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METABOLOMIC ANALYSIS AND BIOLOGICAL ACTIVITY OF PLANTS UNDER ABIOTIC AND BIOTIC STRESSES

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Summary

The interest in the use of the "-omic" approach in analyzing plant tissues has shown explosive growth in recent years. In fact, metabolomic analysis is used to study plant metabolome in various fields of research, including to study plant stress. In general, it is known that plants subjected to various types of stress, both abiotic and biotic, undergo a variation in the plant metabolome. Plant metabolome is the set of all the primary and secondary metabolites found in plant tissues. Metabolomics aims at the extraction, identification, and analysis of these metabolites, and it provides a “fingerprint” of the status of the plant. By comparing the NMR spectra of two different growing conditions, general information about the metabolite changes can be carried out. The proposed project aims to determine the metabolic profile of natural and cultivated plants suffering different types of plant stress. After an Introduction reported in Chapter 1, a comprehensive review of the literature has been produced in Chapter 2 summarizing the papers published in the literature using metabolomics analysis on different plants, methods of analysis and applied stresses. Among these, drought stress is a common phenomenon due to a reduced water availability in some world areas. Drought stress consistently changes the metabolome of plants. Chapter 3 is focused on a review article of *Moringa oleifera* Lam., a drought-resistant plant known for its pharmacological properties. Following, chapter 4 deals with the metabolomics analysis and antimicrobial activity of the plant leaves and seeds under drought stress. Another very common stress is due to fungi attack to either natural or cultivated plants. Chapter 5 reports a field study on lettuce, *Lactuca sativa* L. heavily infested by *Fusarium oxysporum lactucae* (FOL). The effect of foliar fertilization treatments on metabolome were analyzed. Fertilization was based on the use of i) a commercial organic fermented plant material substrate named compost tea; ii) *Arthrospira platensis* named Spirulina; iii) *A. platensis* previously exposed to a natural uptake of FOL DNA to test the idea of self-DNA inhibition as proposed recently.

Metabolomics studies were performed by using Nuclear Magnetic Resonance (NMR) Spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS) followed by chemometrics to determine the metabolite profiles of the studied plants.

Chapter 1 - Introduction: Plant stress in a nutshell

1.1 - What is plant stress?

Plant stress is a common phenomenon that can limit plant growth, development and productivity, and in some cases, it can cause severe damage to plant tissues. Environmental stress factors include abiotic stresses, caused by non-living agents, such as high and low temperature, drought, flooding, salinity, heavy metals, and biotic stresses, caused by living beings, such as pathogens, pests, and herbivores. However, in most of the cases stressed plants can survive, shifting their metabolism towards a new homeostasis, and adapting to the new growing condition (Obata & Fernie, 2012). The stress still causes some mild or severe negative effects on physiological, morphological, and biochemical changes in plants, including decreased photosynthesis, reduced water uptake, and insufficient nutrient absorption (Shulaev et al., 2008). Plant abiotic and biotic stresses are the primary events that lead to reduced crop yields, reduced food security, lower quality of plant products, and in some cases, they can lead to plant death (Xu & Fu, 2022). Understanding the mechanisms of plant stress response is crucial for developing effective strategies to improve plant growth and productivity under stress conditions.

1.2 - Plant stress in current scientific literature

Plant stress has been the subject of an increasing number of articles and reviews since 1990. From 940 articles published in 1995, scientific research on plant stress has resulted in over 23000 articles published in 2022 (Figure 1.1). The rise in interest can also be attributed to the rising importance of -omics technologies, including metabolomics, genomics, proteomics, transcriptomic and other techniques, which allow plant stresses to be categorized from different perspectives.

Metabolomics, as well as other -omics technologies, are useful for their ability to detect molecules derived from metabolic processes. Changes in the levels of certain molecules or classes of molecules can be seen as a specific response of the studied genotype.

Metabolomics has been used in several fields during last decades. Sumner et al. (2003) provides a wide overview of the history behind this technology. The first use of metabolomics dates back to the 1970s, when Horning and Horning (1971) used GC–MS to identify a set of organic compounds, and then created the expression “metabolite profile”, i.e. the qualitative and quantitative characterization of the metabolites that can be found in a solution. In the 1980s, the first applications of NMR were performed by Bales et al. (1984), who used ^{1}H-NMR to characterize human urine by identifying several metabolites, and Nicholson et al. (1984), who used NMR proton spectroscopy to compare serum, blood and urine collected from diabetic and non-diabetic people subjected to fasting. As the interests in this tool was rising, in 1990s roughly a dozen studies were published per year. Sauter et al. (1991) were the first researchers to use metabolomics in plants, using GC-MS to find and identify a wide variety of metabolites extracted from treated and non-treated barley seedlings. The herbicide treatments showed a consistent response in the result of the analysis, providing the first diagnostic approach of metabolomics in plant subjected to a stressful condition. From that moment on, many works focused on plant metabolomics started to flourish, increasing year after year. Most works who study plant stress from a metabolomic point of view are focused on two main objectives. The first objectives are aimed at researching on the role of single or multiple plant metabolites in plant stress resistance. The works are usually conducted by comparing tolerant and non-tolerant genotypes subjected to one or multiple plant stresses, and then they analyze the impact of the stress on the metabolite by remarking increased and decreased metabolites. Moreover, they try to give an explanation to the variation of specific metabolites or classes of metabolites, trying to find a correlation between the variation of metabolites and the ability to resist the stress. The second objectives are aimed at characterizing the metabolome of the plant subjected to the stress, obtaining a metabolomic fingerprint of the stressed plant that can be useful in future studies to further understand how plant stress works, how the plants react to it and how to reduce the impact of the stress and help the plant to recover from it.

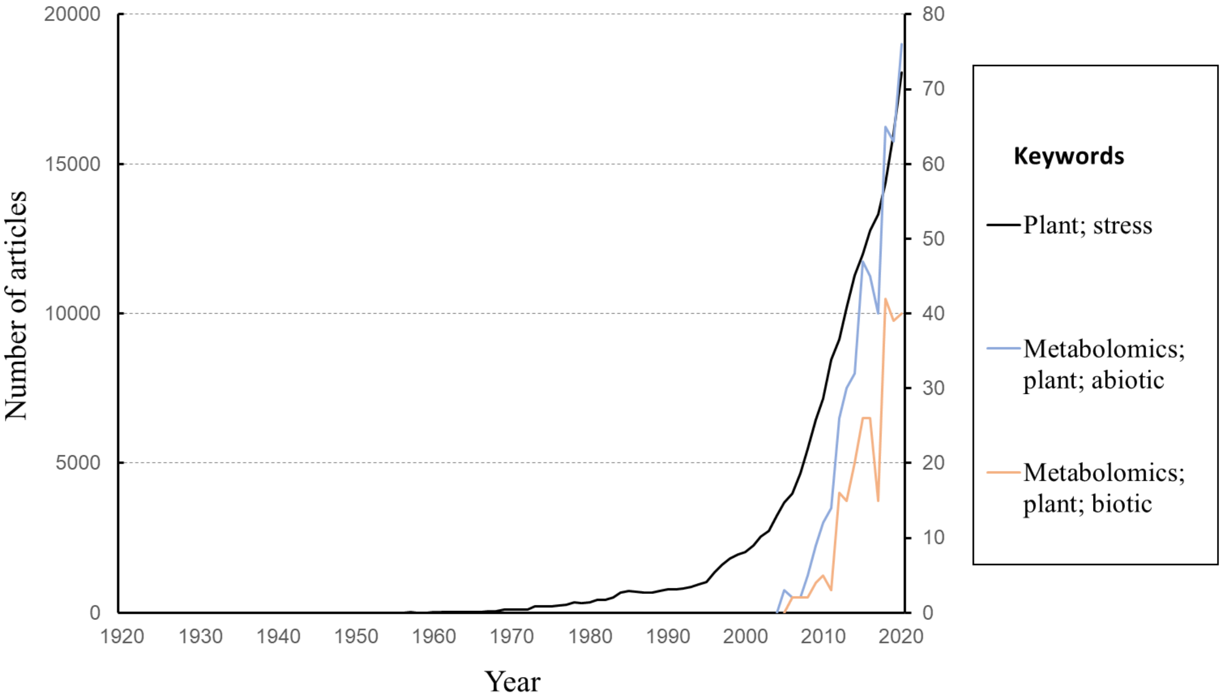


***Figure 1.1**. Number of studies found in Scopus database using different keywords combinations.*

1.3 - Plant-Soil Negative Feedback

Plant-soil negative feedback (PSNF), also known as soil sickness, is a well-known process occurring in nature, noticed by humans especially when cultivating multiple crops in the same field. This phenomenon results in a reduced growth of the same crop if it is repeatedly grown in the same soil portion and the negative effect of re-growth is even stronger when the plant is subjected to extreme environmental stresses (Ehrenfeld et al., 2005). The reasons behind the occurring of this phenomenon have been studied for a long time, and it has been avoided in the past times by using crop rotation. There are multiple factors that can play a role in PSNF. Depletion of soil nutrients is the ability of plants to consume the nutrients present in the soil and replace them with new nutrients coming from the decomposition of the litter (the set of leaves, roots, branches, and other aerial parts that fall from the plant). For some plants, the litter quality is not high enough to replace the nutrients available in the soil, and the next generation of plants cannot find the needed nutrients (Bennett & Klironomos, 2019). Another important factor can be the presence of microbes and pathogens that can affect the growth of the next generation of plants. When a culture dominates a field area, the presence of the roots and of the relative exudates can select a specific set of microorganisms that thrive in that soil. As the plants are replaced with other plants of the same species, the previously selected microorganisms present in the soil are ready to attack the plant and reduce its growing potential. At the same time, planting a different plant species on the same soil would often promote the growth of the plant, because the microorganisms present in that soil could not be able to attack a different plant. The same type of impact could be attributed to nematodes and other invertebrates, but they have been much less studies than microorganisms (Bennett & Klironomos, 2019; Kardol et al., 2007). Other studies have investigated the effect of several chemical compounds released by the plant litter, and that can inhibit the plant itself, including organic acids, saponins, coumarins and other organic compounds. Microorganisms transform such compounds and the products of these processes can cause different degrees of auto-toxicity on the next generation of plants. However, the described mechanism does not explain long-term duration of negative feedback, given that these molecules do not persist in soil for a long time, and undergo several degradation processes in a short period.

PSNF is a phenomenon observed mainly in terrestrial environments, while in aquatic environment the effect is not persistent, probably because the chemical compounds are carried away by water. This simple fact, together with the long persistence and the species-specificity of the PSNF effects in the environment led to the raise of a new hypothesis proposed by Mazzoleni et al. (2015a). The group proposed self-DNA to be the main driver of the PSNF, hypothesizing that DNA produced by the plant and released in the soil by plant litter can inhibit the growth of the next generation of plants of the same species. To prove this hypothesis, they set up an experiment, exposing 9 different plant species to their own self-DNA and to a heterologous DNA (both fragmented and non-fragmented). They found that root growth was inhibited by the addition of the self-DNA, and the growth inhibition was dependent on the concentration of the DNA supplied. Only the fragmented (<500 bp) self-DNA showed the inhibitory effect. They also found a species-specific inhibitory effect of decomposed plant litter on plant growth and development, providing evidence of the role of self-DNA as one of the drivers of PSNF. Moreover, the application of a metabolomic approach to plants subjected to this conspecific inhibitory effect showed an interesting trend regarding the nucleic acids, that were negatively correlated with their root growth. They have subsequently hypothesized that such inhibitory effect of the fragmented conspecific extracellular DNA could be due to short sequences of nucleotides that can interfere with the translation process from mRNA to proteins, or can make the genome less stable (Mazzoleni et al., 2015a). This hypothesis is supported by the fact that there are some nucleic acid-based drugs whose functionality is based on the action of small oligonucleotides that interfere with the formation of specific proteins (Mazzoleni et al., 2014). Then, the same group decided to expand the application of the self-DNA inhibitory capacity to other organisms belonging to other living kingdoms: the species Bacillus subtilis, *Physarum polycephalum*, *Scenedesmus obliquus*, *Trichoderma harzianum*, *Acanthus mollis*, *Sarcophaga carnaria*, were tested by exposing them to self-DNA and heterologous DNA, both compared with the control. The results confirmed that the mechanism of self-DNA inhibition can be considered as a general mechanism occurring in all living beings, since the experiments found a similar inhibitory effect in each tested species (Mazzoleni et al., 2015b).

More investigations were conducted on *A. thaliana* leaves extracts, to further understand the metabolomic shift in the early plant stress response. *A. thaliana* plants were divided in four different sample groups: control, noself treatment with *Zea mays* DNA, noself treatment with *Clupea harengus* DNA and self-treatment. After the appearance of the first true leaves the plants were treated: control treatment consisted of distilled water, the two noself treatment consisted in the addition of fragmented DNA from *Z. mays* and from *C. harengus*, and the self-treatment consisted in the addition of *A. thaliana* DNA. All the DNA added was at a concentration of 200 ng/µL. The plants were grown for 15 hours and sampled after 1, 10 and 15 hours. The treatments were monitored by metabolomics analysis, obtained with an integrated approach of ^{1}H-NMR and LC-MS, which allowed the identification of several primary and secondary metabolites. No general trends were found for most of metabolites, except for nucleosides. A significant increase of ribonucleosides adenosine, guanosine, cytidine and uridine was noticed in the self-DNA treatments. Moreover, an accumulation of the following nucleotides was found in the same treatment: adenosine monophosphate (AMP), guanosine monophosphate (GMP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), the dimeric cyclic dinucleotides c-di-AMP and c-di-GMP, and the methylated adenosine monophosphate, N6-methyl-AMP (m6A). Conversely, no differences in nucleosides content were found for the no-self treatments compared with the control. Such increase could be explained with a dramatic reduction of the metabolism level of the plant. Thus, the nonself-treatment shows no variation of nucleosides, probably because the treatment does not hamper plant metabolism, and the nucleosides are used for translation and transcription processes (Lanzotti et al., 2022).

1.4 - The role of metabolomics

Metabolomics is a recent technology that focuses on the analysis of metabolomes, which are the collection of all the small metabolites (less than 1500 Dalton) produced by the metabolic processes (Obata & Fernie, 2012). This approach is used to determine the metabolites profile of bacteria, fungi, animals, plants and could virtually be suitable for every type of organisms. The outcome of metabolomic analysis is the molecular characterization of the metabolome extracted from the entire organism, from one of their tissues, or from single cells, and the analysis of them provides a metabolomic fingerprint, i.e. a snapshot of the current state of the organism. The metabolome is the final product of gene expression and protein activity of the plant, and comparing fingerprints of control and treated samples allows for the characterization of the effect of the treatment. (Xu & Fu, 2022). There are two types of metabolomic analysis. Targeted metabolomic analysis is performed using chromatographic techniques to separate a specific class of metabolites and focus the analysis only on few metabolites or on that single class of metabolites. On the other hand, untargeted metabolomics detects a large number of metabolites without the need for chromatographic separation, and helps in building a comprehensive overview of the set of metabolites contained in the organism or the tissue (Katam et al., 2022). After the separation of the metabolites, the analysis is usually performed by spectroscopic techniques: the two most commonly used approaches are Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS). No single analytical technique can cover the whole metabolome possibly present in the analyzed sample. Therefore, to obtain a comprehensive metabolome, two or more techniques can be used, each suitable to detect a different set of metabolites. However, when metabolomics is used for this purpose, it can only highlight which metabolites are changing after the stress condition has been applied. It cannot give information on how the stress is modifying the metabolism of the plant, and which metabolic pathway is enhanced or suppressed by the stress. Therefore, metabolomics is often integrated with genomic, transcriptomic, or proteomic approaches that helps to reach a deeper understanding of the metabolic shift that is obtained by the application of a plant stress.

Choosing the right technique among the different chromatographic and spectroscopic methods is not easy, and the choice should be made based on the set of molecules or class of metabolites that are expected to be found in the plant tissue. Plants contain countless metabolites, divided into primary and secondary metabolites. Primary metabolites are the molecules that are directly involved in the main growing processes of the plant, e.g. tissues development and reproduction, and are generally involved in the production of the main macromolecules, such as carbohydrates, lipids, and proteins, that are important for basic biological processes. Secondary metabolites are not involved in these processes, but are produced to help plant interaction with the surrounding environment, and therefore they usually have a protection function, for example antioxidant molecules, antimicrobial molecules or self-produced pesticides.

A typical metabolomic workflow consists of few steps. Sample collection, extraction, analysis of the extracts, data processing, and statistics are usually performed in this order to complete the experiment. Sample collection is a critical step, especially when performing an in-field experiment. The harvesting method should be accurately planned before starting with the cultivation of the plant, and when possible, the different groups of plants (i.e. plants subjected to different treatments) should be cultivated in triplicate sets, and randomly positioned in the cultivated site. Analogously the choice of the plant or the tissue to harvest should be randomized. These precautions must be taken to avoid biases due to the eventual differences in soil composition and irrigation in the chosen field. The extraction methods are a key step that need to be validated with the evaluation of their efficiency. To choose the best extraction protocol, several aspects of the extractions need to be evaluated, such as the solute-solvent interactions, the dissociation of the solute or analyte from the starting matrix, the effect of the pH that could result in the same molecule differently protonated in different samples, the contact time, and the diffusion of the metabolites in the different phases generated by the polarity of the solvents used. Depending on the objectives of the experiment, the analytical method is then chosen based on the molecules of interest and by evaluating advantages and disadvantages of each technique. Each of them has different sensitivity, selectivity, execution speed, and other important parameters (Obata & Fernie, 2012; Sumner et al., 2003).

^{1}H-NMR is the technique used primarily when in need of a fast method that can provide a complete overview of the metabolome. Some advantages of this technique are: the wide range of applicability, since it can be used with almost any organism; the ability to analyze the sample without destroying it, with the possibility to recover it and use it for other analysis; the very easy sample preparation needed due to the fact that, most of the times, no further steps are required apart from the extraction and the dissolution of the extract in the appropriate solvent for the NMR analysis (Katam et al., 2022). The disadvantages of this technique are the relatively low sensitivity and resolution, that allows the characterization of a small numbers of metabolites if compared with techniques based on Mass Spectroscopy.

LC-MS and GC-MS are often used for their high sensitivity, since they rely on the Mass Spectroscopy part that allows the identification and quantification of metabolites that are present in very low concentrations. They are more suitable than NMR for the targeted metabolomic approaches because of the ability to detect all the target molecules that the experiment requires, even at very low concentrations. GC-MS is limited to the analysis of samples that can be heated up and volatilized with derivatization, and although the outstanding sensitivity and ability to separate the analytes, it cannot be used in every situation. LC-MS is used in the remaining cases, when the metabolites under studies are thermolabile, and their quantity need to be very accurate. The chromatography separates the analytes very well and the various choices of ionization and detection methods makes this technique suitable for almost every type of substance, with a high accuracy and resolution, although it’s a quite expensive technique. (Razzaq et al., 2019; Sumner et al., 2003).

Once the data have been collected, they have to be processed and interpreted. From the obtained spectra, metabolites are annotated and identified using the information available in scientific literature. The set of raw data acquired from the spectroscopic technique is usually very large. So, in order to make them simpler and easier to visualize, many tools can be used to process the raw data. The analysis of the data output is very complex, requiring advanced multivariate statistical methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA), orthogonal partial least squared-discriminant analysis (OPLS-DA), or analysis of variance (ANOVA), depending on the obtained data and on the objectives of the study. After the data have been processed, they are interpreted and used to describe the changes related to the applied stress.

Chapter 2 - Metabolomics in plant stress

2.1 - Why metabolomics to understand plant stress?

As discussed earlier, plant stress can be deciphered and interpreted with the use of a metabolomic approach. During last decades, a large number of studies have tried to characterize each single plant stress or a combination of multiple stresses by highlighting the metabolites that were up-regulated or down-regulated as a consequence of the applied stress. When a sufficient amount of literature about each stress was published, researchers tried to understand how the metabolomic shift was correlated with the stress application.

In this regard, we conducted a review work that resumes the state of the art of the use of metabolomics in the study of plant stresses. 151 studies were considered, which showed the effects of several plant stresses on the metabolome of different tissues from many plant species. The list of the studies was carried out by searching for some keywords in Scopus database, and then analyzed one by one. General and specific information about each plant stress was carried out (Table 2.1, Figure 2.1A and 2.1B)

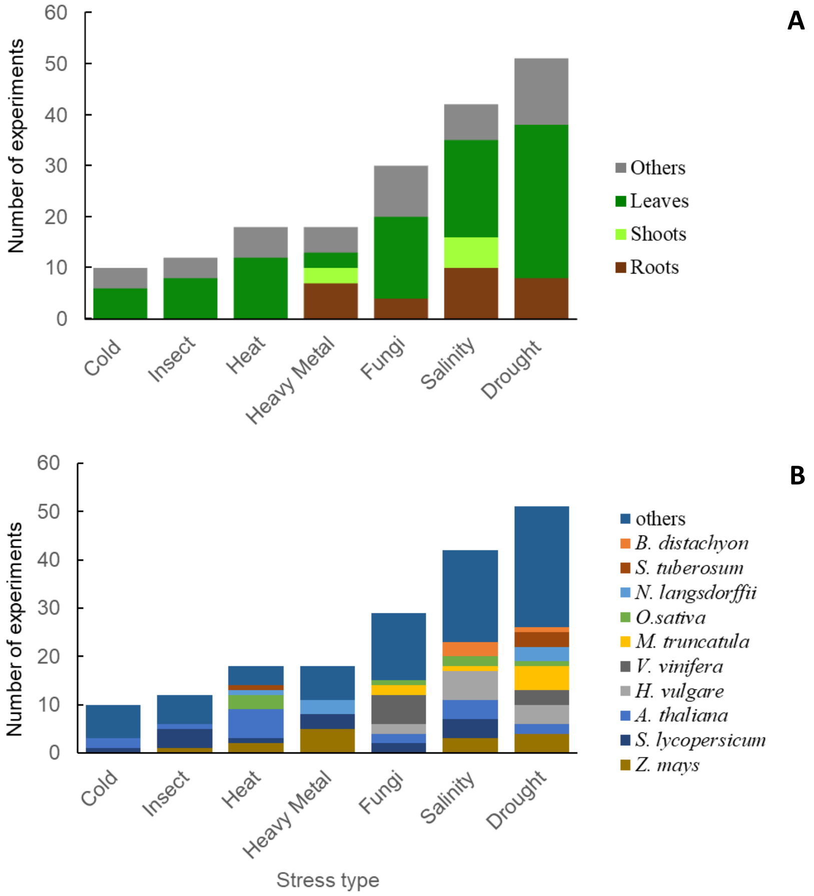


***Figure 2.1**. Bar plots showing different type of studies performed in plant metabolomics: **A)** studies classified according to different plant tissues and sorted by type of applied stress. **B**) Studies clustered by plant species and sorted by type of applied stress*

2.2 - Drought stress

Reduced water availability is a common phenomenon in some world areas, and the impact of this stress is growing due to the effects of climate change. When plants are subjected to drought stress, their reaction can depend on multiple factors, primarily on the plant species. But some common changes are found: the first physiological response is a general adaptation towards improving water usage efficiency: loss of cellular turgor, leaf abscission, reduction of leaf area, closure of stomata and root growth. Because of these changes, a decrease in photosynthesis efficiency is also noticed, which causes an overall reduction of plant development and yield (Chaves & Oliveira, 2004; Xu & Fu, 2022).

Drought stress also seems to decrease the activity of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), with a mechanism that is correlated with the change of stomatal closure inducted by drought stress, although the mechanism has not been clarified yet (Flexas et al., 2006).

Besides these physiological changes, drought stress consistently changes the metabolome of plant tissues. The effects of such harsh condition on the plant metabolome, in our work, was correlated with the rise of amino acids levels, such as β-alanine, leucine, methionine, proline, ornithine, tryptophane, valine, and also the accumulation of sucrose and mannitol. However, the trends of the mentioned amino acids were very complex. For example, proline increased in some studies and decreased in others. But since proline showed an increase way more often than a decrease, we assumed that a general increase of proline was found after the application of drought stress.

The increasing trend of proline was evident. Proline is an important molecule when dealing with plant stresses, because of its osmoprotectant capacity. That is the reason why proline often increases after the application of a stress. Although in some studies proline decreases after drought stress, in the vast majority of the articles we revied, proline strongly accumulated after the application of drought stress. The osmoprotectant activity that proline exerts is achieved thanks to the small size of the molecule, which can adjust the osmotic potential of plant cells subjected to drought stress. The osmotic pressure created in plant cells is important to help the plant in draining water from the soil. Therefore, these small metabolites are needed to balance the osmotic potential once the water quantity inside the cell is reduced, causing the variation of the concentration of the metabolites. Proline is not the only small amino acid that can work as osmoprotectant. There were more amino acids showing an increasing trend, the most consistent ones were valine and tryptophane. Organic acids and sugars can exert a similar function, thanks to their relatively small size. Interestingly, there was a decreasing tendency for organic acids, but an increase of ferulic acid was observed. An explanation to this tendency might be that after exposure to drought stress, plants need a carbon source and organic acids might be suitable for that. Therefore, organic acids seem to be useful in both ways. This is an example of how complex plant metabolism is. Jasmonic acid is another organic compound that was observed to increase consistently after drought stress. It is a phytohormone useful in signalling mechanisms. Jasmonic acid was found to help plants to withstand drought, salinity and cold stressed plants (Feng et al., 2020), as the active form of it, methyl jasmonic acid, is able to upregulate specific genes, especially those involved in proline production. Jasmonic acid also upregulated the production of polyamines, sugars and sugar alcohols, other osmolytes that exert similar protecting functions (Bandurska et al., 2003; Gális et al., 2006). In a similar way, abscisic acid is a plant hormone that mitigates the consequences of plant stress. Several of the analyzed articles showed an increase of abscisic acid after drought, and it seems that the role of this phytohormone is important in the regulation of stomatal closure process and in the gene expression (Ali et al., 2020). Various articles have highlighted a correlation between abscisic acid content and stomatal conductance, which is a parameter used to measure gas exchange that has emerged from various studies. There is some evidence that shows the role of abscisic acid in the transcription of various metabolite-related genes, such as proline, polyamines, sugar and sugar alcohols, all of them being very important in the mitigation of drought stress (Pattanagul et al., 2011; Alcázar et al., 2006; Kumar et al., 2012; Sarafraz-Ardakani et al., 2014). Polyamines have also showed an important activity in improving drought stress resistance. Putrescine, spermidine and spermine have been studied to investigate this aspect (Jorge et al., 2016; J. Yang et al., 2007). Thanks to the large amount of data we collected, we could extrapolate some trends for specific tissues and in specific plant species as well: for example, after drought stress application, phenylalanine increased more in leaves than in the other tissues, whereas in leaves, citric acid decreased. Drought stress caused a specific increase of alanine, threonine, valine, and myo-inositol when only the root tissue was considered. Finally, we found specific trends for: *M. truncatula*, in which drought stress caused the accumulation of cysteine, pyruvic acid, stearic acid and the decrease of asparagine and citric acid; *S. tuberosum*, in which multiple studies showed an accumulation of galactinol; *Z. mays*, with an increasing trend for asparagine.

Table 2.1. *Fold-changes of the principal metabolites that specifically increased or decreased after exposition of each stress typology, to give a general overview of the metabolomic shift tendency.*

Stress	**Metabolite**	**Fold Change**	**Plant species**	**Tissue**	**Reference**
Drought					
	Proline:	15-fold ↑	*Medicago truncatula*	Leaves	Filippou et al. (2016)
		28.6-fold ↑	*Arachis hypogaea,*	Nodules	Furlan et al. (2017)
		16.0-fold ↑	*Arachis hypogaea*	Nodules	Furlan et al. (2017)
		282.3-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
	Tryptophane:	23.8-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
		7.31-fold ↑	*Zea mays*	Leaves	Obata et al. (2015)
	Valine:	6.2-fold ↑	*Medicago truncatula*	Leaves	Filippou et al. (2016)
		5.03-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
	Jasmonic acid:	4.60-fold ↑	*Citrus reshni, Citrus sinensis x Poncirus trifoliata*	Roots	Argamasilla et al. (2014)
		2.4-fold ↑	*Zea mays*	Leaves	Benevenuto et al. (2017)
Salinity					
	Proline:	22.10-fold ↑	*Arabidopsis thaliana*	Leaves	Kempa et al. (2008)
		3.67-fold ↑	*Oryza sativa*	Shoots	Soda et al. (2018)
		1.47-fold ↓	*Cynodon dactylon*	Leaves	Shi et al. (2015)
	GABA:	19-fold ↑	*Arabidopsis thaliana*	Leaves	Kempa et al. (2008)
		4-fold ↑	*Oryza sativa*	Shoots	Soda et al. (2018)
	Serine:	1.62-fold ↑	*Cynodon dactylon*	Leaves	Shi et al. (2015)
		2.09-fold ↑	*Oryza sativa*	Shoots	Soda et al. (2018)
	β-alanine:	1.83-fold ↑	*Hordeum spontaneum*	Roots	Shen et al. (2016)
		2.67-fold ↑	*Hordeum vulgare L.*	Roots	Shelden et al. (2016)
	Galactose:	1.95-fold ↑	*Glycine soja*	Roots	Li et al. (2017)
	Sucrose:	1.74-fold ↑	*Hordeum vulgare L.*	Roots	Shelden et al. (2016)
		1.21-fold ↑	*Arabidopsis thaliana*	Leaves	Kempa et al. (2008)
	Salicylic acid:	2.75-fold ↑	*Poplar x canescens*	Roots	Luo et al. (2009)
		2.62-fold ↑	*Aeluropus lagopoides*	Shoots	Paidi et al. (2017)
Heat					
	GABA:	2.63-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
		1.46-fold ↑	*Glycine max*	Seeds	Chebrolu et al. (2016)
	Threonine:	4.43-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
		1.46-fold ↑	*Glycine max*	Seeds	Chebrolu et al. (2016)
	Valine:	6.51-fold↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
		1.24-fold ↑	*Arabidopsis thaliana*	Seeds	Brito et al. (2020)
		1.2-fold ↓	*Arabidopsis thaliana*	Leaves	Wienkoop et al. (2008)

	Myo-inositol:	4.33-fold ↑	*Agostis stolonifera*	Leaves	Jespersen et al. (2017)
		1.90-fold ↑	*Arabidopsis thaliana*	Leaves	Wienkoop et al. (2008)
	Galactinol:	21-fold ↑	*Arabidopsis thaliana*	Leaves	Wienkoop et al. (2008)
Cold					
	Proline:	17.6-fold ↑	*Arabidopsis thaliana*	Leaves	Wienkoop et al. (2008)
		10.2-fold ↑	*Arabidopsis thaliana*	Aerial Tissues	Hildebrandt (2018)
		1.47-fold ↓	*Cynodon dactylon*	Leaves	Shi et al. (2015)
	Raffinose:	13.6-fold ↑	*Arabidopsis thaliana*	Leaves	Wienkoop et al. (2008)
		2.86-fold ↑	*Miscanthus*	Aerial Parts	Le Gall et al. (2017)
Heavy metal					
	Threonine:	5.78-fold↑	*Chrysopogon zizanioides (Pb stress)*	Roots	Pidatala et al. (2018)
	Abscisic acid:	4.09-fold ↑	*Nicotiana langsdorffii (Cr stress)*	Plant Sample	Fuoco et al. (2013)
		1.91-fold ↑	*Nicotiana langsdorffii (Cd stress)*	Plant Sample	Fuoco et al. (2013)
	Salicylic acid:	13-fold ↑	*Nicotiana langsdorffii (Cr stress)*	Plant Sample	Fuoco et al. (2013)
Fungi					
	Proline:	7.46-fold ↑	*Epipactis helleborine*	Leaves	Lallemand et al. (2019)
		3.73-fold ↑	*Epipactis purpurata*	Leaves	Lallemand et al. (2019)
		2.47-fold ↑	*Vitis vinifera*	Berries	Hong et al. (2012)
		1.71-fold ↑	*Vitis vinifera*	Berries	Hong et al. (2012)
	GABA:	1.56-fold ↑	*Vitis vinifera*	Berries	Hong et al. (2012)
		3.75-fold ↑	*Epipactis helleborine*	Leaves	Lallemand et al. (2019)
		4.17-fold ↑	*Epipactis purpurata*	Leaves	Lallemand et al. (2019)
	Mannose:	7.26-fold ↓	*Cephalanthera damasonium*	Leaves	Lallemand et al. (2019)
		13.45-fold ↓	*Epipactis helleborine*	Leaves	Lallemand et al. (2019)
		5.46-fold ↓	*Epipactis purpurata*	Leaves	Lallemand et al. (2019)
	Abscisic acid:	3.55-fold ↑	*Sorghum bicolor*	Leaves	Tugizimana et al. (2019)

2.3 - Salinity stress

High salinity of soils is a phenomenon that results in sodium accumulation, disturbing the ionic balance and then modifying the metabolism of the plant and other characteristics, such as cellular turgor, growth, photosynthesis, and it influences protein synthesis, lipid and energy metabolism (Skliros et al., 2018). Plant growth is reduced in consequence of salinity stress, especially because it affects leaf expansion and stomata closure. Regarding photosynthesis, several studies found a correlation between increase of NaCl levels and a decrease of photosynthetic activity, due to several factors that are related to reduction of water and CO_2 uptake and reduction of enzymatic activity. (Parida & Das, 2005). Salinity stress affected the content of several amino acids accumulated in the analyzed plant tissues. As explained in the drought stress section, amino acids are useful for the plant as osmoprotectant. Proline mainly increased after salinity stress exposure, showing a very consistent increasing trend. Proline seems to increase water uptake from soil, and it can stabilize and protect cell membrane, enzymes and proteins, according to the study of Arif et al. (2020). Moreover GABA, serine and β-alanine increased in almost every study that detected them. Although several studies proved that polyamines help plants to recover from stress, in the studies we analyzed, polyamines, in particular putrescine, showed a decreasing trend when salinity stress was submitted. Galactose, sucrose, and trehalose increased every time that salinity stress was induced, supporting the strong evidence that sugars and sugar alcohols are involved in plant protection from this stress. Their function in this case is to facilitate the growing process, inactivate Reactive Oxygen Species (ROS), maintain cell turgor, isolate sodium ions, stabilize cellular membrane, protect protein structure from denaturation and aggregation Arif et al. (2020). In particular, the ability of trehalose has long been investigated. Trehalose can form a glassy structure that preserves enzymes functionality and membrane structure, not only in plants but also in other organisms (Abdallah et al., 2016). It also mitigates the variation of osmotic pressure thanks to its osmoprotectant capacity, and inhibits the photo-oxidation process (Skliros et al., 2018).

On the other hand, organic acids showed a general decreasing trend after salinity stress exposure. Despite the fact that organic acids might be useful as osmoprotectant to restore the needed osmotic pressure in plant cells, their production was not stimulated. The decreasing trend, which is similar to the one found in drought stress, might be explained with the need of the plant to use organic acids as carbon source, that will supply the lack of CO_2 caused by stomatal closure. Another hypothesis could be that salinity stress causes a loss of positive ions such as calcium, potassium, magnesium, and that the reduction of organic acids quantity - which can be found in their negative ion form – is necessary to stabilize the ionic balance (Hinsinger et al., 2003; M. A. Khan et al., 2000). However, our review found that caffeic acid and pyruvic acid increased, while fumaric acid and shikimic acid decreased. Shikimic acid is a phytohormone which has been showed to improve photosynthesis, while other studies reported its activity in antioxidative processes and in raising plants tolerance to salinity stress when plants were treated with an exogenous addition of this molecule (Fahad et al., 2015). Some functions of salicylic acid are: excluding and compartmentalizing ions, raising salinity stress resistance; protecting lipids from oxidation processes; adjusting osmotic pressure in plant cells (Singh & Gautam, 2013). We found a strong increase in salicylic acid as a consequence of salinity stress application, confirming the important role of this phytohormone in plant resistance. Glycine-betaine is another important metabolite in salinity stress mitigation. It was showed that glycine-betaine promotes the regulation of the Na^+ /K^+ ratio, helping the cells to better sustain an ionic unbalance due to the presence of ions in the soil. Indeed, as we showed for salicylic acid, the exogenous application of glycine-betaine has improved stress resistance, probably because it is rapidly absorbed when applied on leaves and it can readily be used to reduce ionic unbalance (Ashraf & Foolad, 2007). Glycine-betaine can help proteins by acting similarly to chaperones, promoting protein folding, protein disaggregation, and preserving enzymatic functionality. For this reason, it has a role in avoiding oxidative damage, because this chaperone function of glycine-betaine can protect ROS scavenging enzymes. Moreover, glycine-betaine is also involved in genes expression, which can help in developing stress tolerance (Bourot et al., 2000; Chen & Murata, 2011). The same is true for jasmonic and abscisic acids: they can regulate gene expression and act as signalling molecule for stomatal closure and ion exchange (Kang et al., 2005; Narusaka et al., 2003).

Although some of the analyzed studies showed a variation of glycine-betaine, jasmonic and abscisic acids, the trends were not consistent. However, allantoin and naringenin increased, while caffeoylquinic acid and phosphoric acid were reduced after salinity stress induction. Glucose and cinnamic acid showed an increasing trend, and it became more relevant in leaf tissues only, while β-alanine showed an increasing trend in roots tissues. The amino acids alanine, arginine, asparagine, and glutamate increased in roots only, while fumaric acid, malic acid and succinic acid decreased after salinity stress application. Valine showed an increasing trend in shoots subjected to salinity stress. The analysis by plant species remarked that asparagine increased in *A. lagopoides*, threonine increased in *A. thaliana*, fructose increased and citric acid, raffinose, galactinol, myo-inositol, quinic acid decreased in *B. distachyon*. Arginine, glutamine, valine increased in *H. vulgare*, phenylalanine, threonine, valine increased in *O. sativa*, while threonine and valine were reduced in *T. salsuginea*. Finally, in *Z. mays*, we found an increase of ferulic acid and a decrease of asparagine, glutamine, isoleucine, threonine, linoleic acid.

2.4 - Heat stress

Plants usually have a range of temperature that represents the optimum temperature in which they can grow easily. When the temperature goes far from that range, plants need to adapt through various systems. There is no doubt that plants will be exposed to heat stress in recent future, as the world temperatures are expected to rise. Human activities induced a temperature rise of 1°C since pre-industrial era, and an increase of temperature of 0.1-0.3°C per decade is expected in next decades, according to a recent IPCC report (Allen et al. 2018). Heat stress causes the formation of Reactive Oxygen Species, molecules that cause oxidation of molecules contained in plant cells, including superoxide anion, peroxides, hydroxyl radicals and oxygen molecule in its singlet form.

Although the excessive production of ROS is very harmful, they also have an important function in controlling stress impact in plant tissues (Choudhury et al., 2017). Thus, they are normally produced and deactivated during metabolic processes of plant cells, but when the plant is stressed, they accumulate in larger amounts because the plant struggles to deplete all of them. So, heat stress causes a rapid accumulation of ROS, that causes heat shock protein synthesis (Vacca et al., 2004). Temperature is also a parameter that negatively affects cellular homeostasis, enzymes functionality, growing processes, photosynthesis and plant yield (Hassan et al., 2021; Král'ová et al., 2012). Asparagine, glutamine, GABA, isoleucine, lysine, threonine, valine increased after heat stress submission in all the articles that detected them -valine only decreased in one article-. Sugars and polyols like erythritol, galactinol, mannitol and myo-inositol, also increased. The increase in the amino acid content might be involved with the production of heat shock proteins, like chaperones. Chaperones are very important molecules produced under heat stress, with the purpose of protecting current and newly synthesized proteins from incorrect folding and aggregation (Frydman, 2001; Liberek et al., 2008). Several studies have shown a correlation between production of these proteins and tolerance to heat stress (Hong & Vierling, 2000; Queitsch et al., 2000). Amino acids, as already remarked before, are also useful osmoregulators. Moreover, it was hypothesized that GABA can have a role in protection against oxidative stress (Bouché et al., 2003). Soluble sugars and sugar alcohols have a role similar to the role of the amino acids and their increase after heat stress has been documented previously (Bokszczanin & Fragkostefanakis, 2013). After submitting heat stress, galactinol, mannitol and erythritol also increased in each study that could detect them. Abscisic acid is involved in heat stress as well. The role of this phytohormone is probably to regulate gene expression to make plants more tolerant to heat stress, and therefore, cause the production of heat shock proteins (Ali et al., 2020). Another study shows the ability of absicic acid to produce antioxidant enzymes through gene regulation, and help the plant to defend against ROS (Jajic et al., 2015). In the studies we analyzed, abscisic acid was rarely identified and quantified, and thus we could not estimate the consequence of heat stress on quantity of this molecule. Regarding studies grouped by plants, heat stress caused an increase of raffinose content in *A. thaliana.* Although in previous stress situations proline content was altered, heat stress did not influence the content of proline in a significant way, while other stresses did. This is in agreement with Hua et al. (2001) and Rizhsky et al. (2004).

2.5 - Cold stress

Cold stress is divided in two categories, depending on whether the temperature is higher or lower than the freezing point. We considered only the cold stress occurring at temperatures higher than 0 °C. This stress inevitably slows or stops metabolic processes that usually occur at higher temperatures causing retarded seed germination, delayed growth, withering, leaves yellowing (chlorosis) and wilting, reducing plant yield and also causing oxidative stress (Sharifi, 2010; Yadav, 2009). Low temperature causes solidification of fatty acids contained in the cellular membrane, altering several biological processes, such as photosynthesis and the movements of molecules through the membrane. Similarly to heat stress, cold stress leads to production of some proteins that help the plant to tolerate the stress condition by preventing ice formation and denaturation of proteins (Guy, 1990). This process can protect single organs, or organelles such as chloroplasts (Ul Haq et al., 2019). In our analysis, cold stress induced accumulation of arginine, citrulline, glycine, isoleucine, ornithine, phenylalanine, proline, tyrosine and raffinose. Most of them probably increased for their role as osmoprotectants, very useful in protecting from cold stress as well by regulating of osmotic movements, detoxifying ROS, protecting membrane integrity (Saleem et al., 2020). Proline plays a key role in this stress as well, as it acts as chaperone-like molecule, protecting functionality of many proteins and enzymes, and it seems that high levels of proline are necessary to tolerate cold stress (Saleem et al., 2020; Yadav, 2009). Furthermore, raffinose accumulation seems to be very recurrent in plants response to temperature stress. Apart from their osmoprotectant role, raffinose and other sugar molecules may be useful to avoid cells dehydration and preventing sucrose crystallization. This process helps to create a glassy structure that protects cellular membranes (Crowe et al., 1988; Yuanyuan et al., 2009). Moreover, sugars are important in signalling processes and energy production (Yuanyuan et al., 2009). In our analysis, raffinose consistently increased after cold stress exposure. Abscisic acid can be useful in improving water use efficiency by regulating stomatal closure and shifting gene expression towards the production of the molecules needed to resist, as reported before (Xue-Xuan et al., 2010). Moreover, abscisic acid can control other biological processes such as development of seeds, flowering, accumulation of lipids and proteins, stomata opening (Saleem et al., 2020). The exogenous application of other phytohormones such as salicylic acid can reduce the impact of cold stress by improving seed growth, photosynthesis, osmolyte production, antioxidant activities (Saleem et al., 2020). However, we didn’t find any consistent variation of abscisic acid and salicylic acid. When we only considered leaves, a consistent increasing trend was found for sucrose, that increased in all the cases. Cold stress also led to an increase of glutamine, glutamic acid, valine in the plant *A. thaliana*, which was not registered as a general trend for plants exposed to cold stress.

2.6 - Heavy metal stress

Human activity, in recent decades, caused an increase of heavy metals concentration in soil, such as lead, cadmium, mercury, iron, copper, arsenic, nickel, silver, aluminium, zinc, caused by industrial pollution. Although heavy metals are important for some metabolic processes, when heavy metals overaccumulate, they can cause many problems, because of the strong oxidative potential that can cause damage and metabolic disorders in plant cells, also causing chlorosis, growing inhibition, browning of root tips, difficulty in transferring micronutrients from roots to shoots, alteration in germination process, formation of ROS, disruption of cell lipidic membranes, reduced photosynthetic rate and reduced water uptake due to osmotic stress (Nagajyoti et al., 2010; Syed et al., 2018; Yadav, 2010). Plants avoid intake of heavy metals from soil thanks to an external chelation, using negatively charged molecules such as tartaric acid, aconitic acid, malic acid, oxalic acid, citric acid, malonic acid that blocks metallic ions before they get inside the roots. Moreover, galacturonic acid contained in pectins help cells to retain heavy metals at cellular level (Kosakivska et al., 2021). Chaperones are important in heavy metal stress as well. Some proteins can act as Cu-binding proteins that chelate copper. Metallothioneins are another type of proteins that contain sulphur, that can chelate and sequester metal ions. In our study, increasing trends of threonine, abscisic acid and salicylic acid were noticed in consequence of heavy metal stress. Many other increasing trends for amino acids and sugars were noticed, but not significant enough, also because of the small number of studies considered. The increase of proline after metal stress, probably along with all the other osmolytes, helps plants in osmotic adjustment and more importantly, they promote heavy metals chelation and ROS suppression (Ghori et al., 2019; Hayat et al., 2012; Kholodova et al., 2011). Although heavy metal is very impactful on plant capacity to survive, few trends were found significant in our review, probably because more studies are needed to have a better overview. Abscisic acid increased consistently, and it seems to stimulate the production of molecules rich of -SH groups, called phytochelatin, that are able to block metals and avoid the oxidative mechanisms (Stroiński et al., 2010). Another study suggests that abscisic leads to heavy metals accumulation in roots, by reducing the transportation from roots to shoots, and can also promote antioxidants production to fight the strong oxidative potential of these molecules (Emamverdian et al., 2020).

Salicylic acid showed an increasing trend as well, and according to Nguyen et al. (2021) it can improve glutathione production, antioxidant activity, synthesis of phytochelatin and increase of phytochelatin length. Then, more studies highlighted positive effects of salicylic acid on roots and shoots length and inhibition of lipids peroxidation probably thanks to the mitigation of ROS production, or the sequestration of free heavy metals, but the real mechanism is still under debate (Metwally et al., 2003; Z. S. Zhou et al., 2009). In roots, heavy metal stress increased the content of proline and valine more than it did when the whole plant was considered. In *C. zizanioides*, heavy metals stress caused the increase of glutamic acid, methionine and the decrease of malic acid and fumaric acid.

2.7 - Insect stress

Many insects can grow and develop only thanks to the presence of plants, and they can establish different types of relationship with them. Because of this, plants have adapted to insects with many mechanisms to either avoid them or reduce their impact on plant survivability. Insect oral secretion is very important in insect stress, because it can distinguish between mechanical wounding, which is referred to the simple disruption of the plant tissue, and insect infestation, that is the ability of the insect to use plant nutrients (Devoto & Turner, 2005; Qi et al., 2016). There is a difference in ROS production as well, as ROS levels increase more after herbivory-induced stress than after mechanical wounding. However, some plants did show ROS production after wounding, while others did not (Leitner et al., 2004). Similarly to heat stress, herbivory stress seems to cause a burst of ROS production by the infested plant, trying to defend itself from the herbivore attack (Leitner et al., 2004). When specific molecules secreted by insects are sensed, plants can recognize them, recognize the infestant - in a way similar to the immune system - and act consequently with specific defensive mechanisms, an example of it could be the production of hormonal substances. Jasmonic acid and jasmonates, for example, are important molecules involved in this stress. As stated earlier, they can shift gene expression, help plants in defensive response and reproduction, and ethylene and salicylic acid have a similar role as well (Devoto & Turner, 2005; Gimenez et al., 2018; Howe & Jander, 2008). However, every plant species responds very differently to insect infestation, and it is not easy to find a common pattern because of the wide variability (Wu & Baldwin, 2010). In our analysis, insect stress only showed an increasing trend for the amino acid phenylalanine.

2.8 - Fungi stress

Fungi stress led to the increase of some amino acids, GABA, histidine, isoleucine, methionine, proline, serine and some other compounds, including allantoin, ferulic and abscisic acid, while fumaric acid, coumaric acid and mannose decreased. As explained before, all these metabolites help the plant through their osmoprotectant capacity. Glutamic acid is also a precursor of proline and GABA, as stated in Forde & Lea, (2007). Contradictory results were found for sugars, where some studies reported an increase and others a decrease (Lima et al., 2010; Kaur & Suseela, 2020) and the same was noticed in our analysis, the number of increasing and decreasing trends for sugars is very similar. Mannose was the only sugar that showed a consistent decreasing trend. As explained earlier, phytohormones play an important role, and this is true also in fighting fungi stress, where salicylic acid showed antipathogens activity in local and systemic way, and also ethylene and methyl-jasmonates act against necrotrophic organisms (Patil et al., 2020). Moreover, abscisic acid has a role against both pathogens and insect infection by regulating stomatal closure to avoid further external attacks. And also, abscisic acid can interact with other phytohormones like salicylic and jasmonic acids to build a stronger response with other synergic mechanisms (Ton et al., 2009).

2.9 - Bacteria stress

Changes in plant metabolomics due to microorganisms attacks are much less studied than insect and fungi stress, despite arousing increasing interests over the last years, as showed by the increasing number of articles published in this field. Studies have found that flavonoids could act as phytoalexins or phytoanticipins when plants are subjected to bacterial infection, as reported by two research groups (Gradillas et al., 2020; Miotto-Vilanova et al., 2019). Phytoalexins and phytoanticipins are two classes of antimicrobials produced by plants while fighting infections. Phytoanticipins are already present in plant tissues before the infection occurs, to "anticipate" the infection, while phytoalexins are produced by the plant after the infection occurrence (Osbourn 1996; Tiku, 2018). The two research groups inoculated *Vitis vinifera* and *Rubus* with two different bacteria strains and found an increase of the flavonoid content. However, Pi et al. (2019) showed a different and contrasting result after injection of a strain of *Sinorhizobium fredii* in *Glycine max* seedlings. Regarding amino acids, a study found an increase of proline, arginine and isoleucine and a decrease of phenylalanine after inoculation of wheat streak mosaic virus in *Triticum aestivum* L. This evidence highlights once again the important role of amino acids in every type of stress, and this shift is probably related to the energy metabolism (Farahbakhsh et al., 2019). Increasing amino acids and organic acids trends were found after inoculation of Potato virus Y in *Solanum tuberosum* by (Kogov et al., 2016) as well, while sucrose showed a decreasing trend. Opposite results were found as a consequence of viral infection, with a decreasing trend for alanine, arginine, asparagine, citrulline, glutamine, glycine, histidine, serine and tryptophan (Díaz-Cruz & Cassone, 2018). These contradictory results remark the lack of knowledge about this type of stress.

2.10 - General concepts about multiple stresses

In nature, plants often deal with a combination of two or more stresses (Roy, 2012) and obviously, most of the times, exposing plants to multiple stresses enhance the gravity of the stressful condition (Král'ová et al., 2012; Mittler, 2006). Previous findings have found that proline decreases during exposure to multiple stress, but we did not observe such response. Proline consistently increased after exposition to a combination of drought, salinity, and heat stress. Moreover, it has been hypothesized that sucrose replaces proline in stress tolerance mechanisms when multiple stresses are applied, because proline seems to become toxic to plant cells when the stressful condition reach very severe levels (Rizhsky et al., 2004). However, our results seem to disagree with this hypothesis, since sucrose did not show increasing trends when salinity was combined with other stresses, while in drought and heat combination sucrose increased, but proline did not decrease. Our review work highlighted a decreasing trend for myo-inositol after application of salinity stress combined with drought or heat stress, whereas it did not show a decreasing trend after the application of the single salinity stress. Caffeoylquinic acid also showed a decreasing trend after exposure to combined stresses including salinity, heat and drought stress. The stress combinations most commonly studied are drought/fungi stress, salinity/fungi, drought/heat, salinity/drought, and salinity/heat (Figure 2.2).

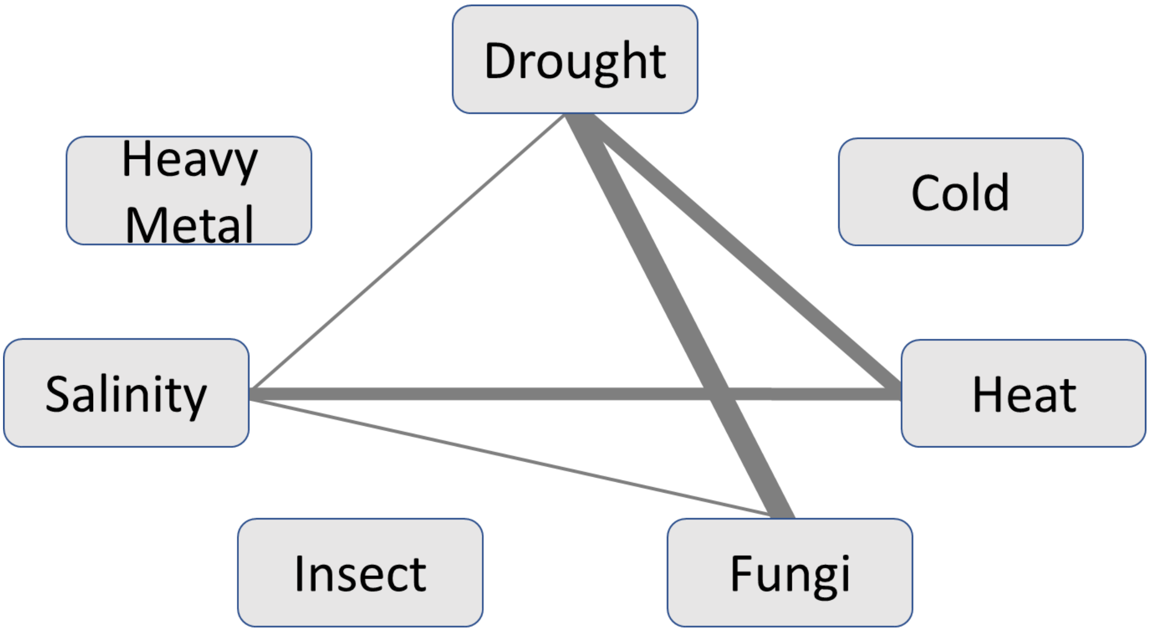


***Figure 2.2**. Graphical representation of studies that worked on stress combinations. Thickness of the lines represents the number of articles that combined the two stresses.*

Chapter 3 - *Moringa oleifera* Lam.: A Phytochemical and Pharmacological Overview of a drought-resistant plant

3.1 - Introduction

Moringa oleifera Lam. is a fast-growing and drought-resistant tree of the Moringaceae family. It is commonly known with several names including moringa, drumstick tree (for the long and slender seedpods), horseradish tree (for the roots taste resembling horseradish), ben oil tree (being rich in behenic acid), and miracle tree (for the medicinal properties) (Olson & Fahey, 2011). *M. oleifera* is the most popular of the thirteen species in the genus Moringa, the unique of the family Moringaceae which includes: *M. concanensis, M. drouhardii, M. arborea, M. borziana, M. hildebrandtii, M. longituba, M. pygmaea, M. rivae, M. ruspoliana, M. ovalifolia, M. peregrine*, and *M. stenopetala*. The species are native to Africa, Arabia, India, Southeast Asia, South America, the Pacific Islands, and the Caribbean (Olson, 2010). It is distributed in Ethiopia, Florida, Philippines, and Sudan (Olson, 2010). *M. oleifera* is one of the most popular plants that can grow in a variety of environments due to its ability to resist to harsh conditions such as high temperatures and limited water availability (Yadava, 1996). Therefore, it grows in a variety of soils including semi-dry, desert, or tropical soils and rainfall conditions (Yadava, 1996). The plant tolerates also different pH levels, ranging from 5.0 to 9.0. However, it prefers neutral pH and well-drained soils. It thrives in temperatures ranging from 25 to 40 °C, though it can withstand temperature swings of −1 to 3 °C and 38 to 48 °C during the coldest and warmest months, respectively (Yadava, 1996). The plant yield related to cutting period and planting density was studied and the data indicated that the highest planting density of 0.2 m ×

0.2 m in combination with the intermediate cutting height of 30 cm produced the highest fresh matter and dry matter yield throughout the whole evaluation period (Zheng et al., 2016). *M. oleifera* is among the food plants richest in nutrients (Alli et al., 2017). It has a high content in essential amino acids, proteins, minerals, vitamins, and polyphenols. It is a rich source of phytochemicals including flavonoids, anthocyanins, isothiocyanates, anthraquinone, alkaloids, essential oils, tannic acid, saponins, steroids, terpenoids, cardiac glycosides (Fahey, 2016). In addition, it is used to treat individuals with extreme malnutrition as well as for its pharmacological (hepatoprotective, antihypertensive, cholesterol-lowering, anti-urolithiasis, antifertility, antidiabetic, and antioxidant activity, nutraceutical properties, and antimicrobial) (Fahey, 2016). Moreover, *M. oleifera* is being used to help to breastfeed mothers improving postpartum milk production (Renityas, 2018). It is also used in Ayurvedic tradition specifically for cancer treatment (Fahey, 2016). *M. oleifera* leaves and buds were used against headache by rubbing them on the temples. Roots and root barks were used as anti-scorbutic (Fahey et al., 2017). The eye diseases were treated with the juice of the leaves added with honey. Dried seeds of *M. oleifera* were used in ophthalmic preparation, venereal affection anti-inflammatory and purgative and as tonic (Fahey et al., 2017). The use in ethnomedicine encouraged scientists to study this plant to determine its chemical composition and to investigate its pharmacological potential. This review is aimed to contribute to the knowledge of *M. oleifera* by providing plant description, phytochemistry and pharmacology.

3.2 - Plant Description

M. oleifera is a short, slender, deciduous, perennial tree that grows to about 10 m tall, slender with drooping branches; branches and stem are brittle, with corky bark. Leaves are feathery, pale green, compound, tripinnate, (30–60 cm long), with many small leaflets, 1.3–2 cm long, 0.6–0.3 cm wide, lateral ones slightly elliptic, terminal ones obovate, and slightly larger (Figure 3.1). Flowers are fragrant, white, or creamy-white, 2.5 cm in diameter, and borne in sprays. The stamens are yellow, and the pods are pendulous, brown, triangular, splitting lengthwise into three parts when dry, and containing about 20 seeds embedded in the pith. The pod has nine ribs on both ends and the seeds are dark brown with three papery wings (Olson & Fahey, 2011).

***Figure 3.1**. Images of Moringa tree, leaves, seeds and flowers.*

The feathery leaves of the tripinnate complex have green curved leaflets that are 1–4 cm long (Figure 3.1). Because of its leaves, the tree is frequently mistaken for a leguminous plant. The alternate twice or thrice pinnate leaves appear at the branch tips in most cases. They have a long petiole with 8–10 pairs of pinnae, each bearing two sets of inverse elliptic leaflets and one at the apex and are 20–70 cm long when young (Olson & Fahey, 2011). The seeds have three papery wings and are oval with a tannish semi-permeable seed arrangement (Figure 3.1). Their arrangements are mostly brown to dark brown but can be white if portions are of low viability. It almost within a week, viable seeds sprout. The body has three white wings that run at 130 intervals from start to finish (Olson & Fahey, 2011). Flowers are prominent, softly fragrant, and are borne on inflorescences 15–25 cm long. They are mostly white to cream in color, 2.5 cm in diameter, and tinged pink in a few varieties (Figure 3.1).

However, the flowers are fragrant and 2.5 cm wide, and they bloom profusely in auxiliary, dropping panicles that are 10-25 cm long. They have a dotted base and are white in color. The direct lanceolate sepals are five-reflexed. The five petals are rumored to be thin. Except for the lowest stamen, they are reflexed and consist of five stamens and five staminodes (Olson & Fahey, 2011).

3.3 - Phytochemistry

Table 3.1 lists all the metabolites that have been found in *M. oleifera* extracts. Many studies have focused their efforts in analyzing the polar and non-polar extracts of *M. oleifera* leaves and seeds to characterize the chemical composition of this plant and try to explain the beneficial characteristics demonstrated by lots of works in recent years. Selected characteristic compounds of the plant are reported in Figure 3.2.

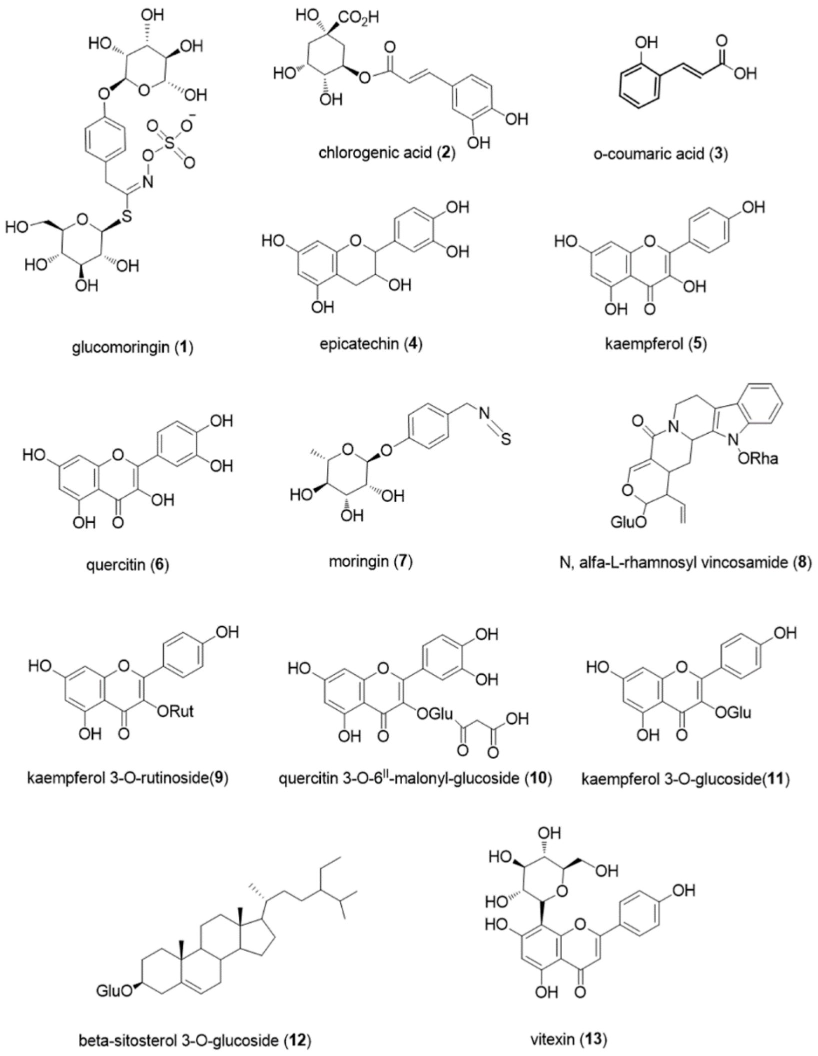


***Figure 3.2**. Bioactive metabolites isolated from M. oleifera.*

***Table 3.1.** List of compounds extracted from Moringa oleifera Lam. leaves and seeds.*

Compounds	**Molecular formula**	**Quantity**		**Method**	**Reference**
		Leaves	**Seeds**		
Caproic acid	$C_6H_{12}O_2$	0.1%	-	GC-MS	(Lee et al., 2017)
Capric acid	$C_{10}H_{20}O_2$	0.1%	-	GC-MS	(Lee et al., 2017)
Lauric acid	$C_{12}H_{24}O_2$	0.72%	0.03%	GC-MS	(Lee et al., 2017)
Myristic acid	$C_{14}H_{28}O_2$	1.13%	0.16%	GC-MS	(Lee et al., 2017)
Palmitic acid	$C_{16}H_{32}O_2$	23.65%	7.92%	GC-MS	(Lee et al., 2017)
		-	Madurai: 6.17%[a]	GC-MS	(Chelliah et al., 2017)
		1.08%[a]	-	GC-MS	(Chuang et al., 2007)
		0,15%	-	GC-MS	(Zhao & Zhang, 2013)

		2,5703%[a]	-	GC-MS	(Bhalla et al., 2021)
Palmitoleic acid	$C_{16}H_{30}O_2$	-	1.49%	GC-MS	(Lee et al., 2017)
Heptadecanoic acid	$C_{17}H_{34}O_2$	0.46%	0.32%	GC-MS	(Lee et al., 2017)
Stearic acid	$C_{18}H_{36}O_2$	1,2051%[a]	-	GC-MS	(Bhalla et al., 2021)
		3.93%	7.10%	GC-MS	(Lee et al., 2017)
Oleic acid	$C_{18}H_{34}O_2$	5.92%	74.50%	GC-MS	(Lee et al., 2017)
		-	Madurai: 5.12%[a]	GC-MS	(Chelliah et al., 2017)
Linoleic acid	$C_{18}H_{32}O_2$	6.84%	0.62%	GC-MS	(Lee et al., 2017)
Arachidic acid	$C_{20}H_{40}O_2$	0.72%	4.28%	GC-MS	(Lee et al., 2017)
Cis-11-Eicosenoic acid	$C_{20}H_{38}O_2$	54.44%	-	GC-MS	(Lee et al., 2017)
Linolenic acid	$C_{18}H_{30}O_2$	-	2.50%	GC-MS	(Lee et al., 2017)
Cis-11, 14-Eicosadienoic acid	$C_{20}H_{36}O_2$	-	0.48%	GC-MS	(Lee et al., 2017)
Cis-5, 8, 11, 14, 17-Eicosapentaenoic acid.	$C_{21}H_{32}O_2$	1.99%	0.60%	GC-MS	(Lee et al., 2017)
Lysine	$C_6H_{14}N_2O_2$	Madurai: 4.10 g/16gN Chennai: 3.30 g/16gN	Madurai: 4.20 g/16gN Chennai: 3.70 g/16gN	HPLC	(Chelliah et al., 2017)
		129.4 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Histidine	$C_6H_9N_3O_2$	Madurai: 2.50 g/16gN Chennai: 3.12 g/16gN	Madurai: 6.30 g/16gN Chennai: 5.70 g/16gN	HPLC	(Chelliah et al., 2017)
		73.9 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Valine	$C_5H_{11}NO_2$	Madurai: 4.55 g/16gN Chennai: 4.75 g/16gN	Madurai: 3.36 g/16gN Chennai: 2.37 g/16gN	HPLC	(Chelliah et al., 2017)
		111.5 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Leucine	$C_6H_{13}NO_2$	Madurai: 4.80 g/16gN Chennai: 4.40 g/16gN	Madurai: 4.80 g/16gN Chennai: 4.70 g/16gN	HPLC	(Chelliah et al., 2017)
		211.5 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Isoleucine	$C_6H_{13}NO_2$	Madurai: 5.90 g/16gN Chennai: 9.10 g/16gN	Madurai: 8.30 g/16gN Chennai: 7.70 g/16gN	HPLC	(Chelliah et al., 2017)
		81.3 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Threonine	$C_4H_9NO_3$	Madurai: 4.10 g/16gN Chennai: 4.50 g/16gN	Madurai: 3.20 g/16gN Chennai: 3.80 g/16gN	HPLC	(Chelliah et al., 2017)
		91.8 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Alanine	$C_3H_7NO_2$	Madurai: 3.23 g/16gN	Madurai: 4.29 g/16gN	HPLC	(Chelliah et al., 2017)

		Chennai: 3.90 g/16gN	Chennai: 3.55 g/16gN		
		121.2 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Aspartic acid	$C_4H_7NO_4$	Madurai: 6.44 g/16gN Chennai: 6.86 g/16gN	Madurai: 6.02 g/16gN Chennai: 6.37 g/16gN	HPLC	(Chelliah et al., 2017)
		195.8 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Serine	$C_3H_7NO_3$	Madurai: 4.25 g/16gN Chennai: 4.66 g/16gN	Madurai: 4.22 g/16gN Chennai: 4.11 g/16gN	HPLC	(Chelliah et al., 2017)
		90.2 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Proline	$C_5H_9NO_2$	128.9 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Glutamic acid	$C_5H_9NO_4$	Madurai: 15.86 g/16gN Chennai: 15.33 g/16gN	Madurai: 14.23 g/16gN Chennai: 14.74 g/16gN	HPLC	(Chelliah et al., 2017)
		268.7 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Glycine	$C_2H_5NO_2$	Madurai: 5.13 g/16gN Chennai: 5.11 g/16gN	Madurai: 5.00 g/16gN Chennai: 4.70 g/16gN	HPLC	(Chelliah et al., 2017)
		102.4 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Arginine	$C_6H_{14}N_4O_2$	Madurai: 4.30 g/16gN Chennai: 3.70 g/16gN	Madurai: 9.44 g/16gN Chennai: 11.00 g/16gN	HPLC	(Chelliah et al., 2017)
		125.4 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Cysteine	$C_3H_7NO_2S$	29.6 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Tyrosine	$C_9H_{11}NO_3$	79.3 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Methionine	$C_5H_{11}NO_2S$	Madurai: 1.90 g/16gN Chennai: 3.40 g/16gN	Madurai: 3.50 g/16gN Chennai: 3.90 g/16gN	HPLC	(Chelliah et al., 2017)
		221.4 mg/100g dw	-	HPLC	(Lalas et al., 2017)
Phenylalanine	$C_9H_{11}NO_2$	Madurai: 4.20 g/16gN Chennai: 4.60 g/16gN	Madurai: 3.50 g/16gN Chennai: 3.90 g/16gN	HPLC	(Chelliah et al., 2017)
		117.2 mg/100g	-	HPLC	(Lalas et al., 2017)
1,30-Triacontanediol	$C_{30}H_{62}O_2$	3,06%	-	GC-MS	(Zhao & Zhang, 2013)
		Madurai: 14.98%[a]	-	GC-MS	(Chelliah et al., 2017)
Octacosane	$C_{28}H_{58}$	Madurai: 8.57%[a]	-	GC-MS	(Chelliah et al., 2017)
Z-14-nonacosane	$C_{29}H_{58}$	Madurai: 8.3%[a]	-	GC-MS	(Chelliah et al., 2017)
2,2-dimethyl-1-oxa-2-silacyclotridecanone-13	$C_6H_{14}OSi$	Madurai: 8.28%[a]	-	GC-MS	(Chelliah et al., 2017)
Nonacosane	$C_{29}H_{60}$	Chennai: 15.55%[a]	-	GC-MS	(Chelliah et al., 2017)

		18,65%	-	GC-MS	(Zhao & Zhang, 2013)
γ-Sitosetrol	$C_{29}H_{50}O$	0,86%	-	GC-MS	(Zhao & Zhang, 2013)
		Chennai: 9.56%[a]	-	GC-MS	(Chelliah et al., 2017)
Campesterol	$C_{28}H_{48}O$	Chennai: 5.00%[a]	Chennai: 1.21%[a]	GC-MS	(Chelliah et al., 2017)
13-docosenamide, (Z)-	$C_{22}H_{43}NO$	-	Chennai: 13.62%[a]	GC-MS	(Chelliah et al., 2017)
6-octadecenoic acid	$C_{18}H_{34}O_2$	-	Madurai: 52.24%[a]	GC-MS	(Chelliah et al., 2017)
Propionamide	C_3H_7NO	0,7335%[a]	-	GC-MS	(Bhalla et al., 2021)
		-	Chennai: 4.48%[a]	GC-MS	(Chelliah et al., 2017)
Ethyl oleate	$C_{20}H_{38}O_2$	-	Chennai: 4.33%[a]	GC-MS	(Chelliah et al., 2017)
Glucomoringin	$C_{21}H_{31}NO_{14}S_2$	16.14–17.3 µmol/g dry weight	-	UPLC	(Lopez-Rodriguez et al., 2020)
		Domesticated: 75.29 µmol/g dry weight Wild: 18.00 µmol/g dry weight	-	HPLC	(Lopez-Rodriguez et al., 2020)
		110-112 µmol/g dry weight	-	HPLC	(Lopez-Rodriguez et al., 2020)
		21.84-22.56 mg/g dry weight	32.88 mg/g dry weight	HPLC	(Lopez-Rodriguez et al., 2020)
		0.77 mg/g dry weight	86.19 mg/g dry weight	HPLC-ESI-MS/MS	(Lopez-Rodriguez et al., 2020)
		33.9-59.4 mg/g dry weight	202 mg/g dry weight		(Lopez-Rodriguez et al., 2020)
Glucomoringin Isomer I	$C_{21}H_{31}NO_{14}S_2$	7.15–8.92 µmol/g dry weight	-	UPLC	(Lopez-Rodriguez et al., 2020)
Glucomoringin Isomer II	$C_{21}H_{31}NO_{14}S_2$	3.85–4.84 µmol/g dry weight	-	UPLC	(Lopez-Rodriguez et al., 2020)
Glucomoringin Isomer III	$C_{21}H_{31}NO_{14}S_2$	18.71–22.58 µmol/g dry weight	-	UPLC	(Lopez-Rodriguez et al., 2020)
Glucosoonjnain	-	Domesticated: 1.16 Wild: 33.79	-	HPLC	(Lopez-Rodriguez et al., 2020)
β-carotene	$C_{40}H_{56}$	33.48 mg/100 g	-	HPLC	(Leone et al., 2015)
		27.22 mg/100 g	-	N/A	(Leone et al., 2015)
		36 mg/100 g	-	AOAC 2004	(Leone et al., 2015)
		39.6 mg/100 g	-	N/A	(Leone et al., 2015)
		37.8 mg/100 g	-	N/A	(Leone et al., 2015)
		17.62 mg/100 g	-	N/A	(Leone et al., 2015)

		20.44 mg/100 g	-	HPLC	(Leone et al., 2015)
		66 mg/100 g	-	HPLC	(Leone et al., 2015)
Lutein	$C_{40}H_{56}O_2$	35.05 mg/100 g	-	HPLC	(Leone et al., 2015)
		102 mg/100 g	-	HPLC	(Leone et al., 2015)
Caffeic acid	$C_9H_8O_4$	0.409 mg/g	-	HPLC	(Leone et al., 2015)
		0.536 mg/g	-	HPLC	(Leone et al., 2015)
Chlorogenic acid	$C_{16}H_{18}O_9$	0.489 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
o-Coumaric acid	$C_9H_8O_3$	6.457 mg/g	-	HPLC	(Leone et al., 2015)
Ellagic acid	$C_{14}H_6O_8$	0.018 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		0.189 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
Ferulic acid	$C_{10}H_{10}O_4$	0.078 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		0.078 mg/g	-	HPLC	(Leone et al., 2015)
		0.128 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
Gallic acid	$C_7H_6O_5$	1.034 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		1.034 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
Epicatechin	$C_{15}H_{14}O_6$	5.68 mg/g	-	HPLC	(Leone et al., 2015)
Isorhamnetin	$C_{16}H_{12}O_7$	0.118 mg/g	-	HPLC	(Leone et al., 2015)
		0.52 mg/g [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		0.72 mg/g [g,h]	-		(Leone et al., 2015)
Kaempferol	$C_{15}H_{10}O_6$	0.04 mg/g	-	HPLC	(Leone et al., 2015)
		2.360 mg/g	-	HPLC	(Leone et al., 2015)
		0.198 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		1.412 mg/g	-	HPLC	(Leone et al., 2015)
		0.8 mg/g	-	LC/MS	(Leone et al., 2015)
		1.23 mg/g	-		(Leone et al., 2015)
		4.59 mg/g	-		(Leone et al., 2015)
		3.92 [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		2.16 mg/g [g,h]	-		(Leone et al., 2015)
		2.25 mg/g	-	HPLC-DAD	(Leone et al., 2015)

		1.75 mg/g	-		(Leone et al., 2015)
		1.05 mg/g	-		(Leone et al., 2015)
		2.9 mg/g [d]	-	LC/MS	(Leone et al., 2015)
		2.3 mg/g	-		(Leone et al., 2015)
		3.5 mg/g	-		(Leone et al., 2015)
		0.3 mg/g [c]	-		(Leone et al., 2015)
		0.16 mg/g [d]	-		(Leone et al., 2015)
Myricetin	$C_{15}H_{10}O_8$	5.804 mg/g	-	HPLC	(Leone et al., 2015)
Quercetin	$C_{15}H_{10}O_7$	0.281 mg/g	-	HPLC	(Leone et al., 2015)
		0.207 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		0.207 mg/g	-	HPLC	(Leone et al., 2015)
		0.807 mg/g	-	HPLC and MS/MS	(Leone et al., 2015)
		3.529 mg/g	-	HPLC	(Leone et al., 2015)
		5.2 mg/g	-	LC/MS	(Leone et al., 2015)
		5.8 mg/g	-		(Leone et al., 2015)
		7.57 mg/g	-		(Leone et al., 2015)
		12.84 mg/g [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		16.64 mg/g [g,h]	-		(Leone et al., 2015)
		9.26 mg/g	-	HPLC-DAD	(Leone et al., 2015)
		6.34 mg/g	-		(Leone et al., 2015)
		7.70 mg/g	-		(Leone et al., 2015)
		5.47 mg/g [b]	-	LC-MS	(Leone et al., 2015)
		9.1 mg/g	-		(Leone et al., 2015)
		15.2 mg/g	-		(Leone et al., 2015)
		0.58 mg/g [c]	-		(Leone et al., 2015)
		0.46 mg/g [d]	-		(Leone et al., 2015)
Rutin	$C_{27}H_{30}O_{16}$	1.674 mg/g	-	HPLC	(Leone et al., 2015)
4-(α-L-rhamnopyranosyloxy)- benzyl	$C_{14}H_{17}NO_5S$	22.56 mg/g [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		21.84 mg/g [h,g]	-		(Leone et al., 2015)
		33.9 mg/g c	-	LC-MS	(Leone et al., 2015)
		59.4 mg/g d	-		(Leone et al., 2015)
4-O-(α-Lacetylrhamnopyra	$C_{16}H_{19}NO_6S$	2.76 mg/g [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		2.16 mg/g [h,g]	-		(Leone et al., 2015)

nosyloxy)-benzyl isomer 1		2.9 mg/g [c]	-	LC-MS	(Leone et al., 2015)
		5.0 mg/g [d]	-		(Leone et al., 2015)
4-O-(α-Lacetylrhamnopyranosyloxy)-benzyl isomer 2	$C_{16}H_{19}NO_6S$	1.80 mg/g [f,g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		1.52 mg/g [h,g]	-		(Leone et al., 2015)
		1.2 mg/g [c]	-	LC-MS	(Leone et al., 2015)
		1.5 mg/g [d]	-		(Leone et al., 2015)
4-O-(α-Lacetylrhamnopyranosyloxy)-benzyl isomer 3	$C_{16}H_{19}NO_6S$	20.16 mg/g [f, g]	-	HPLC-DAD-ESI-MS	(Leone et al., 2015)
		12.76 mg/g [h,g]	-		(Leone et al., 2015)
		17.4 mg/g[c]	-	LC-MS	(Leone et al., 2015)
		50.2 mg/g[d]	-		(Leone et al., 2015)
Toluene	C_7H_8	1,27%	-	GC-MS	(Zhao & Zhang, 2013)
		0.03%[a]	-	GC-MS	(Chuang et al., 2007)
5-tert-Butyl-1,3-cyclopentadiene	C_9H_{14}	0.07%[a]	-	GC-MS	(Chuang et al., 2007)
Benzaldehyde	C_6H_5CHO	0.55%[a]	-	GC-MS	(Chuang et al., 2007)
5-Methyl-2-furaldehyde	$C_6H_6O_2$	0.27%[a]	-	GC-MS	(Chuang et al., 2007)
Benzeneacetaldehyde	C_8H_8O	2.16%[a]	-	GC-MS	(Chuang et al., 2007)
2-Ethyl-3,6-dimethylpyrazine	$C_8H_{12}N_2$	0.12%[a]	-	GC-MS	(Chuang et al., 2007)
Undecane	$C_{11}H_{24}$	0.12%[a]	-	GC-MS	(Chuang et al., 2007)
Isophoron	$C_9H_{14}O$	0.10%[a]	-	GC-MS	(Chuang et al., 2007)
Benzylnitrile	C_8H_7N	1.10%[a]	-	GC-MS	(Chuang et al., 2007)
2,6,6-Trimethyl-2-cyclohexane-1,4-dione	$C_9H_{12}O_2$	0.05%[a]	-	GC-MS	(Chuang et al., 2007)
2,2,4-Trimethyl-pentadiol	$C_{12}H_{24}O_3$	0.09%[a]	-	GC-MS	(Chuang et al., 2007)
2,3-Epoxycarane	$C_{10}H_{16}O$	0.16%[a]	-	GC-MS	(Chuang et al., 2007)
p-Menth-1-en-8-ol	$C_{10}H_{18}O$	0.08%[a]	-	GC-MS	(Chuang et al., 2007)
2,6,6-Trimethylcyclohexa-1,3-dienecarbaldehyde	C_9H_{14}	0.23%[a]	-	GC-MS	(Chuang et al., 2007)
Indole	C_8H_7N	1.20%[a]	-	GC-MS	(Chuang et al., 2007)
Tridecane	$C_{13}H_{28}$	0.16%[a]	-	GC-MS	(Chuang et al., 2007)
Ionone	$C_{13}H_{20}O$	0.13%[a]	-	GC-MS	(Chuang et al., 2007)

		0.03%[a]	-	GC-MS	(Chuang et al., 2007)
1,1,6-Trimethyl-1,2-dihydronaphthalene	$C_{13}H_{16}$	0.41%[a]	-	GC-MS	(Chuang et al., 2007)
Ionene	$C_{13}H_{18}$	0.09%[a]	-	GC-MS	(Chuang et al., 2007)
Damascenone	$C_{13}H_{18}O$	0.28%[a]	-	GC-MS	(Chuang et al., 2007)
Ledene oxide	$C_{15}H_{24}O$	0.60%[a]	-	GC-MS	(Chuang et al., 2007)
2-tert-Butyl-1,4-dimethoxybenzene	$C_{12}H_{18}O_2$	0.39%[a]	-	GC-MS	(Chuang et al., 2007)
(E)-6,10-dimethylundeca-5,9-dien-2-one	$C_{13}H_{22}O$	0.26%[a]	-	GC-MS	(Chuang et al., 2007)
4,6-Dimethyldodecane	$C_{14}H_{30}$	0.29%[a]	-	GC-MS	(Chuang et al., 2007)
3,3,5,6-Tetramethyl-1-indanone	$C_{13}H_{16}O$	0.23%[a]	-	GC-MS	(Chuang et al., 2007)
Dihydro-actiridioide	-	1.21%[a]	-	GC-MS	(Chuang et al., 2007)
2,3,6-Trimethyl-naphthalene	$C_{13}H_{14}$	0.37%[a]	-	GC-MS	(Chuang et al., 2007)
Megastigmatrienone	$C_{13}H_{18}O$	0.57%[a]	-	GC-MS	(Chuang et al., 2007)
1-(2,3,6-Trimethyl-phenyl)-2-butanone	$C_{13}H_{18}O$	3.44%[a]	-	GC-MS	(Chuang et al., 2007)
1-(2,3,6-Trimethyl-phenyl)-3-buten-2-one	$C_{13}H_{16}O$	0.75%[a]	-	GC-MS	(Chuang et al., 2007)
Isolongifolene	$C_{15}H_{24}$	0.56%[a]	-	GC-MS	(Chuang et al., 2007)
Hexahydrofarnesylactone	$C_{18}H_{36}O$	1.30%[a]	-	GC-MS	(Chuang et al., 2007)
Farnesylacetone	$C_{18}H_{30}O$	0.08%[a]	-	GC-MS	(Chuang et al., 2007)
Methyl palmitate	$C_{17}H_{34}O_2$	0.08%[a]	-	GC-MS	(Chuang et al., 2007)
(6E,10E)-7,11,15-trimethyl-methylene-1,6,10,14-hexadeca-tetraene	$C_{20}H_{32}$	0.11%[a]	-	GC-MS	(Chuang et al., 2007)
Phytol	$C_{20}H_{40}O$	0,9664%[a]	-	GC-MS	(Bhalla et al., 2021)
		7.66%[a]	-	GC-MS	(Chuang et al., 2007)
Docosane	$C_{22}H_{46}$	0.28%[a]	-	GC-MS	(Chuang et al., 2007)
1-Docosene	$C_{22}H_{44}$	0.41%[a]	-	GC-MS	(Chuang et al., 2007)
Tetracosane	$C_{24}H_{50}$	1.45%[a]	-	GC-MS	(Chuang et al., 2007)
Pentacosane	$C_{25}H_{52}$	17.41%[a]	-	GC-MS	(Chuang et al., 2007)

		2,14%	-	GC-MS	(Chuang et al., 2007)
Hexacosane	$C_{26}H_{54}$	11.20%[a]	-	GC-MS	(Chuang et al., 2007)
Pyridine	C_5H_5N	0,78%	-	GC-MS	(Zhao & Zhang, 2013)
Hepane,2-methyl-	C_8H_{18}	0,68%	-	GC-MS	(Zhao & Zhang, 2013)
Heptane,3-methyl-	C_8H_{18}	0,50%	-	GC-MS	(Zhao & Zhang, 2013)
Cyclohexane,1,3-dimethyl-,cis-	C_8H_{16}	0,78%	-	GC-MS	(Zhao & Zhang, 2013)
Octane	C_8H_{18}	1,75%	-	GC-MS	(Zhao & Zhang, 2013)
Cyclohexane,1,4-dimethyl-,cis-	C_8H_{16}	0,14%	-	GC-MS	(Zhao & Zhang, 2013)
Octane,2-methyl-	C_9H_{20}	0,17%	-	GC-MS	(Zhao & Zhang, 2013)
Cyclohexane,ethyl-	C_8H_{16}	0,43%	-	GC-MS	(Zhao & Zhang, 2013)
Ethylbenzene	C_8H_{10}	0,83%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,3-dimethyl-	C_8H_{10}	4,63%	-	GC-MS	(Zhao & Zhang, 2013)
o-Xylene	C_8H_{10}	2,82%	-	GC-MS	(Zhao & Zhang, 2013)
Nonane	C_9H_{20}	0,14%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene, (1-methylethyl)-	C_9H_{12}	0,16%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,propyl-	C_9H_{12}	1,04%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1-ethyl-3-methyl-	C_9H_{12}	5,95%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1-ethyl-4-methyl-	C_9H_{12}	2,39%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,3,5-trimethyl-	C_9H_{12}	4,31%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1-ethyl-2-methyl-	C_9H_{12}	2,55%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,2,4-trimethyl-	C_9H_{12}	16,96%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,2,3-trimethyl-	C_9H_{12}	4,14%	-	GC-MS	(Zhao & Zhang, 2013)
Indane	C_9H_{10}	0,39%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1-ethyl-2,4- dimethyl-	$C_{10}H_{14}$	0,64%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1-ethyl-2,3- dimethyl-	$C_{10}H_{14}$	0,99%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,2,3,5-tetramethyl-	$C_{10}H_{14}$	1,59%	-	GC-MS	(Zhao & Zhang, 2013)

Benzene,1,2,4,5-tetramethyl-	$C_{10}H_{14}$	2,12%	-	GC-MS	(Zhao & Zhang, 2013)
Benzene,1,2,3,4-tetramethyl-	$C_{10}H_{14}$	0,58%	-	GC-MS	(Zhao & Zhang, 2013)
Naphthalene	$C_{10}H_8$	1,21%	-	GC-MS	(Zhao & Zhang, 2013)
Dimethoate	$C_5H_{12}NO_3PS_2$	0,52%	-	GC-MS	(Zhao & Zhang, 2013)
Nonadecane	$C_{19}H_{40}$	0,22%	-	GC-MS	(Zhao & Zhang, 2013)
Eicosane	$C_{20}H_{42}$	0,31%	-	GC-MS	(Zhao & Zhang, 2013)
Heptacosane	$C_{27}H_{56}$	7,45%	-	GC-MS	(Zhao & Zhang, 2013)
γ-Tocopherol	$C_{28}H_{48}O_2$	0,75%	-	GC-MS	(Zhao & Zhang, 2013)
Sulfurous acid, hexyl pentadecyl ester	$C_{21}H_{44}O_3S$	0,34%	-	GC-MS	(Zhao & Zhang, 2013)
dl-α-Tocopherol	$C_{29}H_{50}O_2$	1,05%	-	GC-MS	(Zhao & Zhang, 2013)
β-Amyrin	$C_{30}H_{50}O$	4,60%	-	GC-MS	(Zhao & Zhang, 2013)
1,3-Dihydroxyacetone dimer	$C_6H_{12}O_6$	3,8551%[a]	-	GC-MS	(Bhalla et al., 2021)
Acetic acid, ((amino carbonyl)amino)oxo-	$C_3H_4N_2O_4$	3,2396%[a]	-	GC-MS	(Bhalla et al., 2021)
4(1H)-Pyrimidinone, 2,6-diamino-	$C_4H_6N_4O$	2,2433%[a]	-	GC-MS	(Bhalla et al., 2021)
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	4,1801%	-	GC-MS	(Bhalla et al., 2021)
		8,9858%[a]	-	GC-MS	(Bhalla et al., 2021)
2-Hexynoic acid	$C_6H_8O_2$	1,1214%[a]	-	GC-MS	(Bhalla et al., 2021)
Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	$C_7H_{12}O_5$	3,1422%[a]	-	GC-MS	(Bhalla et al., 2021)
3,3-Iminobispropylamine	$C_6H_{17}N_3$	1,9275%[a]	-	GC-MS	(Bhalla et al., 2021)
1-Hexanamine	$C_6H_{15}N$	0,0774%[a]	-	GC-MS	(Bhalla et al., 2021)
1,3-Dioxolan-2-one, 4,5-dimethyl-	$C_5H_8O_3$	6,1627%[a]	-	GC-MS	(Bhalla et al., 2021)
2-Butenethioic acid, 3-(ethylthio)-, S-(1-methylethyl) ester	$C_9H_{16}OS_2$	1,3083%[a]	-	GC-MS	(Bhalla et al., 2021)
Propanamide, N,N-dimethyl-	$C_5H_{11}NO$	3,0349%[a]	-	GC-MS	(Bhalla et al., 2021)
2-Isopropoxyethyl propionate	$C_8H_{16}O_3$	16,8738%[a]	-	GC-MS	(Bhalla et al., 2021)

D-Mannoheptulose	$C_7H_{14}O_7$	2,5622%[a]	-	GC-MS	(Bhalla et al., 2021)
Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)-	$C_7H_{14}N_2O$	4,6738%[a]	-	GC-MS	(Bhalla et al., 2021)
Carbonic acid, butyl 2-pentyl ester	$C_{10}H_{20}O_3$	20,6431%[a]	-	GC-MS	(Bhalla et al., 2021)
Tetra acetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	5,0379%[a]	-	GC-MS	(Bhalla et al., 2021)
α-D-Glucose	$C_6H_{12}O_6$	3,445%[a]	-	GC-MS	(Bhalla et al., 2021)
1H-Cyclopenta(c)furan-3(3αH)-one, 6,6α-dihydro-1-(1,3-dioxolan-2-yl)-, (3αR,1-trans,6α-cis)-	$C_{10}H_{12}O_4$	1,2587%[a]	-	GC-MS	(Bhalla et al., 2021)
3-(1-(4-Cyano-1,2,3,4-tetrahydronaphthyl))propanenitrile	$C_{14}H_{14}N_2$	1,5253%[a]	-	GC-MS	(Bhalla et al., 2021)
Quinolinium, 1-ethyl-, iodide	$C_{11}H_{12}IN$	1,4067%[a]	-	GC-MS	(Bhalla et al., 2021)
N-Isopropyl-3-phenylpropanamide	$C_{12}H_{17}NO$	0,9462%[a]	-	GC-MS	(Bhalla et al., 2021)
1,2-Ethanediamine, N-(2-aminoethyl)-	$C_4H_{13}N_3$	0,8805%[a]	-	GC-MS	(Bhalla et al., 2021)
1,4-Benzenediol, 2-methyl-	$C_7H_8O_2$	4,3169%[a]	-	GC-MS	(Bhalla et al., 2021)
Ethene, ethoxy-	C_4H_8O	0,5981%[a]	-	GC-MS	(Bhalla et al., 2021)
Dihydroxyacetone	$C_3H_6O_3$	2,4651%[a]	-	GC-MS	(Bhalla et al., 2021)
Glycerine	$C_3H_8O_3$	1,8656%[a]	-	GC-MS	(Bhalla et al., 2021)
Erythritol	$C_4H_{10}O_4$	0,5327%[a]	-	GC-MS	(Bhalla et al., 2021)
Monomethyl malonate	$C_4H_6O_4$	2,5684%[a]	-	GC-MS	(Bhalla et al., 2021)
4,5-Diamino-6-hydroxypyrimidine	$C_8H_{12}N_8O_2$	0,6434%[a]	-	GC-MS	(Bhalla et al., 2021)
Furan, 2,3-dihydro-4-methyl-	C_5H_8O	0,2105%[a]	-	GC-MS	(Bhalla et al., 2021)
Catecholborane	$C_6H_5BO_2$	0,6806%[a]	-	GC-MS	(Bhalla et al., 2021)
2-Fluoropyridine	C_5H_4FN	0,8121%[a]	-	GC-MS	(Bhalla et al., 2021)
1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	1,4375%[a]	-	GC-MS	(Bhalla et al., 2021)

3,4-Furandiol, tetrahydro-, trans-	C_4H_8O	0,1746%^{a}	-	GC-MS	(Bhalla et al., 2021)
1-Nitro-β-d-arabino furanose, tetraacetate	$C_{13}H_{17}NO_{11}$	0,5114%^{a}	-	GC-MS	(Bhalla et al., 2021)
1,8-Diamino-3,6-dioxaoctane	$C_6H_{16}N_2O_2$	0,1172%^{a}	-	GC-MS	(Bhalla et al., 2021)
1,7-Diaminoheptane	$C_7H_{18}N_2$	1,7997%^{a}	-	GC-MS	(Bhalla et al., 2021)
N,N-Dimethylacetamide	C_4H_9ON	0,454%^{a}	-	GC-MS	(Bhalla et al., 2021)
2-Oxoglutaric acid	$C_5H_6O_5$	0,5466%^{a}	-	GC-MS	(Bhalla et al., 2021)
Oxazolidine, 2-ethyl-2-methyl-	$C_6H_{13}NO$	0,9008%^{a}	-	GC-MS	(Bhalla et al., 2021)
Heptanal	$C_7H_{14}O$	0,7112%^{a}	-	GC-MS	(Bhalla et al., 2021)
6-Methoxy-3-pyridazinethiol	$C_5H_6N_2OS$	0,4293%^{a}	-	GC-MS	(Bhalla et al., 2021)
3-Piperidinol	$C_5H_{11}NO$	0,5971%^{a}	-	GC-MS	(Bhalla et al., 2021)
1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-	$C_6H_{14}O_3$	21,1909%^{a}	-	GC-MS	(Bhalla et al., 2021)
Benzeneacetonitrile, 4-hydroxy-	C_8H_7NO	3,4763%^{a}	-	GC-MS	(Bhalla et al., 2021)
Benzenebutanal, γ, 4-dimethyl-	$C_{12}H_{16}O$	0,395%^{a}	-	GC-MS	(Bhalla et al., 2021)
2(4H)-Benzofuranone, 5,6,7,7α-tetrahydro-4,4,7α-trimethyl-	$C_{11}H_{16}O_2$	0,4491%^{a}	-	GC-MS	(Bhalla et al., 2021)
Ethanamine, N-ethyl-N-nitroso-	$C_4H_{10}N_2O$	5,2161%^{a}	-	GC-MS	(Bhalla et al., 2021)
Propanoic acid, 2-methyl-, octyl ester	$C_{12}H_{24}O$	15,0279%^{a}	-	GC-MS	(Bhalla et al., 2021)
3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	3,2947%^{a}	-	GC-MS	(Bhalla et al., 2021)
d-Glycero-d-ido-heptose	$C_7H_{14}O_7$	0,3814%^{a}	-	GC-MS	(Bhalla et al., 2021)
D-erythro-Pentose, 2-deoxy-	$C_5H_{10}O$	0,3314%^{a}	-	GC-MS	(Bhalla et al., 2021)
N-Methoxy-1-ribofuranosyl-4-imidazolecarboxylic amide	$C_{10}H_{15}N_3O_6$	0,5345%^{a}	-	GC-MS	(Bhalla et al., 2021)

Formamide, N,N-dimethyl-	C_3H_7NO	0,5847%[a]	-	GC-MS	(Bhalla et al., 2021)
d-Talonic acid lactone	$C_6H_{10}O_6$	0,5651%[a]	-	GC-MS	(Bhalla et al., 2021)
Sorbitol	$C_6H_{14}O_6$	0,482%[a]	-	GC-MS	(Bhalla et al., 2021)
Allo-Inositol	$C_6H_{12}O$	0,8749%[a]	-	GC-MS	(Bhalla et al., 2021)
		0,5189%[a]	-	GC-MS	(Bhalla et al., 2021)
		1,1254%[a]	-	GC-MS	(Bhalla et al., 2021)
D-chiro-Inositol, 3-O-(2-amino-4-((carboxyiminomethyl)amino)- 2,3,4,6-tetradeoxy-α-D-arabino-hexopyranosyl)-	$C_{14}H_{25}N_3O_9$	1,595%[a]	-	GC-MS	(Bhalla et al., 2021)
Muco-Inositol	$C_6H_{12}O_6$	2,0264%[a]	-	GC-MS	(Bhalla et al., 2021)
Inositol	$C_6H_{12}O_6$	2,0545%[a]	-	GC-MS	(Bhalla et al., 2021)
Cyclohexane, 1-methyl-4-(2-hydroxyethyl)-	$C_9H_{18}O_1$	0,4822%[a]	-	GC-MS	(Bhalla et al., 2021)
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O$	0,8519%[a]	-	GC-MS	(Bhalla et al., 2021)
Phenol, 2-methyl-	C_7H_8O	0,3737%[a]	-	GC-MS	(Bhalla et al., 2021)
(1S)-Propanol, (2S)-((tert.butyloxycarbonyl)amino)-1-phenyl-	$C_{14}H_{21}NO_3$	0,967%[a]	-	GC-MS	(Bhalla et al., 2021)
9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	1,0307%[a]	-	GC-MS	(Bhalla et al., 2021)
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	5,0063%[a]	-	GC-MS	(Bhalla et al., 2021)
4-Allyl-3-(dimethylhydrazono)-2-methylhexane-2,5-diol	$C_{12}H_{24}N_2O_2$	0,4257%[a]	-	GC-MS	(Bhalla et al., 2021)
Benzyl- β -d-glucoside	$C_{13}H_{16}O_7$	0,6056%[a]	-	GC-MS	(Bhalla et al., 2021)
4,6-dimethyl-2-propyl-1,3,5-dithiazinane	$C_8H_{17}NS_2$	0,1698%[a]	-	GC-MS	(Bhalla et al., 2021)
1,3-Benzenediol, 2-methyl-	$C_7H_8O_2$	0,545%[a]	-	GC-MS	(Bhalla et al., 2021)
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	1,4694%[a]	-	GC-MS	(Bhalla et al., 2021)

Abbreviations: a: calculated as peak area; b: Mean value of different samples; c: Mature/old leaves; d: Tender/young leaves; e: Mean value of samples collected in different seasons; f: Vegetative plants; g: Obtained considering a moisture of 75%; h: Flowering plants. Dw: dry weight

3.3.1 - Non-Polar Compounds

Fatty acids are present in very high quantities. In their work, Lee et al. (2017) performed an extraction on *M. oleifera* leaves powder using 70% methanol and then partitioning the outcome with hexane, dichloromethane, ethyl acetate, butanol, and water. Finally, the last part of extraction was conducted by supercritical fluid extraction (SFE). The most abundant fatty acid found in the resulting extracts were cis-11-Eicosenoic acid (also called gondoic acid), a monounsaturated acid contained in many oils extracted from plants, followed by palmitic acid (23.65%), and then linoleic acid and oleic acid (6.84% and 5.92%), and apart from them, the other ones are present in quantities lower than 5%. In seeds extracts, the highest content of fatty acid was registered for oleic acid (74.5%), then palmitic acid (7.92%), stearic acid (7.10%), and then minor fatty acids present in smaller quantities (Lee et al., 2017). These fatty acids are important because they take part in metabolic processes that lead to the synthesis of ω-3 fatty acid, which have important roles in regulation processes of cellular development and immune system (Lalas et al., 2017). As showed by the same group, cis-11-eicosenoic acid, linoleic acid, and oleic acid exhibited an antimicrobial effect on Staphylococcus aureus, avoiding biofilm formation by inhibition of cell growth (Lee et al., 2017; Chelliah et al., 2017). The oily fraction of *M. oleifera* leaves is also very important for cosmetic usage, as they are used as a fundamental part of skin and hair products (Chelliah et al., 2017). Moreover, alkanes and alkanes derivatives are another abundant class of molecules in moringa extracts. Chelliah et al. (2017) conducted a complete analysis on *M. oleifera*, performing different extractions with hexane, methanol and water. The extracts were then analyzed with GC-MS, and several compounds were found, the most abundant of which were 1,30-triacontanediol (14.98%) and octacosane (8.57%), in leaves from Madurai variety, and nonacosane (15.55%) and γ-Sitosterol (9.56%) for Chennai variety. Conversely, in seeds they found 6-octadecenoic acid (52.24%), n-hexadecanoic acid (palmitic acid) (6.17%), oleic acid (5.12%) as major compounds in Madurai variety, and (Z)-13-docosenamide (13.62%), propionamide (4.48%), ethyl oleate (4.33%) in Chennai variety (Chelliah et al., 2017). Most of them are alkanes or their derivatives, and they are synthesized by plants to build up an external waxy layer (also called epicuticular wax) to protect leaves from surface wetting and water evaporation. 6-octadecenoic acid, also called petroselinic acid, and palmitic acid are two fatty acids regularly present in plant oils. γ-Sitosterol belongs to the class of the phytosterols, and it is an isomer of β-sitosterol, well known because there is scientific evidence that proved the antidiabetic properties of γ-Sitosterol in vivo against type II diabetes (Balamurugan et al., 2011; Balamurugan et al., 2012). Similar compounds were found by Zhao & Zhang (2013) that carried on a Soxhlet extraction with hexane. The procedure was conducted for 8 h, and when the dried extract was analyzed by GC-MS analysis, they stated that the most abundant compounds in the extract were nonacosane (18.65%), 1, 2, 4-trimethylbenzene (16.96%), heptacosane (7.45%) (Zhao & Zhang et al., 2013). Essential oils are mixtures of dozens or hundreds of molecules, mainly volatiles compounds belonging to the classes of terpenes, alcohols, aldehydes, ketones, esters, phenols and others, often extracted with hydrodistillation, solvent-free microwave extraction (SFME) or supercritical fluid extraction using supercritical carbon dioxide. Despite a wide utilization of essential oils in alternative medicine and aromatherapy, they possess some important properties, such as: antibacterial activity against gram-positive species, probably because of external membrane disruption due to phenolic compounds activity; antioxidant activity, probably due to secondary metabolites with conjugated double bonds; anti-inflammatory activity, triggered by a wide variety of compounds, including antioxidants. An interesting analysis on essential oils was conducted by Chuang et al. (2007) that extracted essential oils from *M. oleifera* leaves using hydrodistillation and tested the extract on fungi cultures finding out that both essential oils and seeds extracts had a positive effect against fungi development, probably exerting anti-fungal activity by disrupting the cellular membrane. The non-polar extract obtained were run through GC-MS instrument, and the main compounds found were mostly alkanes and alkenes, including hexacosane, pentacosane, tetracosane making up almost 30% of total extracts. Moreover, phytol was identified (7.66% based on peak area) (Chuang et al., 2007). Phytol is a diterpene alcohol very abundant in plants because it is one of the two components of chlorophyll. Phytol is used as a food additive, and a group of scientists have proposed that it can be useful to treat schistosomiasis, a disease caused by a parasite (Dhifi et al., 2016). Lastly, a small quantity of terpenes and terpene derivative were found, with hexahydrofarnesylacetone being the most abundant one (1.30%) and then smaller quantities of linalool oxide, farnsylacetone, isolongifolene α-ionene, and α- and β-ionone, barely present in traces (Chuang et al., 2007).

3.3.2 - Polar Compounds

M. oleifera leaves are very high in protein content, and thus, they are highly rich in amino acids. All the essential amino acids can be found in *M. oleifera* leaves and they contribute to more than 50% of total amino acids content. Amino acids presence in leaves was represented by high quantities of glutamic acid. Chelliah et al. (2017) found 15.86 g over 16 g of nitrogen (g/16 gN) of glutamic acid in Madurai variety and 15.33 g/16 gN in Chennai variety, while Lalas et al. (2017) reported 268.7 mg/100 g of glutamic acid. According to Chelliah et al. (2017), methionine was the least abundant in Madurai variety, which was found in quantity of 1.90 g/16 gN, and histidine was the lowest in Chennai variety, present in 3.12 g/16 gN. Conversely, according to Lalas et al. (2017) the least abundant was cysteine which was present in 29.6 mg/100 g of dried leaves. In seeds, the most present amino acid was glutamic acid (14.23 g/16 gN in Madurai, 14.74 g/16 gN in Chennai), whereas the least abundant was threonine for Madurai variety (3.20 g/16 gN) and valine for Chennai variety (2.37 g/16 gN) (Chelliah et al., 2017; Lalas et al., 2017). Regarding glucosinolates, Lopez-Rodriguez et al. (2020) have collected few interesting studies that performed extractions with 70% and 80% methanol, and by using HPLC and UPLC they found different isomers of glucomoringin (4-((α-Lrhamnosyloxy)-benzyl)-glucosinolate) (Figure 3.2(1)) in *M. oleifera* leaves and seeds (Lopez-Rodriguez et al., 2020). These compounds belong to the class of the glucosinolates, natural compounds synthesized by plants responsible for pungent flavor and smell. Their basic structure is formed by a sugar (thioglucose group), bound to a central carbon atom, and the carbon is then bound to a side chain, a nitrogen and a sulphate group, derived from an amino acid. The side chain varies depending on the glucosinolate, determining their biological activity. The main role of glucosinolates is plant defense against infections and diseases. There can be three types of glucosinolates depending on the amino acid that participates to the synthesis of them: aliphatic, aromatic or indolic glucosinolates. Aromatic glucosinolates seems to be the main ones that can be found in *M. oleifera* leaf extracts (Lopez-Rodriguez et al., 2020). Moreover, glucosinolates are precursors of isothiocyanates, molecules containing the -N=C=S group that have showed biological activity in vitro, in particular, they showed hypoglycemic effects, antioxidant and anti-inflammatory effects, anticancer and chemopreventive effects, and it seems that isothiocyanates from *M. oleifera* are more stable than the ones found in other crucifers, because they contain rhamnose, which makes the structure more stable (Lopez-Rodriguez et al., 2020). The review written by Leone et al. (2015) shows a series of tables with many phenolic acids, molecules that belong to the class of polyphenols. They are formed by a monohydroxybenzoic acid unit and different side groups bound the phenolic ring depending on the compound and their importance is due to the documented antioxidant, anti-inflammatory, antimutagenic and anticancer activities (Leone et al., 2015). Phenolic acids were extracted with polar solvents, using different percentages of methanol, water, ethanol, and analyzed via HPLC and LC-MS. The major compounds belonging to this category were caffeic acid, chlorogenic acid (Figure 3.2(2)), o-coumaric acid (Figure 3.2(3)), ellagic acid, ferulic acid and gallic acid, all of them present in quantities between 0.018 mg/g of dried leaves (ellagic acid) and 6.457 mg/g of dried leaves (coumaric acid). More polyphenols are listed in the following tables of the same study. The main polyphenols found are epicatechin (Figure 3.2(4)), kaempferol (Figure 3.2(5)), myricetin, quercetin (Figure 3.2(6)). Myricetin, quercetin and kaempferol are three natural polyphenols that belong to the class of flavonoids, molecules that have a common 15-carbon backbone made by two phenyl rings and a heterocyclic ring between them. The difference between these three molecules lies in the different numbers of hydroxyl groups bound to the basic structure, and thus they exert similar effects on human health. Previous studies listed in the review of Salvamani et al. (2014) have shown that quercetin could have several beneficial effects on human health, such as vasorelaxation to lower blood pressure, positive effects on dyslipidemia, on obesity by reducing accumulation of fats and promoting beta oxidation, anti-inflammatory, anti-atherogenic and anti-atherosclerotic effects in in vivo experiments (Salvamani et al., 2014). Antimicrobial activity of quercetin was also reported. In a recent work, Montone et al. (2021) demonstrated the importance of quercetin combined with lactoferrin and hydroxyapatite (LF-HA)—whose beneficial biological effects were widely investigated (Nocerino et al., 2014; Cuomo et al., 2020; Fulgione et al., 2016)—as antimicrobial agent in food industry. Kaempferol shows a similar impact on health, being a powerful agent against cardiovascular diseases endothelial damage, oxidative stress and against arteriosclerosis. Myricetin effects are explained by the same group, who listed several beneficial effects of this flavonoid, such as antioxidant, antiviral, anticarcinogenic, antiplatelet, hypoglycemic, and cytoprotective properties. Similarly to quercetin, it also showed positive effects on hypertension and accumulation of fats in the body of laboratory rats (Salvamani et al., 2014). Quercetin was the most abundant flavonoid in *M. oleifera* extracts. The highest quantity of quercetin found among the studies is 16.64 mg/g of dried weight (dw), whereas for myricetin is 5.804 mg/g (dw), and for kaempferol is 4.59 mg/g (dw). The last relevant molecules considered in Leone et al. (2015) are the glucosinolates, in particular they listed 4-(α-L-acetylrhamnopyranosyloxy)-benzyl-isothiocyanate (commonly known as moringin) (Figure 3.2(7)) and three isomers of 4-O-(α-Lacetylrhamnopyranosyloxy)-benzylisothiocyanate. Totally, they ranged from 21.84 mg/g (dw) to 59.4 mg/g (dw) among the two works considered in the above-mentioned review (Leone et al., 2015). Moreover, some studies have reported cancer chemoprotective activity, allelopathic activity and repellent/insecticidal activity (Vergara-Jimenez et al., 2017). Finally, Bhalla et al. (2021) conducted identification on two different extracts of *M. oleifera*. They found out that carbonic acid, butyl 2-pentyl ester (20.64%), 2-Isopropoxyethyl propionate (16.87%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (8.99%), 1,3- Dioxolan-2-one, 4,5-dimethyl- (6.16%) were the compounds present in highest peak area in the aqueous extract. A methanolic extraction was performed as well, and because of the different polarity, more compounds were identified in the methanolic extraction compared to the aqueous. In methanolic extracts, 1,3-propanediol, 2-ethyl-2-(hydroxymethyl)-(21.19%), propionic acid, 2-methyl-, octyl ester (15.02%), ethanamine, N-ethyl-N-nitroso- (5.21%), and 9,12,15-octadecatrienoic acid were the most abundant. These extracts were also tested to examine their antioxidant activity on free radicals, with the methanolic extract showing a higher capacity of scavenging free radicals than the aqueous extracts (Bhalla et al., 2021). As stated by the authors, this is probably due to higher polyphenolic molecules found in methanolic extract. A high antioxidant activity can be useful because of a high request both by food and cosmetic industry, as they are used for skin treatment and for food enrichment.

3.4 - Pharmacology

The pharmacological studies on *M. oleifera* are listed in Table 3.2. Different plant organs were extracted with a series of organic solvents and the obtained extracts were tested to determine and confirm the bioactivity known by the ethnomedicine. Pure compounds were also tested and showed interesting bioactivities (Table 3.2 and Figure 3.2).

Table 3.2. Pharmacological activity of organic compounds or extracts of *Moringa oleifera* Lam.

Material Tested	Plant Organ	Disease	Model Used	Observed Effect	Active Principle	Quantity	References
Aqueous, ethanolic and methanolic extracts	Leaves	Anti-microbial	Enteropathogenic and orthopedics wounds bacteria and fungi	Inhibitory properties for wounds and certain fungal infections	n.i.	MIC 20 mg/mL enteropathogens and 3.75 to 30 µg/mL orthopedics wounds	(Oluduro, 2012)
Ethanol (80%), methanol (70%), hydroalcoholic extracts	Leaves	Metabolic disease and diabetes	Beta-carotene-linoleic acid system, Liposome peroxidation, and liver microsomes	Antioxidant	quercetin and kaempferol	1 g 80% methanol, and 70% ethanol hydroalcoholic extracts have DPPH equal to 20, 37, 35 mg of pure quercetin	(Siddhuraju & Becker, 2003)
Ethanol solution (90%)	Leaves	Antioxidant	HPLC, MS-MS, chemometrics, DPPH, ABTS, FRAP	Correlation between phytochemical profile and antioxidant potential	kaempferol 3-O-rutinoside, quercetin 3-O-(6″-malonyl-glucoside), kaempferol 3-O-glucoside, and quercetin derivative	n.r.	(Xu et al., 2021)
Ethanolic and methanolic extract	Leaves	Antioxidant	DPPH	Higher antioxidant potential observed for ethanolic and methanolic extract	n.i.	DPPH from 46.32% ± 1.07% to 58.09% ± 0.92% for methanol extract and 56.76% ± 1.48% to 69.72% ± 1.15% for ethanol extract	(Hossain et al., 2020)
Ethyl acetate extract	Leaves	Cardiovascular	RAW Macrophages	Decreased expression of inflammatory markers	phenolics	LC50, 212.73 µg/mL LC10, 57.53 µg/mL	(Kooltheat et al., 2014)
Hot water and ethanol extracts	Leaves	Cancer	Cancer breast cells	Inhibition of NF-kB signaling	n.i.	IC50 0.31 and 0.26 µg/mL, hot water and ethanolic extract, respectively	(Khalafalla et al., 2010)
Hydroalcoholic extract(80% ethanol)	Leaves	NAFLD	Mice	nt Protected HFD-induced liver dam	n.i.	150 mg/kg body weight, administered by gavage	(Das et al., 2012)
Leaf powder	Leaves	Anti-inflammatory	Guinea pigs	Lower cholesterol and triglycerides and reduced inflammation in the liver	n.i.	10% or 15% Moringa enriched diet, 6 weeks	(Almatrafi et al., 2017a)
	Leaves	Cardiovascular	High-fat fed rats	Decrease cholesterol absorption	β-sitosterol	Bread fortified with extract at	(Halabi et al., 2013)

						10% & 15% for 45 days	
	Leaves	NAFLD	Guinea pigs	Decrease expression of CD68, SERBP1c	n.i.	10% or 15% Moringa diets with 0.25% dietary cholesterol to induce hepatic steatosis, 6 weeks	(Almatrafi et al., 2017b)
	Leaves	Protein deficiency	Fish	Protein supply	n.i.	10%, 20%, and 30% of Moringa leaf meal of total dietary protein. The 10% gave the best response	(Richter et al., 2003)
Methanolic extract	Leaves	Obesity	High-fat induced obesity rats	Anti-obesity properties	n.i.	200 mg and 400 mg/kg reduced atherogenic index (1.7 ± 0.6 and 0.87 ± 0.76, respectively	(Bais et al., 2014)
N, α-L-Rhamnosyl vincosamide	Leaves	Cardiovascular	Isoproterenol (ISO)-induced cardiac toxicity in rats	Increasing the levels of troponin-T, creatine kinase-MB, lactate dehydrogenase and glutamate pyruvate transaminase	N, α-L-Rhamnosyl vincosamide	40 mg/kg for 7 days, oral administration n	(Panda et al., 2013)
			Tri-phenyl tetrazoliumchloride (TCC)				
Total Alkaloids	Leaves	Antihypertensive	Frog hearth	Negative inotropic effect on the heart	Total Alkaloids	3 to 48 ng	(Dangi et al., 39)
			Guinea pigs	Calcium channel Blocking effect			
Water extract (polyphenolics) Hydroalcoholic extract	Leaves	Antioxidant	DPPH	Gold metal ions reduction (Au+ to Au°) to form nanoparticles	n.i.	5 mL extract + 100 mL 1 mM aqueous gold chloride soln	(Tiloke et al., 2018)
		Cytotoxic and anticancer	Male Wistar rats	Decreasing tumor markers (α-fetoprotein and carcinoembryonic antigen) and increasing apoptosis		150 mg/kg–250 mg/kg Male Wistar rats, 7–14 days	
Water extract	Leaves	Antioxidant	DPPH	Improvement extraction for antioxidant tests	tannins	T 80.54°C for 12.19 min. Protein 17.4%, anti-oxidant activity 10.3 µg/mL, tannin 7.8%	(Wahyuni et al., 2020)
	Leaves	Antioxidant	DPPH	Tannins were determined as the active principle for the activity	tannins	n.r.	(Vergara-Jimenez et al., 2017)
	Leaves	Cardiovascular and Diabetes	Starch and phosphate buffer solution	Inhibition of α-glucosidase	quercetin	IC50 0.78 ± 0.21 mg/mL (inhibitor sucrose esterase)	(Adisakwattan and Chanathong, 2011)
			Pancreatic lipase solution	Inhibition of cholesterol esterase		At 10 mg/mL inhibited cholesterol micelle formation of 40.22 ± 2.64%	
Aqueous extracts (AE)	Seeds	Anti-allergic, and Immunosup-pressive prop-erties	Human peripheral blood mononuclear cells	Cytotoxic AE and cMOL, not cytotoxic wsMOL	Coagulant Moringa Lectins (cMOL) and Water-soluble Moringa	6.25 µg/mL	(Araújo et al., 2013)
			NCI-H292, HT-29 and HEp-2 cancer cell lines	Weekly/moderately cytotoxic AE, cMOL, wsMOL			

			Murine erythrocytes	Not hemolytic AE, cMOL, wsMOL	Lectins (wsMOL)		
			Lipopolyssaccharide-stimulated murine macrophages	AE, cMOL, and wsMOL regulated the production of nitric oxide, TNF-α and IL-1β			
			Mouse model of car-rageenan-induced pleurisy	AE reduced leukocyte migration		2.000 mg/kg	
Ethanolic extracts	Seeds	Anti-inflammatory, and anti-diabetic	Rats	Bio-accessibility of 1, using the TNO Intestinal Model (TIM-1), determined as 61% and 62% in fasted and fed states, respectively	Isothiocyanate 1	50 mg/kg intravenously with pure 1 or orally gavage with seed extract	(Richter et al., 2003)
Methanolic extract	Seeds	Antiviral	Influenza A virus (H1N1)	Inhibitory effects against the H1N1 virus	Moringa A, Glucomoringin, and Vitexin	IC50 0.26 ± 0.03, 0.98 ± 0.17, and 3.42 ± 0.37 µg/mL for Moringa A, Glucomoringin and Vi-texin, respectively	(Xiong et al., 2020)
		Anti-inflammatory	Raw264.7 cells	Decreasing the levels of TNF-α, IL-6, and IL-1β			
Phenolic extract	Seeds	Anti-microbial	*Bacillus cereus, Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica*	The observed activity was due to the phenolic compounds obtained from defatted seed extract	n.i.	MIC 0.06–0.157%	(Govardhan Singh et al., 2013)
		Antioxidant	DPPH			IC50 0.9 ± 0.05	
Stem powder	Stems	Anti-inflammatory	Keratinocytes and macrophages induced by PGN, TNF-α, or LPS	Suppression of inflammatory factors. Reduction of NLRP3 expression, and inhibition of caspase-1 activation	β-sitosterol	dose range: 7.5 to 30 µM	(Liao et al., 2018)
Ethanolic extracts	Leaves, seeds, pods	Anti-allergic	Rat basophilic leukemia (RBL-2H3) cells	Extracts and compounds inhibited mast cell degranulation by inhibiting beta-hexosaminidase and histamine release from RBL-2H3 cells, and IL-4 and TNF-α release	β-Sitosterol-3-O-glucoside, Glucomoringin and Quercitin	Extracts of leaves, seeds, and pods: IC50 of 1.20 ± 0.29, 2.52 ± 0.33 and 2.52 ± 0.15 µg/mL, respectively	(Abd Rani et al., 2019)

n.r.: not reported

A recent review by Vergara-Jimenez et al. (2017) reports the major studies dealing with the use of *M. oleifera* in prevention and alleviation of several chronic conditions, including hypercholesterolemia, high blood pressure, diabetes, insulin resistance, non-alcoholic liver disease, cancer, and overall inflammation.

3.4.1 - Leaves: Cardiovascular Activity

Going into details, plant leaves were extracted mainly with hydroalcoholic solvents by different research groups and the extract were tested to evaluate their potential in cardiovascular diseases. Panda et al. (2013) isolated from the extracts the alkaloid N, α-L-rhamnosyl vincosamide (Figure 3.2(8)) and studied the protective effect in isoproterenol (ISO)-induced cardiac toxicity in rats. Oral administration of the alkaloid at 40 mg/kg for seven days markedly reduced the ISO-induced increase in the levels of serum cardiac markers such as troponin-T, creatine kinase-MB, lactate dehydrogenase, and glutamate pyruvate transaminase, as well as cardiac lipid peroxidation. A parallel increase in the cellular antioxidants suggested its cardio-protective and free radical scavenging potential, which was later confirmed by in vitro study (Panda et al., 2013). Rats treated with test compound also improved the ISO-induced abnormal changes in ECG, as well as in cardiac histology. A reduction in myocardial necrosis was further evidenced by the tri-phenyl tetrazolium chloride (TTC) stain in isolated test drug pre-treated rats. The work of Halaby et al. (2013) aimed to study the potential effect of fortified bread with *M. oleifera* leaves powder under 5%, 10%, and 15% concentrations to give more protection against hyperlipidemia. Thirty-two male albino rats were used in this experiment. Results indicated that bread fortified with the extract at 10% and even better at 15% when given to rats for 45 days caused reduction of total cholesterol, triglycerides, low-density lipoprotein, and very-low-density lipoprotein. Kidney function was improved and there was significant reduction in uric acid serum, urea, and creatinine compared to the positive control group (Halaby et al., 2013). The study of Adisakwattana et al. (2011) investigated the effect of leaf water extract of *M. oleifera* on inhibition of alpha-glucosidase and pancreatic alpha-amylase related to diabetes mellitus. Moreover, the study also determined in vitro bile acid binding capacity as well as inhibition of cholesterol micellization, pancreatic lipase, and cholesterol esterase activity. Specific inhibition to intestinal sucrase was found (IC50 value of 0.78 ± 0.21 mg/mL) along with a markedly inhibition (40.22% ± 2.64%) of cholesterol micelle formation (Adisakwattana et al., 2011).

3.4.2 - Leaves: Anti-Inflammatory Activity

Kooltheat et al. (2014) prepared an ethyl acetate fraction of *M. oleifera* from fresh leaves that showed high levels of phenolic and antioxidant activities. Since macrophages, TNF and related cytokines play an important pathophysiologic role in lung damage induced by cigarette smoke, the authors examined the effects of MOEF on cigarette smoke extract-induced cytokine production by human macrophages. Human monocyte derived macrophages (MDM) pre-treated with varying concentrations of *M. oleifera* showed decreased production of TNF, IL-6, and IL-8 in response to LPS and cigarette smoke extract. The decrease was evident at both cytokine protein and mRNA levels. Furthermore, the extract inhibited the expression of RelA, a gene implicated in the NF-κB p65 signaling in inflammation. The findings highlight the ability of *M. oleifera* to inhibit cytokines (IL-8) which promote the infiltration of neutrophils into the lungs and others (TNF, IL-6) which mediate tissue disease and damage (Kooltheat et al., 2014).

3.4.3 - Leaves: Antihypertensive Activity

Dangi et al. (2002) studied the antihypertensive activity of the leaves extract. Preliminary studies indicated that a leaves water extract is efficacious in reducing the chronotropic and inotropic effects on the isolated frog heart. The alkaloids obtained by extract fractionation converted into their salt form, were tested for their activity on the isolated frog heart. The total alkaloidal salts were found to have a negative inotropic effect on the frog heart (Dangi et al., 2002). This activity was further characterized by test on the isolated guinea pig ileum. In their study Bais et al. (2014) evaluated the anti-obesity activity of methanolic leaves extract in rats. Chronic administration of high fat diet (HFD) in rats produced hypercholesterolemia leading to an increase in the body weight total cholesterol, triglycerides, and attenuation in the levels of HDL. Treatment of obese rats with the extract for 49 days resulted in a significant decrease in the level of liver biomarkers, organ weight, and blood glucose level (Bais et al., 2014).

3.4.4 - Leaves: Radical Scavenging and Antioxidant Activity

Siddhuraju et al. (2003) studied the radical scavenging capacities and antioxidant activities. All leaf extracts were capable of scavenging peroxyl and superoxyl radicals. Among the three different moringa samples, both methanol and ethanol extracts of Indian origins showed the highest antioxidant activities, 65.1% and 66.8%, respectively, in the beta-carotene-linoleic acid system. Nonetheless, increasing concentration of all the extracts had significantly ($p < 0.05$) increased reducing power, which may in part be responsible for their antioxidant activity. The major bioactive compounds of phenolics were found to be flavonoid groups such as quercetin and kaempferol (Dangi et al., 2002). After this paper several other authors reported studied on the antioxidant activity of the leaves extract (Vergara-Jimenez et al., 2017; Hossain et al., 2020). Xu et al. (2021) correlated the antioxidant activity to some flavonoid glycosides (Figure 3.2(9–11)) while Wahyuni et al. (2020) to the tannin content (Xu et al., 2021; Wahyuni et al., 2020). This last paper reported a new approach based on HPLC, tandem MS spectrometry (MS-MS), and chemometrics tallowing to correlate specific flavonoid glycosides in the extract with the antioxidant activity tested. Tiloke et al. (2018) studied the antioxidant potential of the extract by the synthesis of phytonanoparticles and the fundamental role as a potential antiproliferative agents against cancer. The resultant gold phytonanoparticles are useful in cancer therapies with improved survival rates and quality of life.

3.4.5 - Leaves: Anticancer Activity

Khalafalla et al. (2010) tested different leaves extracts against leukemia and hepatocarcinoma cells in vitro. The extracts could kill majority (70–86%) of the abnormal cells among primary cells harvested from 10 patients with acute lymphoblastic leukemia and 15 with acute myeloid leukemia, as well as a culture of hepatocarcinoma cells (75% death), but most significantly by the hot water and ethanol extracts.

3.4.6 - Leaves: Hepatoprotective and Nutraceutical Activity

Consumption of high-fat diet (HFD) induces non-alcoholic fatty liver disease and may lead to multiple complications affecting human health. In the study of Das et al. (2012) the preventive as also curative hepatoprotective activity effect of *M. oleifera* leaf extract in alleviating HFD induced liver injury in mice has been reported. Results suggested that *M. oleifera* leaf extract treatment protected HFD-induced liver damage as indicated by histopathology and liver enzyme activity compared to only-HFD fed group ($p < 0.05$). Interestingly, early signs of HFD-induced fatty liver were also alleviated by *M. oleifera* leaf extract. Moreover, significant increase in endogenous antioxidant parameters and lower lipid peroxidation were found in liver of all *M. oleifera* leaf extract treated groups (Das et al., 2012). Almatrafi et al. (2017a; 2017b) studied the mechanisms by which *M. oleifera* leaves modulate hepatic lipids on guinea pigs. Low moringa or 15% high moringa diets with 0.25% dietary cholesterol to induce hepatic steatosis. This study demonstrates that *M. oleifera* leaves may prevent hepatic steatosis by affecting gene expression related to hepatic lipids synthesis resulting in lower concentrations of cholesterol and triglycerides and reduced inflammation in the liver. Richter et al. (2003) evaluated the suitability of freeze-dried moringa leaf meal as alternative protein source for Nile tilapia. Three experimental diets were formulated substituting 10%, 20%, and 30% of the total dietary protein of fish with plant leaves. Diets with higher % of moringa leaves significantly depressed growth performance of the fish compared the other diets. Total phenolics, non-hemolytic saponins, phytic acid, neutral detergent fiber, and acid detergent fiber in the 30% diet may have contributed to the poorer growth performance in these groups thus suggesting the 10% substituting the best option.

3.4.7 - Leaves: Antimicrobial Activity

Oluduro et al. (2012) investigated the antimicrobial activities of the leaf extract of on a series of enteropathogenic and orthopaedics' wounds bacteria and fungi using paper disc diffusion method. All the leaf extracts showed little inhibitory effect on the enteropathogens, whereas aqueous and methanolic extracts showed appreciable inhibitory effects on the orthopaedic's wounds bacteria at 30 mg/mL. Ethanolic extract did not show any zone of growth inhibition on the wound bacteria. Minimum inhibitory concentration was 20 mg/mL on all the enteropathogens and ranged from 3.75 to 30 µg/mL on the orthopaedics' wounds organisms. The study showed that leaves possess inhibitory properties thus can serve as an alternative therapy for wounds and certain fungal infections.

3.4.8 - Leaves, Stems, Pods: Anti-Allergic Activity

The anti-allergic activity of the extracts of leaves, seeds, and pods and of the isolated compounds (β-sitosterol-3-O-glucoside (Figure 3.2(12)), glucomoringin and quercetin) was evaluated by Abd Rani et al. (2019) using rat basophilic leukaemia (RBL-2H3) cells for early and late phases of allergic reactions using as positive control the drug, ketotifen fumarate. The early phase was determined based on the inhibition of beta-hexosaminidase and histamine release, while the late phase was based on the inhibition of interleukin (IL-4) and tumor necrosis factor (TNF-α) release. Both extract and pure compounds showed inhibitions (Table 3.2) thus indicating a potential use as anti-allergic drug candidate.

3.4.9 - Seeds: Anti-Inflammatory Activity

Wolff et al. (2020) reported from the ethanolic seed extract high concentrations of a glucosinolate with isothiocyanate functional group (MIC-1, 1) a compound possessing antiinflammatory and antidiabetic properties. Because it was not characterized metabolically, the authors studied its bioaccessibility using a human intestinal model and bioavailability using serum from treated rats. The results suggest that the compound remains largely unmodified during uptake, unlike other isothiocyanates, and has favourable bioaccessibility and bioavailability characteristics for a potential therapeutic agent. The extracts of moringa seeds are used in rural areas of developing countries to treat drinking water because the seeds contain lectins, carbohydrate-binding proteins able to reduce water turbidity because of their coagulant activity. The study by Araújo et al. (2013) evaluated the cytotoxic and anti-inflammatory properties of the aqueous seed extract on NCI-H292, HT-29, and HEp-2 cancer cell lines, and on murine erythrocytes (Table 3.2). In particular, the extracts exhibited anti-inflammatory activity on lipopolysaccharide stimulated murine macrophages by regulating the production of nitric oxide, TNF-α and IL-1β. The aqueous seed extract reduced leukocyte migration in a mouse model of carrageenan-induced pleurisy; the myeloperoxidase activity and nitric oxide, TNF-α and IL-1β levels were similarly reduced. Histological analysis of the lungs showed that the extract reduced the number of leukocytes.

3.4.10 - Seeds: Antiviral Activity

Xiong et al. (2020) isolated eleven compounds from methanol seeds extract, including two previously unknown and nine known compounds. These compounds were authenticated as a carbamate, three phenylglycosides, four phenol glycosides, two nucleosides, and one flavonoid. Antivirus activity analyses revealed that Moringa A, glucomoringin, and Vitexin (Figure 3.2(13)) possessed strong inhibitory effects against the H1N1 virus, having IC50 values in the range of IC50 = 0.26 ± 0.03, 0.98 ± 0.17, and 3.42 ± 0.37 μg/mL, respectively. Furthermore, these three compounds could decrease the levels of TNF-α, IL-6, and IL-1β, which occur in hosts because of H1N1 infections.

3.4.11 - Seeds: Anticancer Activity

Five-Fluorouracil (5-FU) is a strong anticancer agent commonly used for the treatment of various malignancies. Famurewa et al. (2019) explored whether moringa seed oil could protect against 5-FU-induced nephrotoxicity and its mechanism of action in Wistar rats. Rats were subjected to prophylactic oral treatment of moringa seed oil (Table 3.2). The data obtained suggested that 5-FU-induced nephrotoxicity by oxidative stress, exacerbation of pro-inflammation and apoptosis. The inhibition of the alterations by moringa seed oil is relevant in the clinical management of 5-FU nephrotoxicity in cancer patients.

3.4.12 - Seeds: Antioxidant Activity

Phenolic compounds, antioxidant and antibacterial activities of defatted seed flour were investigated by Singh et al. (2013). The results showed that extractability of phenolic compounds was significantly higher ($p < 0.05$) in bound phenolic extract (4173.00 ± 32.22 mg gallic acid equivalents (GAE)/100 g) than in free phenolic extract (780.00 ± 14.2 mg GAE/100 g) and it showed higher antioxidant and antimicrobial activities. The IC50 value for DPPH radical scavenging activity was 0.9 ± 0.05 and 14.9

± 0.07 mg/mL for bound phenolic and free phenolic extracts, respectively. Bound phenolic extract was more effective (minimum inhibitory concentration (MIC), 0.06–0.157%) than free phenolic extract (MIC, 0.117–0.191%) against tested bacteria. The data indicated that moringa seeds could be a good source of antioxidants and antibacterials for food and pharmaceutical industries. Finally, Liao et al. (2018) identified β-sitosterol isolated from *M. oleifera* stems as an anti-inflammatory compound on two cell lines, keratinocytes and macrophages induces by PGN, TNF-α, or LPS. β-sitosterol over a dose range of 7.5 to 30 µM, dispersed in the medium of the well as nanoparticles with diameters of 50 ± 5 nm, was able to suppress the secretion of the above-mentioned inflammatory factors.

3.5. Conclusions

M. oleifera is an ancient medicinal plant growing in a variety of environments and climates, able to resist harsh environments that recently received an increased interest in the food industry and as phytopharmaceuticals. The phytochemical analysis of the plant's organs identified metabolites belonging to different classes, including flavonoids, anthocyanins, isothiocyanates, anthraquinones, alkaloids, essential oils, tannic acid, saponins, steroids, terpenoids, and cardiac glycosides. Pharmacological studies confirmed the use of the plant as traditional medicine, showing bioactivity including hepatoprotective, antihypertensive, cholesterol-lowering, anti-urolithiasis, antifertility, antidiabetic, and antioxidant activity, nutraceutical and antimicrobial properties. In addition, most published papers reported biological studies on the plant extracts while only few studies tested pure compounds. Interestingly, these compounds belong to the class of phenolics, including phenylpropanoids, flavonoids, flavonoid O-glycosides, flavonoid C-glycosides, and glucosinolates. Thus, the reported paper reviewing the literature on the phytochemistry and pharmacology of the plant indicates that more research is needed to investigate the chemical and biological properties on a larger scale and to clarify the mechanism of action. This aspect will be crucial for its use in phytopharmaceutical, nutraceutical, and food industries.

Chapter 4 - Chemical Analysis and Antimicrobial Activity of *Moringa oleifera Lam.* Leaves and Seeds under drought stress

4.1 - Introduction

Moringa oleifera Lam., also known as miracle, horseradish or drumstick tree is a plant member of the Moringaceae family able to grow as a short and slender tree. *M. oleifera* is native to Eastern countries, such as Himalaya, India, Pakistan, Asia Minor, Africa, and Arabia (Anwar et al., 2007), but today it is distributed also in other countries due to the plant tenacity making possible the cultivation in different habitats (Figure 4.1). The roots can penetrate deeply into the soil and retain water for long periods, making possible the growth of the plant also in dry and desertic soils and at different rainfall levels (Rani & Arumugam,2017; Chaudhary & Arumugam, 2017). The optimal temperature for *M. oleifera* growth goes from 25 to 40 °C, but it can withstand temperatures ranging from -1 to 3 °C and from 38 to 48 °C, being able to resist to a large variety of environments (Palada et al., 1996). The interest in this plant is mainly derived from its traditional uses. In fact, in traditional medicine, *M. oleifera* parts were used to treat a large variety of conditions, as the plant was believed to possess several properties, such as carminative, anti-inflammatory, laxative, anti-rheumatic, anti-inflammatory activities (Anwar et al., 2007; Anzano et al., 2021). Today, *M. oleifera* is used for many purposes: in human diet and livestock feeding, thanks to the excellent nutritional aspects (high quantities of vitamins, proteins, and amino acids) (Rani & Arumugam, 2017); in medicine, thanks to the properties mentioned earlier; as fuel wood; for soil conservation (used as green manure) and water purification (the seeds are used for clarification of water) (Abdulsalam et al., 2007).

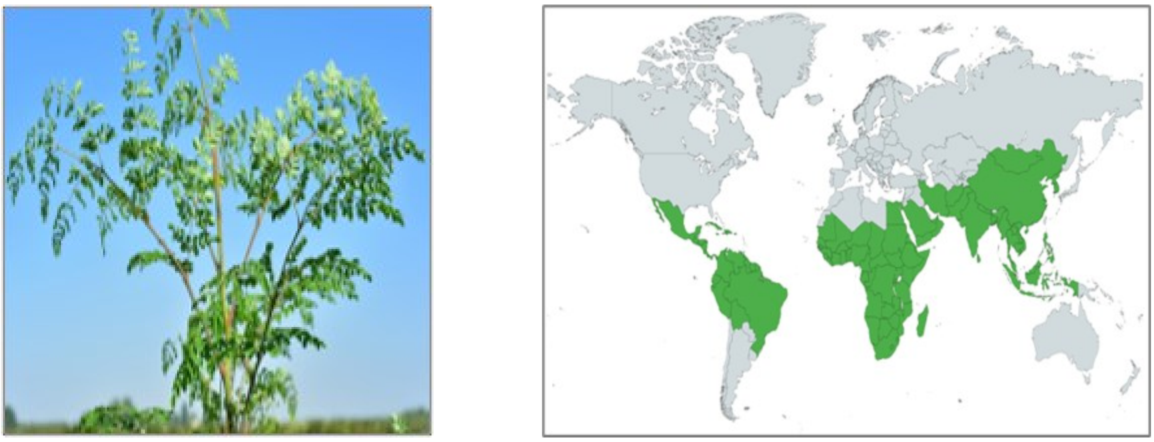

(**a**) (**b**)

Figure 4.1. *(**a**) Moringa oleifera tree; (**b**) Worldwide diffusion of the plant.*

Both polar and apolar leaf and seed extracts contain several relevant compounds belonging to the classes of fatty acids, alkanes, amino acids, glucosinolates, polyphenols, which make *M. oleifera* a very interesting plant from a nutritional and a pharmacological point of view (Anzano et al. 2021, Shakour et al., 2023). Thus, several pharmacological properties have been investigated and attributed to the seeds and leaf extracts, e.g. cardiovascular activity (Panda et al., 2013), anti-inflammatory activity (Kooltheat et al., 2014), antihypertensive activity (Dangi et al.,2002), radical scavenging and antioxidant activity (Araújo et al., 2013; Xu et al., 2021), anticancer activity (Khalafalla et al., 2010; Tiloke et al., 2018), hepatoprotective and nutraceutical activity (Almatrafi et al., 2017b; Richter et al., 2003), anti-allergic activity, antimicrobial (Abd Rani et al., 2019) and antiviral activity (Xiong et al., 2020). The aim of this study was to explore the qualitative and quantitative aspects of the metabolite profile of *M. oleifera* leaves and seeds and to evaluate the antimicrobial activity of their polar and apolar extracts. The interests in plant metabolite profiling approaches are rapidly growing as they can give a wide overview of the metabolites present in a plant extract

(Sumner et al., 2003). It is often used to characterize extracts from plant tissues, or to compare extracts from plants grown in different conditions, as it provides a “fingerprint” of the status of the plant. Here, we performed an untargeted chemical analysis of leaf and seed extracts using an integrated approach of proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy and Gas Chromatography Mass Spectroscopy (GC-MS), followed by the identification of the main components from the obtained spectra. Then, the extracts were tested against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Salmonella enterica* to assess the antimicrobial activity.

4.2 - Results

4.2.1 - NMR analysis of *M. oleifera* polar extracts

Polar extracts of leaves and seeds of *M. oleifera* were analyzed in triplicate through ^{1}H-NMR spectroscopy obtaining a qualitative and quantitative profile of plant tissues analyzed. Peak by peak analysis of the spectra was performed, with the aid of 2D NMR experiments (COSY, HSQC and HMBC), and by comparison with standard compounds available in laboratory and reported in the literature. The main metabolites identified from the NMR spectra are listed in Table 4.1, where they are divided in chemical structural classes.

Table 4.1. *^{1}H-NMR chemical shifts, assignment and multiplicity at 600 MHz in D_2O of organic compounds detected in the polar extracts of M. oleifera leaves and seeds.*

Compound	Assignment	^{1}H (ppm)	Multiplicity (J (Hz)]	*Leaves*	*Seeds*
		Organic acids			
Citric acid (CI)	α,γ- CH_2	2.50**	d (15.0, 15.0]	x	x
	α’,γ’-CH_2	2.68**	d		
Fumaric acid (FU)	α-CH	6.61*	s		x
Malic acid (MA)	β’-CH_2	2.43	dd (15.7, 8.9]	x	x
	β-CH	2.78	dd (15.7, 3.7]		

	α-CH	4.33*	dd (8.9, 3.7]		
Succinic acid (SU)	α-CH_2	2.42**	s	x	x
Acetic acid (AC)	α-CH_3	1.96*	s	x	x
Amino acids					
Alanine (Ala)	β-CH_3	1.51*	d (7.0]	x	x
Asparagine (Asn)	⍰-CH	2.84	dd (17.4, 3.8]	x	x
		2.98*	dd (4.0, 13.0]		
Isoleucine (Ile)	δ-CH_3	0.93	t (7.0]	x	x
	γ'-CH_3	1.05*	d (7.0]		
γ-aminobutyrate (GABA)	β-CH_2	1.92	m	x	x
	α-CH_2	2.34	t (7.0]		
	γ-CH_2	3.04*	t (7.0]		
Leucine (Leu)	δ-CH_3	1.01*	d (7.0]	x	x
Glutamic acid (Glu)	β, β'-CH	2.08, 2.16*	m	x	
Phenylalanine (Phe)	CH-4	7.35	t (7.0]	x	x
	CH-2,6	7.45*	m		
Threonine (Thr)	γ-CH_3	1.36*	d (7.0]	x	x
Tryptophane (Trp)	CH	7.74*	d (7.5]	x	
Tyrosine (Tyr)	CH-3,5	6.86**	d (7.0]	x	x
	CH-6,8	7.10**	d (7.0]		
Valine (Val)	γ'-CH_3	1.03	d (7.0]	x	x
	γ-CH_3	1.08*	d (7.0]		
Carbohydrates					
β-Glucose (β-Glc)	CH-1	4.60*	d (8.0]	x	x
α-Glucose (α-Glc)	CH-1	5.21*	d (4.0]	x	x
Sucrose (Suc)	Glc CH-1	5.43*	d (3.8]	x	x
	Fru CH-3'	4.20	d (8.5]		
myo-Inositol (Myo)	CH-4	3.34*	t (9.5]	x	x
	CH-2,5	3.58**			
	CH-3,6	3.66**			
Other compounds					
Adenosine (Adn)	CH-2	8.25	s	x	x
	CH-8	8.36*	s		
Caffeic acid (Caf)	α-CH	6.42*	d (16.0]	x	
Flavonoids (Fla)	CH	6.53*		x	
Choline (Cho)	$N(CH_3)_3^+$	3.24*	s	x	x
Ethanolamine (Eta)	β-CH_2	3.15*	bt (7.0]		x
Glucomoringin (GMor)	CH_3	1.19	d	x	x
	Glc CH-1	4.33	d (8.0]		
	Rha CH-1	5.57*	d (2.0]		
Glucosinolates (GSin)	Rha CH-1	5.55*	d (2.0]		x
	Rha CH-1	5.53*	d (2.0]		
Quercetin (Que)	CH	7.65*		x	
Trigonelline (Tri)	CH-4	8.12	t	x	x
	CH-3,5	8.88	t		
	CH-1	9.17*	s		

* Signal used for quantification; ** Signal partially overlapped

In the high field region of the leaves spectra (Figure 4.2a), diagnostic signals for the methyl groups of the branched amino acids are showed. Leucine (Leu), isoleucine (Ile) and valine (Val) were identified using the three doublets at δ 1.01 (J=7.0 Hz), δ 1.05 (J=7.0 Hz) and δ 1.08 (J=7.0 Hz) respectively. Threonine (Thr) was also identified by a doublet at δ 1.36 (J=7.0 Hz), corresponding to the γ-CH_3 group, while the β-CH_3 group of alanine (Ala) was assigned through a doublet at δ 1.51 (J=7.0 Hz). This signal was shifted downfield compared to the previous ones because of the proximity to the nitrogen atom. Asparagine (Asn) was identified through the double doublet resonating at δ 2.98 (dd, J=4.0 and 13.0 Hz), whereas the β-CH and β'-CH groups of glutamic acid (Glu), used for its identification, resonated as multiplets at δ 2.08 and δ 2.16, respectively. The amino acid γ-aminobutyrate (GABA) was identified using the triplet at δ 3.04 (J=7.0 Hz). In the low field region of the ^{1}H-NMR spectra of leaves (Figure 4.2c), all the characteristic groups belonging to aromatic amino acids were found. Going into details, the multiplet at δ 7.45 and the triplet at at 7.35 were assigned to phenylalanine (Phe), the doublets at δ 6.86 and 7.10 belonged to tyrosine (Tyr) and the doublet at δ 7.74 (J= 7.5 Hz) was indicative of tryptophan (Trp), all of them corresponding to aromatic protons at different positions of the skeleton (see Table 4.1). The signals used for the identification of five organic acids were found across all the spectral regions (Figure 4.2a,b,c). Acetic acid (AC) was identified through the singlet at δ 1.96 found in the high field region of the spectra and corresponded to the α-CH_3 group. malic acid (MA) was identified by a double doublet resonating at δ 4.33 (J=8.9 and 3.7 Hz), while fumaric acid (FU) showed the characteristic singlet at δ 6.61, both of them being associated to a α-CH group. Signals detected and associated to citric acid (CI) and succinic acid (SU), resonating respectively at δ 2.50 and 2.60, were overlapped, and the quantification of these two metabolites was not possible. The region of the spectra between δ 3.35 and δ 4.10 (Figure 4.2b) was very crowded due to the high number of signals belonging to sugars and sugar alcohols that were not useful for compound identification. The characteristic signal of the anomeric proton (H-1) of α-glucose (α-Glc) resonated at δ 5.21 (d, J = 4.0 Hz), while the anomeric proton of β-glucose (β-Glc) appeared at δ 4.60 (d, J = 8.0 Hz) (Figure 4.2b and Table 4.1). Sucrose (Suc) was identified using the anomeric proton signal resonating at δ 5.43 (d, J=3.8 Hz) due to its Glucose moiety. The triplet resonating at δ 3.34 (J=9.5 Hz) was assigned to myo-Inositol (Myo).

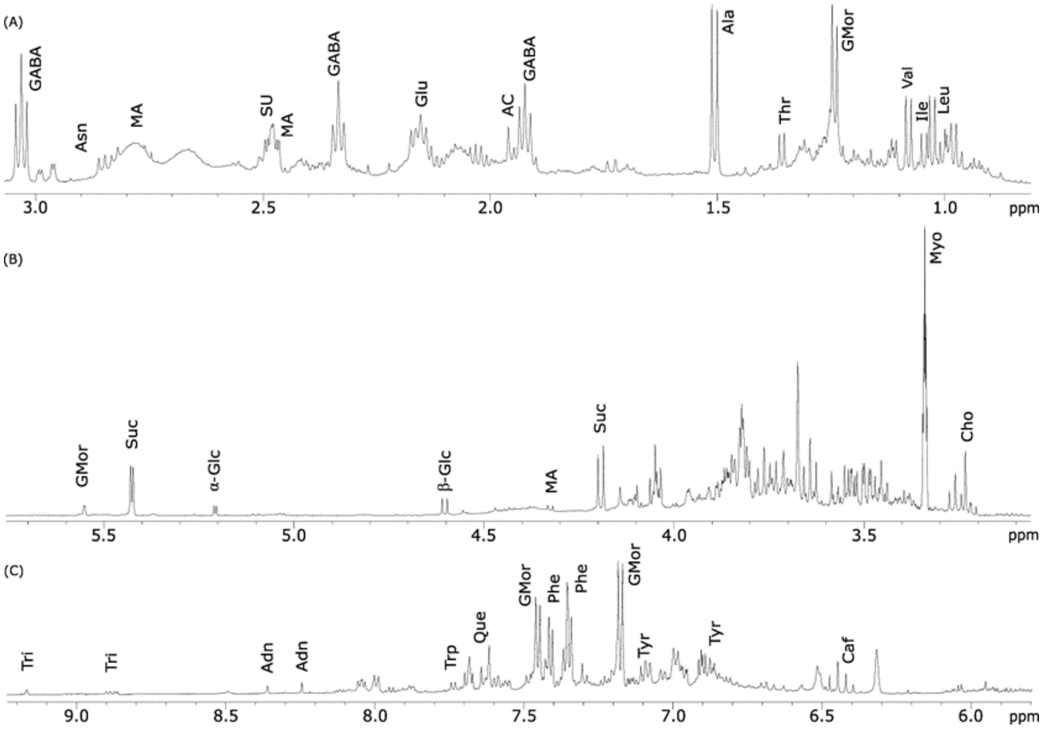


Figure 4.2. *^{1}H-NMR spectrum of M. oleifera polar leaf extract acquired in D_2O at 600 MHz. Spectral regions between (A) 0.5-3.1 ppm vertically expanded (x 5); (B) 3.1-5.7 ppm; (C) 5.8-9.2 ppm vertically expanded (x 2).*

Among other compounds, choline (Cho) presence was showed by the characteristic singlet corresponding to its methyl groups, resonating at δ 3.24, and shifted downfield in the spectra because of the presence of nitrogen (Table 4.1). Glucomoringin (GMor) was identified using the doublet (J=2.0 Hz) resonating at δ 5.57, corresponding to H-1 of its rhamnose residue. In the low-field region between 5.7 and 9.2 ppm, caffeic acid (Caf) was identified by a doublet resonating at δ 6.42 (J=16.0). Signals for flavanoids (Fla) and in particular for quercetin (Que) were found at δ 6.53 and δ 7.65, respectively, both of them only present in spectra of leaves. Lastly, the nucleoside adenosine (Adn) showed a singlet at δ 8.36 (s, CH-8), while the presence of trigonelline (Tri) was revealed by the characteristic signals resonating at δ 9.17 (s, CH-1) and 8.88 (t, CH-3,5) (Figure 4.2c and Table 4.1). Figure 4.3 shows the ^{1}H-NMR spectrum of the seeds polar extract with compounds identification. A close similarity with the NMR spectrum of leaves (Figure 4.2) was evident although there were some differences in the metabolite profiles (Table 4.1). Going into details, signals for flavonoids and, particularly, quercetin were undetectable as well as that for caffeic acid (Figure 4.3). The amino acids phenylalanine and glutamic acid were not found in the seed extracts while the signal identifying fumaric acid were additionally present. The aromatic amino acids were undetectable in the seed extracts. Interestingly, in the aromatic region appeared additional signals at δ 7.15 and 7.37 (each d, J=7.0 Hz) ascribable to glucosinolates (GSin) whose presence was confirmed by the signals at δ 5.55 and 5.53 corresponding to H-1 of rhamnose (Figure 4.3b). Amon other compounds, ethanolamine (Eta) was identified using the broad triplet resonating at δ 3.15 (β-CH_2, J=7.0) (Table 4.1). The differences in the quantified metabolites between leaf and seed extracts are showed in Figure 4.4. Acetic acid and malic acid were the only two organic acids that could be quantified. Acetic acid was slightly more abundant in the leaf extracts. Malic acid quantity was roughly double in seeds compared to the malic acid content of leaves. All the amino acids were more abundant in leaf extracts than in seed extracts. Glutamic acid was the amino acid present in the larger quantity, and along with phenylalanine they showed the largest difference between the two extracts. The aromatic amino acids phenylalanine and tryptophane were undetectable in seed extracts. (Figure 4.4). Conversely, all the carbohydrates were more abundant in seeds, especially for glucosinolates and glucomoringin, that showed the largest difference among the two extracts. Monosaccharides and disaccharides followed the same trend, but the difference was smaller. Considering the relative and the absolute quantity, carbohydrates were also the most abundant molecules in the leave and seed extracts overall. Regarding the other compounds, caffeic acid and flavonoids were only detected in leaf extracts. Moreover, the quantity of quercetin was larger in leaf extracts, while ethanolamine and trigonelline showed a comparable quantity in the two extracts. Finally, choline was present in a larger amount in the seed extracts.

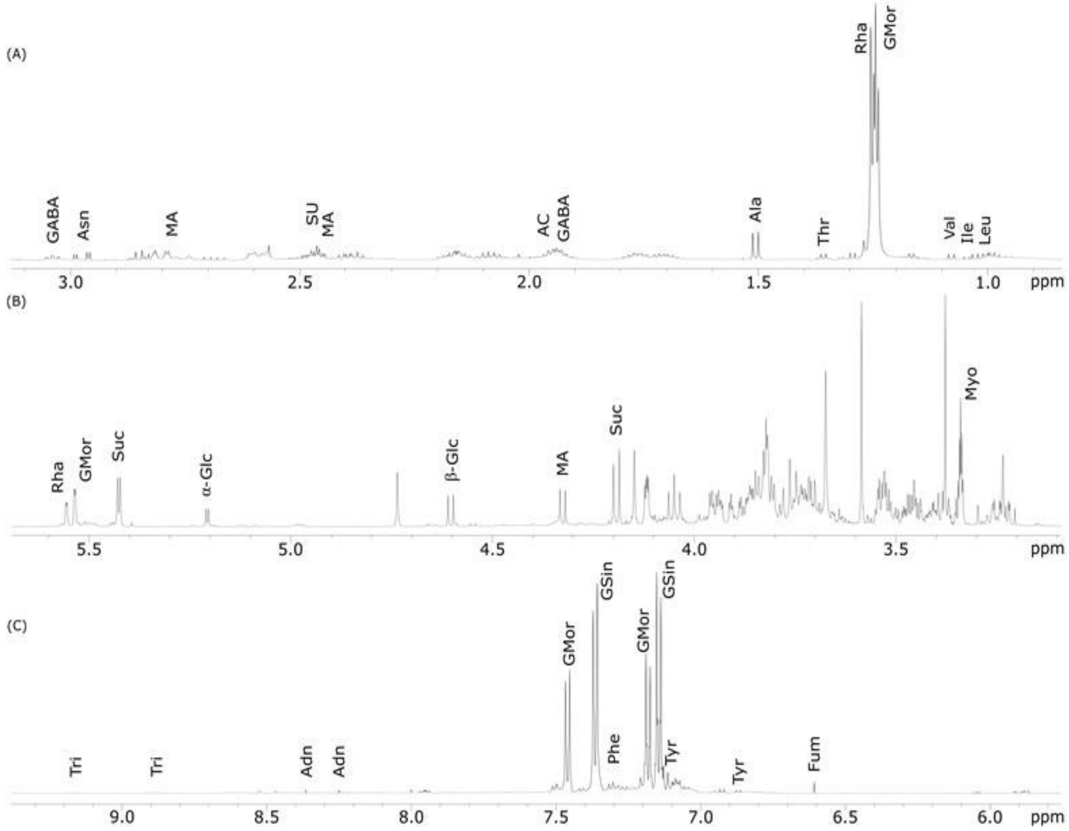


Figure 4.3. *^{1}H-NMR spectrum of M. oleifera polar seed extract run in D_2O at 600 MHz. Spectral regions between (A) 0.5-3.1 ppm; (B) 3.1-5.7 ppm; (C) 5.8-9.3 ppm vertically expanded (x 3).*

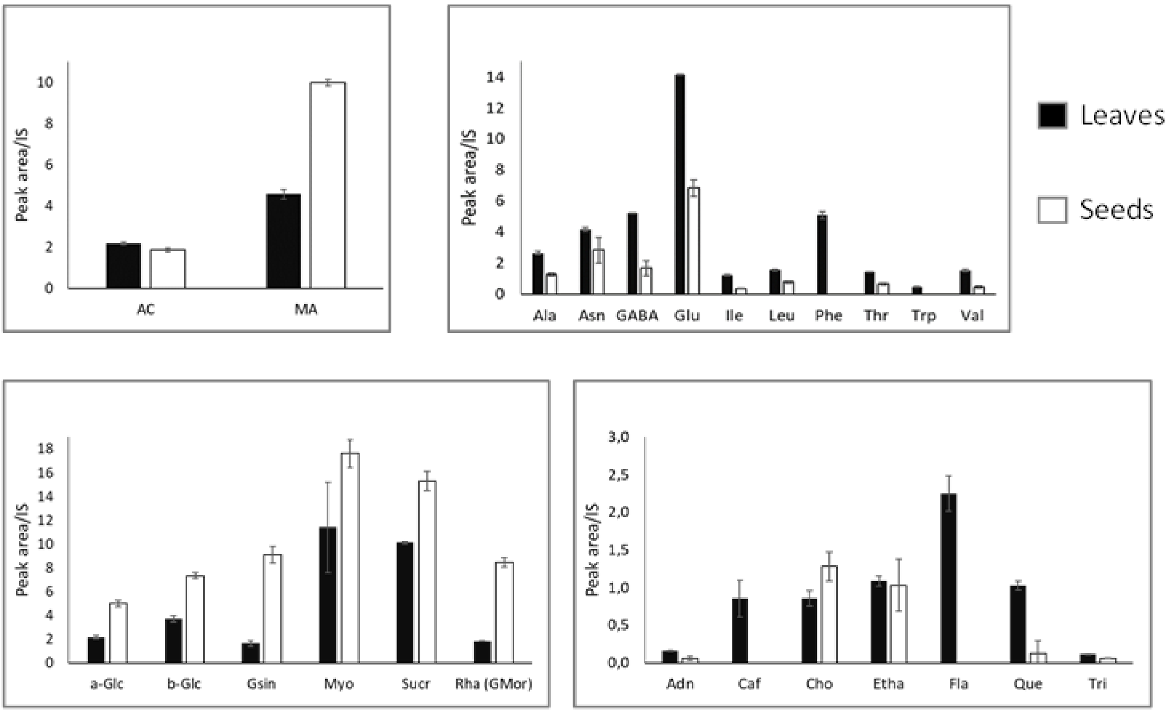


Figure 4.4. Quantitative data of the metabolites identified in leaves and seeds polar extracts. Top-left, organic acids; top right, amino acids; bottom-left, sugars; bottom-right, other compounds. Showed data refers to the mean and standard deviation of three replicates.

4.2.2 - GC-MS analysis of *M. oleifera* apolar extracts

GC-MS spectroscopy was used to analyze the apolar extracts from leaves and seeds from a qualitative and quantitative point of view. All the identified metabolites were present in a larger quantity in apolar seed extract compared to apolar leaves extract (Figure 4.5).

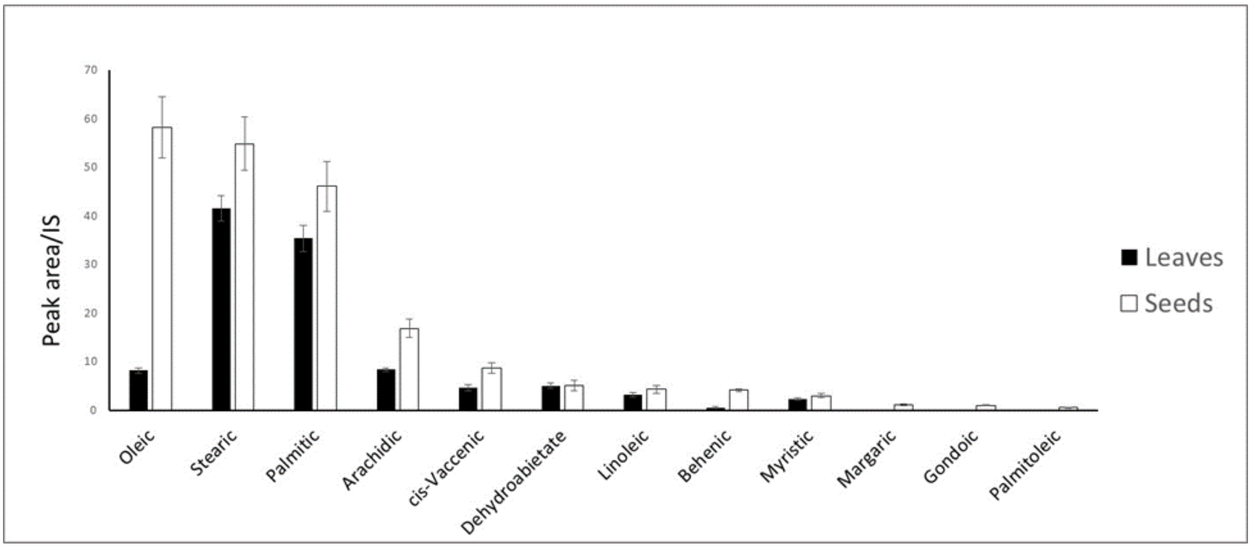


Figure 4.5. *Quantitative data of the metabolites identified in leaves and seeds apolar extracts. Showed data refers to the mean and standard deviation of three replicates.*

Oleic acid is the one present in the largest amount in the apolar seed extract, followed by stearic acid and palmitic acid. Oleic acid is also the fatty acid that shows the largest concentration difference between leaves and seed apolar extracts. On the contrary, stearic acid was the most abundant fatty acid in leaves, followed by palmitic acid. Arachidic acid was also quite abundant, especially for seeds apolar extract. All the other detected fatty acids, including cis-vaccenic, linoleic, behenic, myristic, lauric, margaric, gondoic, palmitoleic, and lignoceric acid, were present in minor amounts. Gondoic and palmitoleic acid were only detected in seed extracts. Oleic acid represents the major metabolite among the unsaturated fatty acids in the seeds (58.18 ± 6.32), while it accounted only for 8.17 ± 0.57

in the leaves. (Figure 4.5). In addition to fatty acids, two terpenoids were identified as methyl Abiet-8-en-18-oate, present only in leaves, and Methyl dehydroabietate, detected in both apolar extracts.

4.2.3 - Antimicrobial activity

Polar and apolar extracts obtained from leaves and seeds of *M. oleifera* were tested for antimicrobial activity against two Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and two Gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica*) pathogens (Figure 4.6).

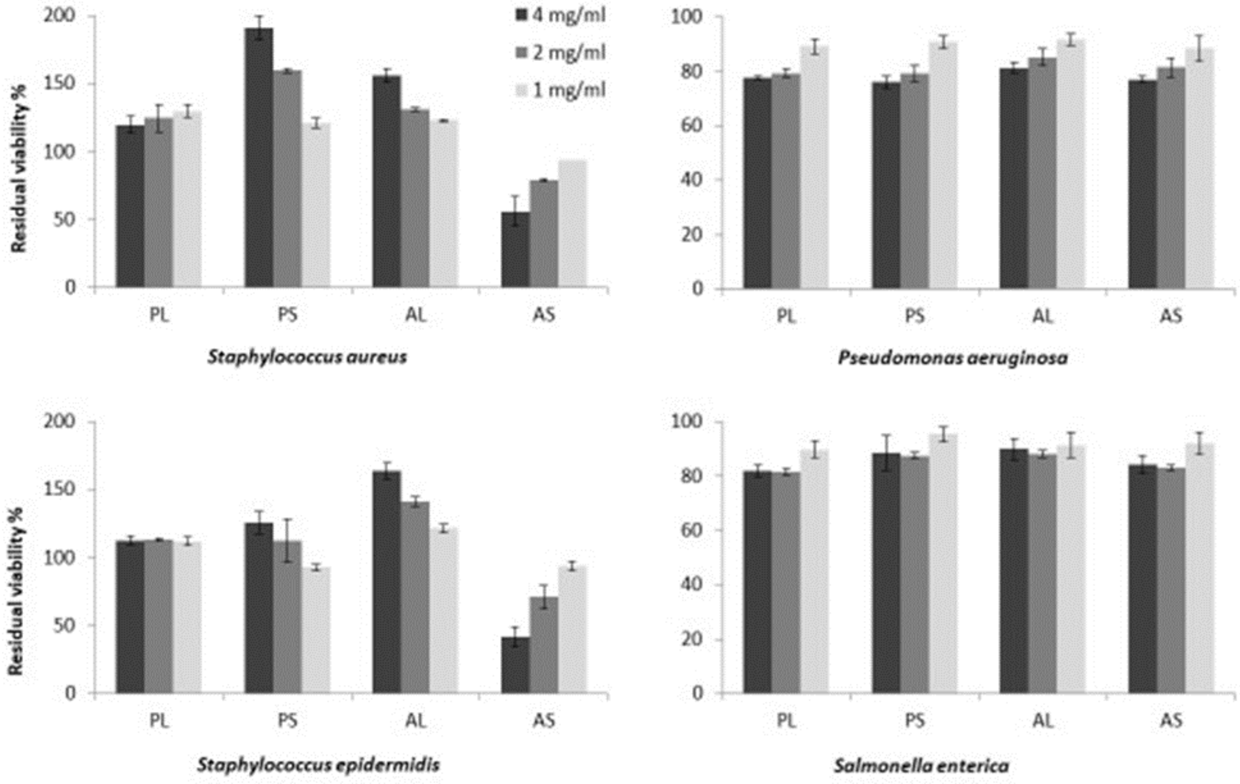


Figure 4.6. *Antimicrobial activity of the M. oleifera extracts: PL = polar leaves extract; PS = polar seeds ectract; AL = apolar leaves extract; AS = apolar seeds extract, tested at 4 mg/mL, 2 mg/mL and 1 mg/mL. Bacterial viability was assessed my measuring the optical density at 600 nm, and expressed as percentage of residual viability compared to the untreated samples. Showed data refers to the mean and standard deviation of three replicates.*

Data reported in Figure 4.6 show that neither polar nor apolar extracts obtained from leaves and seeds affected the microbial growth of the tested Gram-negative bacteria. Whereas apolar extracts obtained from seeds showed a clear dose-dependent antimicrobial effect against the tested Gram-positive bacteria (Figure 4.6). In detail, a reduction of more than the 50% in microbial viability was obtained at a concentration of 4 mg/mL on both the tested *Staphylococcus* species.

4.3 - Discussion

Chemical characterization of *M. oleifera* was carried out using an integrated approach based on ^{1}H-NMR and GC-MS analyses. Regarding the polar fraction extracted from leaves and seeds, twenty-nine metabolites were identified and quantified (Table 4.1 and Figure 4.4). The main metabolites are amino acids, eleven of which were annotated. Generally, they were more abundant in leaves than in seeds. Six carbohydrates were identified and all of them were more abundant in seed than in leaf extracts. Only five organic acids were identified, but signals for succinic and acetic acids were overlapped, and thus, only three of them were quantified. Lastly, among other compounds adenosine, caffeic acid, choline, ethanolamine, quercetin, trigonelline and the general class of the flavonoids were identified and quantified, and all of them were more abundant in leaves except for choline. The chemical composition found by the methods used in this study is similar to the one found by a previous article (Mahmud et al., 2014). The GC-MS analysis resulted in the identification of twelve metabolites, all of them being more present in seeds than in leaves (Figure 4.5). Oleic acid is the main fatty acid, most than six times more abundant in seeds than in leaves, and with cis-vaccenic, linoleic, gondoic and palmitoleic acid, it represents the unsaturated fraction of the oil. Conversely, the saturated fraction is composed of stearic, palmitic, arachidic, behenic, myristic and margaric acid. The antimicrobial assay showed the apolar fraction of the seeds exerts significant antimicrobial activity against the Gram-positive bacteria *S. aureus* and *S. epidermidis*, while it was not active against *S. enterica*. and *P. aeruginosa* (Figure 4.6). These results agree with the literature (Dinesha et al., 2018; Lalas et al., 2012) reporting antimicrobial activity for *M. oleifera* seed oil against *S. aureus* (Dinesha et al., 2018). Similar activity has been reported for *Moringa peregrina* oil (Lalas et al., 2012). Conversely, no antimicrobial effects were found for aqueous extracts from leaves (Moyo et al., 2011). However, studies have detected antimicrobial activity for polar extracts from leaves and seeds, but at concentrations higher than the ones used in our study (Jabeen et al., 2008; Oluduro, 2012; Ndhlala et al., 2014). Moreover, other studies found no antimicrobial effects against *S. aureus* for seeds petroleum ether extract (Saadabi & Abu Zaid, 2011; Ruttarattanamongkol & Petrasch, 2015). The different effects between the apolar fractions of seeds and leaves could be due to the remarkable difference in fatty acids content of the two fractions. It is well known that free fatty acids and monoglycerides can exert an antimicrobial effect especially against gram-positive bacteria (Kodicek & Worden, 1945; Galbraith et al., 1971; Desbois & Smith, 2010; Churchward et al., 2018) that is dependent on the number of double bonds, since unsaturated fatty acids seem to be more active than saturated, and there is a correlation between the number of double bonds possessed by a fatty acid and its antimicrobial activity (Desbois et al., 2018). The antimicrobial activity of fatty acids also depends on the length of the carbon chain. The mechanism of action is still under debate, but it seems due to the detergent properties of free fatty acids that can be disruptive for the cellular membrane integrity or for the functionality of the enzymes involved in the electron transport chain.

4.4 - Materials and Methods

4.4.1 - Chemicals and Solvents

n-Hexane and methanol were obtained from Delchimica Scientific Laboratories (Naples, Italy). Deuterium oxide (D_2O, 99.8 atom% D), used in NMR experiments, was obtained from ARMAR

Chemicals (Döttingen, Switzerland). Dimethyl - 4 - silapentane sodium sulphonate (DSS), used in NMR experiments, was purchased from Merck (Darmstadt, Germany).

4.4.2 - Plant material

Moringa oleifera dried leaves and seeds were bought from a local shop, in Nablus, Palestine. Leaves and seeds were grinded using a kitchen mixer to obtain a fine powder that was subjected to further extraction procedure and analyses.

4.4.3 - Metabolite extraction

The metabolite extraction was performed following the method described in de Falco et al. (2022). 4 g of powdered leaves and seeds were extracted with 50 mL of n-hexane, stirring the solution for 1 h. Then, the apolar extracts obtained were filtered, transferred into vials, and dried at room temperature. The remaining pellets were subjected to polar extractions using 50 mL of a methanol:water solution (1:1), stirring the solution for 1 h. The supernatants were then separated by filtration and centrifuged at 3000 rpm for 10 min, RT. The extracts were then dried using a rotary evaporator (30 °C). Both apolar and polar extracts were stored at 4 °C waiting for further analyses. The procedure has been performed in triplicate for each plant material analyzed.

4.4.4 - ^{1}H-NMR analysis

10 mg of the polar extracts were solubilized in 600 µL of D_2O and transferred into a 5 mm NMR tube. Then DSS was added as internal standard at a concentration of 0.2 mg/mL. The NMR spectra were

recorded at 298°K on a Varian Unity Inova spectrometer operating at 600 MHz. The ^{1}H-NMR experiments were performed with 128 transients and 16 K complex data point. The recycle time was set to 5 s, and a 45° pulse angle was used. Chemical shifts were referred to DSS signal (Δ 0.00 ppm). All spectra were processed using iNMR program (www.inmr.net), phased and baseline corrected. In total, 12 spectra were acquired. Quantification was performed by signal integration relative to the internal standard, DSS, as described in Lanzotti et al. (2022). The region of the solvent peaks was excluded from the analysis. Spectral peak assignments of the detected compounds were obtained based on pure standards purchased by Sigma-Aldrich, and on combined comparison with data reported in the literature and in Human Metabolome Database (HMDB). All spectra were manually phased, and baseline corrected.

4.4.5 - GC-MS analysis

To obtain stable and volatile compounds, apolar extracts were derivatised by using a methanolysis reaction before GC-MS analysis. For this purpose, an aliquot of each apolar extract (0.5 mg) was transferred into a vial and dissolved in 1 mL of methanol + hydrochloric acid 1 N. The vials were vortexed and left at 50°C overnight, then the reaction mixtures were dried under nitrogen, solubilised in n‑hexane and analysed by GC-MS. According to the method described in Grauso et al. (2019a). 1 μL of derivatised samples were injected in a pulsed splitless mode into an Agilent‑7820A GC system with 5977E MSD operating in electrospray ionisation (EI) mode at 70 eV (Grauso et al., 2019a). The system was equipped with a 30 m × 0.25 mm inner diameter (i.d.) fused‑silica capillary column with 0.25µm HP-5MS stationary phase (Agilent Technologies, Cheadle, UK). The injection temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. Separation of the non-polar extract was achieved using a temperature program of 80°C for

1 min, then ramped at 10°C/min to 320°C and held for 1 min. Both chromatograms and mass spectra were evaluated using the MassHunter Qualitative Analysis B.07.00 (Agilent Technologies, Santa Clara, CA, USA). Mass spectra of all detected compounds were compared with fatty acid methyl esters (FAME) as standard compounds and with spectra obtained by the National Institute of Standard and Technologies library NIST MS search. Data were processed with the AMDIS (Agilent Technologies) software to deconvolute co-eluting peaks. The relative amounts of separated metabolites were calculated from total ion chromatography (TIC) by the computerised integrator and by comparison with internal standard, 1-oleoyl-rac-glycerol, added to the apolar extract as described in Grauso et al. (Grauso et al., 2019b).

4.4.6 - Antimicrobial activity assay

The study included the following species: *Stapyloccocus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Salmonella enterica*. Isolates were obtained from patients hospitalized at the Medical School of the University of Naples Federico II. Specimens were analyzed using PCR assay as described in Romanelli et al. (2011). The antimicrobial effect of *M. oleifera* polar and apolar extracts from leaves (PL, AL) and polar and apolar extracts from seeds (PS, AS) were evaluated. Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 200 µL of bacterial culture diluted in Tryptic Soy Broth (TSB) to a final concentration of 1 x 10^6 colony forming units (CFU) mL^{-1} in absence and in presence of tested extracts. Each extract was first solubilized in dimethyl sulfoxide (DMSO) and then added to the culture medium and tested at concentrations of 4, 2 and 1 mg mL^{-1} (DMSO final concentrations ≤ 2,5% v/v). Proper controls in DMSO were included in the experiments. After overnight incubation at 37°C, the antimicrobial activity was optically evaluated comparing treated and untreated samples, by measuring microbial growth at 600 nm wavelength with a microplate reader.

4.5 - Conclusions

The untargeted metabolite profiling approach on polar and apolar extracts of *M. oleifera* leaves and seeds allowed the characterization of the metabolite profile, and the further identification and quantification of several primary and secondary metabolites by using a combination of ^{1}H-NMR and GC-MS analyses. Moreover, both polar and apolar extracts from leaves and seeds were tested to assess their antimicrobial activity. Only the seeds apolar extracts showed a dose-dependent inhibition against *S. aureus* and *S. epidermidis*, and it seems to be due to the remarkably different fatty acid content the two apolar extracts. These findings will be useful for further studies aimed to explore the many other biological potential of this very interesting plant.

Chapter 5 - Metabolomics and chemometrics of lettuce, *Lactuca sativa L.* infected by *Fusarium oxysporum lactucae* under different fertilization treatments.

5.1 - Introduction

In the last decade, metabolomic approaches have been used as a tool to understand the impact of environmental stresses by monitoring the qualitative and quantitative change of metabolites extracted either from whole plants or single tissues (Fiehn, 2002). ^{1}H-NMR metabolomics is one of the most used approaches, as it provides a fast and wide overview of the metabolite content of plant extracts with a relatively easy and fast sample preparation (Sumner et al., 2003). The outcome of the metabolomic analysis is a 'fingerprint' that gives information about the current growing condition of the plant. Several studies tried to describe the metabolite changes resulting from stress conditions, and it seems that different stresses cause either an increase or decrease in specific metabolites (Anzano et al., 2022).

Lettuce, *Lactuca sativa* L., is a well-known food plant worldwide due to its use in both fresh salads and cooked soups. It is a member of the Asteraceae family, and it was first described in 1753 by Carl Linnaeus in the second volume of his Species Plantarum. The whole plant is rich in a milky sap that flows out from any wounds of the basal stem (Pignatti, 1982). Lettuce has also been reported in folk medicine for several therapeutic uses such as a carminative, diuretic, emollient, febrifuge, hypoglycemic, hypnotic, parasiticide and sedative (Noumedem et al., 2017). Lettuce is generally cultivated as an annual crop, requiring relatively low temperatures to prevent it from early flowering.

It can suffer from numerous nutrient deficiencies, as well as be plagued by several insects and pests, fungal and bacterial diseases (Kim et al., 2016).

Due to the importance of lettuce as a food crop, different studies have been done on this plant species using metabolomic approaches. The study published by Sobolev et al. (Sobolev et al., 2005), was based on a combination of 1D and 2D NMR approaches, and provides a detailed characterization of L. sativa polar and non-polar extracts. More recently, the paper by Yang et al. (2018) characterized the metabolome of 30 different lettuce cultivars, with large genetic diversity with provenances from different continents, including America, Asia, and Europe. The reported data allowed differentiation of the analyzed plants, showing different compositions of secondary metabolites. In the same year, van Treuren et al. (2018) performed an extensive metabolomic analysis on 150 different accessions. The identification of many metabolites, through a LC-MS approach, resulted in a clear cluster structure, separating all the cultivated lettuce genotypes from another group of the wild ones. Main differences were found in the relative abundance of chlorogenic, chicoric and caftaric acids, reported to be significantly higher in the wild types.

Several works focused on the effects of specific biotic and abiotic stresses on lettuce leaf extracts. Zhou et al. (2021) found that exposure of *L. sativa* to low nitrogen treatment increased the accumulation of phenolics, sugars, and organic acids (citric and isocitric acid). They also found a decreasing trend for malic and fumaric acids. A mixed response has been found for amino acids with an increasing or decreasing trend for each compound compared to the control. Higher levels of flavonoids and of three amino acids (valine, isoleucine and threonine) were also detected in *L. sativa* leaves by Wei et al. (2021) in plants under thermal stress. Another recent study by Matamoros et al., (2021) reported on the effects of different fertilization treatments on the lettuce metabolome using LC-MS. Five different treatments were compared, including sewage sludge, swine manure, chemical fertilization, mixed organic fraction of municipal solid waste (compost derived from parks, gardens, and kitchen wastes), and a control with no fertilization. Sewage sludge and chemical fertilization resulted in higher levels of amino acids, probably due to higher nitrogen supply. Conversely, treatments with swine manure, the organic fraction of municipal solid waste, and control, corresponding to lower nutrient levels, led to a higher organic acids content, and increased levels of many sugars.

The need for reduction in chemical pesticides in modern agriculture is strongly increasing the interest in organic-based products such as compost from different source materials, also because these compounds have been reported to have suppressive effects on different plant pathogens, especially fungal diseases (Bailey & Lazarovits, 2003; Bonanomi et al., 2007, 2010; Sarma et al., 2010; Termorshuizen et al., 2006), but also nematodes (Thoden et al., 2011). The idea of pathogen control finds correspondence in traditional practices (Lazarovits & Lazarovits, 2001) and specific applications have been reported to fight fungal pathogens, such as Phytium, Rhizoctonia, and Sclerotinia (Pane et al., 2011). Additionally, the control of Fusarium wilt has been attempted in flower plants (Pinto et al., 2010) and tomato, coupling beneficial Trichoderma in combination with sheep manure (Barakat et al., 2009). Moreover, there is a strong interest for organic materials from all sorts of different natural sources, including seaweeds (W. Khan et al., 2009), and microalgae (Braun & Colla, 2023; González-Pérez et al., 2022), as well as fish emulsions (Abbasi, 2011), with reported biostimulant effects when applied in either foliar or seed treatments. Among microalgae, a renowned species is *Arthrospira platensis*, commonly named spirulina, which is widely cultivated and used as a biostimulant for both plant fertilizers and animal food integrator products. It is known that plants react to abiotic and biotic stresses with different metabolic responses (Anzano et al., 2022). In particular, when infected by pathogens plants change their metabolic profile. Here, we present the results of a first study aiming to assess the effects of organic foliar treatment on the metabolic profiles of common lettuce in a field characterized by high infestation levels of the fungal pathogen

Fusarium oxysporum lactucae (FOL). Untreated lettuce was used as control plant. Three different treatments were designed: a first with a commercial organic plant material mixture used for the preparation of a so-called compost tea (CT), i.e., a supernatant of an aerobic fermentation of such an organic substrate; a second with *Arthrospira platensis* (spirulina—SP), as biofertilizer and a biostimulant product (Braun & Colla, 2023; González-Pérez et al., 2022); and a third with the same *A. platensis* previously exposed to a natural uptake of FOL DNA (NAT), to test the idea of self-DNA inhibition as proposed by Mazzoleni et al. (Mazzoleni, et al., 2015a; Mazzoleni et al., 2015b).

Three questions were addressed as aims of this work: (i) is foliar organic fertilization reducing the occurrence of lettuce disease by FOL? (ii) is lyophilized *A. platensis* (spirulina) added to foliar applications, producing comparable effects to the use of generic compost-tea made of mixed plant organic matter? (iii) does *A. platensis*, when exposed to pathogen DNA, show changes in its biostimulant effect on lettuce infected by FOL?

5.2 – Results

5.2.1 - Plant Analysis

Figure 5.1 schematically describes the experimental setting of the work. Control plants (C) were supplied with only pure water. Compost tea (CT) treatment used as starting material a commercial product Stimol C® by G-AGRO. The product solubilized in water was aerobically fermented to give the final fertilization solutions used for plants. Lettuce was hand planted in parcels.

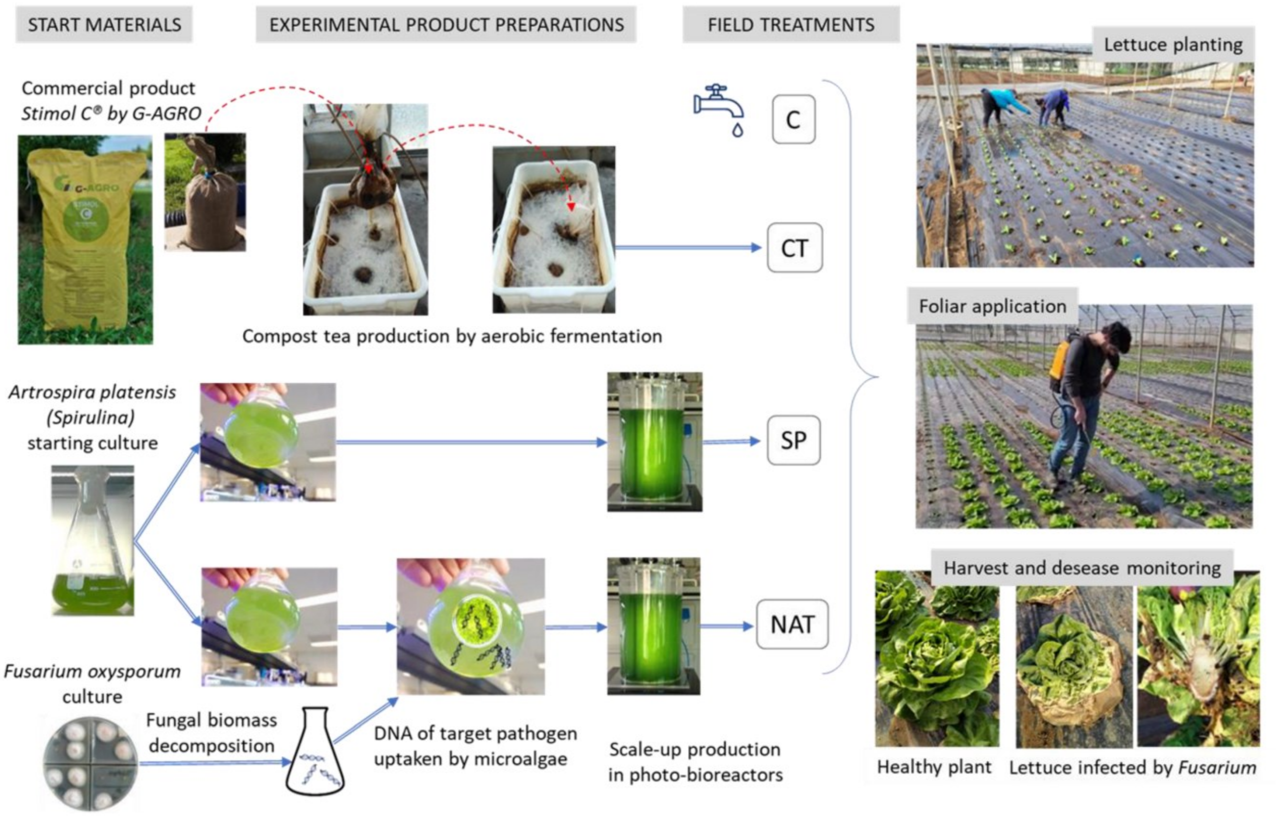


Figure 5.1. *Schematic description of the experimental design of Lactuca sativa organic fertilization. Control plots were irrigated with pure water. Treatments were done by foliar application with a water solution of commercial compost tea (CT), spirulina (SP), and spirulina previously exposed to natural uptake of Fusarium DNA (NAT).*

The organic fertilization produced in the observed experimental conditions some beneficial effects on lettuce growth and disease control (Figure 5.2). In particular, the average number of plants killed by *F. oxysporum* infection was found to be significantly higher in the control plants compared to the treated plants (Table 5.1). Some beneficial effect, though less significant, was also observed in terms of the number of stunting plants (smaller sized with some desiccation of basal leaves, reflecting relatively milder levels of fungal infection. Among the organic treatments, the NAT, i.e., the microalga exposed to the presence of Fusarium DNA, showed a better performance compared to the pure spirulina and to the compost tea (Table 5.1 and Figure 5.2).

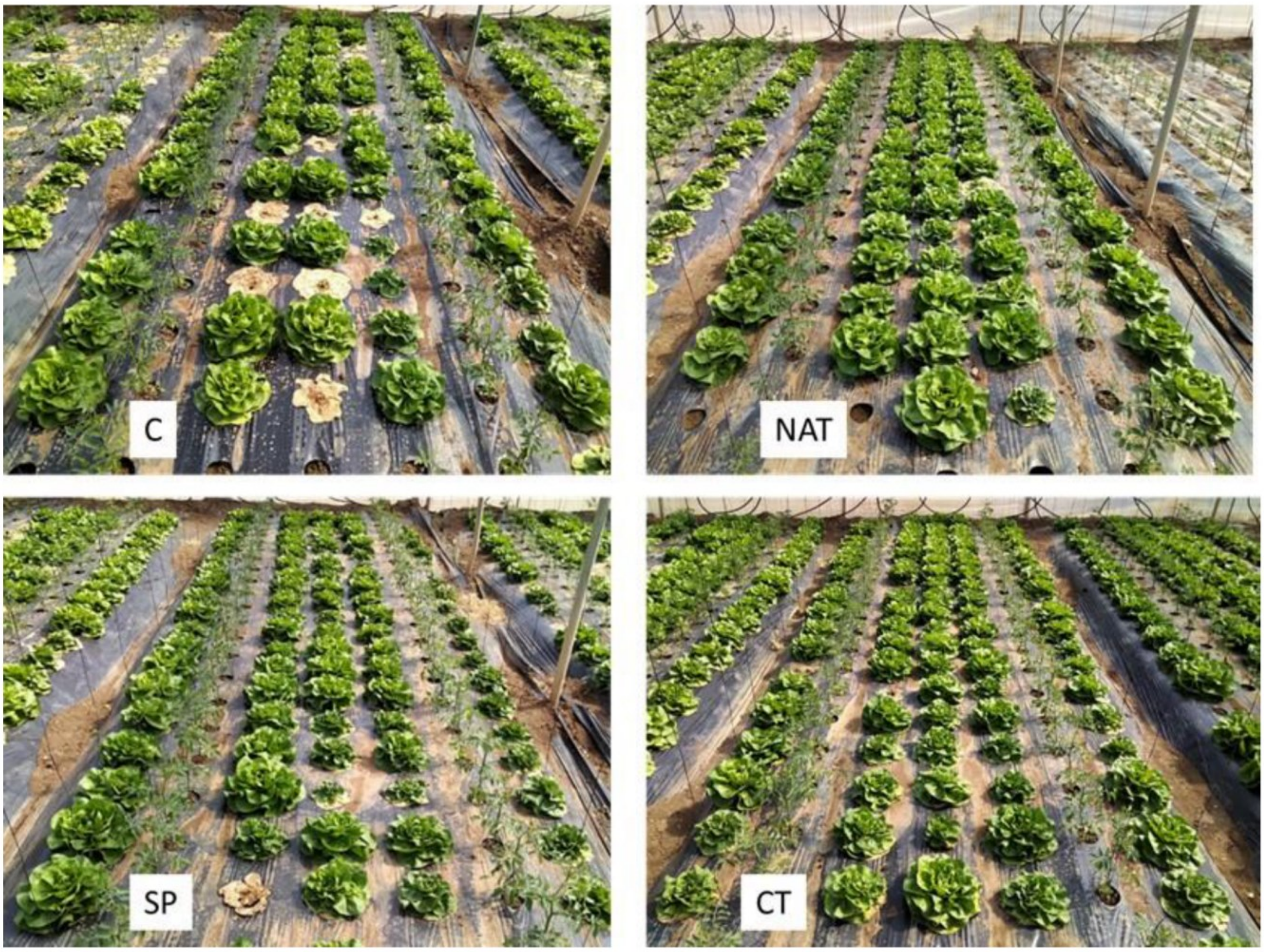


Figure 5.2. *Examples of experimental plots of Lactuca sativa organic fertilization tests: Control (C) irrigated only with water and showing higher occurrence of Fusarium oxysporum attack. Treatments by foliar application with water with added spirulina (SP), spirulina after natural uptake of F. oxysporum DNA (NAT), and commercial compost tea (CT).*

Table 5.1. *Number of plants either dead or with reduced growth because of Fusarium oxysporum infection and weight of individuals in control plots (C) and in different foliar application treatments with compost tea (CT), spirulina (SP), and spirulina previously exposed to natural uptake of Fusarium DNA (NAT). Weight data are averages of random 10 plants and corresponding standard deviation.*

Treatment	**Dead Plants (#)**		**Stunting Plants (#)**		**Weight (g)**	
	AVG	**SD**	**AVG**	**SD**	**AVG**	**SD**
C	18.67	5.51	17.00	5.29	377.40	67.87
NAT	6.00	2.65	10.33	7.51	493.60	73.80
SP	7.33	1.15	13.67	4.16	411.60	103.47
CT	6.67	2.08	17.67	3.06	487.50	106.66

5.2.2 - Metabolomic Analysis

Twenty grams of lettuce leaves afforded, after lyophilization, an average of 1.12 g of dried lettuce powder, resulting in an average moisture content of 94.41%. Samples obtained from the different treatments were analyzed in triplicate and the obtained organic extracts subjected to NMR metabolomics (Figure S1). The NMR data obtained (Figure 5.3, full spectra) showed no significant change among treatments and control plants from a qualitative point of view. However, comparison of the spectra evidenced changes in the metabolite composition (Figure 5.3, expanded low field region).

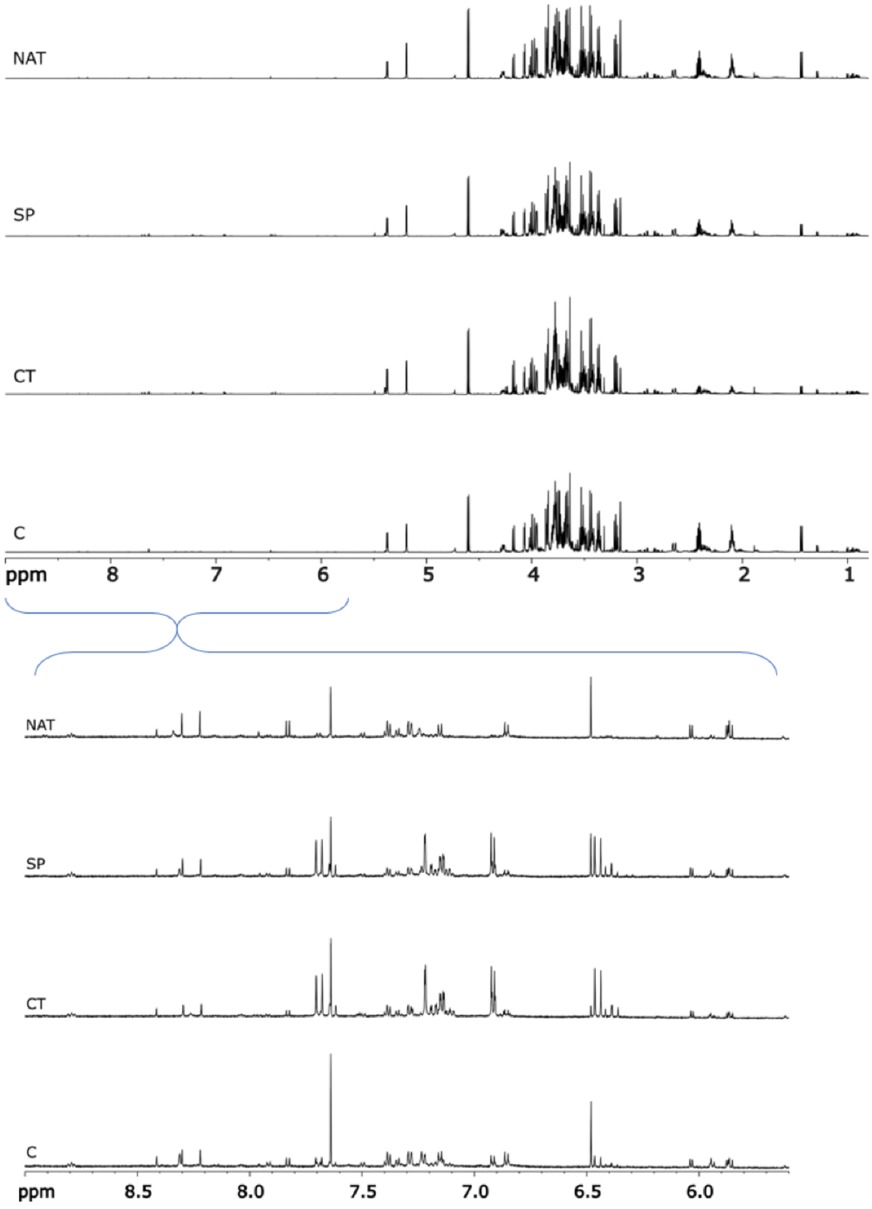


Figure 5.3*. Full ^{1}H-NMR spectra in deuterium oxide (D_2O) at 600 MHz of L. sativa leaf extracts of the control plant (C) and the three treatments (CT, SP and NAT). Only one of the three replicates is shown for each treatment (Top), all replicates are reported in Figure S1. Expanded high field region of the ^{1}H-NMR spectra (Bottom).*

Therefore, we analyzed the spectra peak by peak to identify the main metabolites. Spectral identification was performed with the aid of the 2D NMR spectra acquired, which included COSY, HSQC and HMBC (Figures S2–S4) and by comparison with the data for standard compounds available in the laboratory and reported in the literature. This allowed identification of single metabolites in the extract mixture. Table 5.2 lists the metabolites found using ^{1}H-NMR analysis in the analyzed extracts. Compounds included primary metabolites such as carbohydrates, amino acids, nucleosides, and secondary metabolites such as phenolic acids.

***Table 5.2**. ^{1}H-NMR chemical shifts, assignment, and multiplicity at 600 MHz in D2O of the metabolites detected in all analyzed polar extracts of L. sativa plants*

Compound	Assignment	1H (ppm)	Multiplicity (J (Hz)]
Organic acids			
Acetic acid (Ace)	α-CH_3	1.88*	s
Citric Acid (Cit)	CH_2	2.59*	dd
Formic acid (For)	COOH	8.41*	s
Fumaric acid (Fum)	α-CH	6.48*	s
Malic acid (Mal)	β'-CH_2	2.36	dd (15.7, 8.9]
	β-CH	2.65	dd (15.7, 3.7]
	α-CH	4.28*	dd (8.9, 3.7]
Aminoacids			
Alanine (Ala)	β-CH_3	1.43*	d (7.0]
Asparagine (Asn)	β-CH	2.86	dd (17.4, 3.8]
	β-CH	2.87*	dd (16.9, 7.3]
Aspartic acid (Asp)	β-CH_2	2.76	dd (17.4, 3.8]
γ-aminobutyrate (GABA)	β-CH_2	1.87	m
	α-CH_2	2.26	t (7.0]
	γ-CH_2	2.97*	t (7.0]
Glutamic acid (Glu)	β-CH_2	2.02	m
	γ-CH_2	2.32*	m (7.0]
Glutamine (Gln)	β-CH_2	2.10	m
	γ-CH_2	2.43*	m
Isoleucine (Ile)	δ-CH_3	0.91*	t (7.0]
	γ'-CH_3	1.00	d (7.0]
Leucine (Leu)	δ-CH_3	0.95*	d (7.0]
Phenylalanine (Phe)	CH	7.28	d (7.0]
	CH	7.33*	d (7.0]
	CH	7.38	d (7.0]
Threonine (Thr)	γ-CH_3	1.29*	d (6.4]
Tryptophane (Trp)	CH	7.18	d (7.5]
	CH	7.49*	d (7.5]
	CH	7.69	d (7.5]

Tyrosine (Tyr)	CH	6.85*	d (7.0]
	CH	7.14	d (7.0]
Valine (Val)	γ'-CH$_3$	0.97	d (7.0]
	γ-CH$_3$	1.00*	d (7.0]
Carbohydrates			
α-Glucose (α-Glc)	CH-1	5.19*	d (4.0]
β-Glucose (β-Glc)	CH-1	4.61*	d (8.0]
Fructose (Fru)	CH-4	3.90	dd
	CH$_2$-6	4.00*	dd
myo-Inositol (Myo)	CH-4	3.20*	t (9.50]
Sucrose (Suc)	Glc CH-1	5.38*	d (4.0]
	Fru CH-3	4.18	d (8.5]
Nucleotides			
Adenosine (A)	CH-2	8.21*	s
	CH-8	8.40	s
Citosine (C)	CH-1′	6.03	d (3.5]
	CH-6	7.82*	d (7.0]
Guanosine (G)	CH-8	7.95*	s
Uridine (U)	CH-6	7.91*	d (7.0]
Other compounds			
Chicoric acid (Chi)	CH-2	6.46*	d (16.0]
	CH-3	7.70	d (16.0]
Chlorogenic acid (Chl)	CH-2	6.40*	d (16.0]
	CH-2′	7.10	dd (8.2, 2.2]
	CH-3	7.62	d (16.0]
Choline (Malo)	N(CH$_3$)$^{3+}$	3.15*	s
Ethanolamine (Eta)	β-CH$_2$	3.10*	dt (6.8, 4.0]
Trigonelline (Tri)	CH	8.05	t
	CH	8.80*	t
	CH	9.09	s

The high field region of the spectra contains the methyl groups of the branched amino acids (Figure 5.4a). Specifically, two doublets at δ 0.95 (J = 7.0 Hz) and δ 1.00 (J = 7.0 Hz) were diagnostic in determining leucine (Leu) and valine (Val), respectively. Isoleucine (Ile) was assigned through a triplet at δ 0.91 (J = 7.0 Hz) (Table 5.2). Threonine (Thr) was assigned through a doublet at δ 1.29 (J = 7.0 Hz) because of its characteristic β-CH$_3$. Differently from the other aliphatic amino acids, the β-CH$_3$ group of alanine (Ala) resonated as a doublet at δ 1.43 (J = 7.0 Hz) due to its proximity to the nitrogen atom. Asparagine (Asn) was identified by the β-CH signals resonating at δ 2.86 (dd, J = 17.4 and 3.8 Hz) and 2.87 (dd, J = 16.9 and 7.3 Hz). The double doublet at δ 2.76 (J = 17.4 and 3.8 Hz) was assigned to the β-CH_2 of aspartic acid (Asp). Then, glutamic acid (Glu) and glutamine (Gln) were detected based on β-CH_2 and γ-CH_2 resonating respectively at δ 2.02 (m) and 2.32 (m, J = 7.0 Hz) for Glu and δ 2.10 (m) and 2.43 (m) for Gln. Characteristic signals for γ-aminobutyrate (GABA) were found at δ 2.26 (t, J = 7 Hz, α-CH_2), 1.87 (m, β-CH_2), and 2.97 (t, J = 7 Hz, γ-CH_2).

Aromatic amino acids were identified in the low field region of the ^{1}H-NMR spectra from δ 6.83 to 7.70 (Figure 5.4c) and identified as phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (see Table 5.2).

Among the organic acids, signal characteristics for five were detected across all spectral regions (Figure 5.4a–c and Table 5.2). At high field regions, the α-CH_3 group of acetic acid (Ace) was found at δ 1.88 (s). In the same region citric acid (Cit) was identified by its CH_2 group whose protons resonated as a double doublet at δ 2.59 (J = 15.0, 15.0 Hz). Malic acid (Mal) was identified by the following characteristic signals: δ 2.36 (dd, J = 15.7 and 8.9 Hz, β'-CH_2), 2.65 (dd, J = 15.7 and 3.7 Hz, β-CH), 4.28 (dd, J = 8.9 and 3.7 Hz, α-CH). Finally, in the aromatic region, the acid proton formic acid (For) was identified at δ 8.41 (s, COOH) while a proton characteristic for fumaric acid (Fum) resonated at δ 6.48 (s, α-CH).

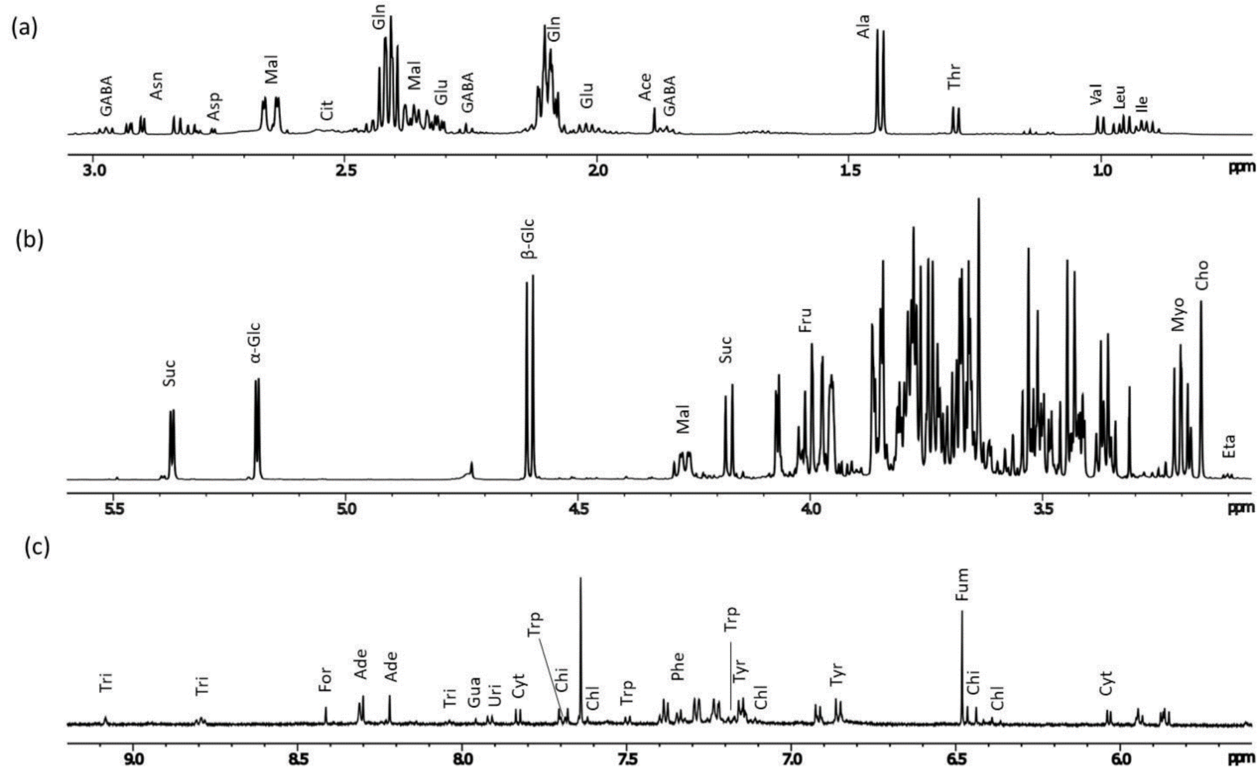


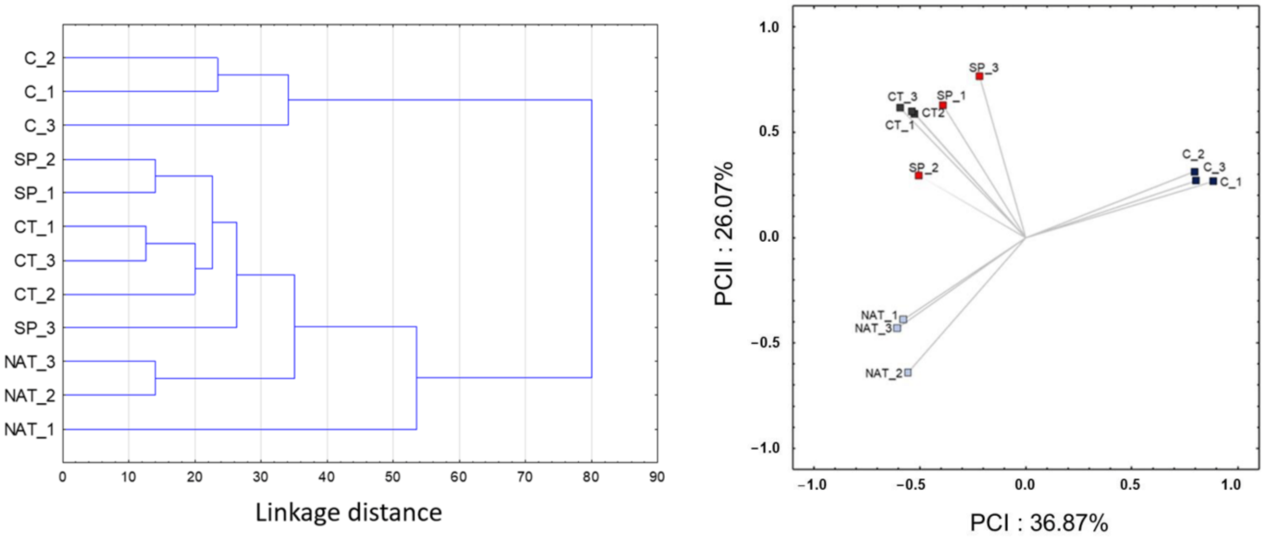
***Figure 5.4**. ^{1}H-NMR spectra in deuterium oxide (D_2O) at 600 MHz of L. sativa leaves extracts (control plant) showing the identified metabolites: (a) spectral region from 0.7 to 3.0 ppm; (b) spectral region from 3.1 to 5.5 ppm; (c) spectral region from 5.6 to 9.5 ppm vertically expanded.*

In the spectra of lettuce aqueous extracts, overlapping signals for sugar protons were observed from 3.33 to 3.87 ppm, which is a very crowded region of the ^{1}H-NMR spectra (Figure 5.4b). Outside this region, the diagnostic anomeric proton signal (H1) of α-glucose (α -Glc) was identified at 5.19 (d, J = 4.0 Hz), while the anomeric proton of β-glucose (β -Glc) was found at δ 4.61 (d, J = 8.0 Hz) (Table 5.2 and Figure 5.4b). Signals identifying fructose (Fru) were found at δ 3.90 (CH-4) and 4.00 (CH2-6). Sucrose (Suc) was identified by the anomeric proton of the glucose moiety resonating at δ 5.38 (d, J = 4.0 Hz). Lastly, the sugar alcohol myo-inositol (Myo) showed a peak at δ 3.20 corresponding to H5. The identification and spectral assignments of the reported sugars (Table 5.2) were obtained both by comparison with NMR data (chemical shifts and coupling constants) of standard sugars (Teresa & Fan, 1996) and by analysis of 2D NMR spectra (Figures S2–S4). Nucleosides were identified in the low field region of the spectra, by their characteristic resonances of the aromatic protons of the heterocyclic rings (Figure 5.4c and Table 5.2). Thus, the purine nucleoside adenosine (Ade) showed two singlets at δ 8.21(CH-2) and 8.40 (CH-8) for the nitrogen base and a doublet at δ 6.03 (d, J = 3.5 Hz) due to the anomeric proton of the ribose moiety. The purine nucleoside guanosine (Gua) was identified by the typical singlet at δ 7.95 (CH-8). The pyrimidine nucleoside cytidine (Cyt) showed a characteristic doublet at δ 7.82 (J = 7.0 Hz) assigned to CH-6, while the same signal for uridine (Uri) resonated at δ 7.91 (J = 7.0 Hz, CH-6). In addition to the polar compounds described above, ^{1}H-NMR spectra from the polar extracts of lettuce leaves indicated the presence of several additional compounds that do not belong to the classes mentioned above. The presence of choline (Cho) was showed by the characteristic methyl singlet resonating at δ 3.15 and confirmed by comparison with pure standard (Figure 5.4b). In addition, the presence of ethanolamine (Eta) was demonstrated by its characteristic double triplet resonating at δ 3.15 (β-CH_2, J = 6.8, 4.0) (Figure 5.4b). The phenolic acid chicoric acid (Chi) was identified by the proton signals of the trans double bond resonating as coupled doublets at δ 6.46 and 7.70 (each d, J = 16 Hz) (Figure 5.4c and Table 5.2). The same functional group allowed the identification of chlorogenic acid (Chl) with the protons of the trans double bond resonating at δ 6.40 and 7.62 (each d, J = 16 Hz). Lastly, trigonelline (Tri) was identified by the proton signals at δ 8.05 (CH, t), 8.80 (CH, t) and 9.09 (CH, s) (Figure 5.4c and Table 5.2).

5.2.3 - Chemometric Analysis

A multivariate analysis was performed on the matrix of integrated NMR spectral data reported in supplementary (Figure S1 and Table S1).

Firstly, the dendrogram produced by the numerical clustering (Figure 5.5a) shows a strong separation between the group of control plants and the general cluster aggregating all treated plants. Moreover, in the latter group, the three different treatments maintained a clear segregation with sub-groups of plants irrigated with compost-tea (CT) aggregated together and joining to those treated with spirulina (SP) with high similarity (linkage distance < 26). The NAT treatment showed a lower level of homogeneity, with their cluster grouping with all the others at a lower similarity level (linkage distance > 50).



(**a**) (**b**)

Figure 5.5. *Numerical clustering (a) and principal component analysis (PCA) (b) of the three replicates of integrated NMR spectral data of control (C) and treated plants (CT, SP and NAT). The classification dendrogram clearly aggregates the control group separately from all fertilization treatments. The ordination plot also shows a clear separation along the first principal component of the C versus treated plants, whereas the second component segregates the NAT from the other organic fertilization treatments.*

Secondly, the ordination plot obtained by PCA (Figure 5.5b) was highly informative regarding the similarity and trends of variation of the spectral data of plants in the different fertilization treatments. The first principal components, accounting for over 36% of the total variability, clearly separated the control (C) from all organic fertilization treatments, whereas the second axis, still highly relevant because accounting for 26% of variability, showed an aggregation of CT and SP separated by the NAT group. The complete PCA plots including the variable vectors are reported in Figure S5).

5.2.4 - Metabolomic Treatment Comparison

A general comparison of the three different treatments with the control plants showed the same number of metabolites in the analyzed samples with differences related to their quantity. Thus, no marker metabolites were observed linked with specific treatments. The integration of the ^{1}H-NMR spectra allowed acquisition of quantitative data of the identified metabolites (Table S1) and evaluation of their change in the analyzed samples (Figure 5.6).

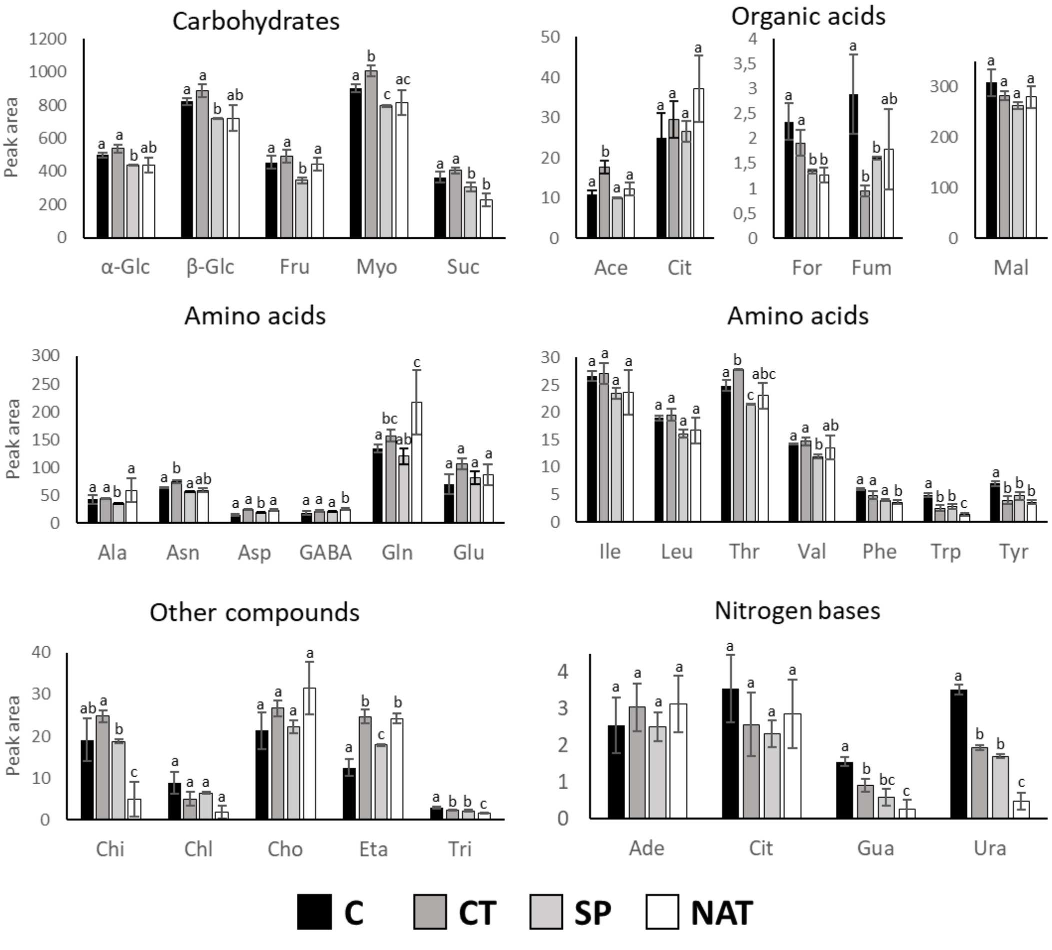


Figure 5.6. *Absolute abundance (µmol/g dry leaves) of the metabolites identified by ^{1}H-NMR in L. sativa leaves extracts. Displayed data refers to the mean and standard deviation of three replicates. The vertical bars represent the four treatments: C = control; CT = compost T, SP = spirulina, NAT = spirulina + Fusarium DNA*

The treatment with CT produced an increase in all the identified carbohydrates compared to control plants. The same increase was observed for acetic and for citric acids. The other organic acids were significantly decreased by CT treatment, especially fumaric acid that decreased its concentration by about 67% compared to C. For amino acids a general trend was observed, with all of them slightly increasing, the increase being higher for glutamic acid (+35% compared to C). The only exceptions were the aromatic amino acids, that showed a slightly decreasing trend. Nucleosides mostly decreased, while for other compounds an increasing trend was observed for the phenolic acid chicoric acid, and for choline and ethanolamine, the latter doubling its quantity, in contrast to chlorogenic acid that almost halved.

Treatment with SP had the opposite effect on carbohydrates, decreasing their quantity overall, compared to the control. Fumaric and formic acids showed a large decrease after SP treatment. Malic acid only slightly decreased, while the other organic acids remained constant. Regarding amino acids, they were not particularly influenced, all of them remaining almost constant except threonine, tryptophan and tyrosine that showed a slightly decreasing trend. Moreover, SP treatment influenced nucleoside content with a large decrease in cytidine, uridine and guanosine. In detail, guanosine decreased by 62.5% and uridine by 51%, both compared to C. Among other compounds, ethanolamine showed an increasing trend, while chlorogenic acid slightly decreased.

NAT treatment resulted in a slight modification of carbohydrate content, with a slight decrease in sucrose, but influenced organic acid content to a greater extent, especially citric acid, that increased (+33%), and formic and fumaric acids, both of which almost halved (−54% and −38%). The only amino acid that dramatically increased after NAT treatment was glutamine (+38% compared to C). However, glutamic and aspartic acids, alanine and GABA slightly increased also, while isoleucine, phenylalanine, tryptophan and tyrosine showed a decreasing trend. Going into detail, tryptophan decreased by 73% and tyrosine by 48%, the others showing a minor decrease. Regarding nucleosides, NAT treatment only caused decreased nucleosides, with uridine showing the largest compared to C (86%). Moreover, decreasing trends were observed for chicoric and chlorogenic acids, while ethanolamine and choline increased by 32% and 48%, respectively, compared to control plants.

5.3 - Discussion

To our knowledge, this is the first report of the characterization of the metabolome of lettuce leaves by NMR metabolomics under organic foliar fertilization treatment, studied under field conditions. Comparison of the three different organic foliar fertilization treatments and the control plants have been performed by using a fast and efficient protocol previously validated in other metabolomics analyses of food plants (de Falco et al., 2018, 2022).

Regarding the agronomic results, it should be noted that the control plants in this study were cultivated in a soil loaded with the fungal pathogen FOL and showed a significant occurrence of the disease during the crop cycle. In the control plots about 20% of the lettuce plants died and a similar number of plants reported stunted growth. All fertilization treatments, especially NAT, reduced the occurrence of the fungal disease in terms of decreasing numbers of both dead and stunted plants. Moreover, the average weight of individual lettuces increased with all fertilization treatments. Among these, NAT again performed better.

Regarding the metabolomic study, several primary and secondary metabolites were identified and quantified in the organic extracts of the analyzed samples by using NMR metabolomics. The data agree with previous studies reporting the chemical composition of lettuce (Matamoros et al., 2021; Sobolev et al., 2005; van Treuren et al., 2018; X. Yang et al., 2018) thus confirming the method used as an efficient and valid protocol for analysis. The data obtained indicate that fertilization treatments did not significantly affect the general plant metabolite composition. However, evident metabolite trends were observed and some agreed with previous studies.

Carbohydrates were the main compounds in the extracts. They showed a slight increase with CT treatment while the microalgae (both SP and NAT) produced a detectable decreasing trend. According to Zhou et al. (2021), stress conditions may increase sugar production, thus we can relate the effect of NAT and SP with the known biostimulation reported for spirulina. An increase from stress was also observed by the same authors for the aromatic amino acids Tyr and Trp, while a decrease was observed for the amino acids, Glu and Asp. Our data totally agree with this finding, showing higher levels of Tyr, Trp, and Phe in the control plant, while lower levels were observed for Glu and Asp.

Similar effects of stress conditions were found by Wei et al. (2021) for the amino acids Val, Ile, and Thr. Our data also agrees with their findings, demonstrating an improvement of the physiological status of lettuce plants after fertilization. Concerning the organic acids, as recently reported by Matamoros et al. (2021), increased levels of organic acids, mainly fumaric acid, are triggered by stressful conditions or lack of nutrients with involvement of the TCA cycle and related energy production. Our findings of reduced amounts of the organic acids Fum, For, and Mal after fertilization treatment confirm the healthiness of the fertilized plants compared to the untreated control plant. The reported results are interesting because on one hand, as expected, they confirmed that plants treated by organic foliar fertilization do have a physiological response reflected by variations in their metabolome. Our observations confirmed other reported published effects (Matamoros et al., 2021; Sobolev et al., 2005; van Treuren et al., 2018; Wei et al., 2021; X. Yang et al., 2018; W. Zhou et al., 2021), showing variations of specific metabolites reflecting a reduction in either biotic or abiotic stresses compared to control. On the other hand, results were very surprising when showing a higher similarity between SP and CT versus NAT treatment. In fact, considering that both SP and NAT treatments were based on lyophilized pellets of the same microalgal species (*A. platensis*) it was reasonably expected that SP and NAT would have shared a higher similarity in their effects on the treated lettuce plants. On the contrary, exposure to FOL DNA in the preparatory steps of the natural microbial library (NAT), evidently induced a variation in the spirulina biochemical characteristics that were somehow stabilized and carried over, during the scale-up process, up to producing different effects and metabolic reactions in the treated plants. Cyanobacteria are well known for their high transformation capability (Wendt & Pakrasi, 2019). This seemed to be confirmed by the fungal DNA observed effects, but the modes of action and the mechanisms of the possible integration in the microalgal genome, remain to be investigated in further work. In this work, the control plants were affected by FOL, i.e., the same fungal pathogen whose self-DNA was used in the preparation of the natural microalgae of the NAT treatment. So, our results, although being produced by a still preliminary study, represent a first proof of concept of the biocontrol idea based on the use of self-DNA of a pathogen species, as proposed by Mazzoleni et al. (Mazzoleni et al. 2014; Mazzoleni et al., 2015a; Mazzoleni et al., 2015b). In other words, while the general biostimulation by organic fertilization may act as a strengthening of the treated plants, some additional beneficial effects seem to be associated with the combined effect of plant biostimulation with a pathogen self-DNA inhibition.

5.4 - Materials and Methods

5.4.1 - Field Site and Experimental Design

The experiment was performed at the farm “Azienda De Vita Rosario” located in Battipaglia, Campania Region, Italy (40°34039.600 N 14°58027.500 E). The site is located in an alluvial plain with highly fertile soils extensively used for horticulture in both open field cultivation and greenhouses. The climate is typical Mediterranean with rains concentrating in winter and usually above 1000 mm/year and mild average temperature, almost never freezing in winter and steadily above 30 °C in summer. Light insolation is comparable to optimal conditions as reported for lettuce cultivation (Kosma et al., 2013).

The lettuce plants were provided by the nursery of Citro Giuseppe, associated with the farm “Finagricola”. The *Lactuca sativa* cultivar was selected as being not resistant to *Fusarium oxysporum lactucae* (FOL). The experiment was organized according to a randomized block design with three blocks including 100 individual plants each in 5 rows (three central and two laterals in each block). Three different treatments of foliar application included: (1) CT: a commercial compost tea named Stimol-C® produced by GWA—Gima Water & Air S.r.l. and made of mixed plant materials and cow manure from biological farms. The physical and chemical characteristics before starting the aerobic fermentation are the following: humidity 13%, pH: 6.8, organic C: 27%, humic and fulvic acids: 8.2%, organic N: 2.3%, C/N ratio: 11.7, salinity: 12.4 dS/m. (2) SP: *Artrospira platensis* (spirulina) selected strain by M2M Engineering, cultivated in a photobioreactor to produce the organic pellet lyophilized and used in the foliar application treatment. (3) NAT: The same strain of *A. platensis* used in the SP treatment was exposed in its preparation to a natural uptake from a culture medium enriched with *F. oxysporum* fragmented DNA before its scale-up in the photobioreactor to produce the organic pellet used in the foliar application treatment.

5.4.2 - *Fusarium Oxysporum Lactucae* (FOL) Phytopathogenic Analyses

Plants with wilting symptoms, collected from different parcels, were transported to the laboratory and examined. Small fragments were aseptically excised from discolored vascular tissues (see Figure 5.1 bottom right inset) placed on Petri dishes of Difco potato dextrose agar, with lactic acid added or not (0.7 mL of 25% lactic acid for 100 mL of PDA medium). Plates were incubated at 24°C and analyzed after one week. Fungal colonies with white abundant aerial mycelium (see Figure 5.1

bottom left) showing a purple–red color on the reverse side of the plates, were observed. Typical micro- and macro-conidia seen at 400 × optical microscope magnifications allowed the identification of the colonies as *F. oxysporum* in all the cases that were examined.

5.4.3 - Natural Microbial Library Preparation

The *F. oxysporum* mycelium was grown in pure culture in petri dishes and flasks. The mycelium was ground in liquid nitrogen and then suspended in a double volume of SDS buffer at pH 8.0. The sample was centrifuged and aerobically decomposed in highly controlled conditions to increase the DNA concentration. The DNA concentration and fragmentation level in the samples were checked using standard extraction methods and evaluated by QUBIT (Thermo Fisher, Third Avenue Waltham, MA, USA) fluorimeter and electrophoresis in 1% agarose gel. The samples were sheared using a Bioruptor Plus (Diagenode, Seraing (Ougrée) Belgium, EU) sonicator and then lyophilized. The production of natural libraries to obtain biomass for trials (NAT treatment), comprised several upscaling steps, from small volume flasks in the laboratory in climatic chambers in controlled conditions, to medium scale photobioreactors in controlled conditions for continuous production. The cultures were grown in the first steps of production in small laboratory scale in flasks in a climatic chamber in controlled conditions of temperature (25°C) with irradiation intensity 200 $\mu mol/m^2s$ and a photoperiod cycle of 12 h day/12 h night, with cells grown in medium substrate containing random DNA fragmented in classic normal medium. The growth medium used was the classic Zarrouk medium for *A. platensis*. Cultures were grown and scaled-up in still 2 L flasks in a climatic chamber and after in the small-scale photobioreactor M2M-PBR-10, with a volume of 10 L in the laboratory. The conditions in the photobioreactor were fully controlled and optimized, with an optimized LED lighting spectrum, with the temperature of the culture at 25°C, lighting 12 h day/12 h night, irradiation 400 $\mu mol/m^2s$. The cultures were then scaled-up in two medium scale photobioreactors (M2M-PBR-150), with culture volumes of 150 L, irradiance 400 µmol/m^{2}s 12 h day/12 h night LD. Photobioreactors are optimized and controlled for continuous and fully controlled production of microalgae with LED lighting spectra optimized for cultivation. Biomass is continuously produced in the photobioreactor, and periodically harvested, dried, and stored in closed packaging for application in field trials. The process for production of microalgal biomass for the basic foliar fertilization treatment (SP) was identical to that described above for the NAT protocol, with the same and parallel steps of scaled-up growing volumes and systems used: flasks, photobioreactors, lab scale, and photobioreactor medium scale. Timing, harvested amounts, and preparation of dried pellets to be used in the foliar application were the same.

5.4.4 - Data Pre-Processing

Data from ^{1}H-NMR (nuclear magnetic resonance) were pre-processed before statistical analysis. In detail, the spectral region was first normalized to total area, to minimize small differences and subsequently, mean-centered applied to minimize the problem of heteroskedasticity in the data. Further, because of the high dominance of most abundant peaks, data were pareto scaled. The process of pretreatment was applied for both polar and apolar data.

5.4.5 - Multivariate Data Analysis

Data from NMR spectra were analyzed using multivariate statistical procedures. Prior to data transformation cluster analyses were run to observe the similarity among sample groups, according also to quantitative information of metabolites within each spectrum. Cluster analyses were made by creating a contingency matrix based on Euclidean distance and a successive hierarchical dendrogram built based on complete linkage methodology. After data normalization and transformation both polar and apolar spectra were analyzed by principal component analysis (PCA) to examine the intrinsic variation in the dataset, and specifically, in metabolic composition of the sample material. Moreover, the methodology allows observation of specific associations between sample groups and metabolite signals according to graphical disposition in multidimensional space. Both cluster analysis and dendrograms, and PCA, were performed by means of Statistica 10 software (StatSoft: Tulsa, OK, USA).

5.4.6 - Chemicals

First-grade dichloromethane and methanol were purchased from Delchimica Scientific Laboratories Glassware (Naples, Italy). Deuterium oxide (99.8 atom %D) and dimethyl 4-silapentane sodium sulfonate (DSS) was obtained from ARMAR Chemicals (Döttingen, Switzerland), chloroform-d (99.8 atom %D) containing 0.03% (v/v) TMS was purchased from Sigma-Aldrich (Milan, Italy). Pure standard amino acids, chlorogenic acid, and nucleosides were used as references (Sigma-Aldrich, Milan, Italy).

5.4.7 - Metabolite Extraction Procedure

Young lettuce leaves (20 g) were harvested from each treatment plot after 30 days since the experiment start and immediately frozen at -80°C. The leaves were then freeze-dried by lyophilization for two days and ground in a mortar, obtaining a fine green powder. One hundred milligrams of each powdered sample were collected in Eppendorf tubes and 1 mL of dichloromethane was added to each tube to perform a non-polar extraction. The mixtures were sonicated for 10 min and then centrifuged at 7000 rpm for 10 min, RT. The supernatants were collected into 10 mL vials and dried at room temperature. The remaining pellets were dried at room temperature for 2 h, then a polar extraction was performed, by adding 1 mL of a methanol/water solution (1:1 v/v) to each tube. The mixtures were sonicated again for 10 min, and then centrifuged at 7000 rpm for 10 min, RT. The supernatants were collected into new Eppendorf tubes and dried using a speed vac. These dried extracts were kept at -20°C until NMR analysis was performed.

5.4.8 - NMR Metabolomic Analysis

Dried aqueous fractions were diluted in 600 µL of D_2O (99.8%), while dried organic fractions were dissolved in 600 µL of $CDCl_3$ (99.8%) and transferred into 5 mm NMR tubes. DSS and TMS, both 0.03% (v/v) in D_2O and $CDCl_3$, respectively, were used as internal standards for aqueous and organic fractions, respectively. The pH of the aqueous fractions was adjusted to 6.0 by using potassium dihydrogen phosphate (KH_2PO_4) as a buffering agent and 1 N sodium deuteroxide (NaOD). The NMR spectra were recorded at 298°K with a Varian Unity Inova spectrometer operating at 600 MHz. For each sample, 200 transients were recorded using a spectral width of 12 ppm on 32 K data points and a relaxation delay of 0.04 s. Chemical shifts were referred to DSS and TMS signals (both 0.00 ppm). All spectra were processed using the iNMR program (www.inmr.net), phased and baseline corrected manually. Quantification was performed by signal integration relative to the internal standard, DSS and TMS. The region of the solvent peaks was excluded from the analysis. Spectral peak assignments of organic acids, amino acids, carbohydrates, chlorogenic acid and its derivatives were obtained based on pure standards purchased from Sigma-Aldrich and interpretation of 2D NMR experiments. Spectral peak assignments of these and the other detected metabolites were obtained by two-dimensional (2D) NMR experiments, including ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C

heteronuclear single-quantum correlation (HSQC) and comparison with data reported in the literature. The COSY spectra were acquired with a spectral width of 6130 Hz in both dimensions, 8 K data points, and 512 increments with 32 transients per increment. The HSQC spectra were acquired with spectral widths of 8000 Hz in the F2 dimension and 25,000 Hz in the F1 dimension, a data matrix with a size of 1 K × 256 data points, and 64 transients per increment. The obtained values showed a very good repeatability, with a coefficient of variation among replicates < 2.5% for all signals.

5.5 - Conclusions

Our findings suggest the possibility of many further studies, firstly to confirm the reported observations, but also to investigate the mechanism of uptake and incorporation of the fungal DNA in the microalgae and its potential optimized use for pathogen control. Further experimental work will have to focus on better definition of the administration protocol and dosages of the foliar application treatments. Likely, the anticipation of the treatments in earlier phases of the plantation will enhance the beneficial effects, reinforcing plant resistance to the pathogen infection and thus better inhibiting disease development.

General conclusions

Understanding plant stress is one of the major challenges towards the improvement of plant sustainability. Metabolomic approaches showed to be an effective way to measure the metabolites variation, but the complexity of plant/stress interaction makes this challenge very hard to face, especially when the experiments are conducted in field conditions. The review work we performed by studying a large number of articles shed light on this problem. Moreover, both the variability due to the applied stress and the stressed plant was wide, but some common trends were observed. However, each detected metabolite can increase or decrease according to different factors, and the number of metabolites to monitor during stress occurrence can be very wide. The large number of biological questions on plant metabolism requires that answers should be sought using the most versatile techniques available. Our goal was the development and use of a method capable to detect most classes of organic compounds in the plant tissues without compounds purification. The approach we developed to evaluate the chemical composition of natural and cultivated plants under abiotic and biotic stresses was based on spectroscopic techniques and multivariate data analysis. It was appropriate for a fast and comprehensive analysis of primary and secondary metabolites, allowing the determination of a metabolomic fingerprint of each species and the evaluation of the different distribution of the metabolites in the different part of the plant object of study. Furthermore, the approach also allowed to detect changes in the metabolite profiles after stress application and fertilization treatments.

In conclusion, the approach used was proved to be suitable for a rapid investigation of different plant species containing a wide range of organic molecules.

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Appendix A: Metabolomics and chemometrics of lettuce, Lactuca sativa L. infected by Fusarium oxysporum lactucae under different fertilization treatments - Supplementary Material.

Figure S1:

^{1}H-NMR spectra in triplicates of the *Lactuca sativa* leaves extracts treated with commercial compost tea (CT), Spirulina (SP), and Spirulina + Fusarium DNA (NAT). C indicates control plants. Spectra were registered in D_2O at 600 MHz.

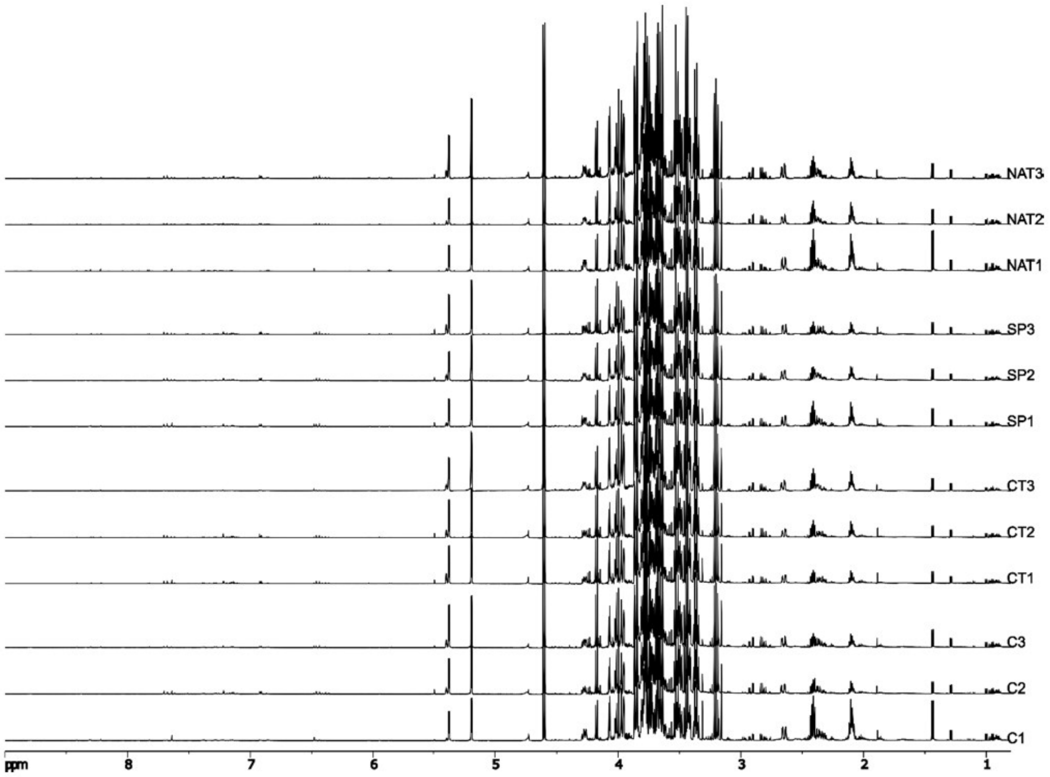

Figure S2: 2D COSY of the control plant registered in D_2O at 600 MHz.

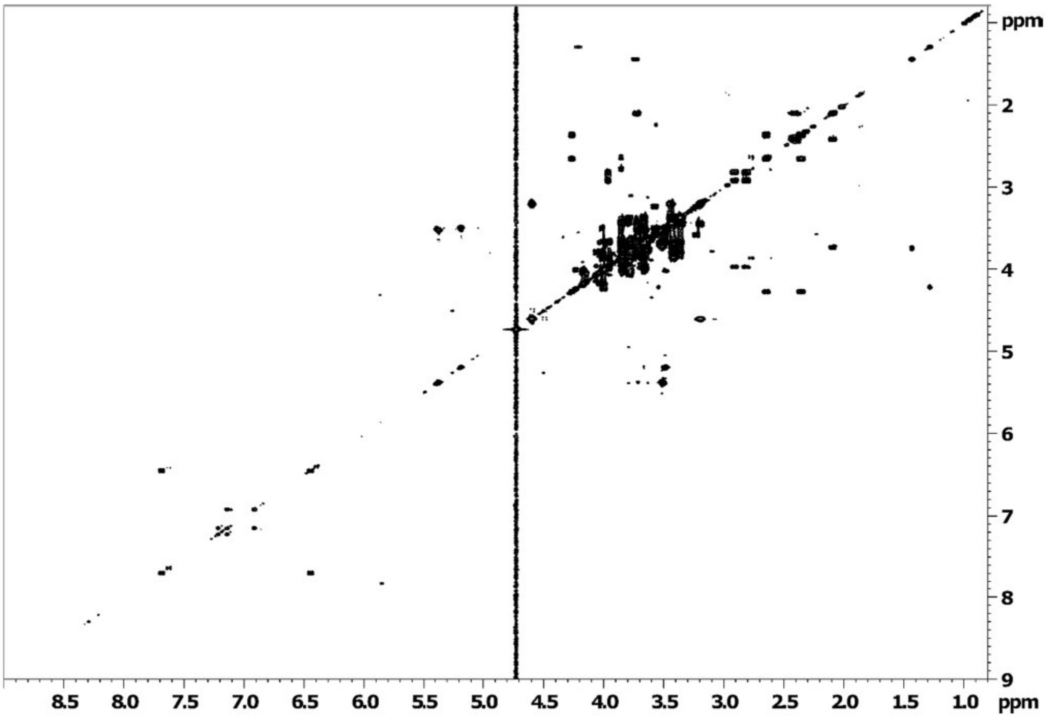

Figure S3: 2D HSQC of the control plant registered in D_2O at 600 MHz

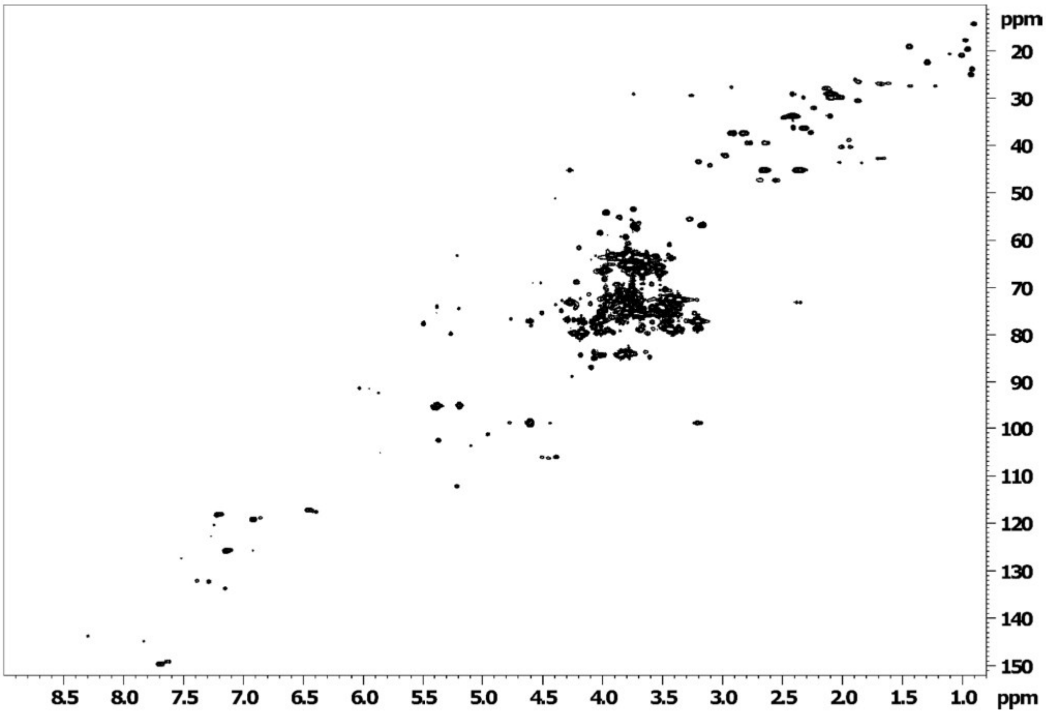

Figure S4: 2D HSQC of the control plant registered in D_2O at 600 MHz

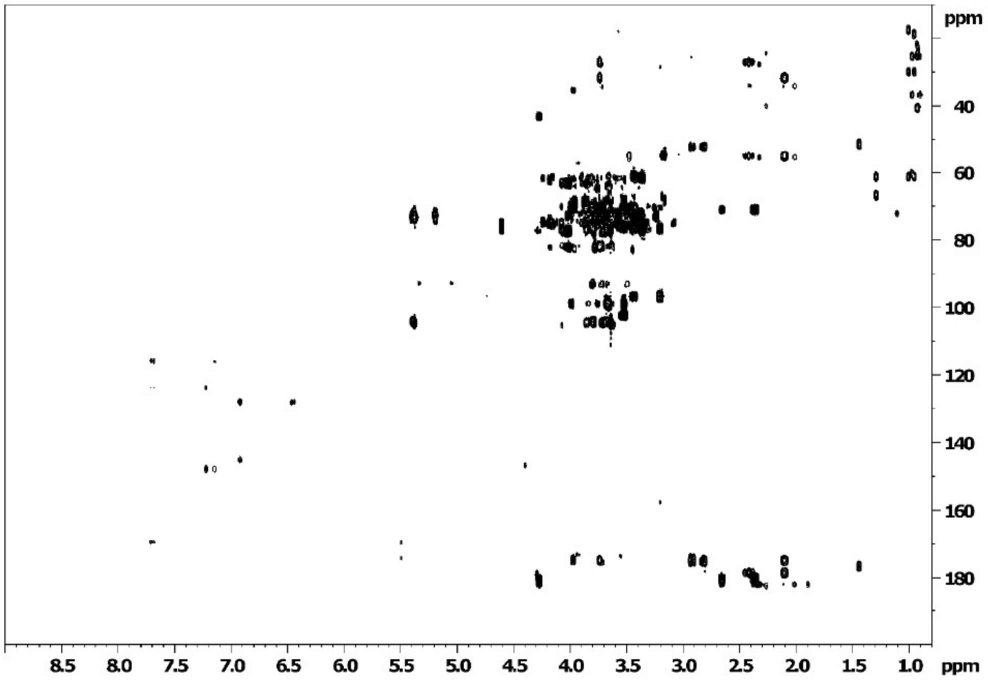

Figure S5: Principal Component Analysis (PCA) plots of first and second axis of all treatments and corresponding NMR spectral signals.

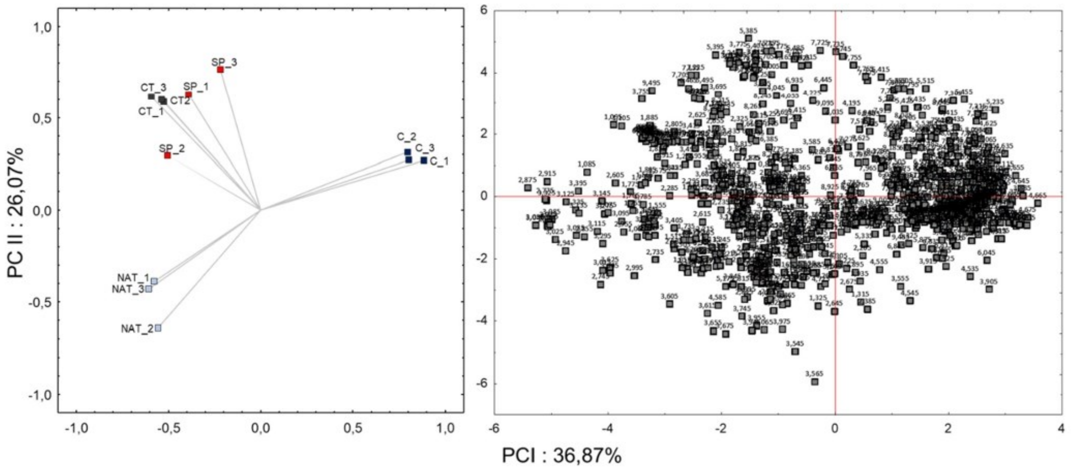

Table S1: Quantitative data (µmol/g dried leaves) of the detected metabolites in the analysed samples. AVG= average; SD= standard deviation

	C	**CT**	**SP**	**NAT**
Compound	**AVG±SD**	**AVG±SD**	**AVG±SD**	**AVG±SD**
Organic acids				
Acetic acid	10.94±0.90	17.75±1.59	10.06±0.02	12.30±1.65
Citric Acid	24.89±6.31	29.58±4.52	26.55±2.69	37.18±8.16
Formic acid	2.34±0.37	1.91±0.26	1.34±0.04	1.27±0.15
Fumaric Acid	2.90±0.79	0.95±0.10	1.61±0.02	1.79±0.81
Malic acid	307.48±26.51	281.75±8.47	263.10±6.87	279.13±20.90
Amino acids				
Alanine	42.85±7.45	43.92±1.15	35.38±1.69	59.32±21.63
Asparagine	64.41±0.90	74.77±3.33	57.59±0.44	59.10±3.57
Aspartic acid	15.42±2.12	24.66±1.67	18.96±1.29	23.35±1.94
GABA	18.62±4.10	21.69±1.70	20.48±1.03	25.15±1.78
Glutamic acid	70.58±17.91	107.06±9.76	81.79±11.64	87.82±18.61
Glutamine	134.70±6.92	157.13±11.21	120.22±14.76	216.60±57.79
Isoleucine	26.61±0.87	27.06±1.96	23.39±0.97	23.56±4.07
Leucine	18.94±0.48	19.53±1.13	16.12±0.66	16.69±2.29
Phenylalanine	5.99±0.19	4.87±0.78	3.97±0.15	3.56±0.35
Threonine	24.86±1.01	27.82±0.07	21.58±0.01	23.02±2.33
Tryptophan	4.91±0.30	2.50±0.56	2.81±0.39	1.32±0.29
Tyrosine	6.99±0.43	3.95±0.71	4.77±0.68	3.63±0.37
Valine	14.25±0.03	14.70±0.78	11.96±0.34	13.55±2.18
Carbohydrates				
α-Glucose	499.68±15.12	538.54±24.02	438.73±1.55	437.76±44.95
β-Glucose	822.37±23.84	887.30±38.88	721.72±0.94	721.22±78.10
Fructose	455.43±38.89	490.97±40.03	345.82±17.26	444.27±38.10
Myo-Inositol	903.42±25.24	1006.79±32.33	796.19±7.07	814.37±75.36
Sucrose	364.62±32.72	407.41±17.00	306.03±25.06	228.88±38.64
Nucleosides				
Adenosine	2.56±0.76	3.04±0.65	2.52±0.39	3.14±0.77
Cytidine	3.56±0.92	2.58±0.87	2.31±0.37	2.87±0.93
Guanosine	1.55±0.12	0.91±0.17	0.58±0.23	0.25±0.26
Uracil	3.53±0.13	1.95±0.08	1.71±0.06	0.48±0.24
Other compounds				
Chicoric Acid	19.12±5.09	24.84±1.42	18.82±0.56	5.07±4.14
Chlorogenic acid	8.89±2.54	5.01±1.64	6.48±0.25	1.88±1.54
Choline	21.36±4.35	26.67±1.97	22.22±1.55	31.59±6.30
Ethanolamine	12.46±2.03	24.73±1.72	17.96±0.26	24.23±1.16
Trigonelline	2.92±0.20	2.38±0.04	2.23±0.23	1.65±0.14