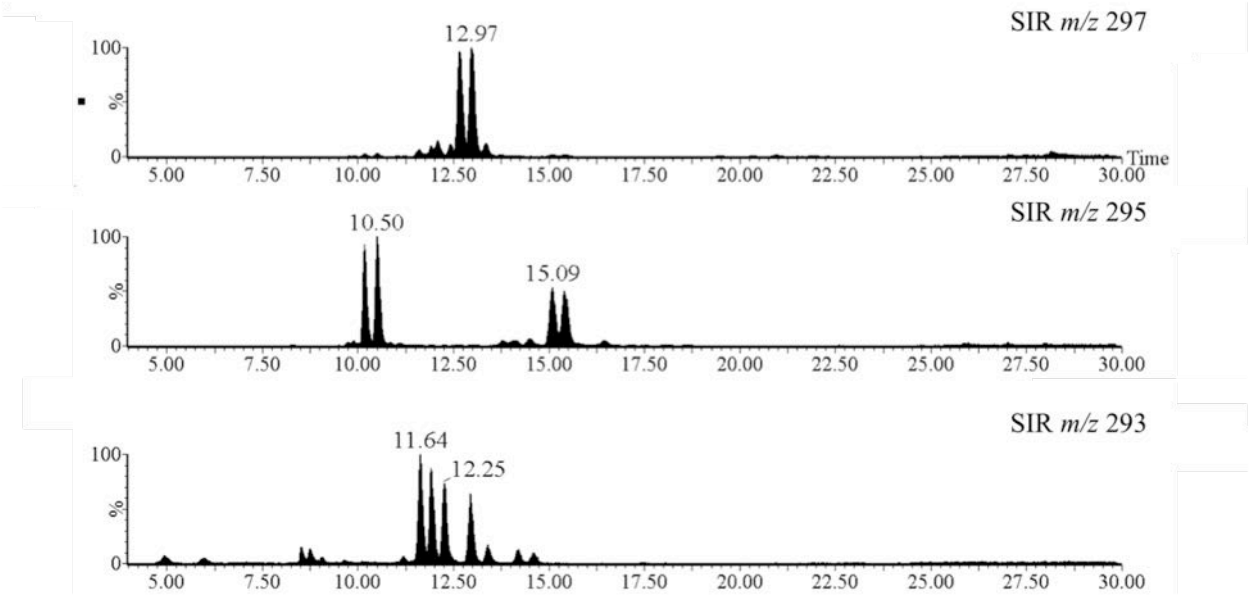


Figure 7S: Separation of minor oxylipins present in *A. brasiliana* extract (m/z 297 and m/z 295), and comparison of chromatographic behaviour with compounds **8-11** (m/z 293

Compound	Precursor ion	Fragment ions
1	293.21150 (calc. 293.21220)	275.20083 ($C_{18}H_{27}O_2$, calc 275.20165); 235.17035 ($C_{15}H_{23}O_2$, calc 235.16992)
2	293.21126 (calc. 293.21220)	275.20152 ($C_{18}H_{27}O_2$, calc 275.20165); 211.13364 ($C_{12}H_{19}O_3$, calc 211.13397); 183.13884 ($C_{11}H_{19}O_2$, calc 183.13905); 109.06499 (C_7H_9O, calc 109.06589)
3	293.21187 (calc. 293.21220)	275.20150 ($C_{18}H_{27}O_2$, calcd 275.20165); 249.22255 ($C_{17}H_{29}O$, calc 249.22155); 224.14190 ($C_{13}H_{20}O_3$, calc 224.14179); 195.13892 ($C_{12}H_{19}O_2$, calc 195.13905); 113.09744 ($C_7H_{13}O$, calc 113.09720)
4	311.22122 (calc. 311.22169)	275.20150 ($C_{18}H_{27}O_2$, calcd 275.20091); 249.22255 ($C_{17}H_{29}O$, calc 249.22155);
5	295.22699 (calc. 295.22787)	277.21681 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23770 ($C_{17}H_{31}O$, calc 251.23804); 195.13875 ($C_{12}H_{19}O_2$, calc 195.13905); 113.09716 ($C_7H_{13}O$, calc 113.09720)
6	295.22681 (calc. 295.22787)	277.21702 ($C_{18}H_{29}O_2$, calcd 277.21730); 251.23769 ($C_{17}H_{31}O$, calc 251.23804); 171.10238 ($C_9H_{15}O_3$, calc 171.10267)
7	295.22661 (calc. 295.22787)	277.21725 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23817 ($C_{17}H_{31}O$, calc 251.23804); 195.13875 ($C_{12}H_{19}O_2$, calc 195.13905); 113.09716 ($C_7H_{13}O$, calc 113.09720)
8	293.21133 (calc. 293.21220)	275.20138 ($C_{18}H_{27}O_2$, calc 275.20165); 249.22211 ($C_{17}H_{29}O_2$, calc 249.22240); 113.09730 ($C_7H_{13}O$, calc 113.09720)
9	293.21109 (calc. 293.21220)	275.20131 ($C_{18}H_{27}O_2$, calc 275.20165); 249.22216 ($C_{17}H_{29}O$, calc 249.22240); 113.09719 ($C_7H_{13}O$, calc 113.09720)
10	293.21137 (calc. 293.21220)	275.20132 ($C_{18}H_{27}O_2$, calc 275.20165); 249.22142 ($C_{16}H_{25}O_2$, calc 249.22240); 197.11782 ($C_{11}H_{17}O_3$, calc 197.11830) 185.11787 ($C_{10}H_{17}O_3$, calc 185.11830)
11	293.21147 (calc. 293.21220)	275.20007 ($C_{18}H_{27}O_2$, calc 275.20165); 249.22294 ($C_{16}H_{25}O_2$, calc 249.22240); 197.11790 ($C_{11}H_{17}O_3$, calc 197.11830) 185.11832 ($C_{10}H_{17}O_3$, calc 185.11830)
12	295.22712 (calc. 295.22787)	277.21661 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23724 ($C_{17}H_{31}O$, calc 251.23804); 183.10223 ($C_{10}H_{15}O_3$, calc 183.10267); 155.14383 ($C_{10}H_{19}O$, calc 155.14414)

13	295.22724 (calc. 295.22787)	277.21751 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23863 ($C_{17}H_{31}O$, calc 251.23804); 183.13845 ($C_{11}H_{19}O_2$, calc 183.13905)
14	295.22729 (calc. 295.22787)	277.21718 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23784 ($C_{17}H_{31}O$, calc 251.23804); 151.11259 ($C_{10}H_{15}O$, calc 151.11284); 141.12819 ($C_9H_{17}O$, calc 141.12849)
15	295.22689 (calc. 295.22787)	277.21572 ($C_{18}H_{29}O_2$, calc 277.21730); 251.23738 ($C_{17}H_{31}O$, calc 251.23804); 155.14405 ($C_{10}H_{19}O$, calc 155.14414)
16	297.24285 (calc.297.24265)	279.23293 ($C_{18}H_{31}O_2$, calc 279.23295); 171.10273 ($C_9H_{15}O_3$, calc 171.10267)
17	297.24243 (calc. 297.24265)	279.23250 ($C_{18}H_{31}O_2$, calc 279.23295); 155.10737 ($C_9H_{15}O_2$, calc 155.10748)

2.5 Fragmentation pathways of oxylipins in MS experiments

As previously discussed, the fragmentation profile of oxylipins from A. brasiliana was established using high-resolution mass spectrometry. The fragments of hydroxy and keto fatty acids are generated mainly by three mechanisms.

All unsaturated hydroxy fatty acids (Compounds **01**-**03**, **05**-**07**, **12**, **13**, **16** and **17**) dehydrate generating fragments at m/z M-18 Da. These fragments are highlighted with yellow circles in the MS/MS spectra shown in Figures 2.7 and 2.8. The main fragment for unsaturated hydroxy fatty acids are generated by mechanism I (shown in Figure 2.4). According to this mechanism, four fragments (Ia, Ia', Ib and Ib') could be detected in MS/MS spectrum. The intensity of each fragment depends on their chemical structure and the stabilization of the negative charge. They are fragments highlighted in light or dark pink in the MS/MS spectra (Figures 2.7 and 2.8).

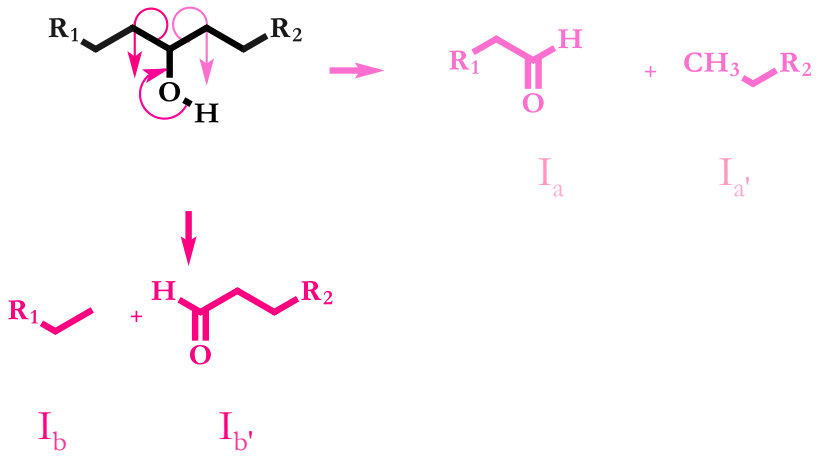


Figure 2.4: Fragmentation pathway of hydroxy fatty acids in MS/MS experiments. The groups R_1 and R_2 depend on the position of oxidation in the carbon chain

In the same way, all polyunsaturated oxylipins with conjugated double bonds (Compounds **3**, **5**, **8-11**) fragment according to mechanism II shown in Figure 2.5. In this case, the cleavage occurs in the double bond near the hydroxyl or carbonyl group. The proposed mechanism involves three 1,5 hydrogen shift, following by another 1,3 hydrogen shift, which can occur in two different positions as pointed by light and dark green arrows in Figure 2.5. Depending on where the negative charge is located, this mechanism generates two detected fragments, which are highlighted in Figure 2.6 by dark and light circles.

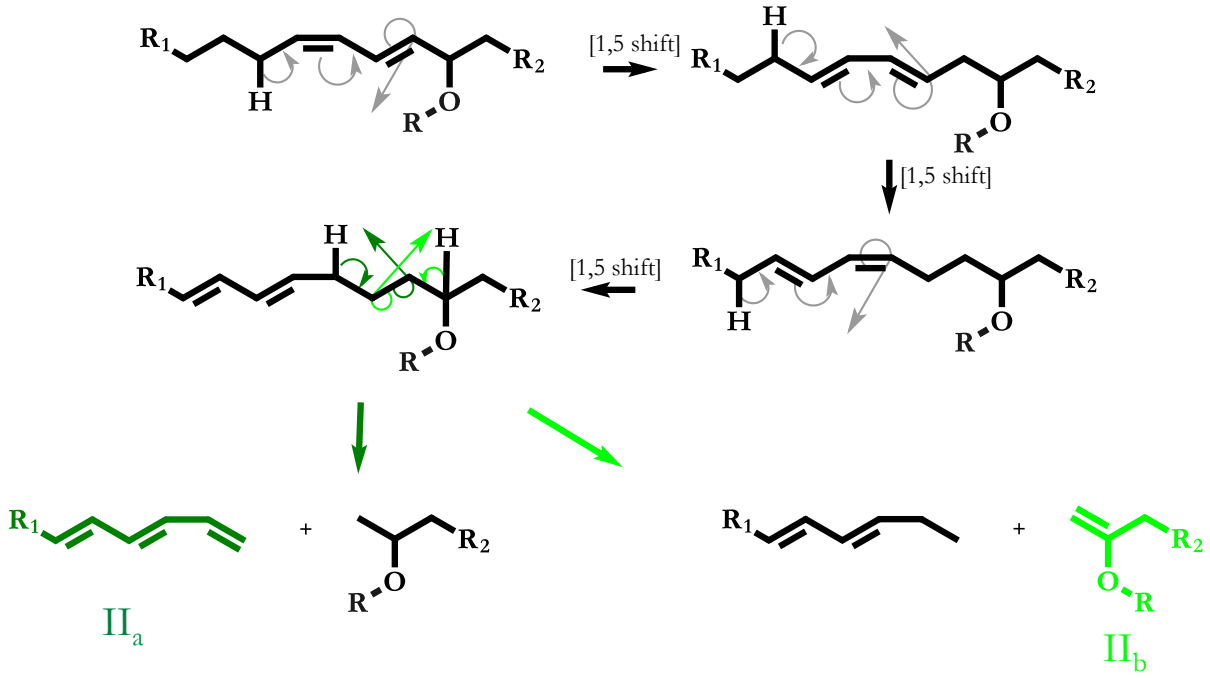


Figure 2.5: Fragmentation pathway of polyunsaturated oxylipins. R represents hydrogen (when the oxylipin is a hydroxy fatty acid) or nothing (when the oxylipin is a keto fatty acid). The groups R_1 and R_2 depend on the position of oxidation and unsaturation in the carbon chain.

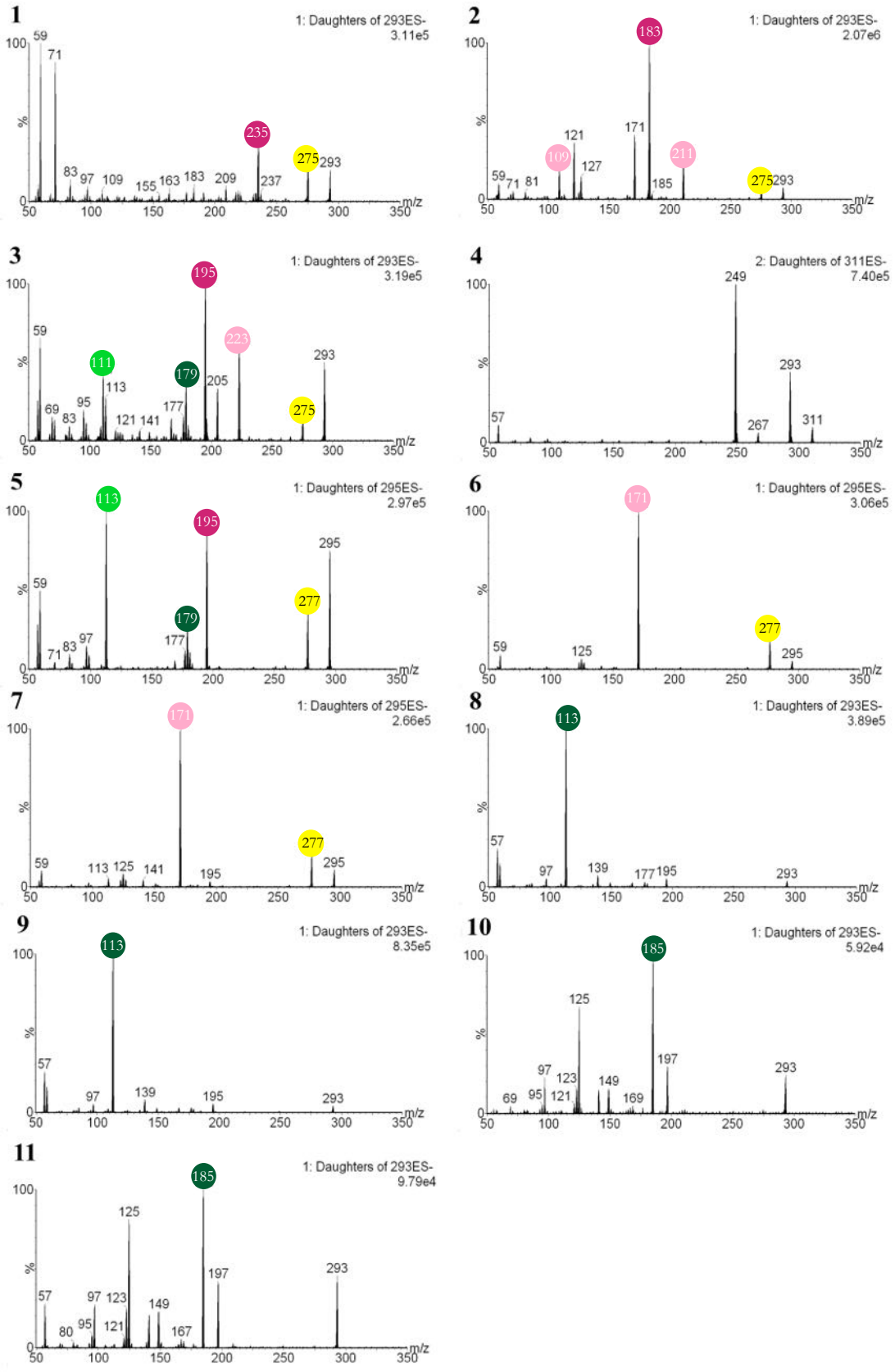


Figure 2.6: MS/MS spectra of oxylipins isolated from *A. brasiliana*. Fragments are highlighted according to the mechanism they are generated (I, II or II)

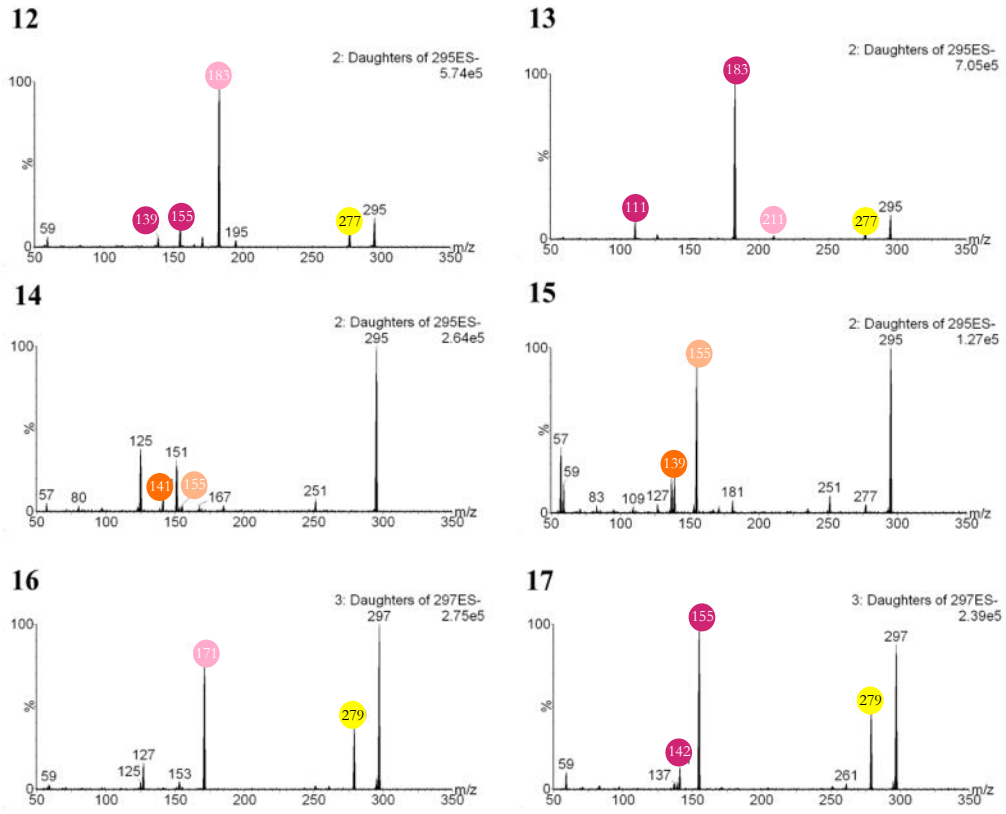


Figure 2.7: MS/MS spectra of oxylipins identified in *A. brasiliana*. Fragments are highlighted according to the mechanism they are generated (I or III)

The mechanism shown in Figure 2.5 does not occur when the keto fatty acid has only one double bond conjugated with the carbonyl group. In this case the fragments seems to be generated by mechanism III (shown in Figure 2.8). Both fragments where detected in the Compounds **14** and **15** as highlighted in Figure 2.7.

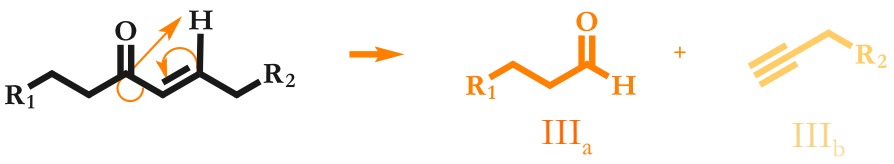


Figure 2.8: Fragmentation pathway of keto fatty acids in MS/MS experiments. The groups R_1 and R_2 depend on the position of oxidation and unsaturation in the carbon chain

All fragments discussed above and generated by mechanism I, II and III are summarized in Figure 2.9 showing that the non-isolated follow one of the three proposed mechanisms.

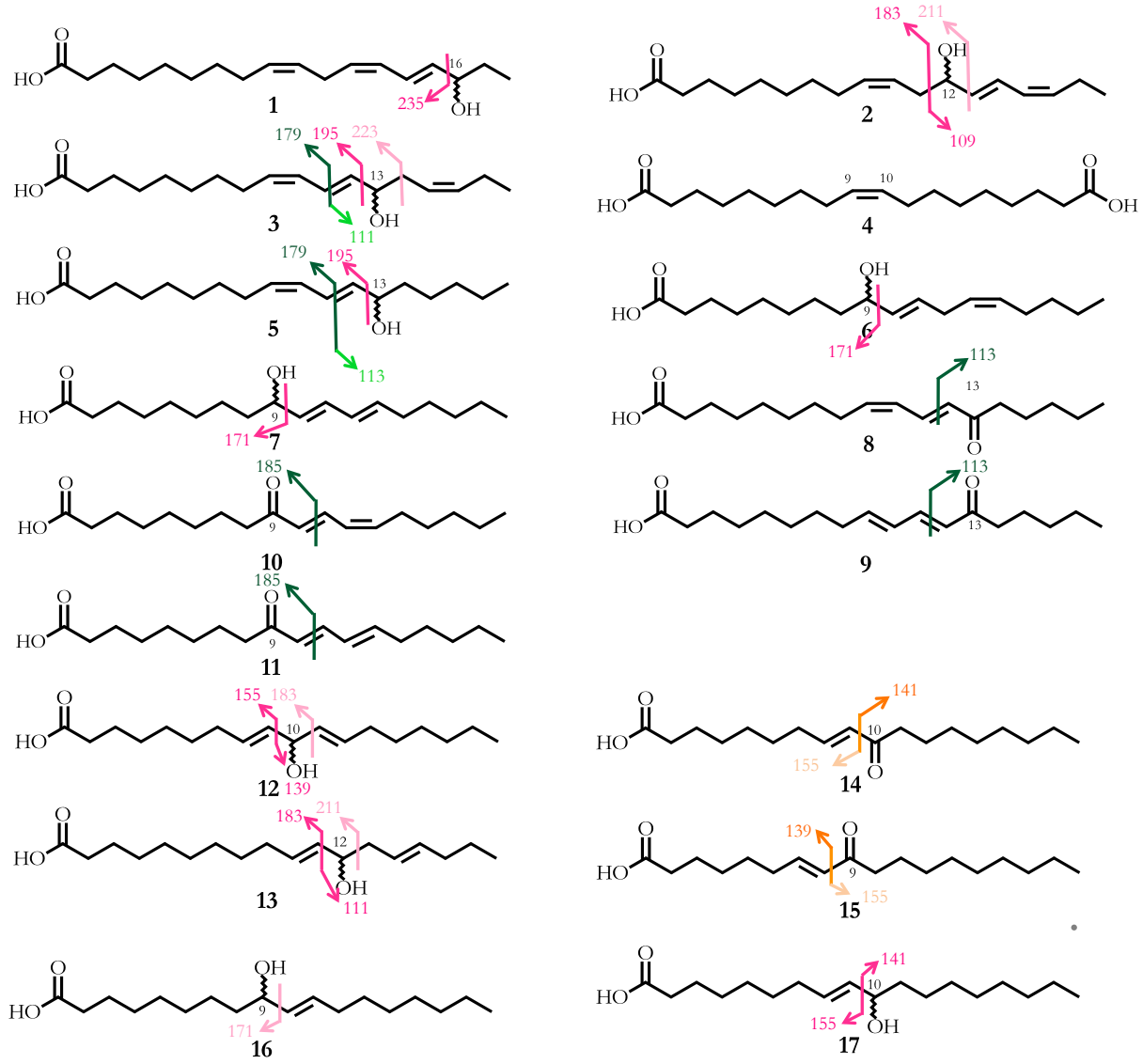


Figure 2.9: Summary of the main fragments from oxylipins detected in *A. brasiliana*

3

Oxylipins: Methods and Concepts

This chapter presents a brief overview of oxylipins in different kingdoms, highlighting the importance of such compounds in ecological interactions. It also presents the development and validation of analytical methods for quantification of oxidized fatty acid and phytohormones

3.1 Introduction

As previously discussed in Chapter 02, the antibiotic compounds found in *A. brasiliana* belong to oxylipins, which constitutes a diverse class of metabolites present in nearly every living organism.[52] These compounds have many biological functions depending on both their molecular structure and the organism they are found.

3.1.1 Oxylipins in animals

In animals, the precursors of oxylipins are usually dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA), eicosapentanoic acid (EPA) and docosahexaenoic acid (DAA), Figure 3.1. Such compounds can be obtained from diet or from elongation of linoleic, linolenic or a-linoleic acid. Due to the high potency, the oxylipins are synthetized *de novo* from the polyunsaturated fatty acids (PUFA).[34,53]

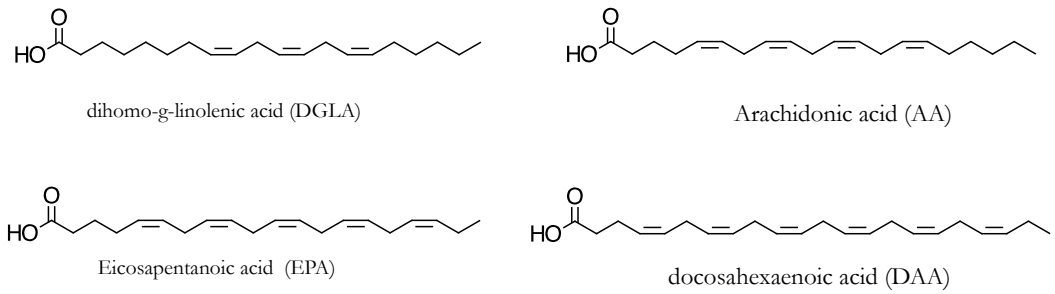


Figure 3.1: Unsaturated fatty acids, precursors of oxylipins in mammalians.

The precursors are released from the cell membrane upon cellular activation and then oxidized by three families of enzyme: cyclooxygenases (COX), lipoxygenases (LOX) or cytochrome P450 (CYP), yielding different compounds as can be seen in Figure 3.2. All these oxylipins have different functions in mammalians and play different roles during the interaction with other kingdoms. The leukotrienes, epoxyeicosatrienoic acids (EET), and prostaglandins are usually related with anti-inflammatory processes in humans.[54,55] Some of these oxylipins are

also found in other kingdoms, for instance, volicitin, which is also found in larvae and supresses the tannin production in plants after the tissue damage, suggesting that it is involved in animal-plant interactions. [56]

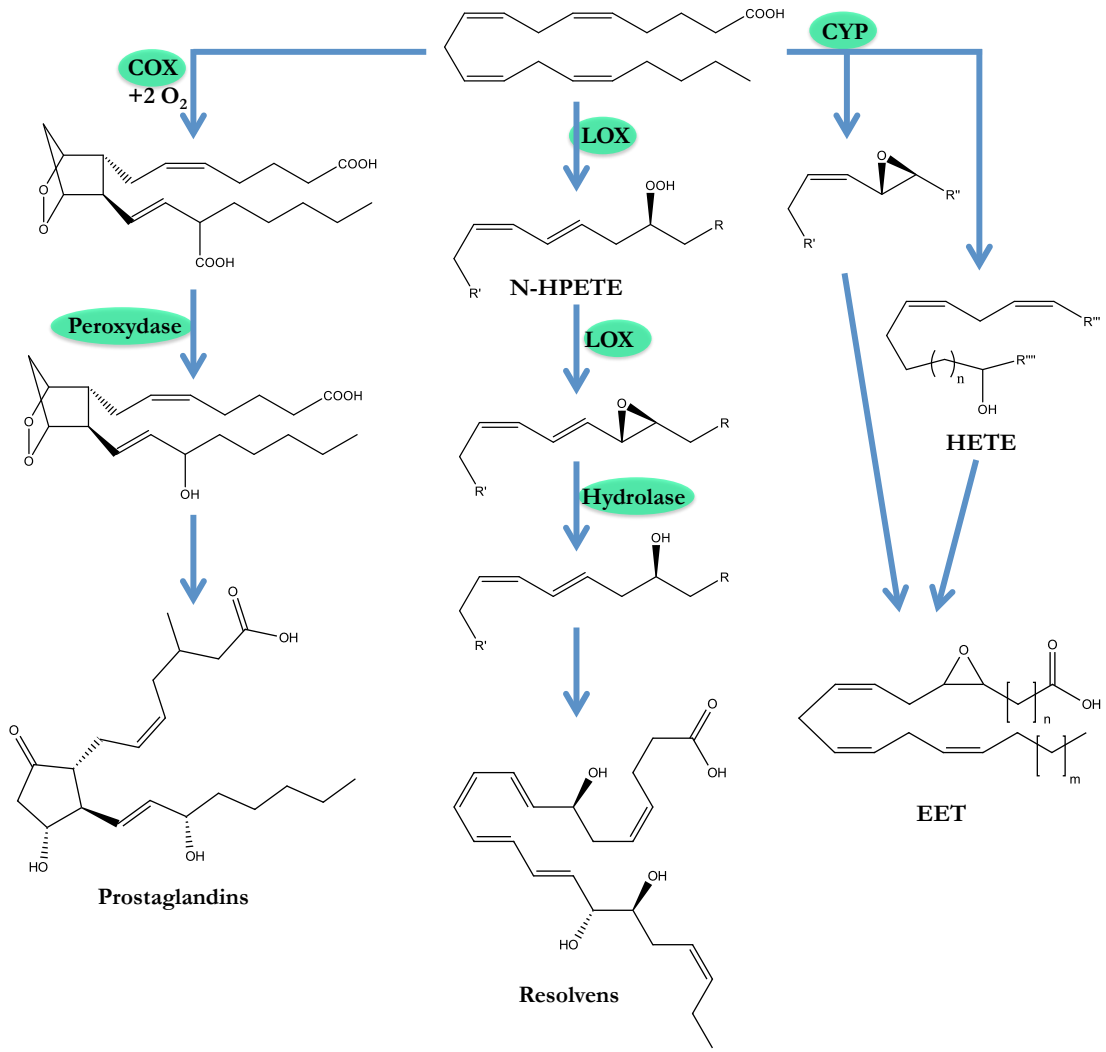


Figure 3.2: Simplified biosynthetic pathway of oxylipins in mammalians

3.1.2 Oxylipins in plants

In plants, the biosynthesis of oxylipins (Figure 3.3) begins with oxidation of unsaturated fatty acids (usually C18 fatty acids) either by chemical (through reactive oxygen species, ROS) or enzymatic reactions (by lipoxygenases, LOX) generating the corresponding hydroperoxide. Then, the biosynthesis can follow

three different paths: the hydroperoxide can be reduced to hydroxy fatty acids by peroxigenases (PXG); it can also be oxidized into a keto group by action of lipoxygenases (LOX); and finally, it can be dehydrated by allene oxide synthase to an unstable allene oxide. This last path leads to the biosynthesis of jasmonates which also act as phytohormones.[30,32,57]

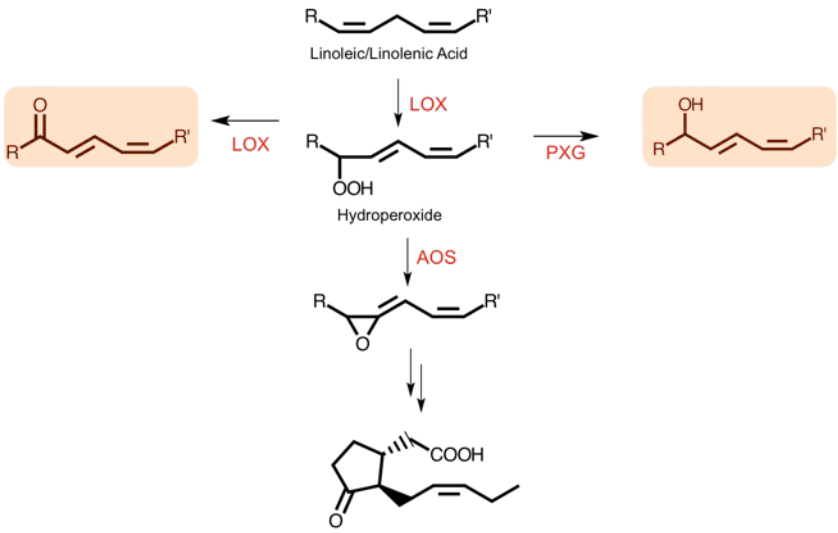


Figure 3.3: Biosynthetic pathway of oxidized fatty acids and jasmonates in plants.

Although plant oxylipins are well characterized, many of the compounds identified during dereplication studies of *A. brasiliana*, (such as compound **1**, **2**, **12**-**17**) have been reported only few times, as can be seen comparing the number of paper published about all 17 oxylipins found in this plant (Figure 3.4).

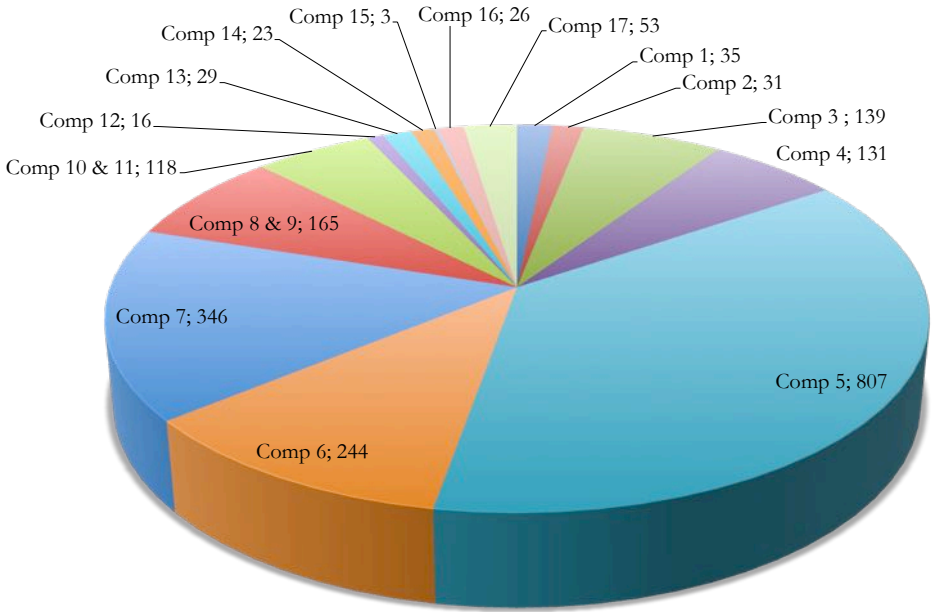


Figure 3.4 Number of papers published about the different oxylipins identified in *A. brasiliana*. (Source: Chemical Abstracts Service; 02.2016) represented by: Compound number ; number of papers

Moreover, for some of these well-studied oxylipins, just little is known about the correlation with plants and microorganism. Many papers are either related to mammalians or to their biological activities in humans. For example, when the 807 papers published about compound **5** are categorized, only 10% of them are associated with plant, and 1% with microorganisms (Figure 3.5).

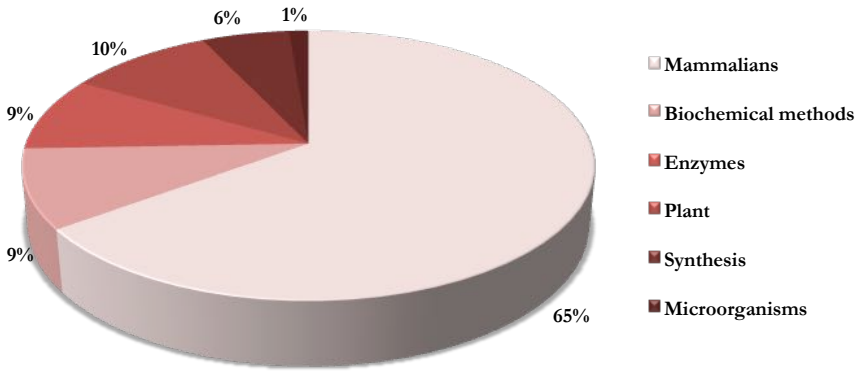


Figure 3.5: Topics associated with the papers published about compound **5**. (Source: Chemical Abstracts Service; 02.2016)

In plants, the most well known oxylipins are the jasmonates derivatives. The comparison between the number of papers published about jasmonates (in this case jasmonic acid) and about the oxidized fatty acids identified in *A. brasiliana* (Figure 3.6) shows that these latter compounds are by far not as intensively studied as other plants oxylipins. Even though, the oxylipins identified in *A. brasiliana* are understudied in comparison with other plant oxylipins, they seem to be involved in intriguing ecological relationships between plant and microorganisms.

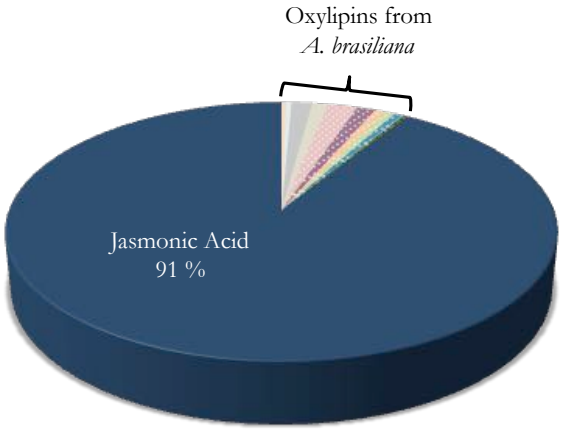


Figure 3.6: Comparison between the number of papers about jasmonic acid and other oxylipins. (Source: Chemical Abstracts Service; 02.2016)

3.1.3 Oxylipins in microorganisms

Undoubtedly, oxidized fatty acids from plants and mammalians are better characterized than those present in microorganism. However, the oxylipins present in fungi and bacteria seems to be a very diverse class of compounds, being produced by enzymes that exhibit unusual catalytic activities. [58]

In the past, it was believed that lipoxygenases were present only in eukaryote organisms. However, 15 years ago the first two plant homologue LOX sequences were described in bacteria.[59] The majority of described prokaryotic lipoxygenases are 13-LOX, but many fatty acids oxidized in other positions have being reported as bacterial metabolites, suggesting that there is a lot to be discovered about oxylipins in these organisms. [58,60]

As discussed for plant and animals, microorganisms also shared some oxylipins with other kingdoms: the prostaglandin E2 (PGE_2), which was first reported as human metabolite is also produced by lager number of pathogenic yeast and fungi (i.e. *Aspergillus fumigatus* and several species of *Candida*). This compound can decrease the pulmonary function in the host when it is infected with pathogenic microorganisms.[61] Since the host and the pathogens are able to produce exact the same molecule, this oxylipin might be involved in an important cross talk between the host (animal) and the pathogen (fungi).[52]

In this way, some compounds found in *A. brasiliana* (**8-11**) have already been reported as compounds which rise up in *Arabidopsis* as plant defence upon infection by *Pseudomonas aeruginosa*.[35] On the other hand, the compounds **16** and **17** have been reported as biotransformation products of *Pseudomonas aeruginosa* rather than as plant-derived metabolites.[35–46] The same occurs with 10-hydroxy 6,12-octadecanoic acid, which is similar to compound **13** and has been reported as a bacterial biotransformation metabolite. [35,62]

Additionally **5**, **6** and **15** were reported as secondary metabolites produced by some microorganisms including fungi. [38,42,44,63] Only three works can be found where such compounds were isolated from plants; however these references also called them unusual plant metabolites.[47–51]

Once the oxylipins seems to be very interesting cross-kingdom signalling molecules, and the role of many oxylipins is not well understood, we decide to investigate the biosynthetic path of the oxylipins present in *A. brasiliana* tissues, trying to figure out whether or not they are involved in ecological interactions between plant and microorganisms. For this, we developed an HPLC-MS method for quantification of oxidized fatty acids in microorganisms, algae plant tissues. Some data also suggest that there is a correlation between the biosynthesis of oxylipins (i.e. jasmonates) and other phytohormones. So, we also developed and validated a method in order to quantify phytohormones in plant tissues, trying to get a broader overview about the correlation of all these signalling molecules during plant-microbe interactions.

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3.3 Quantification of oxylipins in plant, algae and microorganisms

This paper will be submitted to Analytical and Bioanalytical Chemistry.

Quantification of oxylipins in plant tissues, algae and microorganisms

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1 Introduction

Oxylipins are product of chemical or enzymatic lipid oxidation. These compounds have an ancient evolutionary origin, and they are often signalling molecules involved in ecological interaction between organisms from different kingdoms.[52] They are present in mainly all kingdoms, playing different roles according to their molecular structure or the target organism. A lot has been done in the past years regarding the identification of enzymes involved in their biosynthetic routes, and the role of some oxidized fatty acid, especially in mammalians.[53]

Although the oxidized fatty acids have been studied for long time in several organisms, there is a lack of information, including the quantification of these compounds in complex matrixes. Up to now, all papers dealing with direct quantification of oxidized fatty acids are related to biological fluids (i.e. plasma). The quantification of these compounds in plants and microorganisms has been done mainly by indirect measurements of secondary species produced during lipid peroxidation. For instance, lipid peroxidation can be measured by the concentration of malondialdehyde (a secondary product of lipid peroxidation) or hydroperoxide.[64,65] The hydroperoxide mediates the oxidation of ferrous to ferric ions. So the amount of hydroperoxide is measured by a ferric indicator dye, which binds to ferric ions producing a complex with high absorption at 560 nm.[66] The main problem of these indirectly quantification methods is that they are very often unspecific and may provide misleading results

There are only few papers where the free oxidized fatty acids are directly measured.[67–70] In all these cases the sample preparation involves very laborious protocols. So, here we present the first very direct, selective, sensitive, precise and accurate method to quantify 11 oxylipins (previously reported by TRAPP et al.) in plant tissues, algae and microorganisms liquid culture medium. Moreover, we evaluate the effect of internal standards on the method accuracy and precision.

2 Material and Methods

2.1 Reagents and Standards

Solvents used during extraction procedures were of analytical grade except for methanol (MeOH). Chromatographic separation was carried out using Acetonitrile HPLC grade purchased from J. T. Baker (Xalostoc, Mexico). All oxylipins standards were isolated as described for Trapp et al. [71] and the deuterated standards: (KODE and HODE) were purchased from OlChemIm Ltd (Olomouc, Czech Republic).

2.2 Apparatus

A Waters ACQUITY®UPLC® (Waters Corporation, Milford, MA,USA) coupled to a Waters TQD mass spectrometer was used to develop and validate the analytical method for quantification of oxylipins. Chromatographic separation was carried out in a Luna Phenyl-Hexyl column (150 x 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany). Formic acid (0.001%, v/v) and acetonitrile with 0.001% (v/v) of formic acid were employed as mobile phases A and B, respectively. The elution profile was: 0-3 min, 50-80% B in A; 3-8 min, 80-100% B; 8-11 min 100% B; 11-11.1 min 100-50% B in A; and 11.1-16 min 50% B in A. The mobile phase flow rate was 1.0 mL/min. Injection volume was 25 μL. The mass spectrometer was equipped with an Electrospray ionization source, operating in the negative ion mode. Measurements were carried out using the following ionization parameters: source voltage: 4.4 kV, capillary voltage: -48 V, tube lens -113 V, declustering potential 10 V, turbo gas temperature: 300 °C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min.

Selected reaction monitoring (SRM) experiments were used to monitor specific precursor -> product ion transitions for each oxylipin and internal standard. Collision energy was optimized for each compound separately.

During the inter-laboratory reproducibility, the analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen,

Germany) connected to a LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was carried out using the same conditions described above. The ionization parameters used during these analyses were: source voltage: 4,4 kV, capillary voltage: -48 V, tube lens -113 V, desclustering potential 10 V, turbo gas temperature: 300 °C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min

Collision energy, precursor ion isolation width and activation Q were optimized for each compound separately

2.3 Plant and Algae Material

Stem and leave of *A. brasiliana* were collected at São Carlos area in the end of 2014. The tissues were frozen in liquid nitrogen immediately after the collection. Then they were ground and dried in a SpeedVac under reduced pressure at -40ºC. After homogenization, 25 mg of plants were weighted into 1.5 mL tubes and stored at -20 ºC until the measurements.

Prof Dr Armando Augusto Henriques Vieira and Ms Ingritt Caroline Moreira kindly provided two types of algae (*Ankistrodesmus densus* and *Selenastrum bibraianum*) for the previous analyses of oxylipins. The cells of both algae were dried in SpeedVac under vacuum and at -40ºC. Then, the dried cells of each algae were samples in 1.5 mL tubes (25 mg) and stored at -20 ºC until the measurements.

2.4 Oxylipins extraction and analysis

The optimization of oxylipins extraction was performed in two steps. Initially, solvents with different polarities were tested and in the second step the water content and sonication time were evaluated in order to get a high content of oxylipins and low content of chlorophyll.

2.4.1 Extraction using Organic Solvents and Clean-up

In the first step, tubes containing 25 mg of dried plant material were extracted with 250uL of different solvents: ethyl acetate, acetone, diethyl ether, acetonitrile, ethanol and methanol. The samples were kept in ultrasonic bath for 5

minutes, centrifuged at 10,000 g and 4 ºC for 5 minutes. The supernatant was analysed by HPLC-MS/MS using the HPLC method and the SRM.

2.4.2 Aqueous solutions

In the second step, tubes containing 25 mg of dried plant material were extracted with 250 μL of different methanol:water ratios (95, 90, 85 and 80% of methanol). Sonication time was also evaluated: samples extracted with all different methanol: water ratios were kept in the ultrasound bath for different periods (5, 10 15 or 20 minutes). Each extraction procedure was performed in triplicate. After extraction, all samples were centrifuged at 10,000 g and 4 ºC for 5 minutes. The supernatant was analysed by HPLC-MS/MS as described above.

2.4.3 Final method for extraction of oxylipins

The extraction oxylipins, used during the validation of the analytical method, was performed using 1,5 mL tubes containing 25 mg of freeze-dried plant material. Samples were spiked with 10μL of standard solutions (calibration curve or quality control levels) andshacked for 5 minutes. The extraction was performed using 0,240 μL of methanol:water (7:3) and samples were kept in ultrasound bath for 20 minutes. Afterwards, the samples were centrifuged at 10,000 g and 4 ºC for 5 minutes, and filtered using washed cotton. The supernatant was analysed using the method described 2.2.

2.5. Method Validation without internal standard

During this work two analytical methods for quantification of oxylipins were developed and validated: one using deuterated oxylipins (13-KODE and 9-HODE) as internal standard and other without any internal standard. For both method all validation figures were evaluated to access the accuracy and

precision according to the Guidelines on Bioanalytical Method Validation from European Medicines Agency, EMEA and FDA. [72–74]

2.5.1 Limit of detection and limit of quantification

Usually LOD is established using matrix samples spiked with the low amount of standards. However, as none analyte-free matrix was available, the LODs were determined in solvent as three times the noise level. For each matrix, LOQs were defined according to the amount of oxylipins present in ten independent blank samples, which were extracted as described in 2.4.3. For all the LOQ the signal-to-noise ratios were higher than ten.

2.5.2. Calibration curve and linearity

Compounds 1-5 were quantified using the SRM transitions showed in Table 3.2. Since the compounds 6/7, 8/9 and 10/11 are geometric isomers, each pair was quantified using one SRM transitions. The calibration curves were prepared in matrix using a spiking solution containing pure compounds isolated from A. *brasiliana*. For each point of the calibration curve, 25mg of dried plant material were spiked with 10 μL of spiking solution containing all the oxidized fatty acids in different concentrations (0.625, 1.25, 2.50, 12.5, 25.5, 62.5 and 125 μg/mL). Then these samples were extracted as described in 2.4.3. For a workflow sheet see Scheme1S (Supporting material).

2.5.3 Quality controls.

Quality controls (QC) were used to assess the method's repeatability and reproducibility. The quality controls were prepared spiking 25 mg of plant material with 10 μL of spiking solutions with different concentration of all oxylipins: high quality control spiking solution (HQC) containing 112.5 μg/mL of each fatty acid; medium quality control spiking solution (MQC) containing 75 μg/mL; and low quality control spiking solution (LQC) containing 1.87 μg/mL. All these samples were also extracted as described in 2.4.3.

2.5.4 Recovery

Recovery was calculated comparing the amount of each oxylipin present in spiked/extracted and extracted/spiked quality controls. The spiked/extracted quality controls samples were prepared as described in 2.5.3. The extracted/spiked samples were spiked with 10 μL of MeOH:water (7:3) – simulating the addition of spiking solutions – and then extracted as described in 2.4.3. The dry residues were reconstituted in MeOH containing the final concentration of each oxylipin, which corresponds to 2,5 fold less than the spiking solution concentration. (See scheme 1S)

2.6 Method validation using internal standard

All parameters described in 2.5 (linearity, recovery, matrix effect, repeatability and reproducibility) were evaluated during the validation using internal standards. However due to the lake of oxylipin standards, these parameters were evaluated just for 3 oxylipins: 9-HODE (used to quantify compounds 6 and 7); 13-HODE (used to quantify compounds 5) and 13-KODE (for quantification f compounds 8 and 9). Extraction of calibration curve and quality controls samples were performed in the same way as described in 2.4.3. The only difference was that the extraction solution was spiked with 20ng/mL of d3-13-KODE and d4-9-HODE.

2.7 Validation in *Algae*

Initially, 3 samples of both *Ankistrodesmus densus* and *Selenastrum bibraianum* were analysed to establish the basal level of the all oxylipins in algae. Samples containing 25 mg of dried algae tissues were extracted in the same way than described for *A. brasiliana* (item 2.4.3). Once the limits of the method are sufficient for quantification of oxylipins in algae, all the parameter (linearity, recovery and matrix effects) will also evaluated.

2.8 Validation in microorganisms

Quantification of oxylipins in microorganisms was established using Czapeck culture medium. Two strategies for recovery of oxylipins from the liquid medium were evaluated: liquid-liquid extraction and lyophilisation/resuspension. Initially 100mL of Czapeck liquid medium were spiked with 10mg of an oxylipin rich fraction (obtained from *A. brasiliana*). Then, this solution was sampled in 18 samples of 5 ml each.

Twelve of these samples were lyophilized and resuspended in 5 mL of organic solvents: methanol, dichloromethane, ethyl acetate or chloroform (three samples for each solvent). All samples were kept in ultrasound bath for 10 min and centrifuged for 5 minutes at 10.000 g. Then the supernatant was removed and dried under airflow.

In the remaining 6 samples, the oxylipins were removed from the liquid medium by extraction with 5 mL of ethyl acetate or chloroform. The organic phase was removed and dried under airflow.

All the 18 samples were resuspended in 0,5 mL of methanol HPLC grade and analysed using the same method described in 2.2.

3. Results and Discussion

Oxylipins are important compounds in widely range of organisms, such as bacteria, plant, insect and animals. Although some methods have been developed for quantification of these compounds in liquid fluids, none of them directly quantify oxylipins in solid and complex samples. So, here we present the development and validation of an HPLC-ESI-MS/MS based method for direct quantification of 11 oxylipins (Figure 3.7) in plant, algae and microorganisms liquid culture medium.

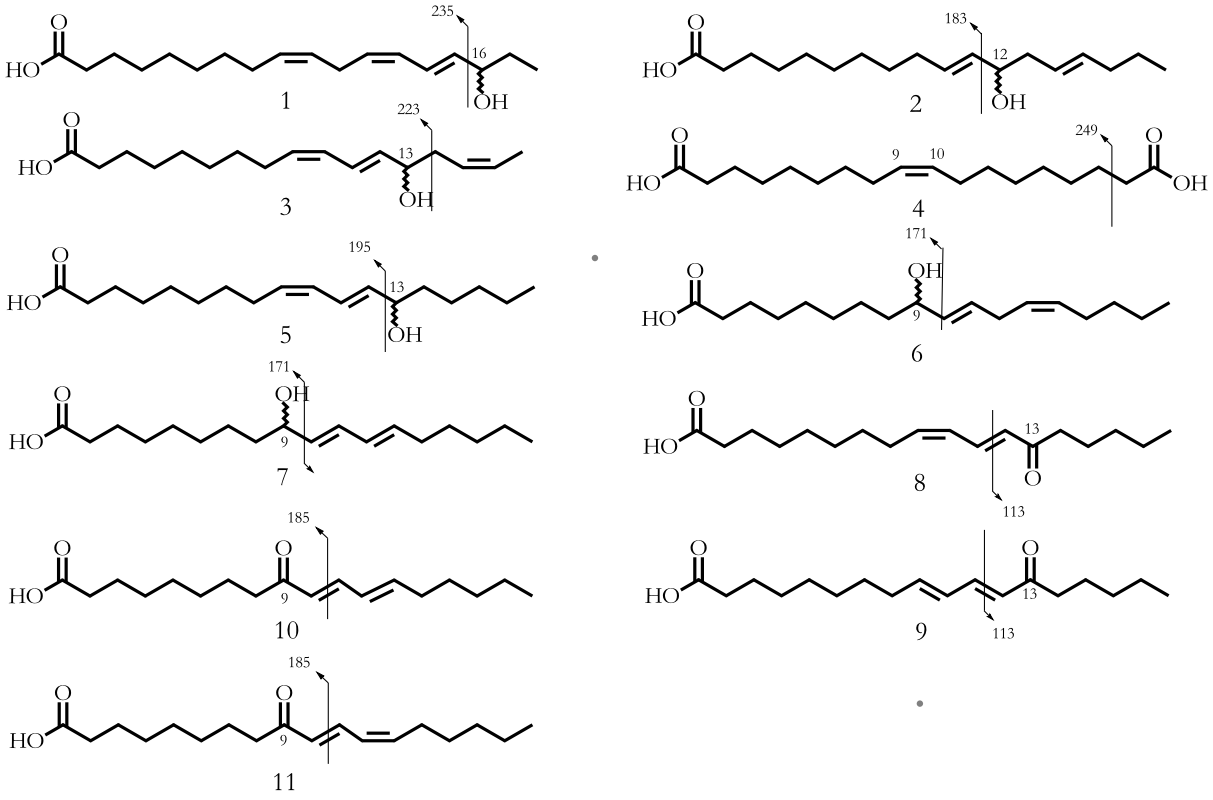

Figure 3.7: Chemical structure of oxylipins quantified in the present study. The arrows represent the fragments used for quantification of each metabolite.

3.4 Development of extraction method

As describe in 2.4.1, several organic solvents were tested to extract the oxidized fatty acids from *A. brasiliana* tissues: ethyl acetate, acetone, diethyl ether, acetonitrile, ethanol and methanol. However all the extraction solution presented high chlorophyll content, and a clean-up process would be necessary to remove it from the samples. During the HPLC-PDA-MS analysis, the chlorophyll present in *A. brasiliana* tissues showed a non-polar nature (data not shown). So, instead of trying to clean the samples up to remove the chlorophyll, we tested aqueous extraction solutions, in order to extract a lower quantity of chlorophyll. Ultrasound was also tested to enhance the extraction of oxidized fatty acids.

After the extraction, all samples were analysed by HPLC-ESI-MS/MS using SRM experiments. The area of the bands present in the SRM chromatograms are summarized in Figure 3.8) for compounds

The results show that independently of the number of insaturations, position of oxidation and the oxidation nature (ketone or hydroxyl group), the better extraction was achieve using methanol:water as extraction solvent. Theses results extend to all other evaluated oxylipins. These results also show that the extraction is improved when the samples are kept in ultra-sound bath. Therefore, methanol:water (7:3) and sonication for 20 minutes were chosen for extraction of oxylipins during to be applied in this method.

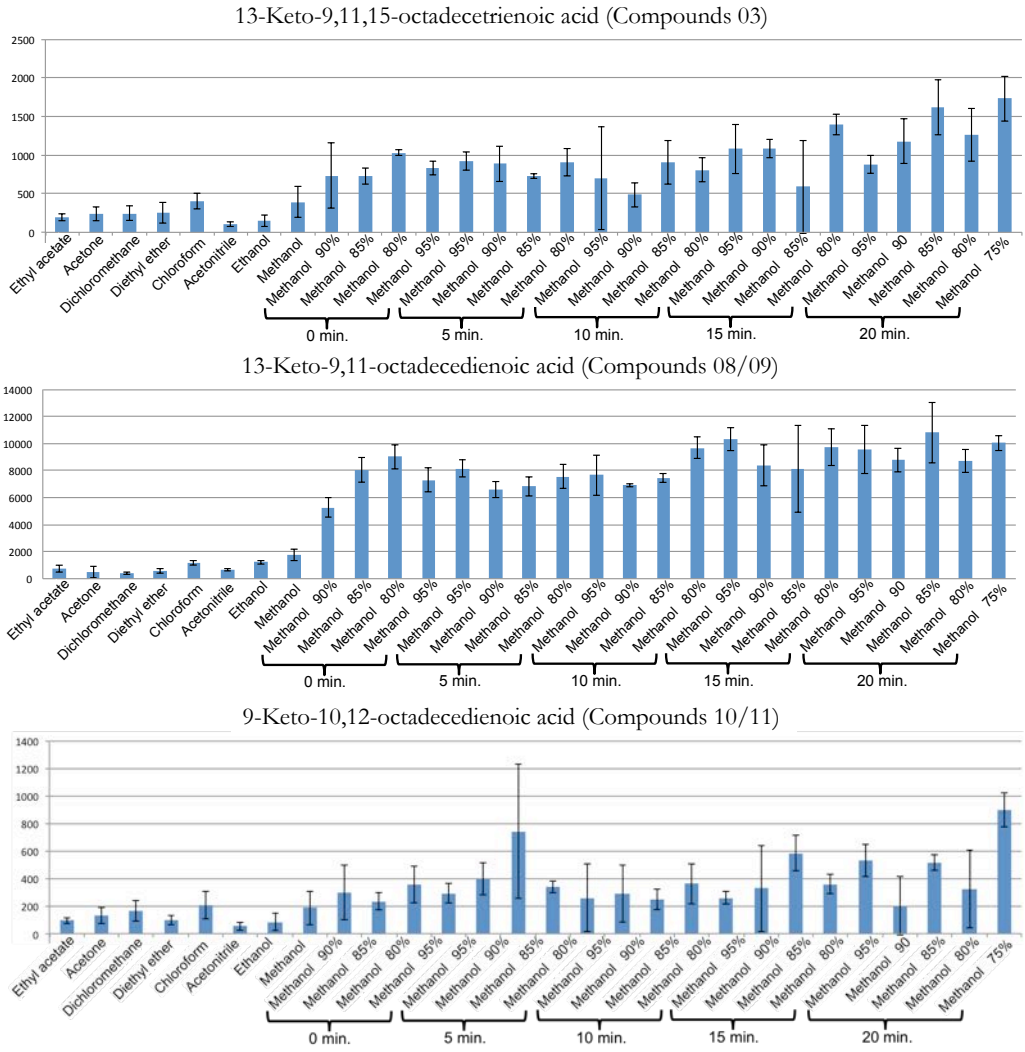

Figure 3.8 Optimization of extraction of some oxylipins from *A. brasiliana*.

3.5 Validation parameters

Once the method for extraction of oxylipins was established, the validation parameters (linearity, reproducibility, repeatability, matrix effect and robustness) were evaluated according to EMEA, FDA and European Commission guidelines. Since there is no blank matrix (all the oxylipins are endogenous in *A. brasiliana*), the quantification was performed using a standard addition method.

3.5.1 Linearity

Linearity was measured using three calibration curves prepared individually and analysed in sequence. Each calibration curve was composed by a blank sample and 7 points, as shown in Table 3.1

Table 3.1: Concentration of oxylipins (μg /mL or μg/g of dried tissue) in the points of calibrations curve and spiking solution

Point	Conc. Spiking solution μg/mL	Final Conc. [μg/mL of solution]	Final Conc. [μg/g of dry plant tissue]
Blank	-	-	-
1	0.625	0.025	0.25
2	1.25	0.050	0.50
3	2.50	0.10	1.0
4	12.5	0.50	5.0
5	25.5	1.0	10
6	62.5	2.5	25
7	125	5.0	50

The SRM transitions, linear regression, weighting factor, R^2 and the basal level of each compound in *A. brasiliana* are shown in Table 3.2. The SRM transitions were defined according to data published by Trapp et at..

Since all R^2 presented in Table 3.2 are bigger than 0.99 we consider that a linear regression is satisfactory for quantification of all oxylipins. However, the best results were achieved when different weighting factors were applied to the calibration curve, depending on the compound that is being quantified.

Table 3.2: SRM transitions, linear fit parameters for quantification of each oxylipin without internal standard.

Compound	SRM transition	Curve	Weighting factor	R^2	Amount in blank samples (µg /g DW)
[1]	293>235	15538,9x+1429,5	$1/x^2$	0,992	0.92
[2]	293>183	30535x + 2753,7	$1/x^2$	0,999	0.90
[3]	293>223	16990,5x +2821,6	1/x	0,998	1.67
[4]	311>249	13755,5x +5278,9	$1/x^2$	0,995	3.84
[5]	295>195	19817,6x + 2582,7	$1/x^2$	0,994	1,30
[6]/[7]	295>171	32167,1x +19816,0	$1/y^2$	0,995	6,16
[8]/[9]	295>113	43352,7x + 14471,0	1/x	0,998	3,34
[10]/[11]	293>185	21105,3x + 9060,6	$1/x^2$	0,989	4,29

DW: Dry weight of *A. brasiliana*

It is important to highlight that the best weighting factor and the regression model were determined evaluating not only the R^2 but also the residues for all calibration points (data not shown). In this case all the residues might be below 20% for the lower calibration curve point and 15% for the others. When these parameters are not satisfied for one of the points of calibration curve, this point is excluded from the calculations. According to the guidelines neither the lowest point nor two consecutive points can be excluded. All these requirements were satisfied during the statistic processing, showing the method is linear for quantification of oxylipins in *A. brasiliana*.

3.5.2 Matrix effect

It is well known that matrix (in this case plant metabolites) can have a pronounced effect in the response of the mass spectrometer during the quantification of some analytes by HPLC-ESI-MS/MS. For this reason, the validation guidelines recommend that for quantification using HPLC-ESI-MS/MS, all samples (calibration curve and quality controls) must be prepared in the matrix.

Although some guidelines accept methods using samples prepared in solvent, it is only allowed once it has been proved that the matrix does not affect the analyser response in different analyte concentrations.

Evaluation of the matrix effect can be done by comparison of the angular coefficient of both calibration curves: prepared in matrix and solvent. If the difference is below 15%, the method can be validated in solvent, otherwise the validation must be carried out in matrix.Curves in solvent and in matrix for all oxylipins are shown in Figure S1 (supplementary information). The angular coefficient (α) for calibration curves prepared in matrix and solvent, as well as relative difference between these values are summarized in Table 3.3. These results show that the matrix do affect the analyser response for half of the oxylipins (samples highlighted in red). So, for these compounds the validation must be carried out in matrix. Therefore, we decided to validate the method in matrix for all oxylipins present in this study.

Table 3.3: Matrix effect for quantification of oxylipins in *A. brasiliana* tissues.

Compound	α_{matrix}	$\alpha_{solvent}$	$\left(\frac{\alpha_{matrix} - \alpha_{solvent}}{\alpha_{solvent}}\right) x100\%$
[**1**]	15019	13928	7.83%
[**2**]	23716	24711	4.03%
[**3**]	15773	14926	5.67%
[**4**]	0.9698	1.0956	11.5%
[**5**]	15100	12885	17.2%
[**6**]/[**7**]	14516	12220	18.8%
[**8**]/[**9**]	42318	32278	30.9%
[**10**]/[**11**]	21464	16018	34.0%

3.5.3 Repeatability and reproducibility

Accuracy and precision were assessed by overall mean, relative standard deviation (RSD%) and relative error (Error%) of independent quality controls samples (QC). In this method the quality control levels were defined as: low quality control (3 fold the concentration of lowest point of the calibration curve (CC)), medium quality control (75% of the concentration of the highest point of CC) and high (90% of the concentration of the highest point of CC).

Method repeatability was assessed by accuracy and precision of two batches of samples prepared by the same analyst in two different days. Up to now, the method repeatability was measured only in intra-laboratory experiments. It was calculated by accuracy and precision within four batches prepared in two different days by two analysts working at Laboratório Micromolecular de Microbiologia.

Each batch was composed by one calibration curve (CC) and 5 samples of each quality control level (LQC, MQC and HQC). The inter-laboratory reproducibility will be assessed comparing these previous results of accuracy and precision with those obtained from batches prepared and analysed at Separare-Núcleo de Pesquisa em Chromatografia.

According to international guidelines, a method based on HPLC-ESI-MS/MS is accurate and precise if the accuracy and precision is below 20% for low quality control level, and 15% for medium and high quality control levels. Giving the results showed in Table 3.4, this method is suitable for quantification of oxylipins in *A. brasiliana* tissues, since relative errors and standard deviations are below 15% for all compounds in both repeatability and intra-lab reproducibility assays.

Table 3.4 Repeatability and reproducibility for quantification of oxylipin in *A. brasiliana* plant tissu

Compound	Nominal conc. (ug/uL FW)*	Within-run n=5			Repeatability n=10			Intra-lab Reproducibility n=20		
		Mean ± SD	RSD (%)	Error (%)	Mean ± SD	RSD (%)	Error (%)	Mean ± SD	RSD (%)	Error (%)
[**1**]	0.075	0.067 ± 0.004	6.08	-10.40	0.073 ± 0.010	12.8	-2.53	0.075 ± 0.009	12.5	-0.15
	3.000	2.963 ± 0.231	7.79	-1.23	2.880 ± 0.212	7.08	-4.01	2.858 ± 0.241	8.05	-4.73
	4.500	4.297 ± 0.121	2.88	-6.44	4.092 ± 0.210	4.68	-0.07	4.246 ± 0.398	8.85	-5.65
[**2**]	0.075	0.075 ± 0.008	10.32	0.27	0.078 ± 0.009	11.8	3.33	0.079 ± 0.009	11.5	4.96
	3.000	2.868 ± 0.171	5.95	-4.39	2.826 ± 0.157	5.25	-5.81	2.814 ± 0.147	4.89	-6.22
	4.500	4.105 ± 0.213	5.20	-8.77	4.153 ± 0.102	4.25	-7.71	4.120 ± 0.249	5.54	-8.43
[**3**]	0.075	0.074 ± 0.008	10.29	-1.33	0.069 ± 0.004	5.93	-7.60	0.072 ± 0.006	8.50	-4.39
	3.000	3.162 ± 0.169	5.34	5.41	2.978 ± 0.249	8.29	-0.73	2.946 ± 0.249	8.32	-1.80
	4.500	4.781 ± 0.084	1.75	-6.24	4.425 ± 0.410	9.10	-1.66	4.430 ± 0.456	10.1	-1.56
[**4**]	0.075	0.075 ± 0.013	8.36	0.00	0.075 ± 0.006	7.73	0.27	0.077 ± 0.008	10.43	2.77
	3.000	2.759 ± 0.196	7.10	-8.02	2.812 ± 0.238	7.94	-6.25	2.872 ± 0.259	8.62	-4.28
	4.500	4.134 ± 0.356	8.62	-8.14	4.108 ± 0.468	10.4	-8.71	4.429 ± 0.511	11.3	-1.58
[**5**]	0.075	0.067 ± 0.005	7.00	-11.33	0.067 ± 0.004	5.43	-10.81	0.069 ± 0.007	9.77	-7.42
	3.000	2.989 ± 0.212	7.08	-0.37	2.969 ± 0.221	7.35	-1.05	2.880 ± 0.197	6.57	-4.02
	4.500	4.628 ± 0.145	3.13	-2.84	4.414 ± 0.253	5.63	-1.80	4.272 ± 0.273	6.07	-5.08
[**6**]/[**7**]	0.075	0.071 ± 0.005	6.40	-5.33	0.071 ± 0.005	6.56	-5.00	0.075 ± 0.007	9.31	-0.08
	3.000	2.717 ± 0.160	5.88	-9.43	2.75 ± 0.150	5.01	-8.33	2.732 ± 0.007	0.23	-8.94
	4.500	3.959 ± 0.112	2.84	-12.03	3.958 ± 0.152	3.37	-12.04	4.011 ± 0.185	4.10	-10.88
[**8**]/[**9**]	0.075	0.072 ± 0.006	8.06	-4.44	0.073 ± 0.004	15.44	-2.22	0.075 ± 0.010	13.2	-0.33
	3.000	2.800 ± 0.251	8.97	-6.65	2.732 ± 0.213	7.11	-8.92	2.826 ± 0.206	6.86	-5.81
	4.500	4.112 ± 0.153	3.71	-8.62	3.999 ± 0.228	5.06	-11.13	4.150 ± 0.233	5.17	-7.77

3.5.4 Robustness

Robustness is capacity of an analytical method to remain unaffected by small variations in the method parameters. It can be measured by comparison of the concentration of quality controls samples analyzed under two different conditions.

Here, we have evaluated the method robustness changing acetonitrile by methanol in the chromatographic method. So, a batch of sample (calibration curve and quality controls) was prepared and analyzed using both methods (standard and modified). After the quantification, the overall means of each quality control level obtained in each method were compared. The correlations between these means are shown in Table 3.5.

Table 3.5: Evaluation of method robustness

Compound	Correlation between the concentration of quality conbtrols		
	LQC	MQC	HCQ
[**1**]	7.70%	6.60%	8.60%
[**2**]	-3.80%	-2.20%	2.70%
[**3**]	0.50%	-13.4%	-14.5%%
[**4**]	8.30%	-9.2%	-11.3%
[**5**]	9.50%	-8.50%	-12.7%%
[**6**]/[**7**]	3.90%	10.5%	13.4%
[**8**]/[**9**]	-4.40%	-10.7%	-8.00%
[**10**]/[**11**]	2.20%	6.60%	12.1%

As recommended by the guidelines, a method is considered robust if the deviations in the quality controls are below 15 %. According to the results presented in Table 3.5, this method is robust considering the change in the chromatographic mobile phase from acetonitrile to methanol

3.6 Validation using internal standards

Although all recommendations were fulfilled and the method was considered precise, accurate and robust for quantification of oxylipins in *A. brasiliana* tissues, some points of the calibration curves and quality control had to be excluded during the validation, because they were considered outliers (points with error or RSD bigger than 20% for LQC level or 15% for other samples). Usually, these outliers are consequence of mistakes made during the sample preparation (i.e. pipetting).

Some guidelines allow exclusion of outliers. EMEA recommends that the back calculated values of calibration points should be within 20% of the nominal value for at least 75% of the calibration standards. The FDA guideline, allows the exclusion of up to 2 points of the calibration curve, since these points are not the lowest and highest concentrations. These two guidelines do not mention exclusion of outliers in quality controls, thought. According to the Guidance on Pesticide Residues Analytical Methods of the European Commission, it is possible to exclude up to 20% of the quality controls samples for each level (REF).

During the validation all these recommendations were accomplished. However, we also evaluated whether or not a method using internal standards could contribute to decrease the number of excluded point during the validation. Since, pipetting is one of the most common mistake made during quantification, the addition of a IS would be helpful to correct them.

Due to the lake of standards, the addition of internal standards was evaluated only for compound [5], [6]/[7] and [8]/[9]. The linear fitting, R2 and weighting used during these evaluations are shown in Table 3.6.

Table 3.6 Linear fitting, weighting and correlation coefficient for oxylipins calibration curves using internal standard method.

Compound	Calibration curve	Weighting	R^2
13-HODE	Y= 0.488678 + 0.00714018*X	1/X^2	0.9869
9-HODE	Y= 0.59446 + 0.0188614*X	1/X^2	0.9855
13-Oxo	Y= 0.116593 + 0.00289745*X	1/X	0.9970

The contribution of internal standard was evaluated by accuracy and precision during repeatability and reproducibility studies. As can be seen in Table 3.7, similarly to what happened for the method without IS, all RSD and errors are below 15%, proving that this is an accurate and precise method.

Table 3.7: Validation parameters for quantification of oxylipins in *A. brasiliana* using IS method. (**n** means the number of samples for each QC level)

Comp.	**Quality control**		**Repeatability 01 n=10**			**Reproducibility n=15**		
	Level	**Conc.**	**Mean**	**RSD %**	**Error %**	**Mean**	**RSD %**	**Error %**
[5]	**LQC**	**0.075**	0.084	12.44	10.51	0.081	7.76	4.67
	MQC	**3.000**	3.060	1.99	2.72	3.027	0.89	1.55
	HQC	**4.500**	4.718	4.85	2.57	4.866	7.52	4.24
[6]/[7]	**LQC**	**0.075**	0.083	10.00	8.35	0.081	8.03	2.52
	MQC	**3.000**	2.774	-7.52	5.34	2.905	-3.25	6.60
	HQC	**4.500**	4.173	-7.28	0.88	4.639	2.99	14.29
[8]/[9]	**LQC**	**0.075**	0.078	4.44	13.4	0.077	2.36	2.19
	MQC	**3.000**	2.814	-6.19	3.64	2.975	-0.85	7.83
	HQC	**4.500**	4.153	-7.72	0.80	4.521	0.48	11.62

Moreover, to compare both methods (with and without addition of IS) we analysed the residues of each quality control sample (LQC, MQC and HQC). These residues are the errors of each sample, comparing the back-calculated and nominal concentrations of each oxylipin. The concentration of the oxylipins in the samples was calculated in three different ways:

a) **Area**: QC samples were quantified based on a calibration curve draw correlating the different amount of oxylipins and the analyser response area.

b) **Ratio**: QC samples were quantified based on a calibration curve correlating the different amount of oxylipins with the ratio standard/internal standard. In this case none weighting factor was applied to the calibration curve

c) **Ratio/weight**: QC samples were also quantified based on calibration curve correlating the different amount of oxylipins with the ratio standard/internal standard. However in this case a weighting factor was applied to the calibration curve

In order to evaluate whether or not internal standards contribute to reduce the outliers during the quantification of oxylipins, we compared the number of CQ samples presenting errors out of the limits established by the guidelines (20% for LCQ and 15 % for MCQ and HQC). The samples were quantified using the three methods discussed above: area, ratio and ratio-weight. The outlier samples are represented by pink symbols in, Figure 3.9. Although the number of outliers does not vary much for compounds 3 and 06/07 (changes from 0 up to 7.5% of the total number of samples), for compounds 8/9, the addition of IS reduces the number of outliers from 20 to 7%, and it goes down to 5% when a weight of 1/x is applied to the calibration curve. These results suggest that the IS can be very helpful to reduce the number of outliers during the quantification of some oxylipins. Now, we intend to evaluate the influence of IS for all oxylipins that are quantified using this method. However, we still need to isolate more standards to do these measurements.

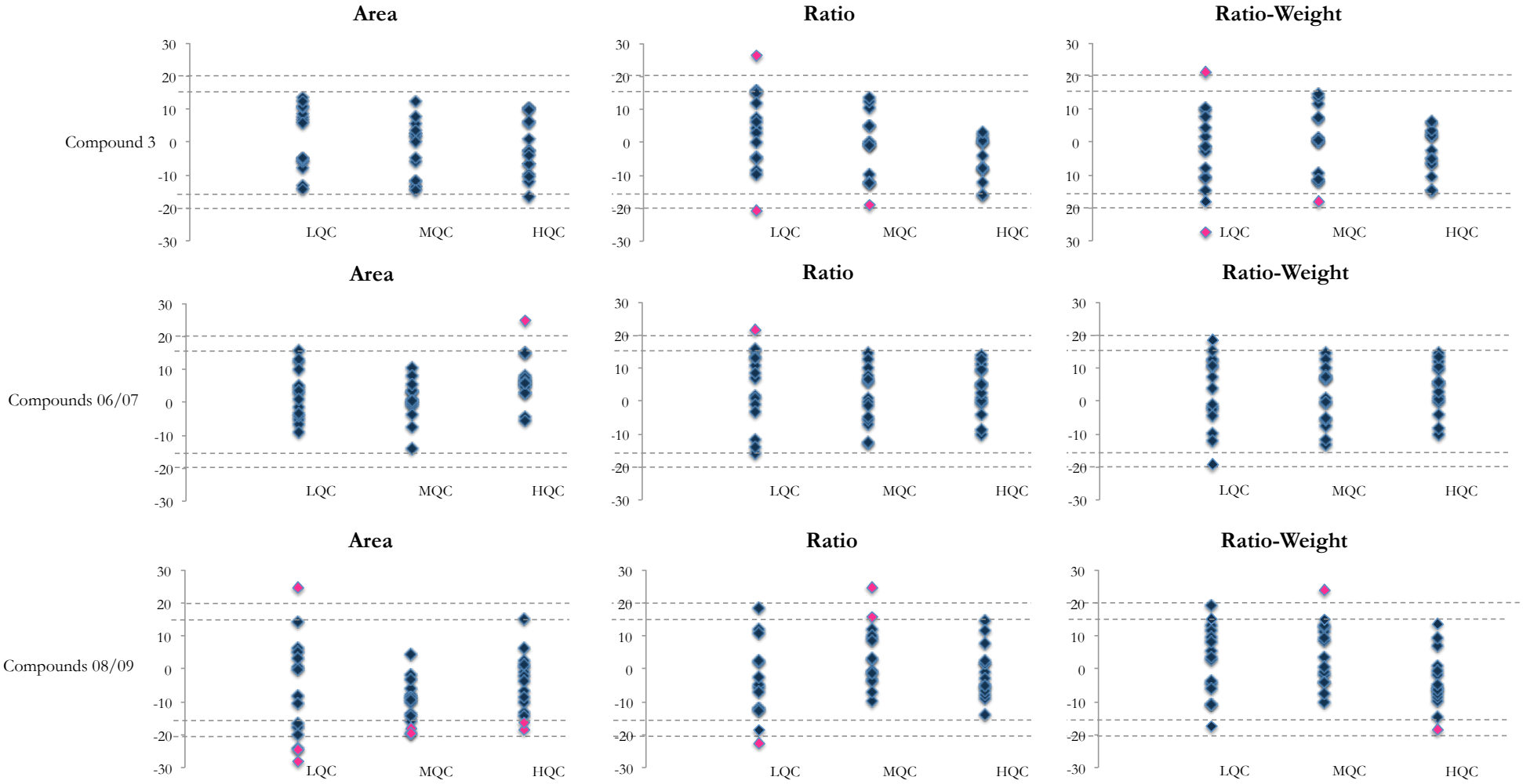

Figure 3.9: Residual plot of quality control samples using distinct analytical and mathematic approaches

3.1 Quantification of oxylipins in Algae

In order to evaluate whether or not the method used for quantification of oxylipins in *A. brasiliana* was also suitable for quantification of oxylipins in algae, we extracted and analysed 3 samples of two types of algae (*Selenastrum bibraianum* and *Ankistrodesmus densus*) using the same protocol that has been used to extract *A. brasiliana* tissues. These analyses were used to determine if both limit of detection and limit of quantification were sufficient to detect oxylipins in algae.

Since both area (Figure 3.10) and signal/noise (Figure 3.11) for all oxylipins present algae are larger than those present in *A. brasiliana*, one can conclude that this method is suitable to detect and quantify oxylipins in both algae. The SRM chromatograms for oxylipins present in diatoms are shown in the Figure S3 (supporting information).

To ensure that this method cannot only quantify oxylipins in algae, but can also do this in a precise and accurate way, we will evaluate the method linearity, recovery and matrix effect. It is going to be done as soon as we finish the isolation of oxylipins standards.

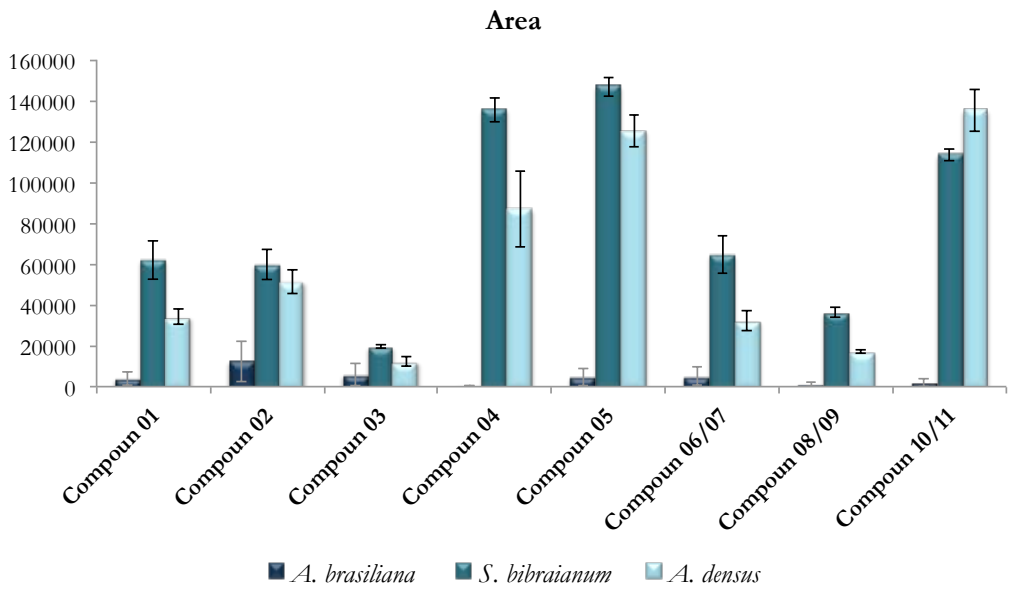

Figure 3.10 Comparison of the area for each oxylipin in *A. brasiliana* and algae.

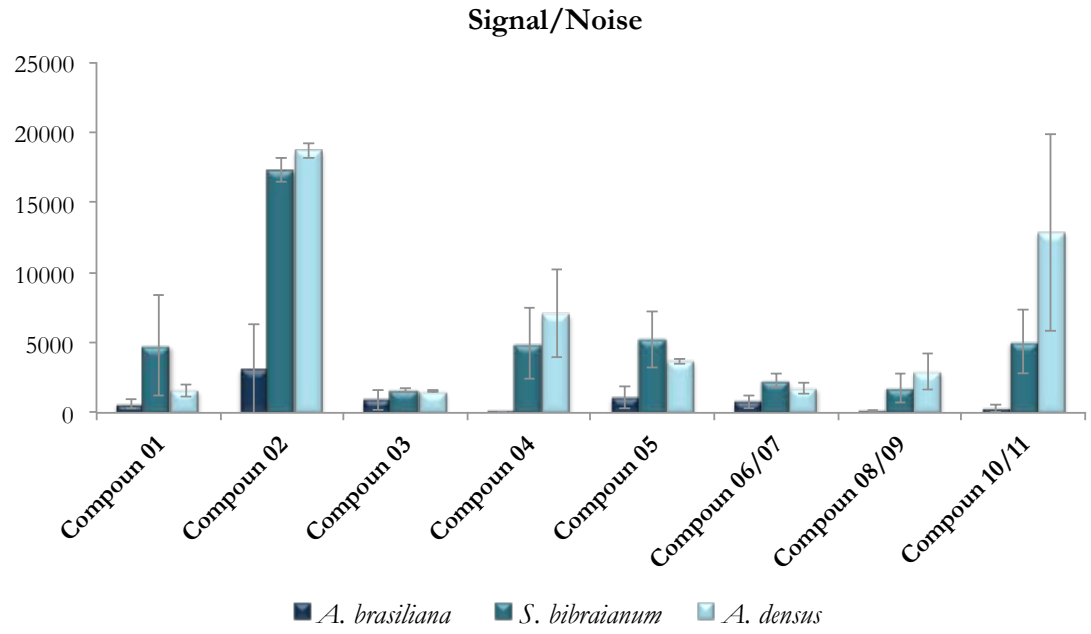

Figure 3.11 Comparison of the signal/noise for each oxylipin in *A. brasiliana* and algae.

3.2 Quantification of oxylipins in microorganisms

Microorganisms can be cultured in both liquid and solid media. In this method the oxylipins will be quantified using Czapeck, a common liquid culture medium. Since the liquid culture medium is very diverse from plant tissue, the optimization of the oxylipins extraction was the first parameter to be evaluated during the method development. Lyophilisation/resuspension and liquid-liquid partition were tested to recover the oxylipins from Czapeck medium:, as described in 2.8.

Figure 3.12 brings the mean area for each oxylipin obtained in each extraction procedure. Although the partition with chloroform presents a high standard deviation (probably, due to pipetting mistakes, once non IS was used during this protocol) it was the chosen one for extraction, since it presents the higher recovery for almost all oxylipins.

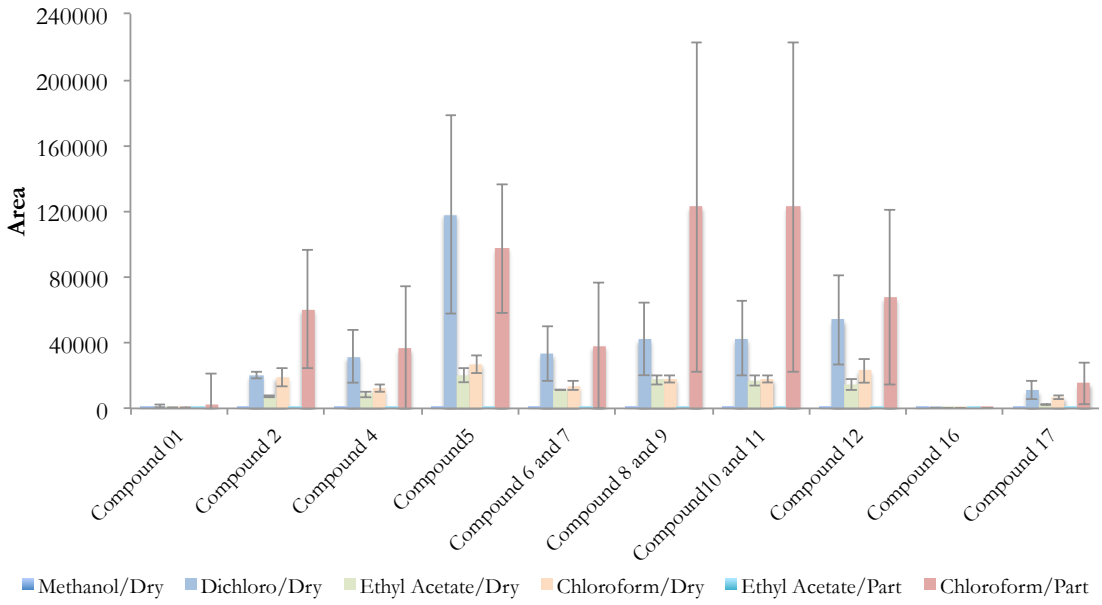

Figure 3.12 Optimization of oxylipin extraction from culture liquid medium

As discussed before, some of the oxylipins detected in A. brasiliana were also reported as biotransformation products. Since we intend to evaluate the ability of endophytes to consume ordinary fatty acids and turn them into oxylipins, quantification of ordinary fatty acids (oleic, linoleic and linolenic) will also be considered in this part of the method development. The extraction of ordinary fatty acids from the liquid culture medium is summarized in Figure 3.13, Although extraction using lyophilisation/resuspension with dichloromethane (Dichloro/Dry) presents the best oxylipin recovery, during this method these fatty acids will be extracted by partition with chloroform, so the same method can be used for extraction of fatty acids and oxylipins. Once we get the oxylipins standards, all the validation parameters will be evaluated for quantification of oxylipins in microorganisms.

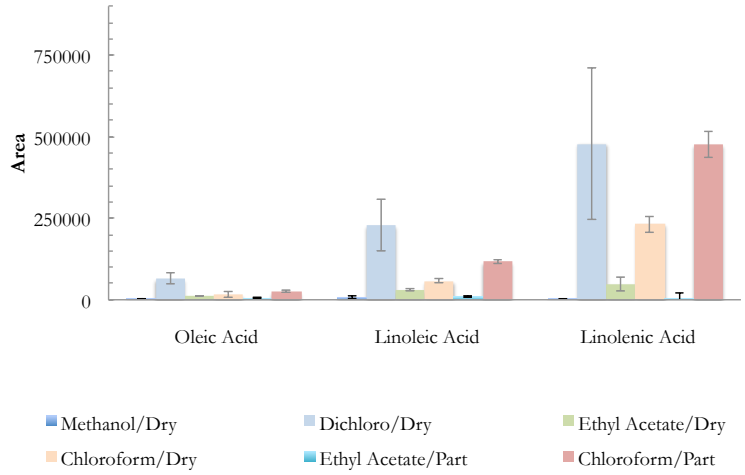

Figure 3.13 Optimization of fatty acid extraction from culture liquid medium

4. Final Considerations

Oxylipins constitute an important cross-kingdom signalling molecule; here we presented the first validated method for quantification of 11 oxylipins in plant tissues, which is also suitable for detection of these compounds in microorganisms and algae. Further experiments will be done to confirm if this method can also quantify accurate and precisely these compounds in algae and microorganism. Moreover, we have shown that the addition of internal standards is useful, but not indispensable, for quantification of oxylipins in *A. brasiliana* tissues.

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Supporting information

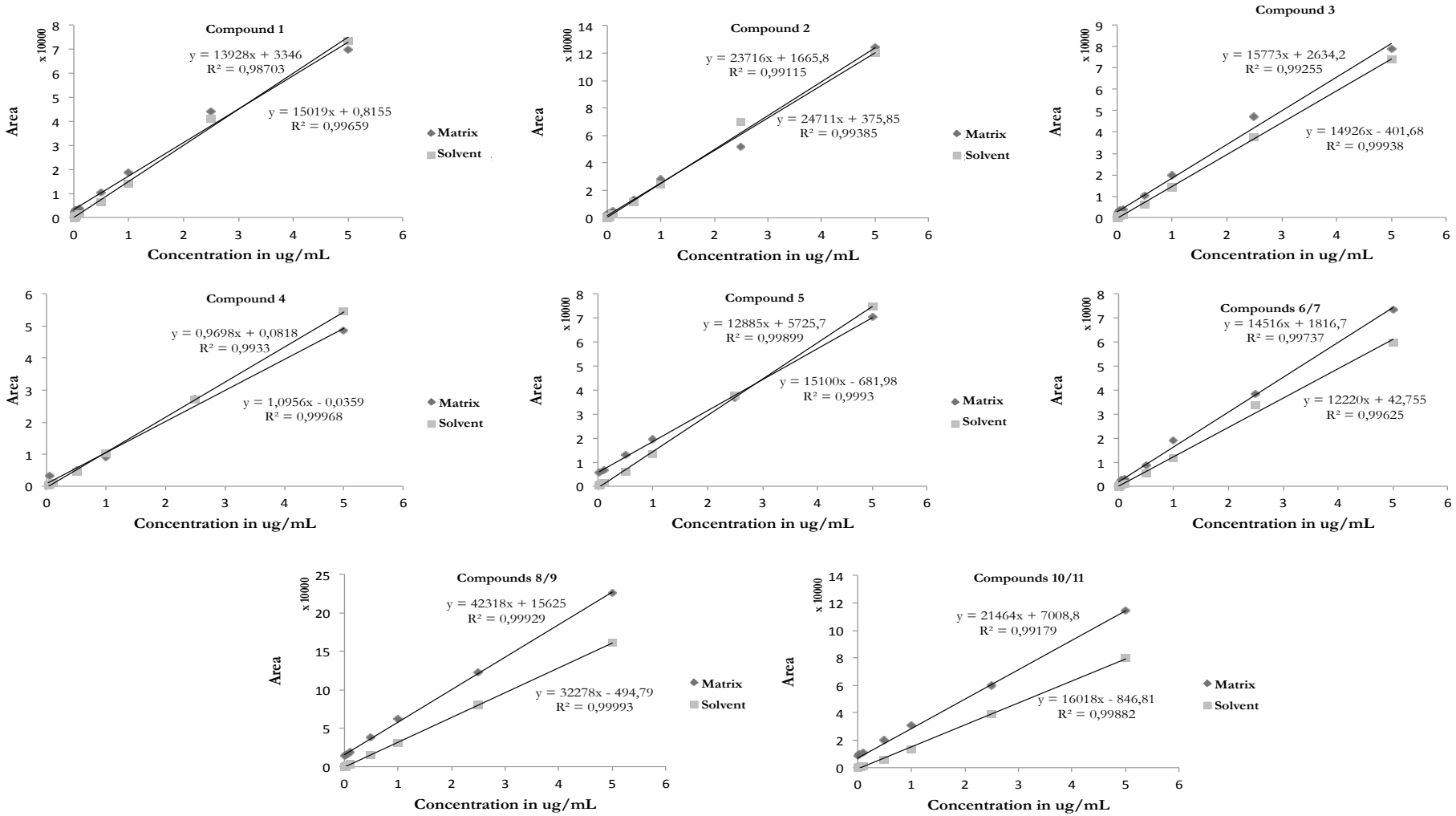

Figure S1: Comparison between calibrations curve for each oxylipins prepared in matrix and in solvent.

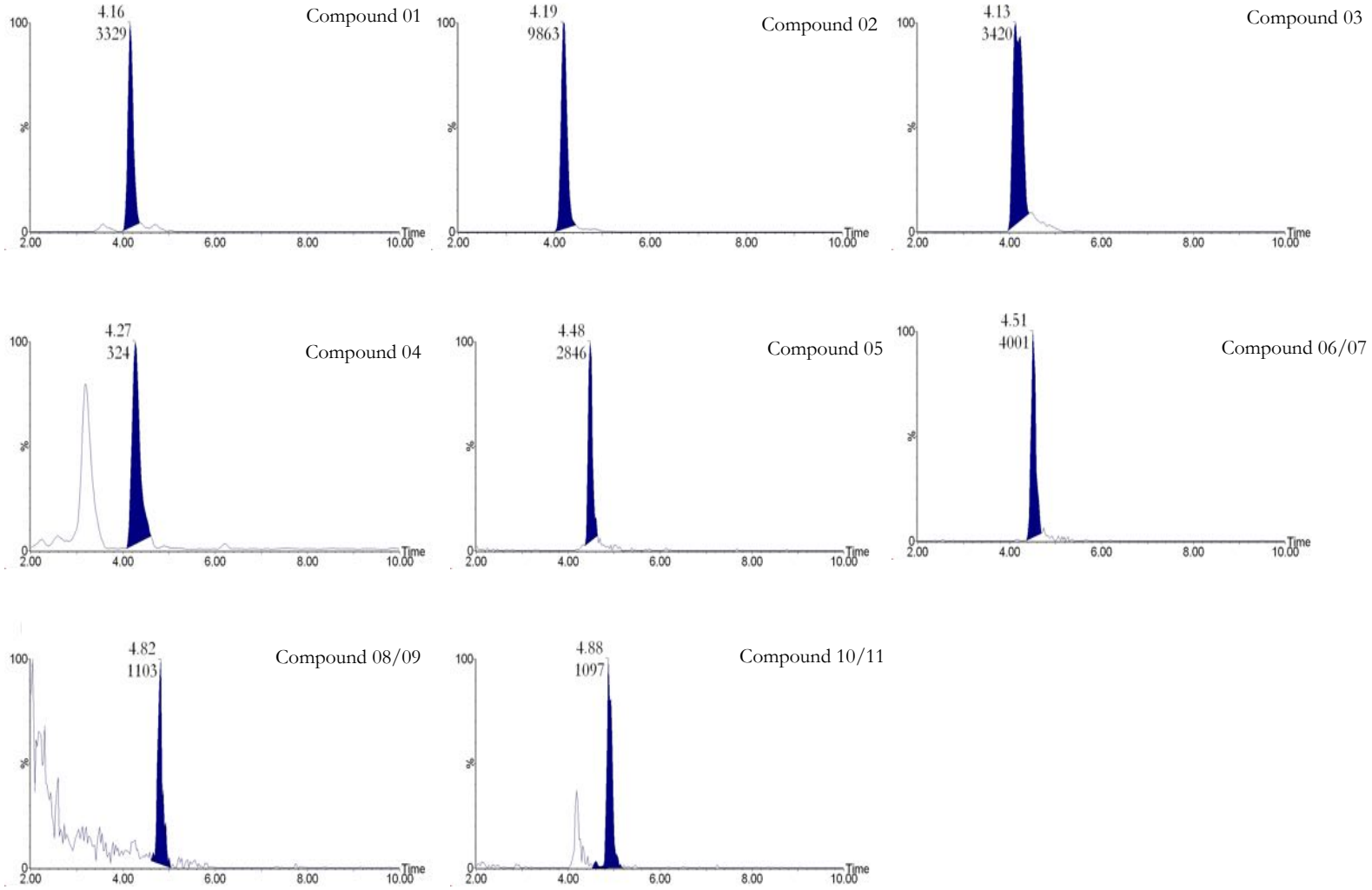

Figure S2: SRM chromatograms for oxylipins present in *A. brasiliana*.

3.3 Quantification of phytohormones in plant tissues

As discussed at the beginning of this chapter, jasmonates constitute some of the most well known plant oxylipins. Moreover, it seems that there is a link between the oxylipins and the activation of some phytohormones biosynthesis.[75] So, here we present a paper about the development and validation of a HPLC-MS/MS method for quantification of some jasmonates and other phytohormones (salicylic acid, abscisic acid, indole acetic acid, 12-oxophytodienoic acid and jasmonic acid-isoleucine conjugate) in plant tissues. This work was carried out during an internship at Max Planck Institute for Chemical Ecology.

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Validated method for phytohormone quantification in plants

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Phytohormones are long time known as important components of signaling cascades in plant development and plant responses to various abiotic and biotic challenges. Quantifications of phytohormone levels in plants are typically carried out using GC or LC-MS/MS systems, due to their high sensitivity, specificity, and the fact that not much sample preparation is needed. However, mass spectrometer-based analyses are often affected by the particular sample type (different matrices), extraction procedure, and experimental setups, i.e., the chromatographic separation system and/or mass spectrometer analyser (Triple-quadrupole, Iontrap, TOF, Orbitrap). For these reasons, a validated method is required in order to enable comparison of data that are generated in different laboratories, under different experimental set-ups, and in different matrices. So far, many phytohormone quantification studies were done using either QTRAP or Triple-quadrupole mass spectrometers. None of them was performed under the regime of a fully-validated method. Therefore, we developed and established such validated method for quantification of stress-related phytohormones such as jasmonates, abscisic acid, salicylic acid, IAA, in the model plant *Arabidopsis thaliana* and the fruit crop *Citrus sinensis*, using an Iontrap mass spectrometer. All parameters recommended by FDA (US Food and Drug Administration) or EMEA (European Medicines Evaluation Agency) for validation of analytical methods were evaluated: sensitivity, selectivity, repeatability and reproducibility (accuracy and precision).

Keywords: phytohormones, HPLC-MS/MS, quantification, *Arabidopsis thaliana*, *Citrus sinensis*, iontrap

INTRODUCTION

Phytohormones constitute a distinct class of signaling molecules in plants. They can be classified according to their chemical structure—jasmonates [jasmonic acid (JA) and derivatives 12-oxo-phytodienoic acid (OPDA)], auxins (in particular indole-3-acetic acid, IAA), cytokinins, gibberellins, abscisic acid (ABA), salicylic acid (SA), brassinosteroids, ethylene—or according to their biological function—regulator of plant growth, development and reproduction or mediators during biotic and abiotic stresses (Santner and Estelle, 2009).

Frequently these molecules act at low concentrations and play key roles in ecological interactions between plants and other organisms (Pozo et al., 2005; Pieterse et al., 2009; Santner et al., 2009) Therefore, quantification of phytohormones is an essential step to understand their functions in plant metabolism and ecological interactions. While the first highly sensitive methods for quantitative phytohormone analyses relied on immunoassays (Weiler, 1984), in the last 15–20 years many methods have been developed for quantification of these compounds, particularly using hyphenated techniques such as GC-MS (Kowalczyk and Sandberg, 2001; Müller et al., 2002, 2006; Engelberth et al., 2003) and LC-MS (Wilbert et al., 1998; Forcat et al., 2008; Pan et al., 2008, 2010; Müller and Munné-Bosch, 2011; Balcke et al., 2012; Liu et al., 2012).

These techniques provide a powerful analytical tool for quantifying secondary metabolites in plant tissues, especially due to their high sensitivity, specificity and reproducibility. However, different approaches might be adopted depending on the separation method (GC or HPLC) and the spectrometer (triple quadrupole, iontrap, TOF) applied during the quantification studies. Moreover, mass spectrometry analyses are strongly influenced by other compounds present in the plant tissues which can suppress or increase the analyte ionization, a fact that is often not considered. Hence, the matrix effect and several other parameters (like analyte stability and recovery) must be controlled during a quantification study and validation strategies should be employed in order to produce reliable analytical methods for quantification of plant metabolites.

Several papers and reviews covering validation of analytical methods have been published (Shabir, 2003; Bliesner, 2006; Chandran and Singh, 2007). As a rule, these papers describe important parameters such as accuracy, precision (repeatability and intermediate precision), specificity, detection and quantification limits, linearity, range, robustness, etc. All this set of

information should be obtained in the same laboratory as a part of repeatability assays. However, for proceeding with reproducibly assays an inter-laboratory experiment is often necessary. Collaborative trials are used to test the performance (generally the precision) of the analytical method demonstrating that it can be used in more than one laboratory, producing reliable and true results (Hund et al., 2000).

In this present paper we describe the development and inter-laboratory validation of an analytical method for quantification of six phytohormones—the auxin indole-3-acetic acid (IAA), ABA, JA, isoleucine jasmonic acid conjugate (JA-Ile), SA, and 12-oxo phytodienoic acid (OPDA)- in *Arabidopsis thaliana* and *Citrus sinensis* using an iontrap mass spectrometer.

MATERIALS AND METHODS

REAGENTS AND STANDARDS

All solvents used during extraction procedures were analytical grade except for methanol (MeOH). Chromatographic separation was carried out using MeOH HPLC grade purchased from Roth (Carl Roth GmbH, Germany) or J. T. Baker (Xalostoc, Mexico). IAA (purity > 99%), ABA (purity > 99%) and SA (>98%) were purchased from Sigma-Aldrich. 12-oxo phytodienoic acid were purchased from Cayman (Biomol GmbH, Hamburg, Germany). JA was synthesized by saponification of commercially available methyl-JA. Jasmonic acid isoleucine conjugate (JA-Ile) was synthetized according to Kramell et al. (1988). Deuterated standards: [^{2}H5] indole-3-acetic acid (d5-IAA), [^{2}H4] salicylic acid (d4-SA) and [^{2}H6] (+)-cis, trans-abscisic acid (d6-ABA) were purchased from OlChemIm Ltd (Olomouc, Czech Republic) and jasmonic-d5 acid 2,4,4-d3 acetyl-2,2-d2 (d5-JA) was purchased from CDN isotopes (Quebec, QC, Canada).

APPARATUS

HPLC-MS/MS analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) connected to a LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was carried out in a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%, v/v) and MeOH with 0.05% (v/v) of formic acid were employed as mobile phases A and B, respectively. The elution profile was: 0–10 min, 42–55% B in A; 10–13 min, 55–100% B; 13–15 min 100% B; 15–15.1 min 100–42% B in A; and 15.1–20 min 42% B in A. The mobile phase flow rate was 1.1 mL/min. Injection volume was 25 μL. The LTQ mass spectrometer was equipped with an Electrospray ionization source, operating in the negative and positive ion modes. Negative measurements were carried out using the following ionization parameters: source voltage: 4.4 kV, capillary voltage: −48 V, tube lens −113 V, declustering potential 10 V, turbo gas temperature: 300°C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min. For positive analyses ionization parameters were set at: source voltage: 4.2 kV, capillary voltage: 29 V, tube lens 45 V, declustering potential 10 V, turbo gas temperature: 300°C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min.

Selected reaction monitoring (SRM) experiments were used to monitor specific precursor ion → product ion transitions for each phytohormone and internal standard. Collision energy, precursor ion isolation width and activation Q were optimized for each compound separately.

During the inter-laboratory reproducibility, the analyses were performed in an Acquity HPLC (Waters Co.) coupled with Quattro Premier XE (Micromass Technology) mass spectrometer, using a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) and the same elution conditions mentioned above. The ionization parameters used during these analyses were: In negative mode (capillary: 3.4 kV, extractor 3 V, source temperature 110°C, desolvation temperature 350°C, desolvation gas flow: 800 L/h, cone gas flow: 10 L/h), and in positive mode (capillary: 3.4 kV, extractor 3V, source temperature 110°C, desolvation temperature 350°C, desolvation gas flow: 800 L/h, cone gas flow: 10 L/h). Cone voltage and collision energy were optimized for each compound individually.

PLANT MATERIAL

A. thaliana was cultured for 4 weeks under short day conditions (10 h light/14 h dark photoperiod), 40% humidity and 23°C. After harvesting, plants were immediately frozen in liquid nitrogen and ground in a GenoGrinder (SPEXSample Prep, München, Germany) for 2 × 30 s at 1500 rpm. After homogenization, 100 mg of plants were weighted into 1.5 mL tubes and stored at −80°C until the measurements.

C. sinensis was cultured in a greenhouse (Araraquara, Brazil) under normal light conditions and temperature average of 26°C (day) and 18°C (night). Light green leaves from small trees were collected, immediately frozen in liquid nitrogen and ground in a mortar. After homogenization, 100 mg of frozen plant material were weighted into 1.5 mL tubes and stored at −80°C until the measurements.

PHYTOHORMONES EXTRACTION AND ANALYSIS

Optimization of phytohormones extraction

Two parameters were evaluated during the optimization of phytohormones extraction: composition of extraction solution and type of plant samples (fresh or dry material). Initially tubes containing 100 ± 1 mg of plant material were either kept at −80°C or dried overnight in a freeze drier at −42°C. The extraction was performed adding 1.0 mL of either ethyl acetate, dichloromethane, isopropanol, MeOH or MeOH:water (8:2) into each tube containing dry or fresh plant material. Samples were shaken for 30 min in the Starlab shaker and centrifuged at 16,000 g and 4°C for 5 min. The supernatant was transferred into a new 1,5 micro-centrifuge tube and dried in speed vac. After drying, 100 μL of MeOH were added to each sample, homogenized under vortex and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was analyzed by HPLC-MS/MS.

In a second set of analyses, the influence of both MeOH:water ratio and addition of acid in the extraction mixture was evaluated. The extraction procedure was performed as described above using 3 different MeOH:water ratios (7:3, 6:4, and 1:1) pure, or containing 0.2% of HCl.

Preparation of standards solutions

Stock solutions of each original phytohormone standard were prepared at 1 mg/mL in MeOH. For deuterated

compounds, stock solutions were prepared in acetonitrile at 100 μg/mL.

Working solutions of original phytohormones standards were prepared diluting stock solutions in MeOH:water (7:3), at different concentration for each phytohormone depending on the range of the calibration curve: ABA and IAA (100 μg/mL), JA and SA (200 μg/mL), OPDA (50 μg/mL), and JA-Ile (40 μg/mL).

The internal standard stock solutions (d5-JA, d6-ABA, d4-SA, and d5-IAA) were combined and diluted (final concentration 10 ng/mL for d4-SA and d5-IAA and 20 ng/mL for d5-JA and d6-ABA) with MeOH:water (7:3) yielding the extraction solution.

Final method for phytohormones extraction

Tubes containing 100 mg of fresh and ground plant material were kept at −80°C, and transferred to liquid nitrogen before the extraction. The samples were removed from the liquid nitrogen and 1 mL of extraction solution containing the internal standards (d5-JA, d6-ABA, d5-IAA, and d4-SA), prepared as described in Preparation of Standards Solutions, were directly added. The samples were briefly mix with a vortex, and spiked with phytohormones standards as described in Method Validation to generate the calibration curve and quality control (QC) samples. The spiked samples were shaken for 30 min in the Starlab shaker and centrifuged at 16,000 g and 4°C for 5 min. The supernatant was transferred into a new 1,5 micro-centrifuge tube and dried in speed vac. After drying, 100 μL of MeOH were added to each sample, vortexed and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was analyzed by HPLC-MS/MS.

METHOD VALIDATION

Limit of detection and limit of quantification

The limits of detection (LOD) and quantification (LOQ) for analytical methods based on HPLC analysis can be expressed in response units (signal-to-noise levels). Usually LOD is established using matrix samples spiked with the low amount of standards. However, as none analyte-free matrix was available the LODs were determined in solvent as three times the noise level.

For each matrix, LOQs were defined according to the amount of phytohormones present in 10 independent blank samples, which were extracted as described in Optimization of Phytohormones Extraction. For all the LOQ the signal-to-noise ratios were higher than 10.

Calibration curve and linearity

The calibration curves were prepared in matrix using three different spiking solutions: spiking solution A containing ABA (at 4, 8, 40, 100, 200, 1000, 3000, and 4000 ng/mL), IAA (2, 4, 20, 50, 100, 500, 1000, 2000 ng/mL), and JA-Ile (0.8, 1.6, 8, 20, 40, 200, 400, and 800 ng/mL); spiking solution B containing SA (at 50, 100, 200, 500, 1000, 2000, 4000, and 8000 ng/mL) and JA (at 25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL); and spiking solution C containing OPDA (at 500, 1000, 2000, 4000, 6000, 7000, 8000, and 10,000 ng/mL). All spiking solutions were prepared (in MeOH:water, 7:3) by serial dilution of working solutions.

Samples for calibration curve were prepared adding 50 μL of each spiking solution (A, B, and C) into the tubes containing 100 mg of ground fresh plant material and extracted as described in Preparation of Standards Solutions. For a flow sheet see **Scheme 1** (Supporting Material).

Quality controls

QC were used to assess the method's accuracy and precision. QCs were prepared spiking plant material with three different levels of each phytohormone (low, medium and high; **Scheme 1**, Supporting Material).

High quality controls (HQC) were prepared spiking 100 mg of plant material with 50 μL of: high spiking solution A (containing 2800 ng/mL of ABA and IAA and 280 ng/mL of JA-Ile); high spiking solution B (containing 5600 ng/mL of SA and 2800 ng/mL JA) and high spiking solution C (containing 2800 ng/mL of OPDA). Medium quality controls (MQC) were prepared spiking 100 mg of plant material with 50 μL of: medium spiking solution A (containing 700 ng/mL of ABA and IAA and 140 ng/mL of JA-Ile); medium spiking solution B (containing 2800 ng/mL of SA and 1400 ng/mL of JA) and medium spiking solution C (containing 1400 ng/mL of OPDA). And low quality controls (LQC) were prepared spiking 100 mg of plant material with 50 μL of: low spiking solution A (containing 14 ng/mL of ABA and IAA, and 2.8 ng/mL of JA-Ile); low spiking solution B (containing 280 ng/mL of SA and 140 ng/mL of JA) and low spiking solution C (containing 450 ng/mL of OPDA). All QC were prepared in quintuplicates.

Recovery

Recovery was calculated comparing the amount of each phytohormone present in spiked/extracted and extracted/spiked QC. The spiked/extracted QC were prepared as described in Quality Controls. The extracted/spiked samples were spiked with 150 μL of MeOH:water (7:3)—simulating the addition of spiking solutions—and extracted as described in Preparation of Standards Solutions. The dry residues were reconstituted in MeOH containing the final concentration of each phytohormone, which corresponds to half of spiking solution concentration.

Validation in Citrus sinensis

Linearity, reproducibility, recovery, and matrix effects were also evaluated for quantification of phytohormones in leaves of orange, *C. sinensis*. Initially, 10 samples were analyzed to establish the basal level of the six phytohormones in *C. sinensis* tissues. Due to the high content of IAA and ABA and low content of OPDA, the range of calibration curves and QC levels were adjusted to better fit to the new matrix.

The calibration curves were prepared in matrix using three different spiking solutions: spiking solution A contained ABA (at 4, 8, 40, 100, 200, 1000, 3000, and 4000 ng/mL), and JA-Ile (0.8, 1.6, 8, 20, 40, 200, 400, and 800 ng/mL); spiking solution B containing SA (at 50, 100, 200, 500, 1000, 2000, 4000, and 8000 ng/mL), JA (at 25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL) and IAA(25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL); and spiking solution C contained OPDA (at 60, 120, 240, 480, 640, 800, 1000, and 1200 ng/mL). All spiking solutions were prepared (in MeOH:water, 7:3) by serial dilution of working solutions. Samples for calibration curves were prepared adding 50 μL of each spiking solution (A, B, and C) into the tubes containing 100 mg of ground fresh plant material and extracted as

described in Preparation of Standards Solutions. For a flow sheet see **Scheme 1** (Supporting Material).

HQC were prepared by spiking 100 mg of plant material with 50 μL of: high spiking solution A (containing 2800 ng/mL of ABA and 280 ng/mL of JA-Ile); high spiking solution B (containing 5600 ng/mL of SA and 2800 ng/mL JA and IAA) and high spiking solution C (containing 840 ng/mL of OPDA). MQC were prepared spiking 100 mg of plant material with 50 μL of: medium spiking solution A (containing 700 ng/mL of ABA and 140 ng/mL of JA-Ile); medium spiking solution B (containing 2800 ng/mL of SA and 1400 ng/mL of JA and IAA) and medium spiking solution C (containing 600 ng/mL of OPDA). And LQC were prepared spiking 100 mg of plant material with 50 μL of: low spiking solution A (containing 14 ng/mL of ABA and 2.8 ng/mL of JA-Ile); low spiking solution B (containing 280 ng/mL of SA and 140 ng/mL of JA and IAA) and low spiking solution C (containing 90 ng/mL of OPDA). All QC were prepared in quintuplicates.

Recovery of phytohormones in *C. sinensis* samples was evaluated for the QC samples as described in Recovery.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

Optimization of ion trap parameters for quantification of phytohormones

Due to their high sensitivity, specificity, and the fact that not much sample preparation is necessary, HPLC-MS/MS experiments, especially those involving SRM, are used as reference for quantitative analyses. These also include phytohormone quantifications.

SRM experiments are based on two stages of ion selection. The precursor ion (a protonated or deprotonated target molecule) is selected in the first stage of tandem mass spectrometer, fragmented under a controlled process, thereby generating a specific fragment ion, which is then selected in the second stage of tandem mass spectrometer. Hence, the specificity of SRM experiments relies upon the choice of a specific precursor-fragment ion transition, while the sensitivity depends on the yield and stability of both precursor and fragment ions (Kowalczyk and Sandberg, 2001). Moreover, selection of precursor and fragment ions as well as fragmentation mechanism occurs in different ways for distinct mass spectrometers (triple quadrupole, ion trap, time of flight). Therefore, different approaches and parameters optimization are needed depending on which kind of detector is used in the SRM experiments.

Quantification of phytohormones in plant tissues has been so far carried out using either triple quadrupole or Q-trap instruments (Forcat et al., 2008; Pan et al., 2008, 2010; Balcke et al., 2012; Liu et al., 2012), which are well known for their high performance in SRM experiments (Rousu et al., 2010; Tanaka et al., 2011) Ion trap mass spectrometers, on the other hand, are widely available due to their high versatility, capability of doing MS^n, and for its low cost compared with triple quadrupole, which make it an attractive option for compound identification, screening and qualitative analyses. However, they present specific challenges for quantification experiments, since the scan speed and fragmentation mode do not fit the best with SRM experiments. Therefore, many parameters must be carefully adjusted in order to reach good sensitivity in ion trap mass spectrometers, specially the injection time and activation Q (Evans et al., 2000).

During the present work all parameters for ionization, fragmentation and detection of phytohormones (ABA, IAA, SA, JA, JA-Ile, and OPDA) were optimized, in order to achieve good sensitivity and selectivity in an ion trap mass spectrometer. The values of precursor ion isolation width (ISO), collision energy (CID) and activation Q (Act Q) that presented the best sensitivity and the more stable signals for each phytohormone are shown in **Table 1**. Activation Q must be adjusted before choosing the product ion, since it determines the range of product ions that can be generated. Modification in the default value (0.250 for the equipment used in this work) can provide new fragment ions, which can be interesting for quantification (stable and with high intensity). Injection time for all SRM transitions was 100 ms.

Optimization of phytohormones extraction

The efficiency of phytohormones' extraction was evaluated for both dry and fresh plant material using different organic solvents/mixtures [acetate, dichloromethane, isopropanol, MeOH, MeOH:water (8:2), MeOH:water (7:3), MeOH:water (6:4), MeOH:water (1:1)]. The influence of acidification by hydrogen chloride in the phytohormone extraction was also tested. The results are presented in Figure A (Supplementary Material). When the extraction is performed using non-polar organic solvents (ethyl acetate and dichloromethane) there is a clear difference in the extraction efficiency between fresh and dry material. However, for polar and aqueous mixtures such difference decreased drastically. Mixtures of MeOH and water provided higher extraction efficiency for all phytohormones. Here, the ratio of 7:3 was chosen as extraction solution due to its good performance in extracting the phytohormones and the low content of chlorophyll present in the final sample.

During the evaluation of method repeatability, the concentration of OPDA in the QC samples did not fit with the added amount. The concentration present in the QC was always higher than expected. After more detailed analyses it was observed that

Table 1 | Fragmentation parameters for the phytohormones.

	Precursor ion (m/z)	ISO* (Da)	CID** (V)	Act Q***	Fragments (Da)
ABA	263.0	2.0	30	0.250	152.0–154.0
d6-ABA	269.0	2.0	30	0.250	158.0–160.0
IAA	176.0	2.0	20	0.250	129.0–131.0
d5-IAA	181.0	2.0	20	0.250	134.0–136.0
JA	209.0	1.0	25	0.210	58.0–60.0
d5-JA	214.0	1.0	25	0.210	61.0–63.0
JA Ile	322.0	2.0	30	0.250	129.0–131.0
OPDA	291.0	2.0	18	0.250	164.0–166.0
SA	137.0	1.0	28	0.250	92.0–94.0
d4-SA	141.0	2.0	28	0.250	96.0–98.0

**Precursor ion isolation window; **Collision-induced dissociation energy; ***Activation Q.*

such issue occurred due to the increase of OPDA content in the plant samples during the sample preparation. Actually, all the samples were put on ice, spiked with internal standards and extracted by addition of extraction solution containing the internal standards. As the QC were prepared after the calibration curve samples, the increment in OPDA content in the QC was bigger than in the calibration curve samples. Therefore, the changes in the OPDA content in the plants samples were evaluated while QCs were kept on ice. For this purpose, 18 tubes containing 100 mg of fresh and ground plant tissues were transferred from liquid nitrogen onto ice. The OPDA concentration was evaluated for samples kept on ice for 0, 5, 10, 15, 30, and 45 min. For each point, three tubes containing plant material were removed from ice and added with 1.0 mL of extraction solution. The extraction was carried out as described in Final Method for Phytohormones Extraction. The graphs present in **Figure 1** shows the changes in OPDA content.

These data suggest that OPDA content varies quickly in the wounded/ground plant tissues even when the samples are kept on ice. After 5 min it increased by 50% and the amount doubled within the first 10 min. This might be due to remaining enzyme activities releasing lipid-bound OPDA from plastids localized galactolipids, which are well known for Arabidopsis (Stelmach et al., 2001). These results showed clearly the importance of keeping plant tissues frozen as long as possible, even during weighting and before adding the extraction solvents. Therefore, samples must be maintained at very low temperature (−80°C or liquid nitrogen) before the extraction, and the extraction solvent must be added immediately after removing the samples from such conditions.

These results also highlight the importance of the validation studies for quantification methods, since many parameters involved in the extraction and analysis cannot be proper addressed when statistical figures are not evaluated. In this way, the use of QC as defined in validation protocols can be of great value even during method development. For this reason, validation of each assay or test method should be performed on a case-by-case basis, to ensure that the parameters are appropriate for the method's intended use.

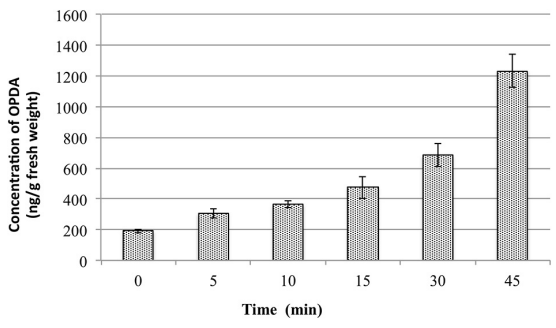

FIGURE 1 | Changes in OPDA content in *Arabidopsis thaliana* leaf samples depending on the time the samples were kept on ice.

VALIDATION OF ANALYTICAL METHOD

The validation studies are conducted in order to demonstrate that the analytical method is applicable for the aimed purpose and to ensure that the obtained values are close to the unknown content of the analyte present in real samples (EMEA, 2006; González and Herrador, 2007; European Commission, 2010). In this work, we evaluated the selectivity/specificity, limits of detection and quantification, linearity, recovery, repeatability and reproducibility of analytical method for quantification of phytohormones in *A. thaliana* and *C. sinensis* tissues.

Selectivity, limit of detection and quantification

Selectivity is defined as the ability of quantification method to discriminate the analyte from the other sample components, giving pure, symmetric and resolved peaks (Green, 1996). For methods which include chromatographic separation, selectivity can be assessed by chromatographic resolution, evaluating whether the peak relative to the analyte is separated from the other peaks present in the matrix. When no blank matrix is available, the selectivity can also be assessed comparing the MS/MS spectrum related to the analyte present in the matrix with the MS/MS spectrum of original standard. If there is no additional peaks MS/MS spectrum for the band correspondent to the analyte in the matrix comparing to MS/MS spectrum of original standards, it suggests that the method is selective.

Therefore, the present method is considered selective/specific for the phytohormones quantification, since the SRM chromatograms present in Figure B (Supplementary Material) contain either only one or well-resolved peaks for all phytohormones. For JA, JA-Ile, and OPDA the peaks are very symmetric and sharp (width less than 30 s). Although for IAA and SA the peaks are broader and not symmetric, the selectivity of the method was also confirmed by the very similar profile of MS/MS spectra related to these bands (**Figure 2**) and the original standards prepared in solvent (Figure B of Supplementary Material).

The limit of detection (LOD) is the lowest analyte concentration, which can be distinguished from the noise in blank samples [it is defined as a concentration with signal/noise (S/N) of 3]. When no analyte-free matrix is available, the detection limit can be calculated in solvent (LOD of the equipment) or by dilution of matrix until reaching an S/N of 3. Since the dilution of the matrix also reduces the matrix effect, thereby not presenting huge advantages compared with the measurements in solvent, in the present work the LODs were evaluated for the HPLC-MS/MS system and the values are shown in **Table 2**.

The limit of quantification (LOQ) is defined as the lowest analyte concentration, which can be quantified precisely and accurately. According to EMA and FDA it corresponds to the concentration of analyte, which yield a peak with S/N of 10. However, as can be seen in **Figure 2**, the amount of every phytohormone in the blank sample yield peaks with S/N of at least 30. Therefore, it is not possible to calculate the LOQ using the conventional definition. For this reason the LOQ for this method was established as the lowest point of the calibration curve (**Table 2**). The SRM chromatogram of this point for every phytohormone is shown in the Figure C (Supplementary Material).

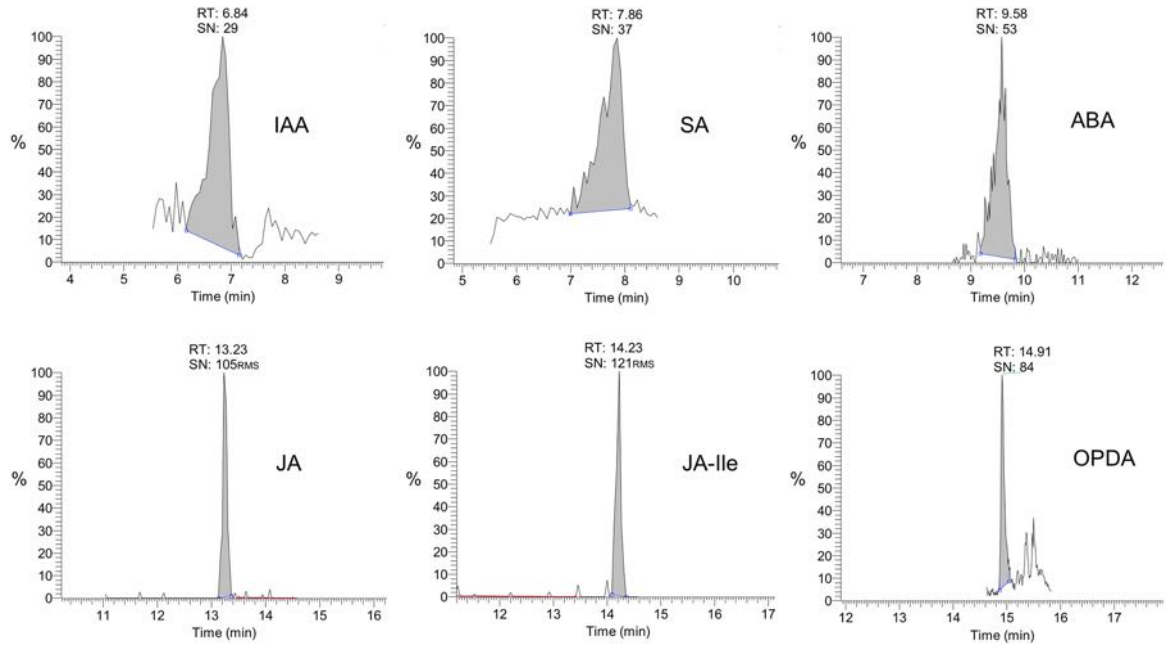

FIGURE 2 | Selected reaction monitoring (SRM) chromatograms for various phytohormones in *Arabidopsis thaliana* leaf extracts.

Table 2 | Parameters of calibration curve for each phytohormone: curve range, regression, weighting, correlation coefficient limit of quantification (LOQ) and amount of each phytohormone present in blank (untreated) *Arabidopsis thaliana* samples.

Analyte	Range (ng/g FW)	Curve*	R^2	LOQ (ng/g)	Amount in blank samples** (ng/g FW)	Matrix effect***
IAA	2–2000	Y = 0.0929916 + 0.0239565*X	0.992	2.0	7.13 ± 1.62	−31%
ABA	2–2000	Y = 0.0726676 + 0.0159863*X	0.998	2.0	5.32 ± 0.88	+11%
JA-Ile	0.4–400	Y = 0.146972 + 0.106572*X	0.993	0.4	1.64 ± 0.23	−25%
JA	12.5–2000	Y = 0.335095 + 0.00835023*X	0.997	12.5	41.32 ± 7.80	+7%
SA	25–4000	Y = 0.80327 + 0.00608683*X	0.989	25.0	123.59 ± 12.89	+46
OPDA	75–2000	Y = 3.03745 + 0.00598094*X	0.998	75.0	447.41 ± 57.21	−87%

**A weighting factor of $1/x^2$ was applied to all curves, except for OPDA, which used a factor of 1/x.*

***Values are average ± standard deviation. Concentrations represent the amount of each phytohormone in plant tissues (ng/g of fresh weight, FW), which is corresponding to the concentration (ng/mL) in the injection solution.*

****Values correspond to $((m_{matrix}/m_{solvent}) - 1)*100\%$.*

The S/N of the first calibration point for all phytohormones is much higher than 10, which is established as the minimum S/N ration for the LOQ. It proves the lowest calibration limit for all phytohormones is above to the LOQ of this method.

Calibration curve and linearity

The range of calibration curves was defined for each compound based on the amount of each compound present in the matrix (**Table 2**) and the changes that might occur during experiments. It is important that the calibration curves include the concentration of the phytohormones present in the blank (untreated control) samples, since it usually corresponds to the control in biological experiments. Hence, the analytical method must be suitable to quantify the amount of each phytohormone in control samples. Here it should be mentioned that the phytohormone concentrations measured in this study are in the same range as published by other groups (e.g., Müller et al., 2002; Pan et al., 2008).

Both correlation coefficient (R^2) and residual plots were used to evaluate the linearity of calibration curve for each phytohormone.

Homoscedasticity tests were performed in order to select the best weighting for the linear regression. In these tests, the residual of each point of calibration curve (difference between the calculated and theoretic values) is plotted against the concentration level. For an adequate regression model (regression and weighting) the residuals are normally distributed along the X-axis

(Almeida et al., 2002). To support the data shown in **Table 2**, **Figure 3** presents the residual plots for the best regression and weighting applied to the calibration curve of each phytohormone. For IAA, ABA, JA-Ile, JA, and SA the weighting factor that fits the best to the linear curve is $1/x^2$. For OPDA, it was 1/x. A linear regression was used in the calibration curve for all phytohormones. Thus, those factors and regression were applied in every analytical curve during the whole validation study.

MATRIX EFFECT

The matrix components can affect the analyte stability, extraction and ionization. As was shown above for OPDA, some enzymes present in wounded *A. thaliana* tissues can modify the basal concentration of OPDA, even when the tissues are kept at low temperature (4°C). In other cases, some enzymes can also degrade the analyte or modify the efficiency of analyte extraction. Moreover, for HPLC-MS/MS methods, some constituents of the matrix affect the efficiency of the analyte ionization, when both have the same retention time. In this case, the matrix interferes can either suppress the analyte ionization (decreasing the response) or enhance it (producing higher responses). The effects of matrix on quantitative methods are not completely understood and varies depending on both analyte and matrix composition.

During the validation, we evaluated the influence of *A. thaliana* constituents on quantification of every phytohormone, analysing the slope (m) of each calibration curve prepared

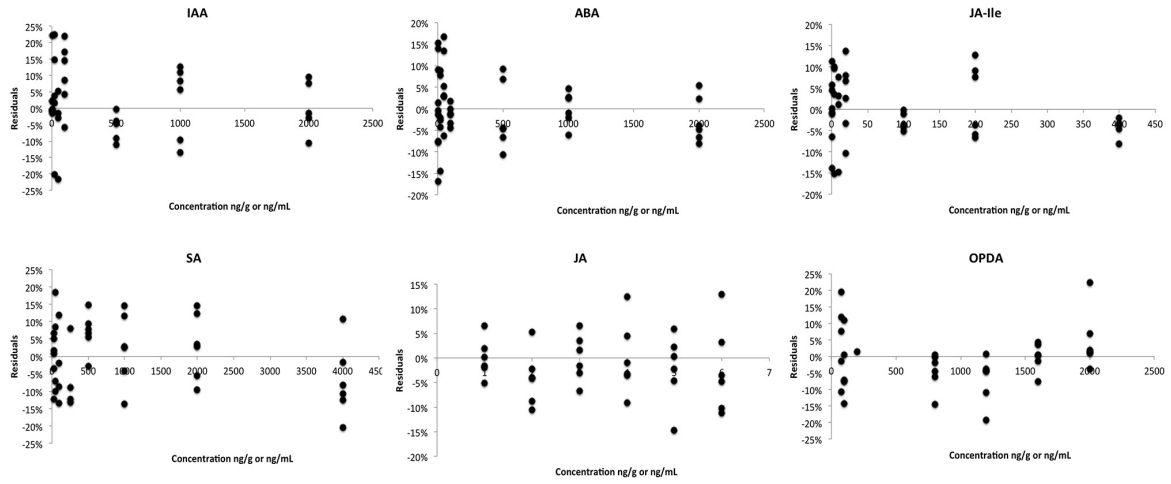

FIGURE 3 | Residual plot associated with the best regression and weighting for calibration curve of each phytohormone.

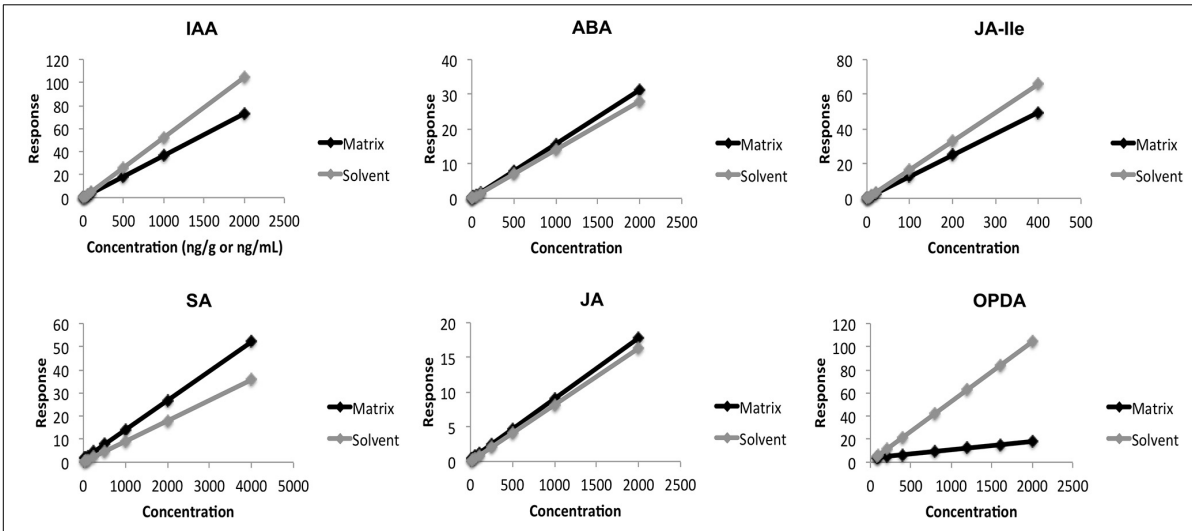

FIGURE 4 | Comparison between calibration curves performed in the matrix (*Arabidopsis thaliana*) and in solvent.

in both solvent and matrix. Comparison between these slopes ($m_{matrix}/m_{solvent}$) showed that the matrix has small influence for quantification of JA and ABA, +7% and +11%, respectively (**Table 2** and **Figure 4**). For the other phytohormones components present in *A. thaliana* affected the measurements in two different ways: increasing the response for SA (+46%) and decreasing it for JA-Ile (−25%), IAA (−31%), and OPDA (−87%). These data proved that the components present in the matrix can indeed influence the response of each analyte in different ways and intensities. Therefore, one of the most reliable ways to evaluate all the matrix effects on quantitative results is using quantification methods (calibration curve and QC) fully developed in the presence of the matrix.

Repeatability, within-laboratory reproducibility, and inter-laboratory reproducibility

Repeatability, within-laboratory reproducibility and inter-laboratory reproducibility (**Scheme 1**) were evaluated in order to define the method's accuracy and precision (EMA and European Commission). They were assessed by overall mean, standard deviation and coefficient of variation for three QC levels (low, medium and high) for independent samples prepared using three spiking solutions.

Repeatability was evaluated by standard deviation and coefficient of variation of three batches (curve and QC) prepared in the same day by the same analyst. While the within-laboratory reproducibility was evaluated comparing the mean, standard deviation (RSD), and coefficient of variation (error) obtained during repeatability measurements and those obtained for samples prepared by a second analyst. The error and standard deviation for practically all QC (low, medium, and high) of all phytohormones were below 15% (**Table 3**). It indicates that this method is precise and accurate for quantification of phytohormone when the measurements are performed in a same laboratory (same equipment, solvents and standards) even when the samples are prepared by different analysts.

Inter-laboratory reproducibility was assessed by collaborative study with Accert Chemistry and Biotechnology Inc., where three new batches of both calibration curve and QC samples were prepared and analyzed using the same extraction procedure as described for repeatability. The error and standard deviation are very low (<10%). It proves that this method is precise and accurate and, hence, it can be used in different laboratories for quantification of phytohormones in *A. thaliana* tissues in order to generate directly comparable data. It is important to highlight that all measurements (calibration curve and QC) must be done in the matrix and for each batch of real samples a calibration curve and the QC (quintuplicate) must be analyzed before samples in order to guarantee the accurateness of the results.

Table 3 | Values of repeatability, within-laboratory reproducibility and inter-laboratory reproducibility obtained during the validation of the method for quantification of various phytohormones (ABA, IAA, JA-Ile, SA, JA, and OPDA) in *Arabidopsis thaliana*.

	Expected conc. (ng/g FW)*	Repeatability *n*=3			Within laboratory reproducibility *n*=6			Inter-laboratory reproducibility *n*=9		
		Mean ± *SD*	RSD (%)	Error (%)	Mean ± *SD*	RSD (%)	Error (%)	Mean ± *SD*	RSD (%)	Error (%)
ABA	7.00	6.67 ± 0.59	8.91	−4.73	6.92 ± 0.56	8.11	−1.15	7.01 ± 0.31	4.37	0.21
	350.00	344.22 ± 11.30	3.28	−1.65	361.58 ± 26.76	7.40	3.31	359.20 ± 22.21	6.18	2.63
	1400.00	1331.25 ± 57.27	4.30	−4.91	1422.62 ± 144.62	10.17	1.58	1401.39 ± 122.85	8.77	0.10
IAA	7.00	7.12 ± 0.71	9.96	1.67	7.29 ± 0.47	6.43	4.09	7.36 ± 0.21	2.86	5.09
	350.00	313.07 ± 8.70	2.78	−10.55	326.16 ± 22.11	6.78	−6.81	332.73 ± 17.28	5.13	−4.94
	1400.00	1306.57 ± 63.12	4.83	−6.67	1378.70 ± 7.32	7.32	−1.52	1353.86 ± 105.62	7.80	−3.30
JA-Ile	1.40	1.57 ± 0.11	6.76	12.34	1.56 ± 0.10	6.36	11.76	1.54 ± 0.03	2.19	10.32
	70.00	70.30 ± 1.85	2.64	0.43	72.24 ± 4.02	5.56	3.20	71.04 ± 3.47	4.88	1.48
	280.00	273.76 ± 3.36	2.36	−2.23	290.30 ± 22.26	7.67	3.68	286.83 ± 22.10	7.71	2.44
SA	140.00	138.46 ± 16.48	11.9	−1.10	138.25 ± 11.59	8.38	−1.25	136.18 ± 3.54	2.60	−2.73
	1400.00	1367.10 ± 43.35	3.17	−2.35	1355.08 ± 84.25	6.22	−3.21	1321.77 ± 56.00	4.24	−5.59
	2800.00	2598.57 ± 112.39	4.32	−7.19	2667.01 ± 145.34	5.45	−4.75	2612.61 ± 139.18	5.33	−6.69
JA	70.00	73.62 ± 3.79	5.15	5.18	70.87 ± 5.63	7.95	−1.25	69.83 ± 3.36	4.82	−0.25
	700.00	691.744 ± 76.12	11.00	−1.18	690.70 ± 63.21	9.15	−1.33	674.38 ± 27.99	4.15	−3.66
	1400.00	1348.90 ± 59.33	4.40	−3.65	1371.20 ± 107.43	7.83	−2.06	1354.27 ± 44.22	3.27	−3.27
OPDA	225.00	220 ± 12.11	5.48	−1.78	233.74 ± 23.54	10.07	3.89	231.11 ± 17.02	7.36	2.71
	700.00	696.42 ± 105.51	15.15	−0.51	711.56 ± 76.76	10.79	1.65	685.69 ± 52.24	7.62	−2.04
	1400.00	1371.79 ± 187.81	13.69	−2.02	1450.72 ± 161.20	11.11	3.62	1411.55 ± 129.21	9.15	0.83

*Corresponds to ng/mL.

Recovery

As described in Recovery, recovery was determined by the ratio between the amount of each phytohormone present in spiked/extracted and extracted/spiked samples. The extracted/spiked samples contained all the matrix interferes and 100% of the phytohormones concentration, since the standards were not subjected to the extraction procedure. On the other hand, in the spiked/extracted samples, the standards were added to the plant samples and the whole extraction procedure was performed afterwards. Thus, the spiked/extracted samples mimicked what happened with the phytohormones during the extraction procedure. The values of recovery for the different QC are shown in the **Table 4**. The overall recovery corresponds to the mean of recovery in different levels. For IAA and ABA the recovery was high, nearly 100%. However, for OPDA the overall recovery was 67.95%. It proved that the matrix affects the recovery distinctly depending on the analyte and on the concentration level. It also shows the significance of performing the calibration curve in the matrix and of validating the analytical method, once the different recoveries were enclosed for the entire range of the calibration curve developed in the matrix.

Quantification of phytohormones in Citrus sinensis

In order to transfer this method to another plant, we choose one of the most the important fruit crops, *C. sinensis*. Thus, statistical parameters such as linearity, repeatability (accuracy and precision), matrix effect and recovery were also evaluated for quantification of phytohormones in leaves of this plant.

The basal level of each phytohormone is shown in **Table 5**. Both range of calibration curve and QC levels had to be modified in order to adjust the quantification method to the content of phytohormones present in citrus. As mentioned above it is important that the calibration curves include the concentration of the phytohormones present in the blank (untreated control) samples, since it usually corresponds to the control in biological experiments.

Linear regression was used for all phytohormones calibration curves with weighting of $1/x^2$ for IAA, ABA, JA-Ile, JA, and SA and 1/x for OPDA. Linearity was assessed by correlation factor (**Table 5**) and matrix effect corresponds to the ratio between the angular coefficient of calibration curve in matrix and in solvent.

This particular matrix (*C. sinensis*) had a small effect in the calibration curve for IAA, ABA, and SA. However, for OPDA, JA-Ile, and JA the matrix had a strong influence in the inclination of the calibration curves (**Table 5** and Figure D, Supplementary Material). The comparison between these results and those presented in **Table 2** (for *A. thaliana*) highlights the importance of performing the calibration curve in the presence of each individual matrix, since they interfere differently in the quantification of each phytohormone.

Table 4 | Percentage of recovery during the extraction of phytohormones in *Arabidopsis thaliana*.

Compound	% of Recovery (Mean + Error)			
	Low*	Medium*	High*	Overall average of recovery
IAA	88.94 ± 12.75	90.98 ± 15.64	97.09 ± 15.15	92.34 ± 4.24
ABA	98.60 ± 11.33	104.50 ± 7.48	105.48 ± 8.52	102.86 ± 3.72
JA-Ile	73.31 ± 13.18	80.99 ± 9.24	77.26 ± 8.50	77.19 ± 3.84
JA	85.35 ± 15.98	75.32 ± 5.71	75.65 ± 12.50	78.77 ± 5.70
SA	86.37 ± 9.31	86.72 ± 9.42	93.71 ± 9.39	88.93 ± 4.14
OPDA	80.05 ± 11.45	63.90 ± 10.97	59.89 ± 5.91	67.95 ± 15.70

**Corresponding to the concentrations given in Quality Controls.*

Table 5 | Parameters of calibration curve for each phytohormone: curve range, regression, weighting, correlation coefficient, limit of quantification (LOQ) and amount of each phytohormone present in blank *Citrus sinensis* samples.

Analyte	Range (ng/g FW)	Curve*	R^2	LOQ (ng/g)	Amount in blank samples** (ng/g FW)	Matrix effect***
IAA	25–4000	Y = 2.09382 + 0.0199062*X	0.989	25	111.47 ± 17.95	−16%
ABA	20–2000	Y = 3.463782 + 0.012662*X	0.994	20	262.07 ± 6.71	+2%
JA-Ile	0.4–400	Y = 0.176041 + 0.0702724*X	0.994	0.4	1.91 ± 0.10	+86%
JA	12.5–2000	Y = 0.74606 + 0.013142*X	0.987	12.5	54.32 ± 9.43	+147%
SA	25–4000	Y = 0.152181 + 0.00719421*X	0.998	25.0	29.60 ± 6.35	−4%
OPDA	30–600	Y = 1.41748 + 0.00743165*X	0.981	30	85.15 ± 1.49	−32%

**A weighting factor of $1/x^2$ was applied to all curves, except for OPDA, which used a factor of 1/x.*

***Values are average ± standard deviation. Concentrations represent the amount of each phytohormone in plant tissues (ng/g of fresh weight, FW), which is corresponding to the concentration (ng/mL) in the injection solution.*

****Values correspond to $((m_{matrix}/m_{solvent}) - 1)*100\%$.*

Table 6 | Values of repeatability (accuracy and precision) obtained during the validation of the method for quantification of various phytohormones (ABA, IAA, JA-Ile, SA, JA, and OPDA) in leaves of *Citrus sinensis*.

	Expected conc.(ng/g FW)*	Repeatability $n = 3$			% Recovery (mean + error)	Overall average of recovery
		Mean ± *SD*	RSD (%)	Error (%)		
ABA	70.00	73.13 ± 3.42	4.68	4.48	84.53 ± 13.04	82.77 ± 8.06
	350.00	369.09 ± 15.06	4.08	5.46	75.63 ± 8.08	
	1400.00	1479.93 ± 73.67	4.98	5.71	88.15 ± 4.83	
IAA	140.00	142.75 ± 9.55	6.69	1.97	55.65 ± 7.97	66.16 ± 8.51
	1400.00	1488.05 ± 23.62	1.59	6.29	70.29 ± 7.42	
	2800.00	3023.73 ± 164.79	5.45	7.99	72.54 ± 5.86	
JA-Ile	1.40	1.43 ± 0.17	11.87	2.38	39.74 ± 8.91	63.23 ± 10.90
	70.00	69.96 ± 2.74	3.92	−0.06	73.36 ± 7.59	
	280.00	300.15 ± 10.17	3.39	7.20	76.59 ± 5.82	
SA	140.00	160.94 ± 2.88	1.79	14.96	61.16 ± 5.56	73.95 ± 5.19
	1400.00	1611.94 ± 106.92	6.63	15.14	81.15 ± 7.58	
	2800.00	3205.07 ± 107.74	3.36	14.47	79.56 ± 3.75	
JA	70.00	76.17 ± 4.97	6.52	8.82	63.61 ± 8.97	75.59 ± 7.40
	700.00	747.05 ± 8.03	1.07	6.72	83.47 ± 7.73	
	1400.00	1496.15 ± 108.73	7.27	6.87	79.68 ± 4.81	
OPDA	90.00	90.67 ± 7.34	8.10	0.74	66.19 ± 10.58	71.00 ± 10.70
	300.00	303.39 ± 34.64	11.42	1.13	74.00 ± 9.77	
	420.00	450.81 ± 60.86	13.50	7.34	72.81 ± 6.57	

*Corresponding to the concentrations given in Validation in Citrus sinensis.

Statistical parameters accuracy (error) and precision (RSD) were also evaluated for quantification of phytohormones in *C. sinensis* and the results are presented in **Table 6**. Basically, all values are lower than 15%, proving that this method is suitable for quantification of phytohormones in citrus.

Recovery was calculated by comparison between spiked/extracted and extracted/spiking samples as described in Recovery. In the same way as discussed for matrix effects, recovery depends on both matrix and the nature of each compound. Recovery of IAA, for example, is strongly different between *Arabidopsis* and *Citrus*. Therefore, to compare the content of phytohormone in different matrix both calibration curve and recovery must be evaluated in every individual matrix.

GENERAL COMMENTS

In the present work we developed and validated a reliable, precise and accurate method for quantification of six different phytohormones (IAA, ABA, SA, JA, JA-Ile, and OPDA) in tissues of two different plants, the model plant *A. thaliana* and the fruit crop *C. sinensis*. As it was possible to transpose the method to a second, independent laboratory, its applicability and reproducibility in different laboratory environments with different set-ups was successfully demonstrated. Moreover, we showed the significance of the validation of the analytical method for the understanding of analyte stability and the matrix effect in the different levels of the analyte concentrations and for different matrixes. This study shows that it is possible to reach comparable standards for phytohormone measurements, independent where the analyses are performed.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00417/abstract

Scheme 1 | Needs to establish a validated method.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

Supporting information

Figure A: Extraction of phytohormones according to different extraction solvents.

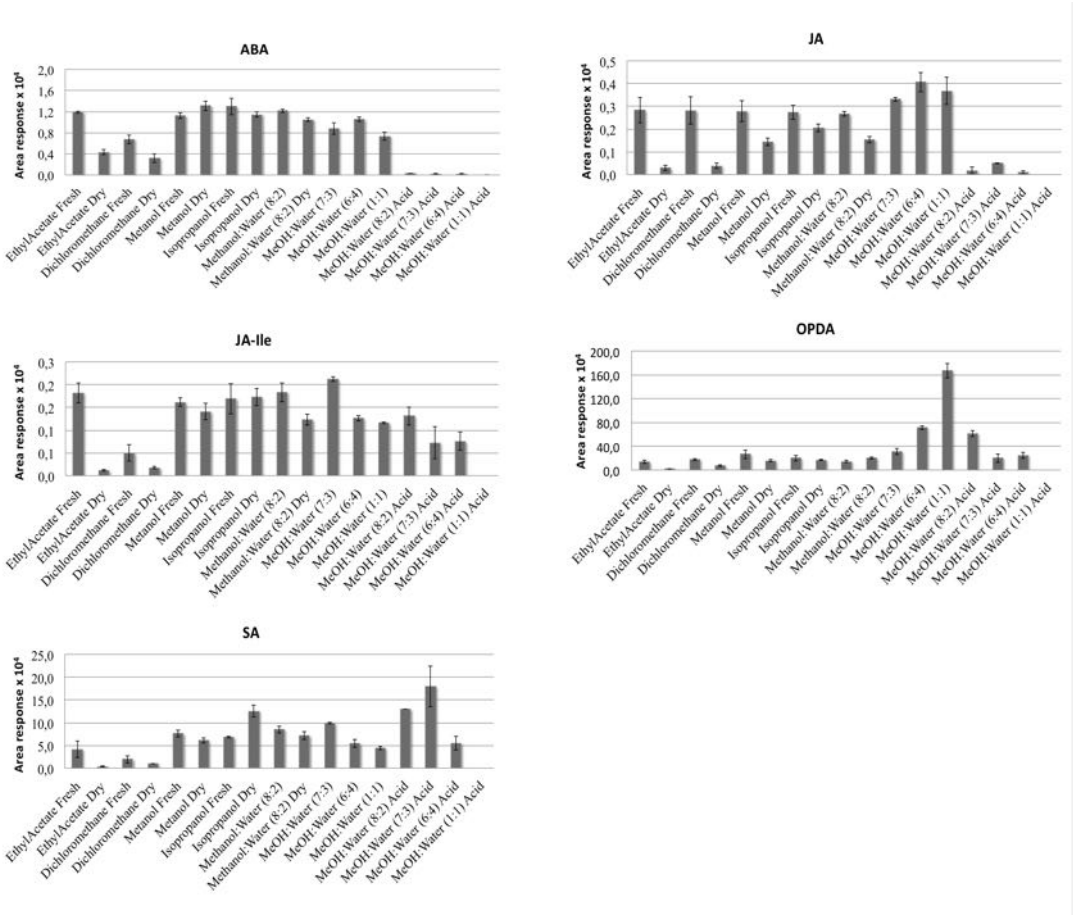


Figure B: Comparison between the MS/MS spectrum of original standards in solvent and in the matrix.

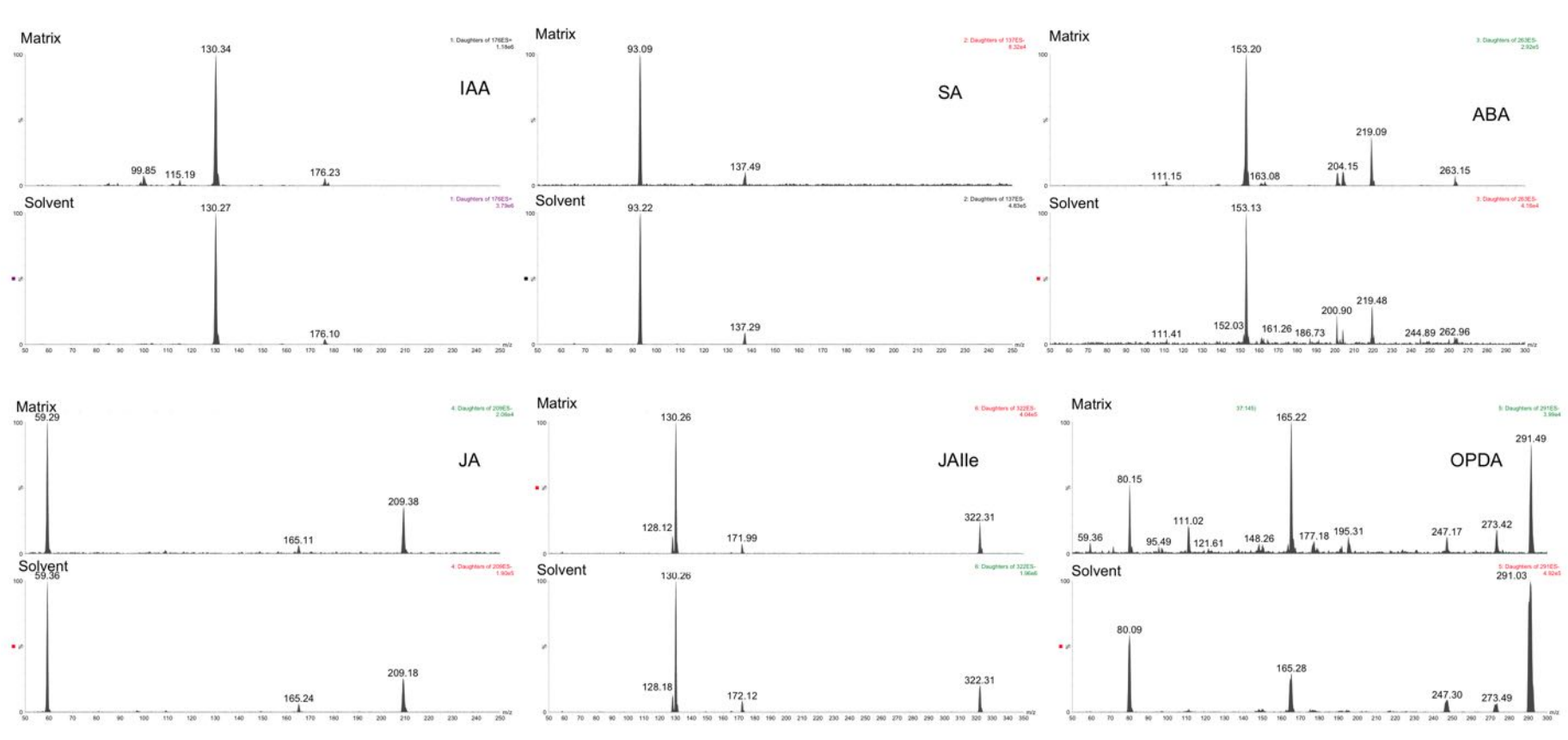


Figure C: SRM chromatograms of the lowest points of calibration curve for each phytohormone. (IAA:, SA:, ABA:, JA:, JAIle:, and OPDA:

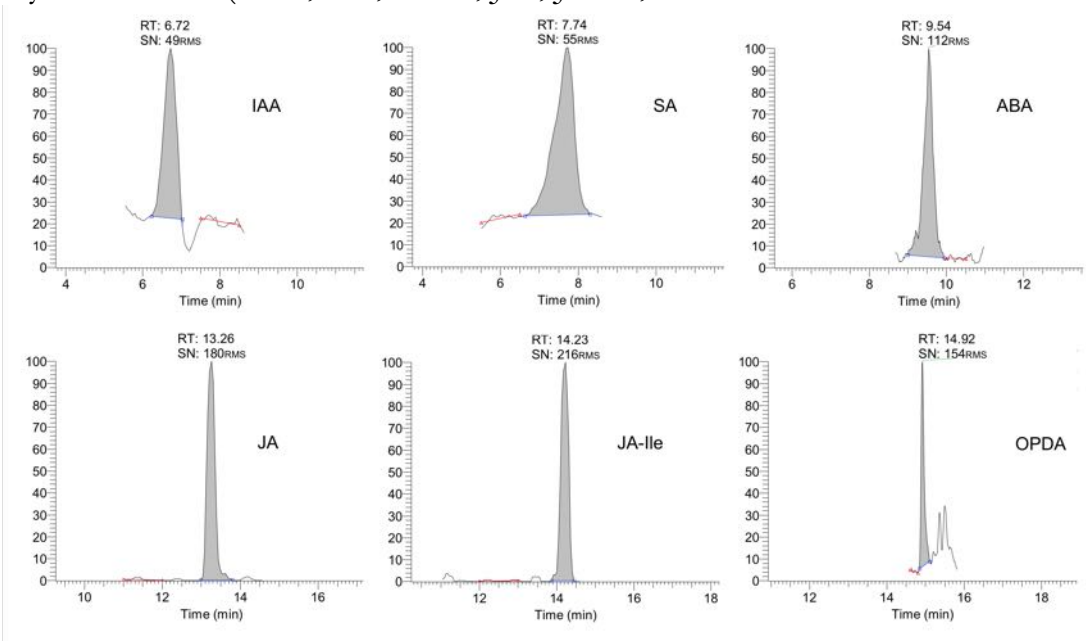
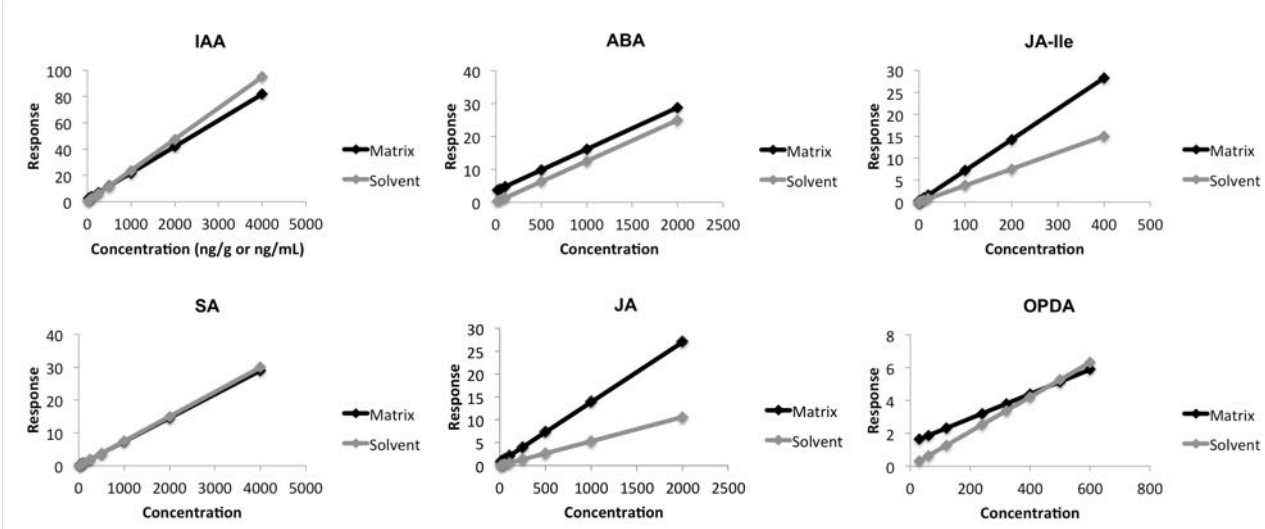
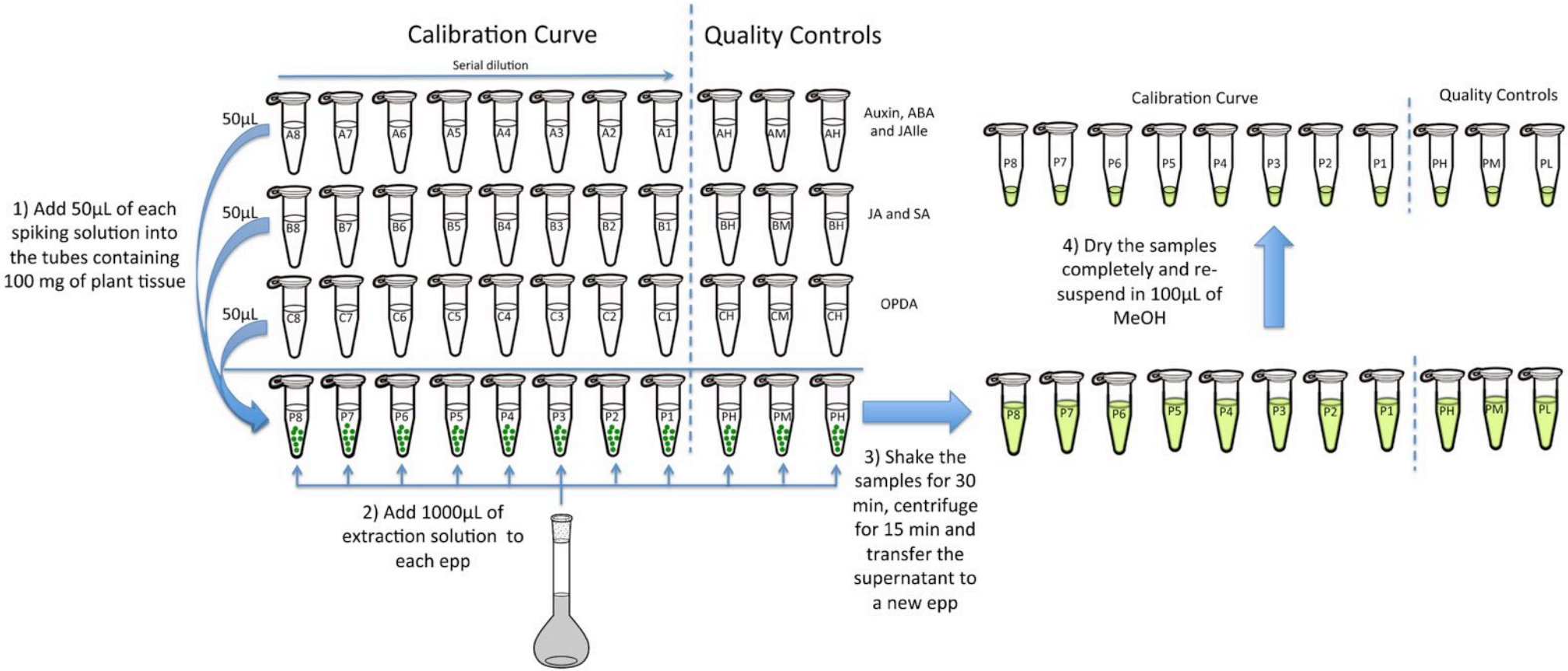


Figure D: Comparison between calibration curves performed in the matrix (*Citrus sinensis)* and in solvent.




Calibration Curve
Quality Controls
Serial dilution
50µL
50µL
50µL
A8 A7 A6 A5 A4 A3 A2 A1 AH AM AH
B8 B7 B6 B5 B4 B3 B2 B1 BH BM BH
C8 C7 C6 C5 C4 C3 C2 C1 CH CM CH
P8 P7 P6 P5 P4 P3 P2 P1 PH PM PH
Auxin, ABA and JAIle
JA and SA
OPDA
1) Add 50µL of each spiking solution into the tubes containing 100 mg of plant tissue
2) Add 1000µL of extraction solution to each epp
3) Shake the samples for 30 min, centrifuge for 15 min and transfer the supernatant to a new epp
4) Dry the samples completely and re-suspend in 100µL of MeOH
Calibration Curve
Quality Controls
P8 P7 P6 P5 P4 P3 P2 P1 PH PM PL
P8 P7 P6 P5 P4 P3 P2 P1 PH PM PL

4

Relationship between microorganisms and oxylipins in *A. brasiliana*

4.1 Introduction

As discussed in the previous chapter, some of the oxylipins found in *A. brasiliana* have already been reported as signalling molecules rising during the infection of *Arabidopsis* by *Pseudomonas*.[31] Some other oxylipins were also described as metabolites produced by bacteria or fungi. Once we had appropriated tools to quantify the oxylipins in plant tissues, we decided to establish axenic cultures of *A. brasiliana*, hoping these plants would be complete bacteria-free. Therefore, we could correlate the absence of bacteria with presence/absence of oxylipins. In this way, methods to detect and quantify bacteria in plant tissues were developed, in order to figure out whether the antibiotic oxylipins found in *A. brasiliana* tissues were related with the presence of endophytic bacteria.

4.2 Material and methods

4.2.1 Establishing axenic culture of *A. brasiliana*

Aseptic cultures of *Alternanthera brasiliana* were establish from surface sterilized seeds collected at São Carlos during the end of 2008. Two protocols for seeds-surface sterilization were tested:

A) Immersion in Tween 20 (10 min), Water (1 min), Sodium hypochlorite 2% (8 min) and Water (8 times);

B) Immersion in Tween 20 (10 mins), Water (1 minute), Ethanol 70% (2 min), Sodium hypochlorite 5% (15 min) and Water (5 times).

The last washing water from both sterilization procedures was sowed in Petri dishes containing BDA agar in order to test the efficacy of the surface sterilization method. After the surface sterilization, seeds were transferred onto Petri dishes containing supplemented MS medium (4.3 mg MS salt, 0.44 mg thiamine, 0.41 mg pyridoxine, 0.50 mg nicotinic acid, 20 mg myo-inositol, 30g sucrose and 8 g agar, in 1 litre, pH 5.7 $\pm$ 0.1) and kept under white light illumination, with daily photoperiod of 16 h at 25 $\pm$ 2 ^{o}C until germination.

After germination, seedlings were transferred to 300 mL Erlenmeyer flasks with the same MS medium described above, prepared with 10 g/L agar. For all following experiments the plants were kept under full daylight period at 25 ± 2 °C.

4.2.2 Development of PCR-based method for bacteria detection in plant tissues

4.2.2.1 DNA extraction

The development of the PCR-based method for bacteria detection in plant tissues included the optimization of DNA extraction, DNA amplification and separation of the amplicons in agarose gel. DNA extraction was performed using two different protocols:

Protocol 1[76]: 100 mg of frozen and ground *A. brasiliana* tissues were weighted in 1.5 mL epp tubes and added with 500 µL of potassium phosphate buffer (10 mM, pH 6.5). The samples were shaken briefly and centrifuged for 20 min at 4 °C and 500*g*. The supernatant was transferred to a new epp tube and pelleted at 4 °C and 15,000g for 30 min. The pellet was added with 500 µL of BCE buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100 and 2 mM mercaptoethanol), incubated at room temperature for 30 min and centrifuged for 30 mins at 4 °C and 15,000*g*. A small portion of autoclaved 0.1 mm glass beads and 500 µL DNA extraction buffer (200 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1% sodium dodecyl sulphate) were added into each tube containing pelleted cells, vortex 3 x for 40 s and incubated at room temperature for 1 h. After incubation, 500 µL of Tris buffered phenol (pH 8) was added to each tube, which were vortex briefly and centrifuged for 10 min at 15,000*g* and 4 °C. The supernatant was removed and mixed with 500 µL of chlorophorm/isoamylalcohol (24:1) and the tubes were centrifuged for 10 min at 15,000*g* and 4° C. The aqueous phase was then mixed with 0.5 vol 1 M NaCl and 0.6 vol isopropanol, for DNA precipitation. The tubes were kept on ice for 1 h and centrifuged for 15 min at 15,000*g* and 4 °C. The pellets

were washed with ethanol 70%, ethanol 95% and rehydrated with 20 μL of RNA-free water.

Protocol 2 (common CTAB procedure): 100 mg of frozen and ground *A. brasiliana* tissues were weight in 1.5 mL epp tubes and added with 500 μL CTAB buffer. The tubes were kept in water bath 55 °C for 1 h. After incubation, 500 μL Tris buffered phenol (pH 8) was added to each tube, vortex briefly and the tubes were centrifuged for 7 min at 16,000*g* at room temperature. Aqueous phase was removed and mixed with 500 μL of chlorophorm/isoamylalcohol (24:1), and the tubes were centrifuged for 7 min at 16,000*g* at room temperature. DNA was precipitated adding 0.08 vol 7.5 M ammonium acetate and 0.54 vol isopropanol to the aqueous phase. The tubes were kept on ice for 1 hour and centrifuged for 15 min at 16,000*g* and 4 °C. Pellets were washed with ethanol 70%, ethanol 95% and rehydrated with 20 μL of RNA-free water.

4.2.2.2 DNA amplification

DNA amplification was carried out in 50 μL mixtures containing 2 μL DNA template, 2.5 mM $MgCl_2$, Taq buffer, 0.1 mM of each dNTP, and 1.25 U of Taq DNA polymerase (Thermo Scientific) and 0.5 mM of each primer 799F/1492R [76]. The reactions were performed in an Eppendorff Mastercycler Pro using the following program: initial denaturation at 95 °C for 3 min, followed by 30 cycles at 94 °C for 20 s, 53 °C for 40 s, and 72 °C for 40 s, with a final extension step at 72 °C for 7 min.

The amplicons were analyzed by electrophoresis using gels with different amounts of agarose (1, 1.5, 1.8 and 2%).

4.2.3 Development of PCR-based method for quantification of bacterial cells in plant tissues

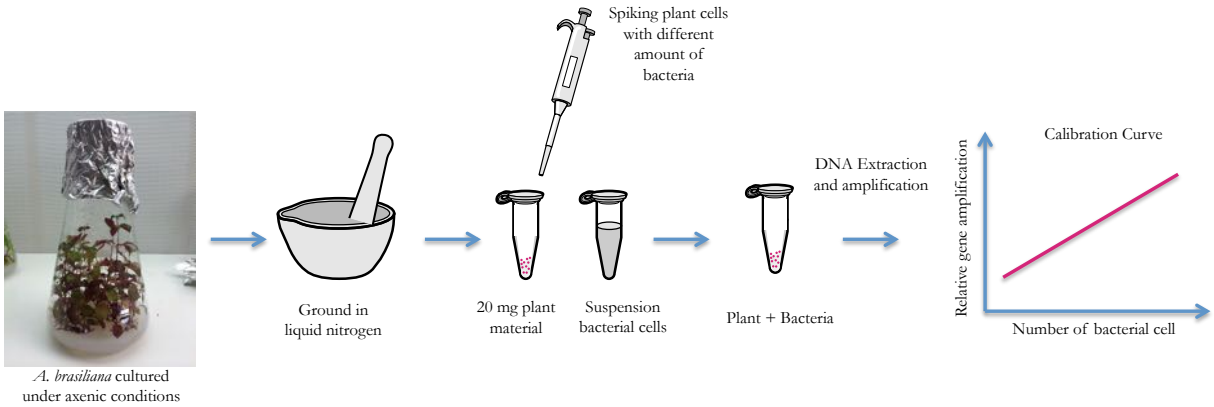
The method for quantification of bacterial cells in plant tissues was based on real time PCR analysis. In this case, two sets of primer pairs were

evaluated for bacterial DNA amplification: 534/783 [77] and 519/907 [78]. A specific primer for amplification of plant DNA (TEFf and TEFr) was used similarly to a "house-keeping gene".

Annealing temperature, final reaction volume, amount of DNA template and primer concentration were optimized, for both primer pairs (plant and bacterial), in order to achieve a specific and sensitive method. The experiments were performed in CFX96 Touch™ Real-Time PCR Detection System (Germany) or Applied Biosystem StepOnePlus (Brazil), using 10uL reaction: 1uL of each primer (6pmol), 2 uL of water, 5uL of Brilliant II SYBR Green Master Mix and 1uL of DNA template.

4.2.4 Calibration curve for quantification of bacterial DNA

After the optimization of DNA amplification, a calibration curve was established using plant samples spiked with different concentrations of bacterial cells. Tubes containing 20 mg of ground and frozen *A. brasiliana* tissues were spiked with different volumes (0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 µL) of *Bacillus subtillis* suspension (ABS $_{625nm}$ = 0.1). Samples were shaken briefly and extracted according to protocol 2 described in 4.2.2. DNA amplification was carried out using the best conditions obtained after the optimization of q-PCR parameters. This procedure is summarized in Scheme 1.



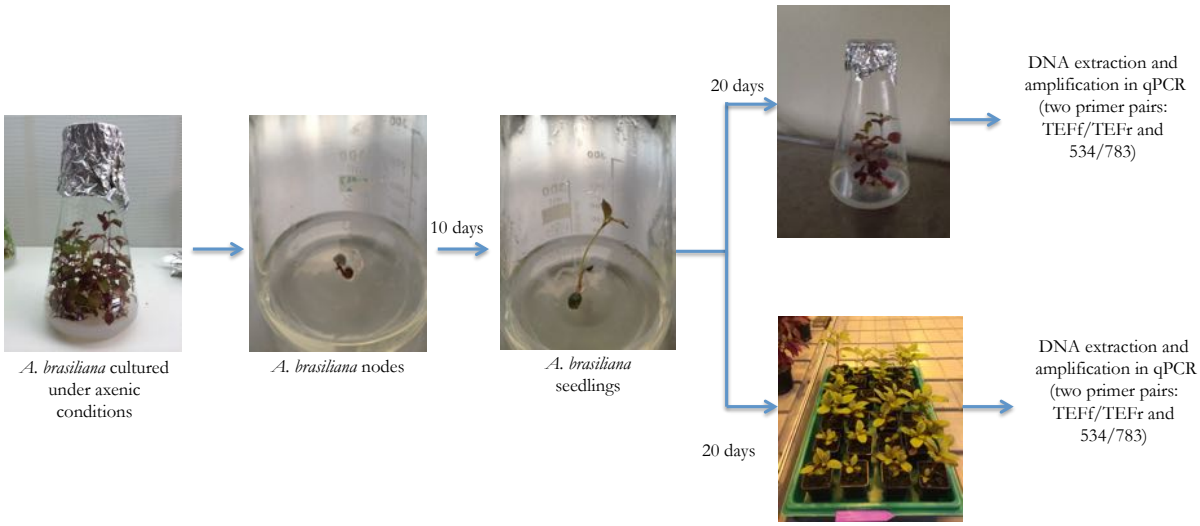
Scheme 1: Procedure for generating the calibration curve during quantification of bacterial cells in plant tissues.

4.2.5 Quantification of bacterial cells in *Alternanthera brasiliana*

Initially, quantification of bacterial cells were performed according to the protocol described in 4.2.4 for normal plants (4 months old) and plants cultured under aseptic conditions (15 days old). However, the results lead us to analyze plants in the same developing stage. Therefore, nodes of plants cultured under aseptic conditions were used to generate new seedlings. These nodes were added to new flasks containing MS medium, and after 10 days half of these seedlings were transferred to pots containing normal soil and half were kept under aseptic conditions. All these plants were kept under the same light, temperature and humidity for 30 days.

Next, the quantification of bacterial cell was carried out using normal surface sterilized and non-surface sterilized *A. brasiliana* tissues as well as plants cultured under aseptic conditions. For normal plants, stem and leaves were cut in small pieces, and half of them had their surface sterilized in order to remove the epiphytic microorganisms. Sterilization was performed by immersion of plant pieces into the following sequence of solvents/solutions: Water (sonication for 5 min), water (1 min), ethanol (1 min.), sodium hypochlorite 5% (3 min), water (1 min) and water (1 min).

Pieces of stem and leave (aseptic, sterilized and non-sterilized) were transferred in 2.0 mL tubes, frozen in liquid nitrogen and ground in a GenoGrinder (SPEXSample Prep, München, Germany) for 2 cycles of 30 s at 1500 rpm using 2 steel beads per tube (30 mm). DNA extraction and DNA amplification were performed as described in 4.2.2 and 4.2.3 respectively. The whole procedure is summarized in Scheme 2.

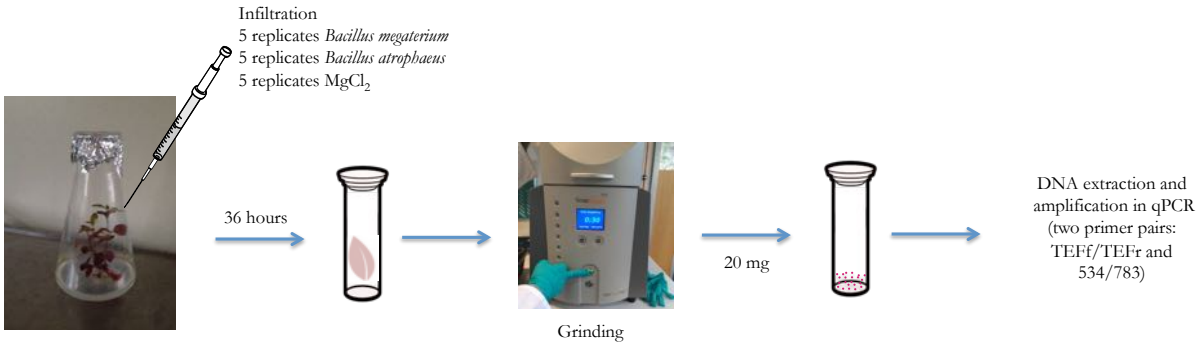


Scheme 2: Procedure for quantification of bacterial cells in *A. brasiliana* cultured under normal and aseptic conditions.

4.2.6 Inoculation experiments

The initial experiment for infiltration of bacteria into the *A. brasiliana* tissues was performed using two different strains of endophytic bacteria previously isolated from *A. brasiliana*: 1) *Bacillus megaterium* and 2) *Bacillus atrophaeus* . Suspension of each strain (10^6 cells/mL) was prepared in $MgCl_2$ (10 mM) as described by Vollenweider [31].

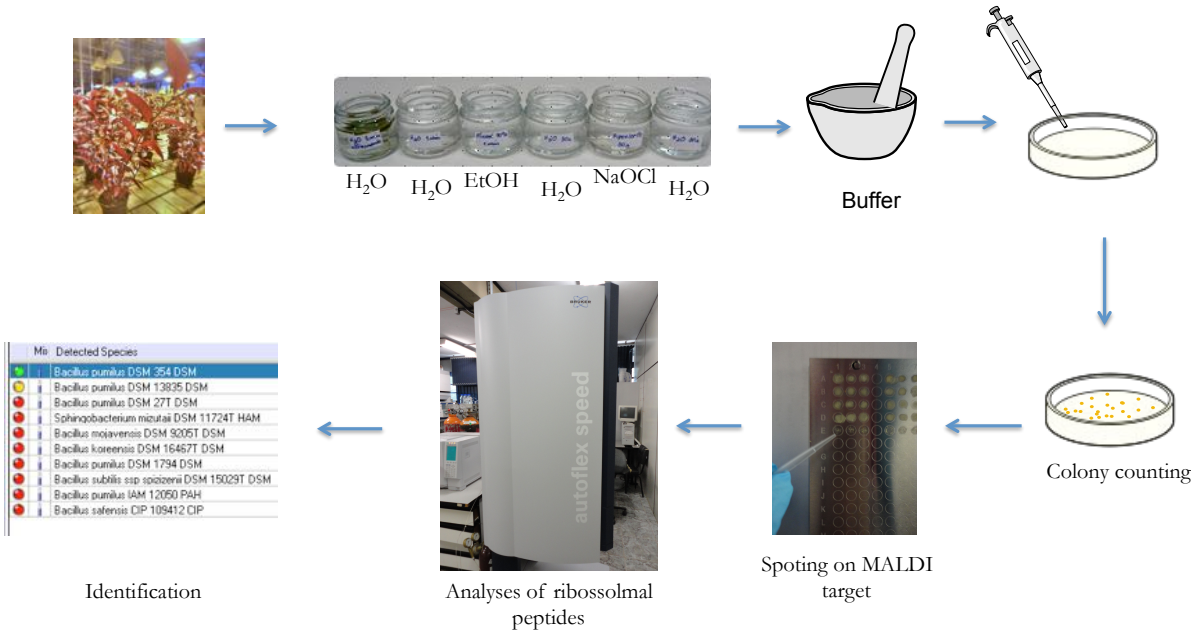
Plants grown under axenic conditions for 30 days were infiltrated with 1 µL of bacterial suspension (8 replicates for each infiltration). 1 µL of 10 mM $MgCl_2$ was used as a negative control. After 36 h of incubation the infected leave (infected with *Bacillus megaterium*, *Bacillus atrophaeus* or $MgCl_2$) were removed frozen in liquid nitrogen and ground. 20 mg of each sample were transferred into a 1.5 mL epp tube and the DNA extraction and amplification were performed as described in 4.2.2 (protocol 02) and 4.2.3 respectively. The remaining plant material was dried in order to perform the quantification of oxylipins. The entire procedure is summarized in Scheme 3.



Scheme 3: Procedure for re-inoculation and quantification of bacteria in *A. brasiliana* tissues.

4.2.7 Qualitative analysis of culturable bacterial community associated with plant

In order to analyse qualitatively the culturable bacterial community associated with *A. brasiliana,* a new protocol is being established in cooperation of the undergraduate student Natalia Ritez Rivaben. In this method, leave and stem of *A. brasiliana* are cut in small pieces with the same weight and submitted to surface sterilization using water, ethanol and sodium hypochlorite. After sterilization (as described in Trapp et at)[71], these pieces were individually crushed in a sterilized mortar containing 10 mL autoclave PBS buffer. The suspension is then transferred into Petri dishes containing different culture media (Muller Hinton, Nutrient Agar and Brain Heart Infusion). These plates are kept at 27°C for 48-72 hours. After this period, the colonies are counted and transferred to a MALDI target. The samples are covered with 2uL of matrix (20mg of hydroxycinnamic acid, in 1mL water:acetonitrile:trifluoracetic acid [50:50:2.5]) and analysed in a Bruker Autoflex Speed MALDI TOF. The profile of the ribosomal peptides of each bacterium is compared to Biotyper database in order to identify the endophytic bacteria. This protocol is summarized in Scheme 4.



Scheme 4: Protocol for qualitative analyses of endophytic bacterial community

4.3 Results and discussions

Different methods are available for detection of bacterial cells in plant tissue: optical transmission microscopy, cultivation and DNA amplification, among others. Some aspects of these techniques are compared in Table 4.1. Microscopy, for example, is a very inexpensive analyze. However, the traditional transmission microscopy is not very sensitive neither very specific. It requires stain approaches and well-trained staff to find bacterial cells in complex samples (i.e. plant tissues). DNA amplification on the other hand, is a very sensitive and comprehensive technique. It can also be very specific when the primer pairs are well designed to detect a certain kind of microorganism. But it is very expensive requiring many reactants such as enzymes, primers, buffers etc.

Table 4.1: Comparison between different approaches for detection of bacteria in plant tissues. + indicates favorable points.

Detection method	Specificity	Sensitivity	Comprehensiveness	Speed	Cost
Microscopy (transmission)	+	+	++	-	+++
Cultivation	++	+	-	++	++
DNA amplification	++	+++	+++	+++	-

The most noticeable point against cultivation techniques for detection of bacteria in plant tissues relies on the lack of comprehensiveness, since it can only detect culturable bacteria. Therefore, during this study we choose the genomic approach (DNA amplification using PCR) for detection of bacterial cells in plant tissues, once we were interested in a fast and sensitive method for detection of both culturable and unculturable bacteria.

4.3.1 Establishing axenic culture of *A. brasiliana*

As described in 4.1 seeds of *A. brasiliana* were sterilized, sown in Petri dishes containing modified MS medium (Figure 4.1a) and kept there until germination (around 20 days). The seedlings were transferred to 300mL Erlenmeyer flasks (Figure 4.1b) containing the same culture medium. After 30 days, the seedlings were transferred to 2000mL Erlenmeyer flaks (Figure 4.1c), where they were maintained for further experiments.

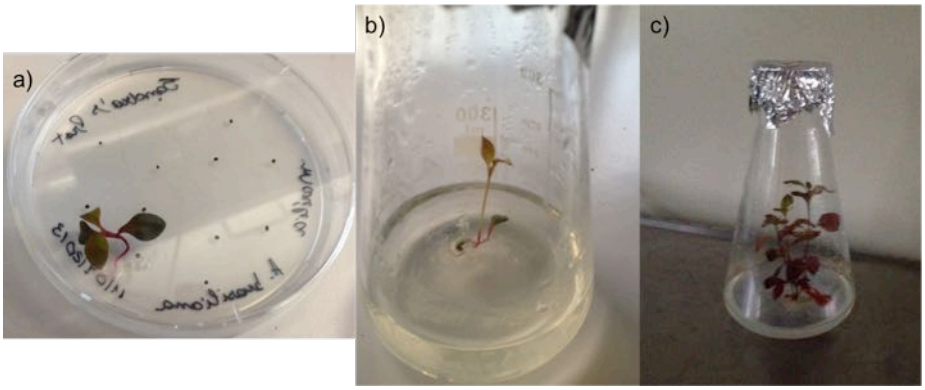


Figure 4.1: Axenic culture of A. *brasiliana*. A) Seed germination; b) first transfer (300 mL Erlemeyer flasks) and c) transfer to 2.000mL Erlenmeyer flasks

In order to evaluate whether the plants cultured under aseptic conditions were bacteria-free, a PCR-based method was developed to amplify bacterial DNA present in plant tissues.

4.3.2 Optimization of bacterial DNA extraction and amplification.

DNA extraction and amplification was performed according as described in 4.3.2 for: a) plant tissues (5 samples); b) plant tissues spiked with bacterial cells (5 samples) and c) bacterial cells (1 sample as positive control). No amplification of bacterial DNA was observed using the amplification conditions described by Nissinen and co-workers (Figure 4.2).[76]

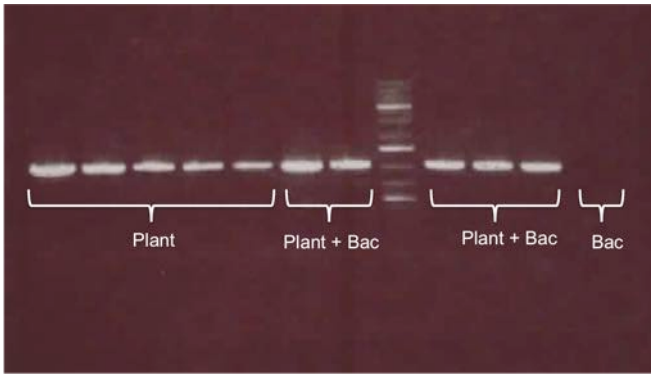


Figure 4.2: Agarose gel with amplified DNA from: Plant tissues (Plant), Plant tissues spiked with bacterial cells (Plant+Bac), bacterial cells (Bac) and and 1kb Plus DNA Ladder from Fermentas.

The bands present in Figure 4.2 correspond to chloroplast and/or mitochondrial DNA. According to endosymbiotic theory, several organelles (e.g. chloroplast, mitochondria) present in eukaryotic cells originated from symbioses between singles-cell organisms. These organelles present many 16S rRNA conserved genes similar to those from bacterial cells. These genes are often amplified by prokaryotes universal primers, which are used for detection of broad range of bacterial cells. However, the amplicons from prokaryotes and eukaryotes genes are composed by different number of base pairs (bp) and can be separated by electrophoresis. Therefore, PCR parameters were optimized to improve amplification of bacterial DNA in presence of chloroplast/mitochondrial DNA.

Influence of several PCR parameters (e.g. annealing temperature and time, primer and enzyme concentration, additives etc.) was evaluated during the optimization of DNA amplification. Table 4.2 shows a comparison between initial and optimized parameters used for amplification of extracted DNA.

Table 4.2: Comparison between initial and optimized parameters used during DNA amplification.

		Initial parameters [79]	Optimized parameters
Composition of reaction solution	Taq polymerase	2.5 unit	1 unit
	BSA	0.5 mg/mL	-
	Template	200 ng	200 ng
	dNTP	2.5 mM of each	2.5 mM of each
	Primers 799r and 1492f	15 pmol of each	15 pmol of each
Amplification Parameters	Initial denaturation	95 °C 3 min	95 °C 3 min
	Denaturation	94 °C/ 20 sec	94 °C/ 20 sec
	Annealing	53 °C/ 40 sec	57 °C/ 40 sec
	Elongation	72 °C 40 sec	72 °C 40 sec

When the extracted DNA was amplified using optimal PCR parameters, a weak band around 700kb related to bacterial DNA was observed in the positive control (samples composed by bacterial cells), Figure 4.3. However these new parameters also improved the amplification of plant DNA (band around 1000kb) and dimerization of primers (band present at the base of agarose gel),

Figure 4.3. Moreover, the separation of bacterial and plant amplicons (around 700 and 1000 kb) is not very effective in agarose 1%.

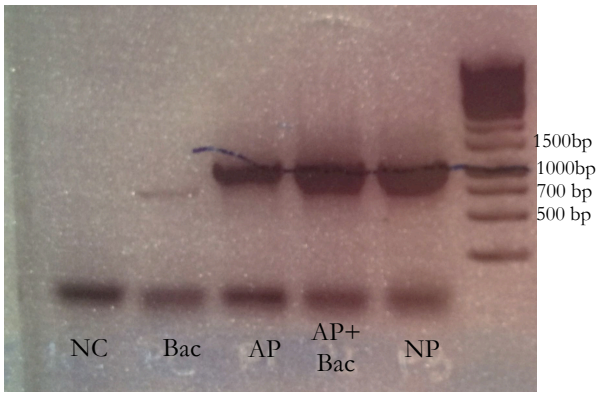


Figure 4.3: Agarose gel with amplified DNA from: Negative control (NC), Bacterial DNA (PC), Aseptic plant tissues (AP), Aseptic plant tissues spiked with bacterial cells (AP+Bac), Normal plant tissues (NP) and 1kb Plus DNA Ladder from Fermentas.

Then, three different agarose concentrations were tested in order to improve the separation between plant and bacterial amplicons (1.5, 1.8 and 2.0 %). The best separation was achieved with 2.0% of agarose, as can be seen by comparison between the bands around 700 kb (bacterial DNA) and 1000 kB (plant DNA) presented in Figure 4.4. Primer dimers cannot be seen in this picture since they ran out of the gel due to the long running time. However, they were still present in these samples.

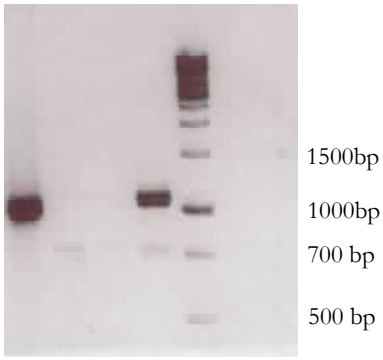


Figure 4.4: Amplicons from plant and bacterial DNA, electrophoretic separated in agarose 2% gel. Aseptic plant tissues (AP), Bacterial DNA (Bac), Aseptic plant tissues spiked with bacterial cells (AP+Bac), 1kb Plus DNA Ladder from Fermentas and Negative control (NC).

Primer dimers are generated when two complementary regions present in the primers are annealed. Since normal polymerase enzymes present some activity even at room temperature, when the polymerase is added to the PCR reaction solution containing the primers dimers, it starts to amplify it. The amplification of the primer dimers by DNA polymerase leads to a competition for PCR reagents inhibiting the amplification of target DNA.

Amplification of primer dimer can be prevented by optimization of PCR parameters (concentration of primers, magnesium chloride, nucleotides, ionic strength and annealing time and temperature). However, modification in these parameters usually decreases the amplification of target DNA, as well. As the PCR parameters has been previously optimized for amplification using 534f/783r primer set, another approach was adopted to reduce the primer dimer: addition of polymerase enzyme after the first denaturation step. In this case the enzyme just comes in contact with the DNA when the strands of target DNA were already denatured and are ready for amplification, reducing the consumption of PCR reagents in the amplification of primer dimers. Figure 4.5 shows the agarose gel with the products generated by this procedure. The presence of more intense bands corresponding to bacterial DNA (around 700 kb) indicates the addition of Taq polymerase after the initial denaturation step improved the amplification of target DNA. Thus, this procedure was applied in further experiments.

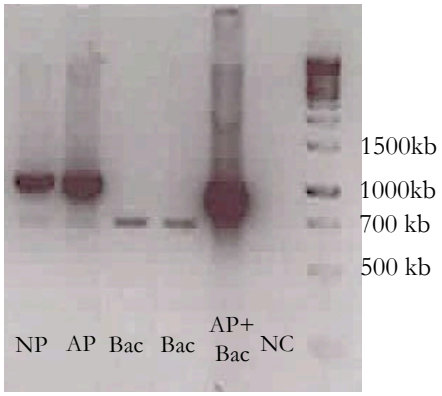


Figure 4.5: Products of amplification of plant and bacterial DNA, by addition of Taq polymerase after the initial denaturation step. Non-aseptic plant tissues (NP); Aseptic plant tissues (AP); Bacterial DNA (Bac), Aseptic plant tissues spiked with bacterial cells (AP+Bac), Negative control (NC), and 1kb Plus DNA Ladder from Fermentas.

4.3.3 Detection of bacteria in normal and aseptic plants

The optimized conditions for DNA extraction, amplification and separation were employed to detect bacterial DNA in normal and aseptic plants. Amplified DNA from plants cultured under aseptic conditions (AP), normal plants (NP), negative control (NC) and positive control (bacterial cells in two different concentration 01 and 02) are shown in Figure 4.6.

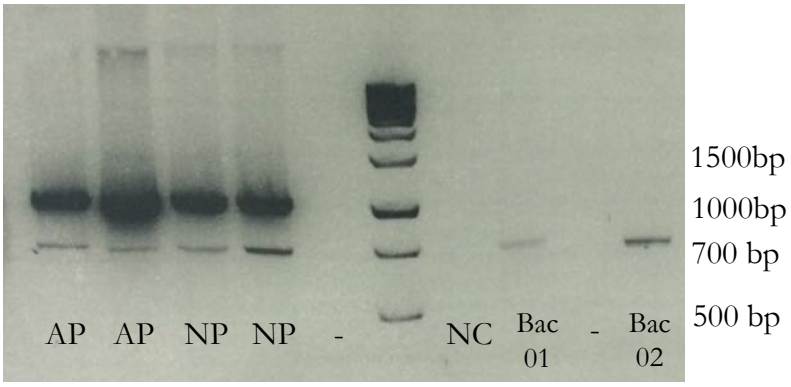


Figure 4.6: Agarose gel with amplified DNA from: Aseptic plant (AP); Surface Sterilized Normal Plants (NP), 1kb Plus DNA Ladder from Fermentas, Negative control (NC), Bacterial DNA extracted from: 10 uL and (Bac 01) and 50 uL (Bac 02) of *Bacillus* sp. suspension cell 0.1 ABS

These data show that endophytic bacteria are present in both normal and aseptically cultured plants, proving that it was not possible to get *A. brasiliana* completely bacteria-free to use as negative control in ecological studies about the correlation between bacteria and oxylipins. Since the plants cultured under aseptic conditions are not completely bacteria-free, from now on they will be called axenic cultures, what means these plants are free of external contaminations (epiphytic microorganisms) but still contain endophytic bacteria.

Before trying new procedures to get bacteria-free plants (i.e. by callus or free cells culturing), we analyzed the plants by fluorescence microscopy using Syto 9 stain. In this technique the Syto 9 is able to stain bacteria mitochondrial DNA. Then the sample is irradiated with laser (at 488 nm) transferring energy to Syto 9. When the irradiation is removed, chloroplast presents an auto-fluorescence at 650nm, while the complex mitochondria-Syto9 emit radiation between 505-530nm. In this way, it is possible to detect bacterial cells and discriminate them from plant chloroplast.

Using co-focal microscopy it is possible to analyze several slices of the plant tissues. Once bacterial and chloroplasts are found in the same slices, one can conclude the bacteria are living inside the cell. So, as can be seem in Figure 4.7, both bacteria (pointed by small white arrows) and chloroplasts are found in the same slice suggesting that these microorganisms are true endophytes, and probably is not possible to get rid of them by generating free cells or callus culture.

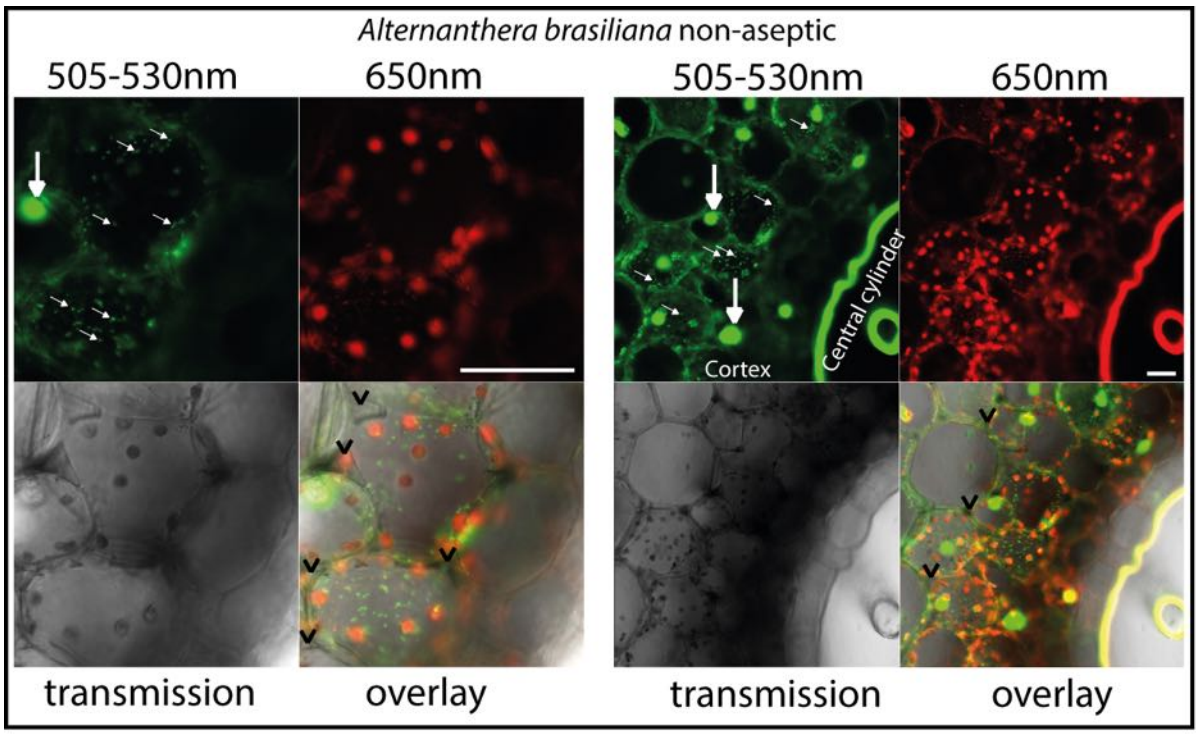


Figure 4.7: Microscopy of *A. brasiliana* plant tissues. Small arrows indicate bacterial cells, and the big white arrows indicate nucleus from plant cells.

Since both axenic and normal plants contain endophytic bacteria, it seems that getting plant completely absent of bacteria is a tough task. Therefore, we looked at the oxylipins in both axenic and normal plants trying to understand whether or not there was a correlation between the presence of bacteria with oxylipins.

Plant samples were extracted and analyzed according to the method for quantification of oxylipin described in Chapter 3. The MRM chromatograms of *A. brasiliana* (Figure 4.8) prove that despite of some small differences the oxylipins are present in both axenic and normal plants.

As normal and axenic plants contain both endophytic bacteria and oxylipins, the presence and absence of oxylipins and bacteria cannot be direct correlated. Then, the concentration of both bacterial cells and oxylipins were evaluated in order to check if there was a correlation between the amount of these two factors (oxylipins and bacterial cells). In this way, a q-PCR based method for quantification of bacterial cells was developed as described below.

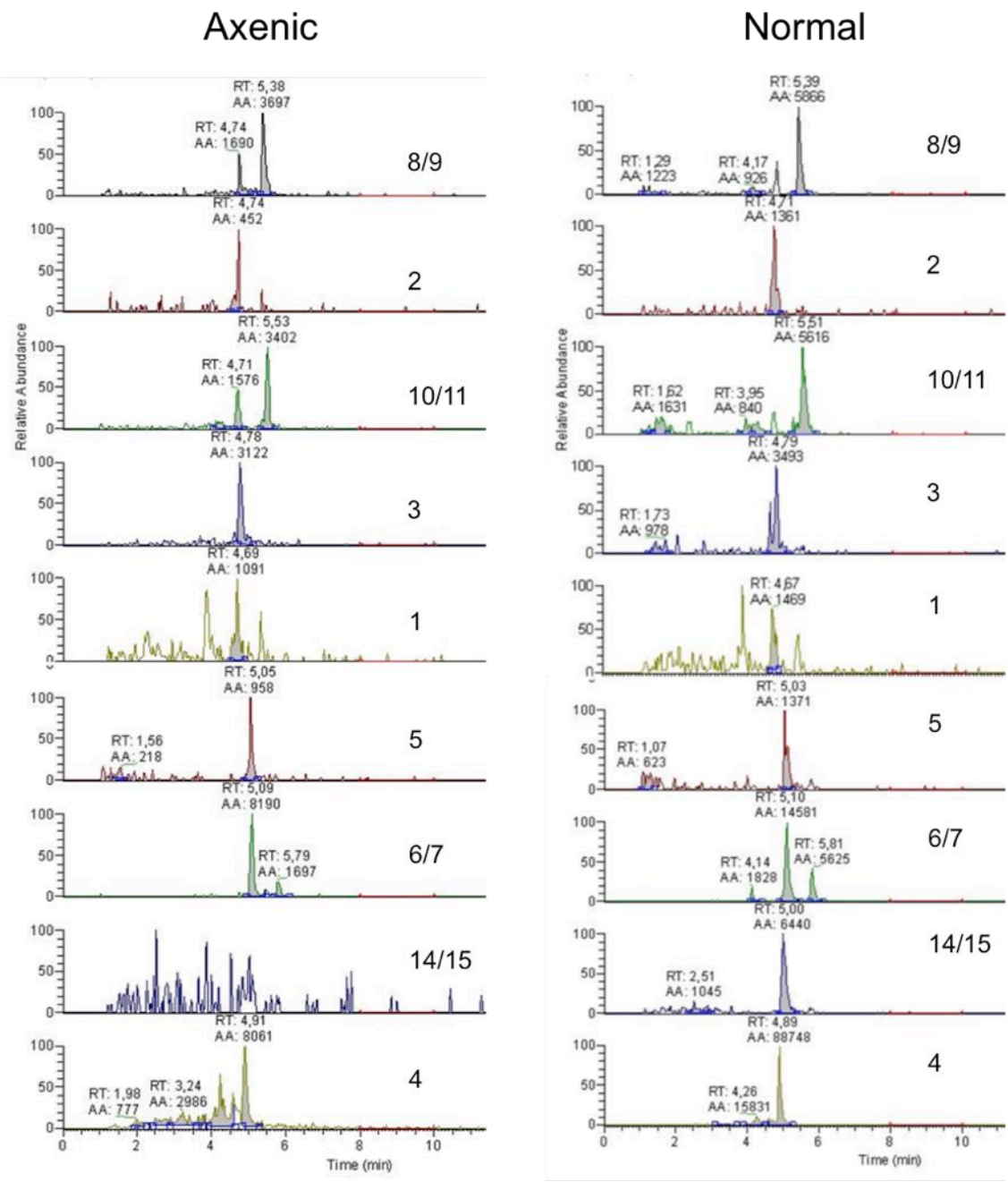


Figure 4.8: Oxylipins MRM chromatograms for axenic and normal *A. brasiliana* extracts.

4.3.4 Quantification of bacterial cell in plant tissues

Quantification of bacteria in environmental samples can be performed in many different ways (e.g. colony or cell count, molecular analysis, flow cytometry, etc). Focused on the same points discussed in beginning of this chapter, we developed a q-PCR based method to quantity bacterial cells in *A. brasiliana*

tissues, since it quantifies both culturable and unculturable bacteria with high sensitivity.

Primers to be used in DNA quantification must generate small amplicons (around 300 kb) with high specificity and reproducibility. In this way, the primer pair set used previously, during the detection of bacterial DNA in plant tissues, was not suitable for a quantification of bacterial DNA based on real time PCR analysis, since it generates amplicons with approximately 700kb.

Thus, two primer pairs were tested for quantification of bacterial DNA: 534/783 and 519/907 [77,78,80,8180]. Specificity in amplifying bacterial DNA was the first parameter evaluated for these two primer pairs. Samples of plant tissues and bacterial cells were extracted according to protocol 2 and amplified as described in 4.2.3. PCR amplification products were analyzed in 3% agarose gel. The results are shown in Figure 4.9.

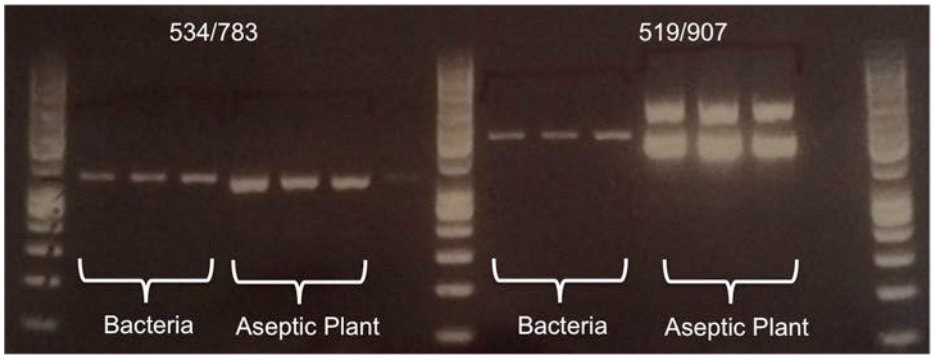


Figure 4.9: Agarose gel with products from amplification of DNA present in plant and bacterial cell samples using two different primer pairs: 534f/783r and 519f/907r.

Although the 519/907 seems to be more sensitive (giving more intense bands), it is not selective for bacterial DNA, presenting two bands in the agarose gel corresponding to plant and bacterial DNA. Even though the amplicons can be separated in agarose gel, this primer is not appropriated for qPCR analyses, since different amplicons cannot be distinguish by this technique. Therefore, the quantification of bacterial DNA in plant tissues was performed using 534/783 primer pair.

4.3.4.1 Optimization of amplification parameter for bacterial and plant DNA

DNA extraction varies from sample to sample, which represents a problem for quantification measurements. In this way, a plant housekeeping gene (TEFf-TEFr), which is constant expressed in plant tissues, was used as internal standard to correct any variation in the DNA extraction. The q-PCR parameters (concentration of primer pairs and annealing temperature) were experimentally optimized for both primer sets 534f-783r (target bacterial DNA) and TEFf-TEFr (plant housekeeping gene).

According to amplification curves (Figure 4.10) the optimal annealing temperature (those which amplifies the target DNA in the lower number of amplication cycles) is around 56ºC for 534f/783r and 53ºC for TEFf/TEFr.

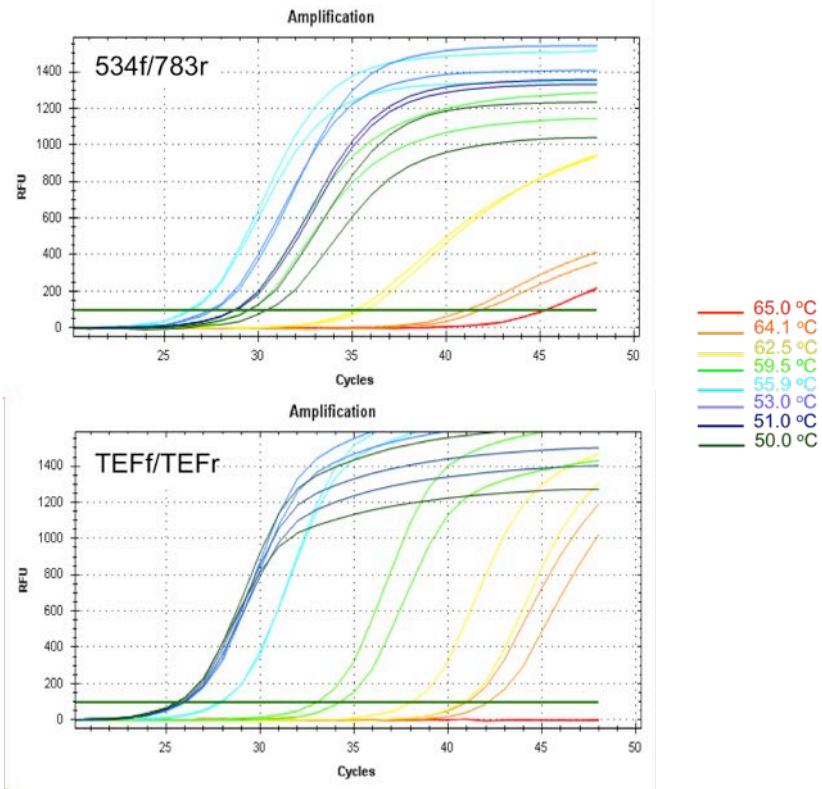


Figure 4.10: Amplification curves for 534f/783r and TEFf/TEFr primers at different temperatures.

Figure 4.11 shows the melting peaks for each amplification curve presented in Figure 4.10. Since every melting curve present just one peak, corresponding to a specific amplicon, the method is considered specific for amplification of bacterial DNA at all tested annealing temperature. For TEFf/TEFr, temperatures over 62.5oC generate un-specific products (probably due to the dimerization of primers) what is confirmed by the peak at low melting temperature (between 75-80°C)

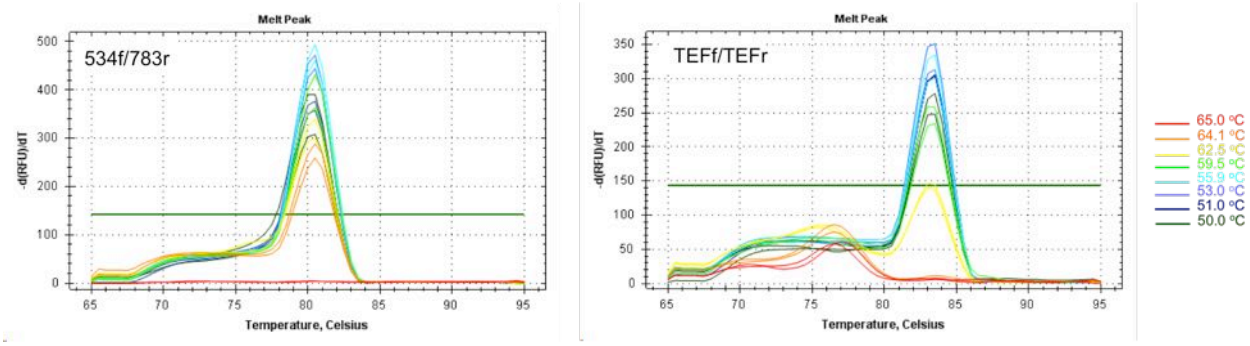


Figure 4.11: Melting peaks for amplicons generated during amplification of bacterial (534f/783r) and plant (TEFf/TEFr) DNA, at different temperatures.

The optimization of primer concentration is exemplified by amplification and melting curves shown in the Figures 4.12 and 4.13, respectively. Concentration around 15-20 pmol is ideal for both primers: 534f-783r and TEFf/TEFr, showing only specific products (one peak in the melting curve) and low number of PCR amplification cycle. Therefore, all the further quantification experiments were performed using 15 pmol of each primer and 56°C and 53°C as annealing temperature for 534f/783r and TEFf/TEFr, respectively.

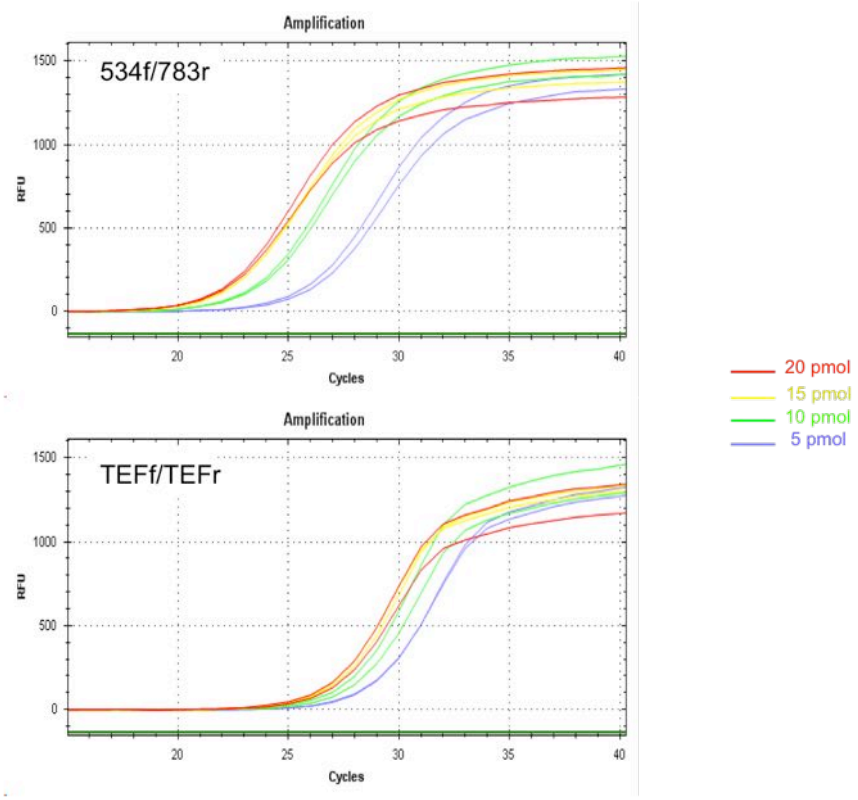


Figure 4.12: Amplification curves for 534f/783r and TEFf/TEFr primers at different primer concentrations.

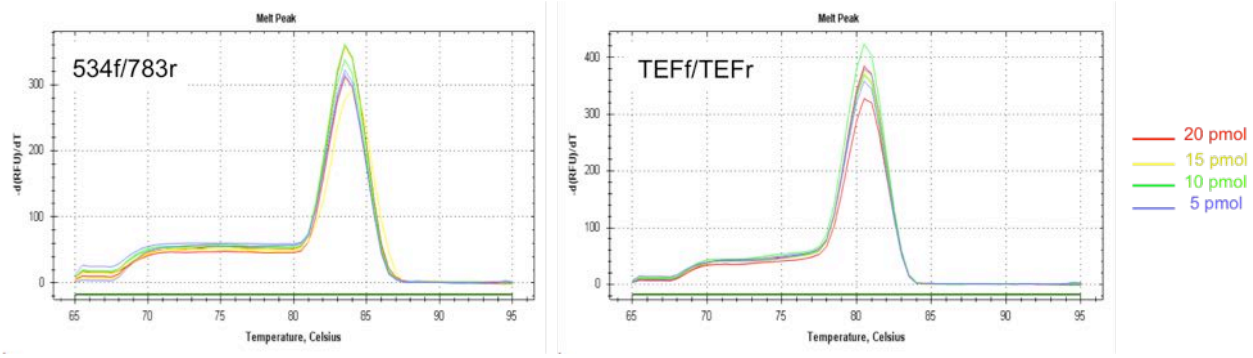


Figure 4.13: Melting peaks for amplicons generated during amplification of bacterial (534f/783r) and plant (TEFf/TEFr) DNA, at different primer concentrations

4.3.4.2 Calibration curve

Many papers report quantification of bacterial cells based on a standard curve generated by dilutions of PCR products [77,82,83]. This approach is common used for quantification of gene expression where the calibration curve is

generated from serial dilution of purified PCR products produced by the amplification of the target DNA. In this case, the absolute concentration of bacterial cells is assessed by combination of the number of PCR copies, DNA concentration, size of the amplicon and assumption that the average molecular mass of a double-stranded DNA molecule is 660 g/mol. [77,84]

This method uses some approximations and does not considerer the analytical parameters involved in the extraction procedure such as recovery, accuracy, precision etc. It is hard to overcome these issues for quantification of gene expression, since it is not possible to generate a calibration curve with the gene expressed in different levels. However, another approach can be used for quantifying bacterial cells. Since a calibration curve can be established using samples with different concentrations of bacterial cells. Therefore we proposed a new q-PCR based method for quantification of bacterial cells in plant tissues.

The calibration curve was generated using plant samples spiked with different amount of bacterial cells. So, a homogeneous sample of *A. brasiliana* tissues was aliquot in 20mg, and for each point of the calibration curve 3 samples were spiked with different amount of bacteria (1.5×10^4, 4.5×10^4, 1.5×10^5, 4.5×10^5 cells). The samples were subjected to DNA extraction according to protocol 2. Plant and bacterial DNA were amplified according to optimal PCR parameters, described in 4.3.2.

The calibration curve (Figure 4.14) correlates the relative gene expression with the amount of bacterial cell added in plant tissue (being TEFf/TEFr the reference gene). Values presented in this graph correspond to the mean between 3 replicates and the respective errors.

This curve shows a good correlation between the amount of bacterial cells (added to *A. brasiliana* tissues) and the relative gene expression (R^2=0.97781). Therefore, this method was used for quantification of bacterial cell in *A. brasiliana* tissues.

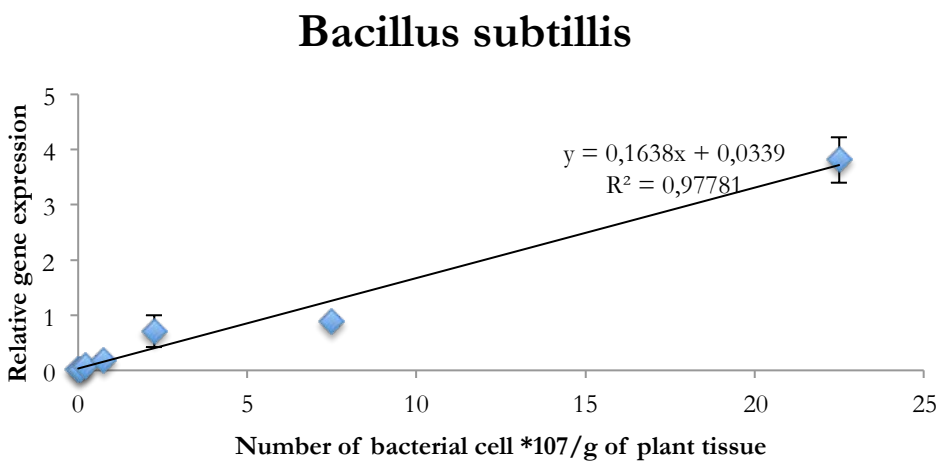


Figure 4.14: Calibration curve for bacterial cell in *A. brasiliana* tissue.

We strongly believe this is a well-designed approach for quantification of bacterial cells in plant tissues. Since it represents a straight correlation between the amount of bacterial cells and the gene expression. Moreover, this method embraces all parameters that can influence the extraction of bacterial DNA. It can be theoretically applied for quantification of both broad range of bacteria (using universal primers, such as 534f/783r) or specific strains (using genera or specie-specific primers). Therefore, this method will be tested with specific primers (for *Bacillus* genera) and for model plants as *Arabidopsis thaliana*. This will be also validated (evaluation of its repeatability and reproducibility), compared with previously published methods, and submitted as a original paper to the Journal of Microbiological Methods.

4.3.5 Quantification of bacteria in different *A. brasiliana* tissues

The first experiments for quantification of bacterial cells in plant tissues were performed with both normal and aseptic plants. For normal plants, stem and leaves were analyzed separately, what was not possible for aseptic plants since the plants were too small and there was not enough plant material to separate stem and leaves. Normal plants were also subjected to surface sterilization in order to

compare the endophytes to the whole bacterial community associated with A. *brasiliana*. This experiment was performed using three biological replicates.

The concentration of bacterial cell in different plant tissues is presented as box plots in the Figure 4.15. Although the groups (leave, leave surface sterilized, leave non-surface sterilized, stem, stem surface-sterilized, stem non-surface sterilized and sterile plants) present high and non-homogeneous variance, these data suggest a big difference between the amount of bacterial cells present in normal and axenic plants. Such huge difference might occur due to the different sizes and stage of development between sterile (around 15 days old) and normal plants (around 4 months old). I this case, the large variance and the small sample size (triplicate) did not allow discrimination between surface and non-surface sterilized plants.

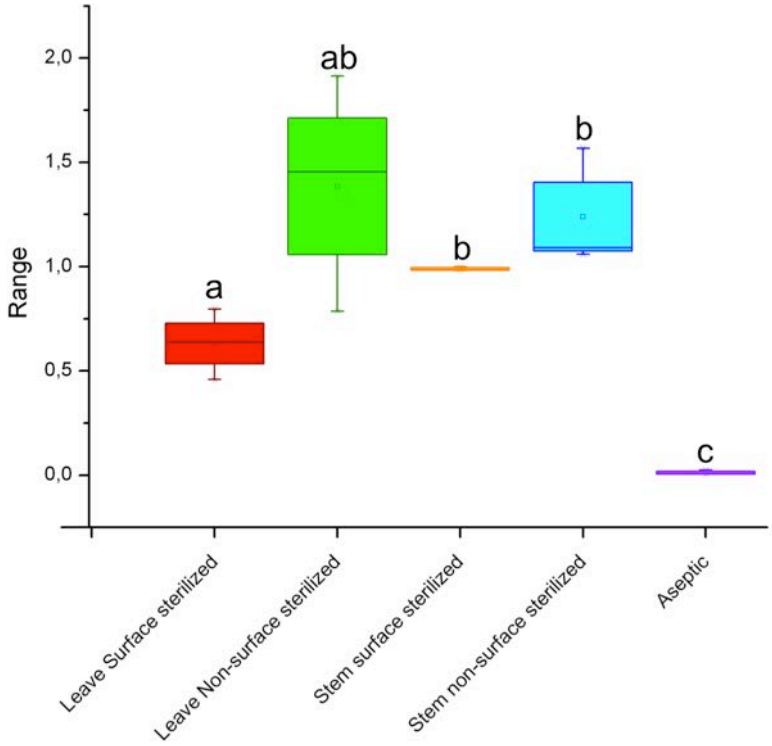


Figure 4.15: Box plot with relative amount of bacterial cells in *A. brasiliana* tissues. Means were compared by ANOVA ($P < 0.05$)

Therefore, a new experiment was designed using plants in the same stage of growth (30 days) and groups with bigger sample size. This new experiment was performed using seven replicates for normal and axenic plants. Plants cultured under normal conditions were also subjected to a surface sterilization to verify the

amount of endophytic bacteria associated with these plants. All plants were extracted and the concentration of bacterial cell in each tissue is shown in Figure 4.16.

Comparison between these data with those presented above, indicates that the big difference between the content of bacterial cells in aseptic and normal plants was related to the different stages of development of normal and aseptic plants used before. Indeed, the number of endophytes associated with normal and axenic plants is different, although it is not so prominent as described before. It suggests that plant might control the number of bacteria living inside the tissues.

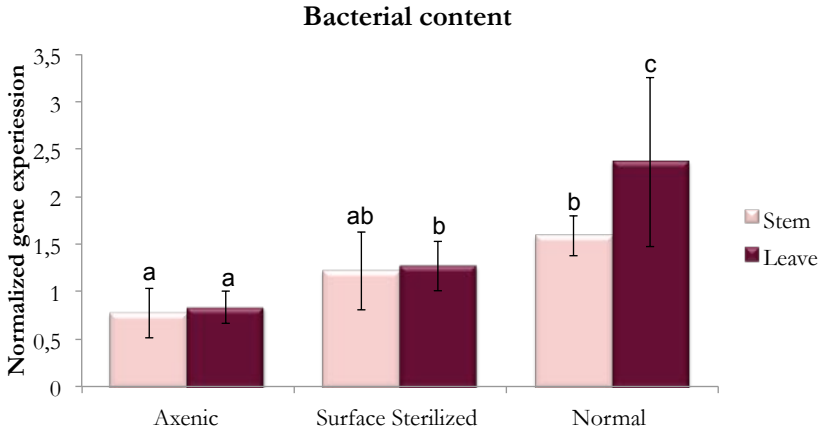


Figure 4.16: Comparison between the amount of bacterial cells in stem and leaves from *A. brasiliana* cultured under normal and aseptic conditions. Means were compared by ANOVA (P< 0.05)

Once, the content of endophytic bacteria is different between normal and axenic plants, all these plants were also analysed by a LC/MS-MS, in order to determine whether this difference in bacterial cells is reflected in the amount of oxylipins present in *A. brasiliana* tissues. However, the method for quantification of oxylipins previously developed, employed samples composed by both stem and leaves. Therefore, the bacterial cells concentration was also evaluated in samples combining leave and stem. In this case, both axenic and normal surface-sterilized plants were evaluated. The results are shown in *Figure 4.17*.

The means (7 samples) were compared for significance using analysis of variance (ANOVA; P<0.05), confirming that the amount of endophytic bacteria

present in plants cultured under normal conditions is bigger than that present in plants cultured under aseptic conditions.

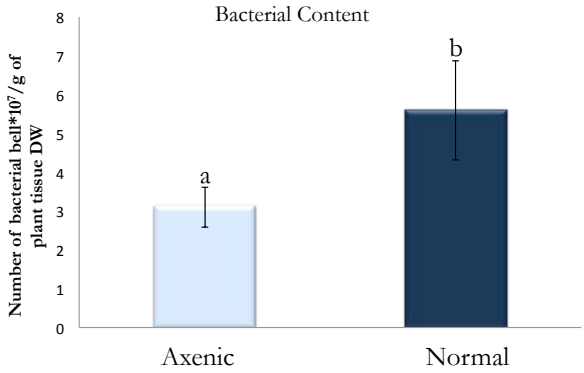


Figure 4.17: Concentration of bacterial cell in different *A. brasiliana* tissues, cultured under different conditions. Means were compared by ANOVA (P< 0.05)

4.3.6 Quantification of oxylipins in *A. brasiliana* tissues

These experiments were developed at the Max Planck Institute for Chemical Ecology, and at that time we did not have all oxylipins standards to transfer the validated method to that laboratory. So, the quantification method was validated only for some oxylipins (for 13-HODE, 9-HODE and 13-Oxo), which we were able to purchase from OlChemin. Therefore, the content of these oxylipins in *A. brasiliana* tissues was evaluated in two ways: quantitatively (for those compounds we had standards) and comparatively for the other oxylipins. The graph with relative content of oxylipins in axenic and normal *A. brasiliana* plants is shown in Figure 4.18 . These data were analyzed by ANOVA test (P<0.05)

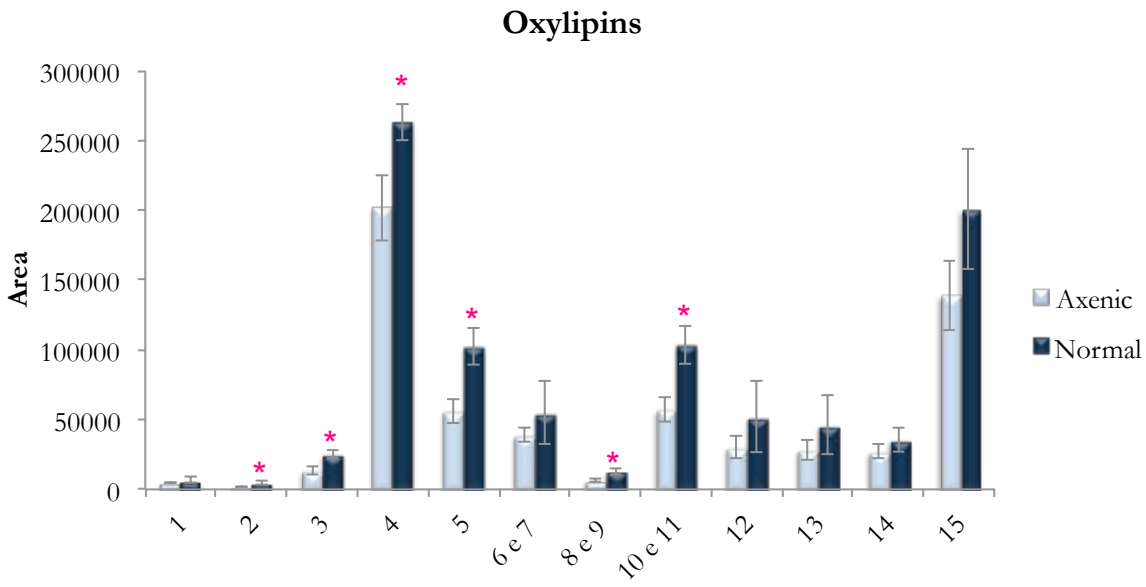


Figure 4.18 Relative content of antibiotic oxylipins in normal and axenic *A. brasiliana* plants. * Indicates significant difference in the amount of certain compound between normal and axenic plants.

Although, these are the comparative results, since the analytical parameters (precision, accuracy, recovery etc) had not been evaluated for all these compounds due to the lack of analytical standards, it suggests that there is a difference in the content of compounds **2**, **3**, **4**, **5**, **8/9** and **10/11** between normal and axenic plants. This difference in the relative content of some oxylipins between normal and axenic plants is confirmed quantitatively for compound **5** (13-HODE), **6/7** (9-HODE) and **8/9** (13-KODE) (Figure 4.19).

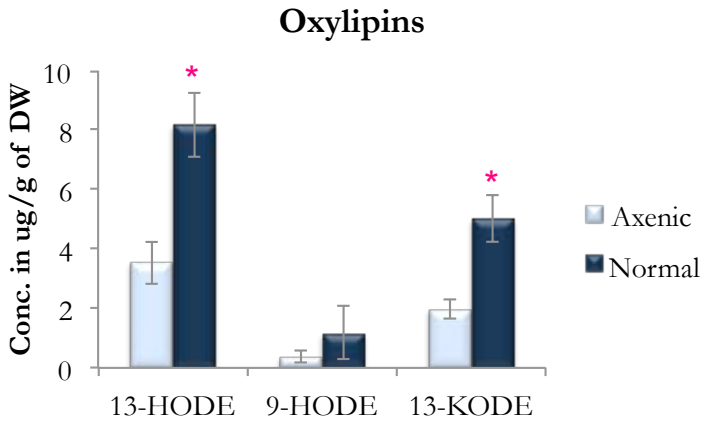


Figure 4.19: Concentration of oxylipins in *A. brasiliana* tissues.

These results suggest a correlation between the content of both endophytic bacteria and oxylipins in *A. brasiliana* tissues. According to previous results (described in the manuscript published in Phytochemistry) compounds **5**, **6/7** and 14/15 are also produced, in vitro, by some of endophytic bacteria isolated from *A. brasiliana* tissues. Trying to get further information about the correlation between oxylipins and endophytic bacteria, we re-inoculated two different strains of *Bacillus* into *A. brasiliana* tissues. This experiment was performed using an oxylipin-producer (*Bacillus megaterium*) and non-oxylipin producer bacteria (*Bacillus atrophaeus*) to evaluate the influence of these bacteria in the content of oxylipins in *A. brasiliana* tissues.

4.3.7 Re-inoculation experiments

Re-inoculation was performed as described in 4.2.7. Each treatment (inoculation of *Bacillus megaterium*; *Bacillus atrophaeus* and MgCl2) was performed in seven replicates. Three samples were not inoculated (they were kept as non-injured control). All these 24 samples were analysed by q-PCR (for quantification of bacterial cells) and by mass spectrometry (for quantification of oxylipins and phytohormones).

Although after 36 hours of inoculation, no statistical significance was observed in the number of bacterial cells within the plant tissue (Figure 4.20), the quantification of oxylipins and phytohormones provided some intriguing results.

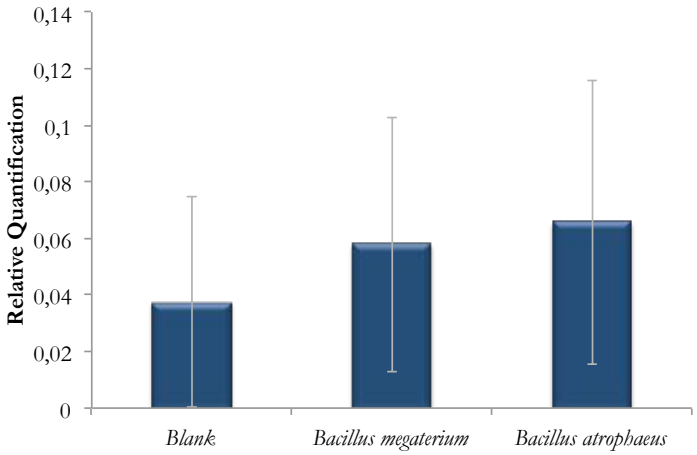


Figure 4.20 Relative amount of bacterial cells 36 hours after the re-inoculation of bacteria into *A. brasiliana* tissues

Many of the oxylipins found in *A. brasiliana* have been already described as stress-response molecules. The activation of lipoxygenases by wounding or infection have also been reported in several plants, what increases the concentration of the oxidized fatty acids. Interestingly, after the inoculation of bacterial cells into plant tissues, only **compound 04** (9*Z*-octadecendioic acid) had the concentration increased, as can be seen in Figure 4.21. This compound has been reported in more that 130 papers. It has also been cited in more than 200 patents. However, it has never been described as a stress-mediating molecule during tissue damaging. Among the 130 papers there is only one reporting that the quantity of this compound increases by around 0,4 in resistant strains of *Arabidopsis* during *Fusarium* attack.[85] However, here the concentration of this compound increased surprisingly 27-40 folds depending on the treatment (Figure 4.21). Although, many other experiments are required to understand whether or not the endophytes contribute for oxylipins production in *A. brasiliana*, these results bring a new molecule to be evaluated during experiments dealing with stress response in plants

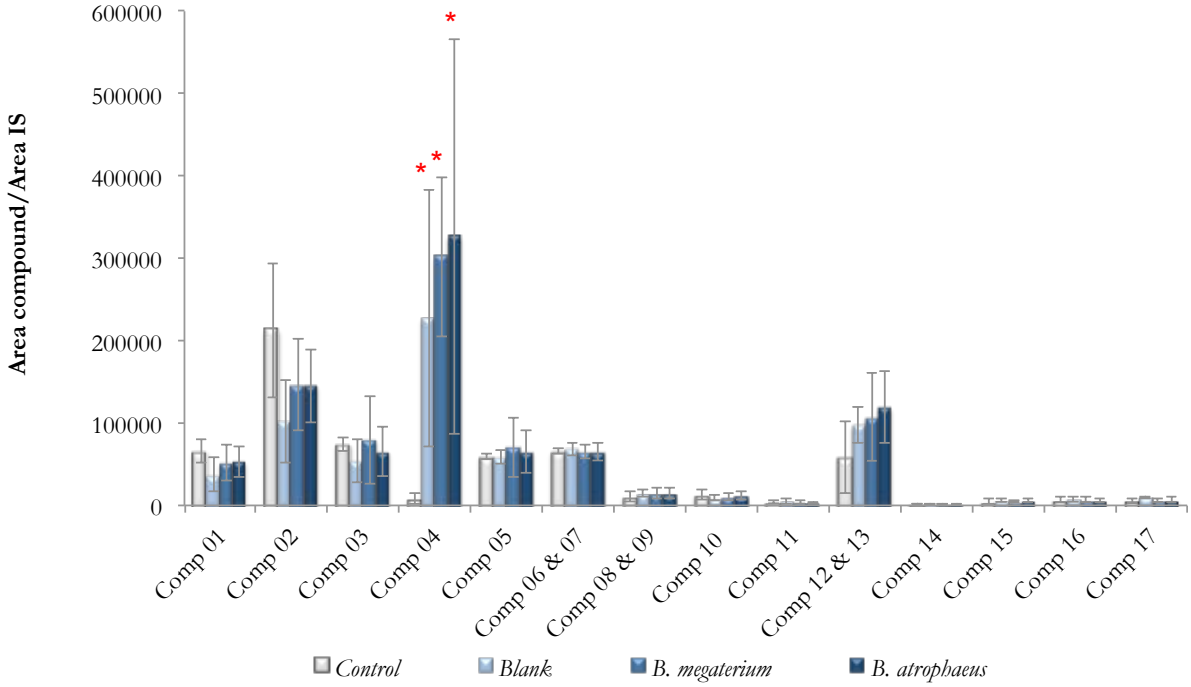


Figure 4.21: Relative amount of oxylipins during re-inoculation experiments

Concerning phytohormones, 36 hours after the tissue damage, only the concentration of salicylic acid and isoleucine-jasmonic acid conjugate had increased significantly. Salicylic acid is a molecule related with activation of defence mechanisms in plant. It activates both local and system acquired resistance. It has been demonstrated that it is related to the biosynthesis of jasmonates during wounding in rice. [86]

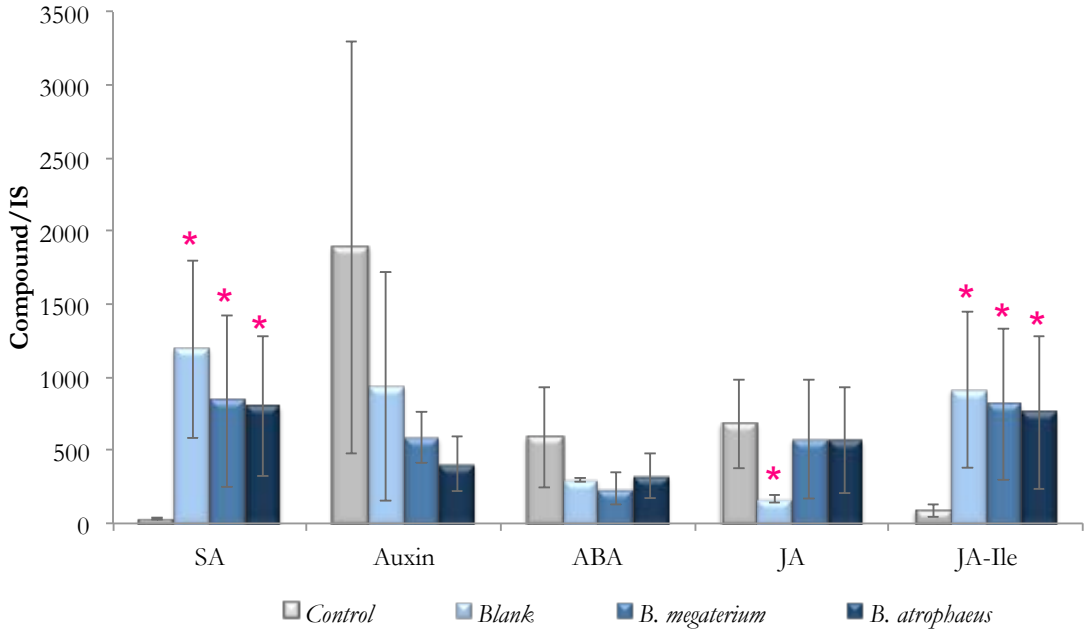


Figure 4.22: Concentration of phytohormones after re-inoculation experiments

Although, it is not possible to establish a direct correlation between SA and JA, the behaviour of jasmonic acid during this re-inoculation experiment is also interesting: in control samples (those inoculated with $MgCl_2$) the concentration of JA decreases significantly, what is not observed for samples inoculated with bacterial suspension. It is well known that plant defence system is through a network of signalling cascades, including jasmonates and salicylic acid. The antagonism between JA and SA has also been reported many times in rice, maize and *Arabidopsis*.[75,85] However, nothing has been published about different responses of JA during wounding and infiltration of microorganisms as we reported here. Although, many other experiments have to done in other to understand the role oxylipins, phytohormones and endophytes in *A. brasiliana*, the results presented during this project show that more factors (i.e. new molecules, biosynthetic links) can be involved in the biosynthetic cascade of oxylipins and phytohormones.

4.3.8 Qualitative analyses of culturable microbiota associated with A. brasiliana

A higher content of oxylipins was detected in plants cultured under normal conditions than in those cultured under axenic conditions. So, we have speculated that these two factors (oxylipins and bacteria) could be correlated and we developed some tools to quantitatively analyse these factors. Unfortunately, the correlation between them could not be established in a qualitative way yet.

Vollenweider and co-workers, have shown that the concentration of some oxylipins increase much more when *Arabidopsis* is infected by certain kind of bacteria.[31] So, we are now developing a method to identify culturable bacteria associated with different *A. brasiliana* samples, trying to understand if the increase of oxylipin content could be related to a specific type of bacteria. This work has being done in cooperation with the undergraduate student Natalia Ritez Rivaben. The isolation of bacterial strains associated with different plant tissues is described in 4.2.7. We are still optimizing the amount of plant tissues, the culture media and incubation time in order to get as much strains of bacteria as possible.

The preliminary results shown in Figure 4.23, suggest that the culture medium has a strong influence on the type of bacteria will be isolated. Moreover, it seems that the microbiota associated with leaves a stem of *A. brasiliana* are slightly different.

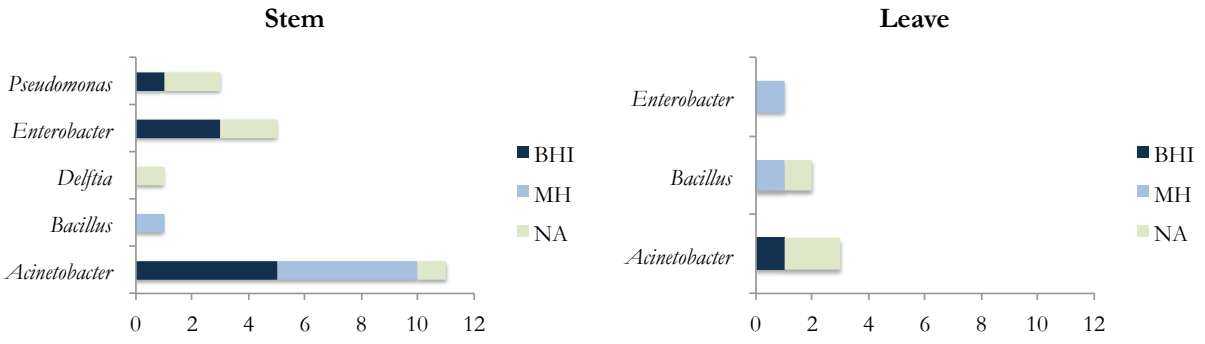


Figure 4.23: Culturable microbiota associated with *A. brasiliana*

As mentioned before, this method is being optimized, and it has to be validated in order to ensure that the differences do reflect the difference in the associated microbiota. However it is a promising technique to study quali and quantitatively the culturable microbiota associated with plants.

4.4 References

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5

Final Considerations

Alternanthera brasiliana is a plant widely used in Brazilian folk medicine, and many papers had confirmed its antibiotic properties. However, there was no association of this biological activity with the compounds present in *A. brasiliana*. Using dereplication strategies we were able to perform an efficient bioguided assay, isolating and identifying just the compounds responsible for the A. brasiliana antibiotic activity.

Once the antibiotic compounds were identified as oxylipins, we realized that there was something even more interesting about them, than only their antibiotic activity. These compounds seem to be involved in an intriguing ecological relationship between *A. brasiliana* and its endophytes. In order to investigate whether there was a correlation between oxylipins and endophytes axenic cultures of A. brasiliana were established. The PCR analyses revealed that, even the plants cultured under axenic conditions were full of bacteria. Moreover, microscopy analyses proved that these bacteria were leaving no only inside the tissue but also inside the cells. Suggesting they are real endophytes, which are vertical transmitted via seeds.

Since, it was not possible to get plants absent of bacteria, a method for quantification of bacterial cells using q-PCR was developed. Moreover, we also developed and validated an analytical method for quantification of phytohormones and another one for quantification of oxylipins, trying to get a better overview of these factors (oxylipins, phytohormones and bacterial cells).

During the validation of these two methods, it has been shown, trough matrix effect, repeatability and reproducibility, the importance of the evaluation of analytical parameters before quantifying compounds in complex samples. It has been highlighted, for instance that the quantification using calibration curves prepared in solvent can give misleading results due to the influence the matrix can have over some compounds. It was also shown that the concentration of endogenous compounds (i..e OPDA) can vary a lot during the sample preparation.