# Variation of yield and composition of essential oils from Mint and Basil in response to mycorrhizae bio-elicitor and hydric stress

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“Para além da curva da estrada
Talvez haja um poço, e talvez um castelo,
E talvez apenas a continuação da estrada.
Não sei nem pergunto.
Enquanto vou na estrada antes da curva
Só olho para a estrada antes da curva,
Porque não posso ver senão a estrada antes da curva.
De nada me serviria estar olhando para outro lado
E para aquilo que não vejo.
Importemo-nos apenas com o lugar onde estamos.
Há beleza bastante em estar aqui e não noutra parte qualquer.
Se há alguém para além da curva da estrada,
Esses que se preocupem com o que há para além da curva da estrada.
Essa é que é a estrada para eles.
Se nós tivermos que chegar lá, quando lá chegarmos saberemos.
Por ora só sabemos que lá não estamos.
Aqui há só a estrada antes da curva, e antes da curva
Há a estrada sem curva nenhuma. “

- Alberto Caeiro (em "Poemas Inconjuntos"; Heterónimo de Fernando Pessoa)

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To all: **Thank you very much**!

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## List of abbreviations

**CC** Classes of Compounds

**DB-1** Gas-liquid chromatographic capillary column with methylsilicone immobilized phase

**DB-17HT** Gas-liquid chromatographic capillary column with phenylmethylsilicone immobilized phase

**EO** Essential oil

**EOMT** Eugenol-O-methyltransferase

**GC-MS** Gas chromatography associated with mass spectrometry

**ISSC-MAP** International Standard for Sustainable Wild Collection of Medicinal and Aromatic Plants.

**MS** Mass spectrometry

**PAL** Phenylalanine ammonia lyase

**R** Statistic program for natural sciences

**RI** Retention index

**SD** Standard deviation

# Resumo

Hoje em dia, perante a realidade das alterações climáticas, manifestas, por exemplo, nas mudanças nos padrões de precipitação e temperaturas extremas, torna-se necessário encontrar novos métodos de cultivo de plantas medicinais e aromáticas (MAP), uma vez que o clima tem grandes implicações para o metabolismo vegetal. O aproveitamento da água toma particular importância em climas áridos ou semi-áridos como é exemplo o da região Mediterrânica. Uma consequência comum associada à ocorrência de *stress* hídrico é a diminuição da biomassa que, no contexto do cultivo, resulta numa poupança de água que não compensa a perda de rendimento. No entanto, na última década, a aplicação de fungos micorrízicos arbusculares (AMF) tem sido considerada uma estratégia importante para contrariar os efeitos do *stress* hídrico, melhorando simultaneamente o rendimento e qualidade das culturas. No presente estudo, uma experiência fatorial baseada no *randomized complete block design* com três fatores foi realizada para investigar o efeito de AMF e do *stress* hídrico sobre a composição do óleo essencial (OE), rendimento, características fisiológicas e morfológicas da menta (*Mentha* sp.) e manjericão (*Ocimum basilicum* L. cv. Genovese Gigante). Os fatores incluíram a inoculação de um AMF (*Glomus intraradices*), um nível de irrigação (*stress* hídrico leve (60% de capacidade de campo)) e a interação de ambos, AMF e *stress* hídrico. Adicionalmente examinaram-se os efeitos da secagem das plantas e da colheita em duas alturas diferentes (inverno e final da primavera) na produtividade e qualidade dos óleos essenciais.

Inicialmente, as sementes foram germinadas e as plantas transplantadas e transferidas para uma estufa, permitindo melhor controlo das condições do meio. No decorrer da experiência, diferentes características funcionais foram registradas para obter informações relacionadas à influência do AMF e do *stress* hídrico no desenvolvimento das plantas. Após cada colheita, o rendimento dos óleos essenciais foi registrado e finalmente estes foram analisados com o auxílio de CG e CG-EM.

Em todas as amostras os principais constituintes dos OE para a menta foram o óxido de piperitenona (22%-91%) e para o manjericão o Eugenol (1%-51%) e o Linalool (18%-60%). A maior quantidade de óxido de piperitenona (91%) foi obtida na 1ª colheita com plantas sob *stress* hídrico e posteriormente secas. Relativamente aos compostos do manjericão, a maior

quantidade de Eugenol (51%) foi obtida na 1ª colheita com plantas sob *stress* hídrico e destiladas em fresco enquanto a maior quantidade de Linalool (60%) foi obtida na 2ª colheita com plantas micorrizadas (sem *stress* hídrico) e posteriormente secas. As condições de *stress* diminuíram a altura e a biomassa das plantas, enquanto as plantas inoculadas apresentaram uma atenuação dos efeitos adversos do *stress* hídrico. Como tal, a inoculação micorrízica resultou numa melhoria relativamente aos parâmetros de crescimento, bem como nas características fitoquímicas e fisiológicas da menta e manjericão. Em conclusão, os resultados deste estudo poderam ser úteis para se avaliarem novas possibilidades de melhorar a produtividade, o manejo do cultivo e a qualidade da menta e manjericão em países mais quentes e permitindo um menor *input* de agroquímicos.

## Abstract

Nowadays, faced with the reality of climate change and increasing threat of unstable precipitation and temperature increase, it is necessary to find new methods of cultivation of medicinal and aromatic plants (MAP), since the climate has great implications for the plant metabolism. The exploitation of water is particularly important in arid or semi-arid climates such as the Mediterranean. A common consequence associated with the occurrence of water stress is the reduction of the biomass that in the context of the crop production, results in the saving of water but does not compensate the loss of profit. However, in the last decade, the application of arbuscular mycorrhizal fungi (AMF) has been considered an important strategy to counteract the effects of water stress while improving crop yield and quality. In the present study, a factorial experiment based on randomized complete block design with three factors was performed to investigate the effect of AMF and water stress on the essential oil (EO) composition, yield, and physiological and morphological characteristics of mint (*Mentha* sp.) and basil (*Ocimum basilicum* L. cv. Genovese Gigante). The factors included AMF inoculation (*Glomus intraradices*), an irrigation level (mild water stress (60% Field capacity)) and the interaction of both AMF and drought stress. In addition, the effects of plant drying and harvesting at two different stages (winter and late spring) on yield and quality of essential oils were examined.

Initially, the seeds were germinated, and the plants transplanted and transferred to a greenhouse, allowing a better control of the environment conditions. During the experiment, different functional characteristics were recorded to obtain information related to the influence of AMF and hydric stress on plant development. After each harvest, the yield of the essential oils was registered and finally the latter were analyzed with the aid of GC and GC-MS.

The main EO constituents for mint were Piperitenone oxide (22%–91%) and for basil Eugenol (1%-51%) and Linalool (18%-60%). Overall, the highest amount of Piperitenone oxide (91%) was obtained in the 1$^{st}$ harvest with plants under hydric stress and posteriorly dried and the highest amount of Eugenol (51%) was obtained in the 1$^{st}$ harvest with plants under hydric stress, distillated in fresh. In addition, the highest amount of Linalool (60%) was obtained with mycorrhizal plants (without hydric stress) from the 2$^{nd}$ harvest and posteriorly dried. Drought conditions decreased the height and biomass, whereas AMF plants ameliorated the adverse effect of drought conditions. In general, mycorrhizal inoculation resulted in an improvement in the growth parameters as well as the phytochemical and physiological characteristics of mint and basil. In conclusion, the results of this study might be useful to improve the productivity, cultivation management and quality of mint and basil in warmer countries and with less input of agrochemical

# Variation of yield and composition of essential oils from Mint and Basil in response to mycorrhizae bio-elicitor and hydric stress

## 1. Introduction

Several research articles have been published concerning the effect of hydric stress or mycorrhizae on essential oil-bearing plants. However, literature related to the interaction of both biotic and abiotic factors like the latter mentioned is scarce. According to Jayne et al (2014), which conveyed a meta-analysis of the research articles that used both arbuscular mycorrhizae fungi and water stress, there are present in literature 54 eligible studies from 1983 to 2014 (Jayne et al., 2014). The interaction of different factors simultaneously resembles the influence plants are subjected to in nature. Still, the isolated study of these parameters is relevant to the understanding of the interaction of both. Furthermore, biotic and abiotic factors can affect the secondary metabolism which might be interesting to obtain differentiated and novel natural products.

This study started by the postulation that certain procedures applied during cultivation may affect the production of natural products. In that sense it was chosen two type of "inducers", drought stress and AMF.  The hydric stress level used in this experiment may be described as a mild hydric stress and its selection was based on several reports regarding MAP which used this type of water regime. The fungus chosen was *Glomus intraradices*, since it was the first mycorrhiza fully genetically described, it is a fast colonizer, is commonly used and according to some studies it only provokes growth depression in highly administered soils (in terms of micronutrients).

## 1.2 Metabolomics: Volatile Oils and the Environment

In the present days, it is well known that plant specific metabolic composition determines the quality of herbs used for food, medicine or industrial material (Schweiger et al., 2014). In an organism there are different 'spheres' with distinct characteristics that in a whole give the organism the building blocks to survive. Those 'spheres' summarized by systems biology can be described as the genome/transcriptome, proteome and metabolome.

Plants are not at all static or easily predictable systems but are highly dynamic in their chemical composition which is modulated by environmental factors. The 'phytometabolome' according to Schweiger et al (2014) is a snapshot of the complete set of metabolites of a plant, representing the metabolic phenotype (Schweiger et al., 2014). Metabolomics has been suggested to be the ultimate level of post-genomic analyses as it reflects both transcriptional and post-transcriptional regulation since the transcript levels often do not correlate with corresponding protein levels (Allwood et al., 2008; Hall et al., 2002). In short, understanding the metabolome permits to a certain extent to distinguish constitutive production (set of metabolites produced in a 'healthy' state) and "*de novo*" production (when a plant is subjected to traumatic stimuli and presents an induced production where compounds are produced in a higher quantity and/or in a different proportion) (Figueiredo et al., 2008). Thus, as mentioned above, the proportion by which these compounds are synthetized interferes with the quality of the herbs. In that sense metabolomics has great relevance since it aims at determining a sample's profile of these compounds at a specific time under specific environmental conditions (Schweiger et al., 2014).

Two hundred years of modern chemistry and biology have described the role of primary metabolites in basic life functions such as cell division and growth, respiration, storage and reproduction (Bourgaud et al., 2001). However, the role of the secondary metabolites was unknown till the end of the XIX century. The differentiation between "secondary" and "primary" metabolism (and metabolites) was probably first introduced by Albrecht Kossel in 1891 (Mothes et al., 1980). "Secondary products" is synonymous to "natural products", a term more commonly used by chemists (Hartmann, 2007). Further on, for researchers, these compounds turned from waste products (what was thought initially about these metabolites; toxic residues released by the cells with no specific purpose) to 'ecochemicals' (involved in direct and indirect defense, intra and interspecific communication, increased plasticity and other processes).

Known organisms that synthetize such chemicals and that are of health and commercial interest are medicinal and aromatic plants. Medicinal plants or non-timber forest products

(NTFPs), also known as minor forest produce, special, non-wood, minor, alternative and secondary forest products, are useful substances, materials and/or commodities obtained from forests which do not require harvesting (logging) trees (retrieved from 'Glossary of Forestry Terms in British Columbia', 2008). Research on NTFPs has focused on their ability to be produced as commodities for rural incomes and markets and as a key component of sustainable forest management and conservation strategies (retrieved from 'Glossary of Forestry Terms in British Columbia', 2008). Currently, it is estimated that the number of higher plant species used worldwide for medicinal purposes is more than 50.000 (Schippmann et al., 2002). This equates to approximately 20% of the world's vascular flora and constitutes the biggest spectrum of biodiversity used by people for a specific purpose (Hamilton et al., 2006). This spectrum of biodiversity or better said - 'chemodiversity' - is just as much a characteristic of life on Earth as biodiversity; both are entangled. In that sense, among other organisms, plants are an important repository of chemodiversity. The study of the metabolites that entail chemodiversity require not only the understanding, in a reductionist form, of the organism that produces these metabolites, but also its interaction with the surrounding environment. The metabolome might be a direct pathway to that understanding since according to Schweiger et al (2014) a thorough understanding of 'phytometabolome' modulation is necessary to eventually decode mechanisms underlying changes in plant quality (Schweiger et al., 2014). This modulation type of research, posteriorly, may lead to a prediction of desirable chemical profiles, yield increase and maintenance or increase of the quality of the natural product of the study target (in this case, essential oil-bearing plants) and preferably, conservation of biodiversity.

As stated before, the study of the metabolome embraces the study of natural products, or better said the secondary metabolism. Plant secondary metabolites are usually classified according to their biosynthetic pathways (Harborne, 1999). Most of these compounds which are reported to be largely lipophilic products with molecular masses under 300 can be assigned to the following classes (in order of decreasing size): terpenoids, fatty acid derivatives including lipoxygenase pathway products, benzenoids and phenylpropanoids, C5-branched compounds, and various nitrogen and sulfur containing compounds (Dudareva et al., 2004). Nearly all these classes are emitted from vegetative parts as well as flowers (Knudsen et al., 1993) and some are even emitted from roots (Steeghs et al., 2004).

These emitted compounds, mainly terpenoids, are used to maintain communication and fruitful adaptive interaction with their environment (Kumari et al., 2014). Herbivore-challenged plants are known to emit volatiles not only to invite the natural enemies of herbivores (parasitoids and predators), but also to aid neighboring plants by inducing a defense response in them (Pare et al., 1999; Kessler et al., 2001). This complexity of interactions in the plant kingdom exhibits tremendous variation in their chemistry and roles (Gershenzon et al, 2007). As such, the emission of these chemical compounds represent some adaptive solution to the environment; plants are static (in terms of motion) and due to that extremely plastic since environments are highly heterogeneous both in space and time, and organisms must either acclimate to, or escape from, adverse conditions (Gianoli et al., 2007). Since plants are sessile, highly complex traits have developed to guarantee their survival and reproduction in response to the environmental changes (Fig.1).

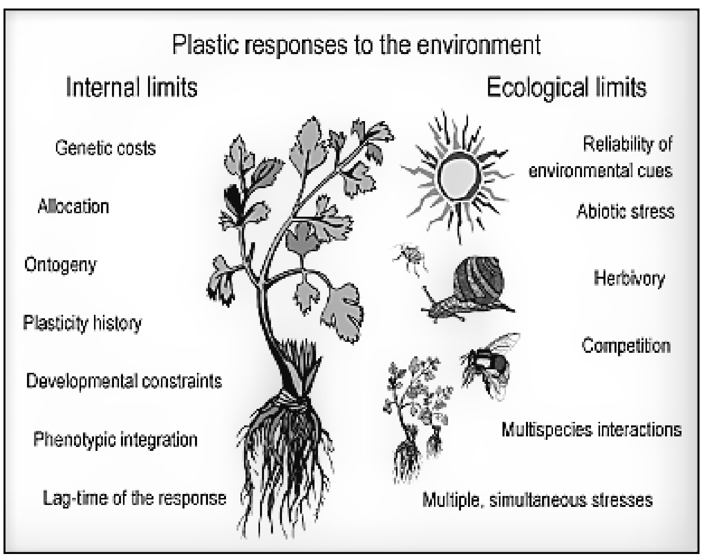



Fig.1: In contrast to internal limits, multiple biotic and abiotic factors, which more often than not exert their influence simultaneously, have been exploited in less detail, despite the growing evidence of their importance (Gianoli et al., 2007).

Furthermore, the adaptations plants present to a certain factor may be inferred by the volatile emissions. Terpenoids, which are to a great extent the main constituents of plant-derived essential oils might be used as a means to understand plant plasticity. The general scheme of the biosynthetic reactions of this group of compounds is shown in Figure 2 (Baser et al., 2015). Basically, through photosynthesis, green plants convert carbon dioxide and water into glucose. The cleavage of glucose produces phosphoenolpyruvate (1), which is a key building block for the shikimate family of natural products (4). Furthermore, decarboxylation of phosphoenolpyruvate gives the two-carbon unit of acetate and this is esterified with coenzyme-A to give acetyl CoA (2). Self-condensation of this species leads to the polyketides and lipids. Acetyl CoA is also a starting point for synthesis of mevalonic acid (3), which is an important starting material for the terpenoids (Baser et al., 2015).

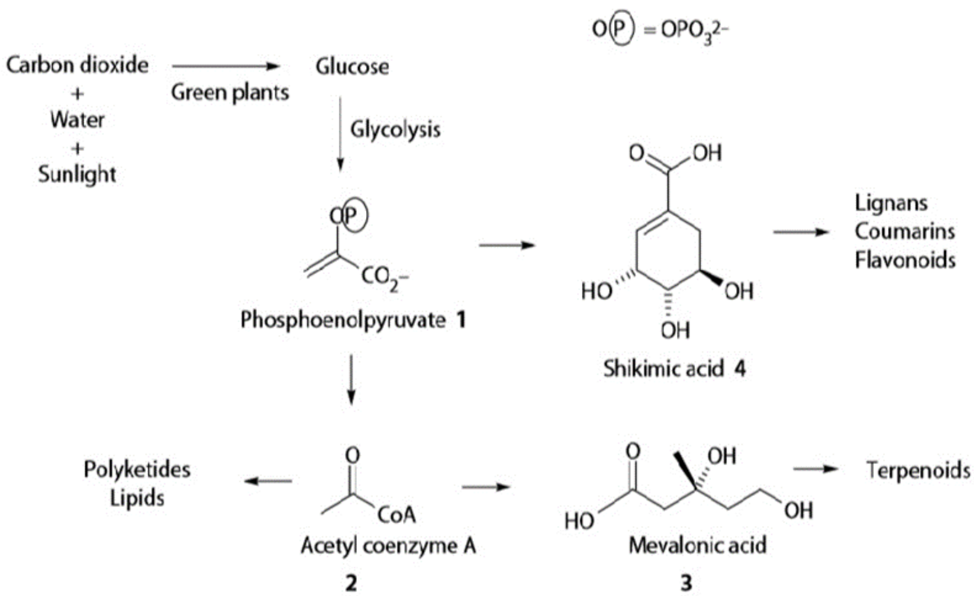



Fig.2: General pattern of biosynthesis of secondary metabolites (Baser et al., 2015).

In resume, the basic pathway of volatile terpenoid biosynthesis is conveniently treated in three phases: (a) Formation of the basic C5 units, (b) condensation of two or three C5 units to form C10, C15 or C20 prenyl diphosphates, and (c) conversing of the resulting prenyl diphosphates to end products. The formation of basic C5 units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) proceeds via two alternative pathways: the already mentioned long-known mevalonate pathway from acetyl-CoA and the methylerythritol phosphate pathway from pyruvate and glyceraldehyde-3-phosphate, discovered only in the last 10 years (Rodríguez-Concepción et al., 2002). The methylerythritol phosphate pathway, localized in the plastids, is thought to provide IPP and DMAPP for hemiterpene, monoterpene, and diterpene biosynthesis, while the cytosol-localized mevalonate pathway provides C5 units for sesquiterpene biosynthesis. However, metabolic ‘‘cross-talk’’ between the two pathways is prevalent (Schuhr et al., 2003), particularly in the direction from plastids to cytosol (Laule et al., 2003).

Hence, these compounds, terpenoids, usually are stored in specialized histological structures. These structures are present for these specific compounds since these compounds might be toxic for the plant itself, causing internal oxidative damage, so the organism adapted in such a way that these metabolites are not in direct contact with the plant but in specific secretory glands.

The methods by which the latter compounds are obtained, which are disclosed as complex mixtures of mainly terpenes (essential oils) is either by hydrodistillation or steam distillation of plants or parts of plants, or by expression of the fresh pericarp of certain citrus. This definition is restrictive: it excludes both the products obtained by extraction with solvent aid, as well as those obtained by any other procedure (pressure gas, “enfleurage”, and others). The designation ‘essential oils’ also embark odorous products that do not pre-exist in the plant but result from an enzymatic reaction on a substrate, a reaction that can only occur after an alteration of the tissues (Bruneton et al., 1991). The latter may happen with the distillation itself. The physical properties of essential oils are that they are liquid at ambient temperature, rarely have color and in general their density is below of water density. Thus, essential oils are soluble in alcohols and in usual organic solvents (Bruneton et al., 1991).

Final remarks are that these generally low molecular weight compounds are a close link to the phenotype in the sense that it permits to understand what are the flexible responses to the environment of the plant when the latter is already developted. Understanding the biosynthetic routes and making correlations with the environment is necessary for the development of standartized natural products, and so, a standartized effect.

## 1.3 Biotic and abiotic factors study as tools for production

Most crop plants grow in environments that are suboptimal which prevents plants from attaining their full genetic potential for growth and reproduction. To make sense of these type of environments influence on plants the study of abiotic and biotic factors have been deepened and modulated in the last decades. Abiotic stresses result from inappropriate levels of environmental factors, such as drought, flood, extreme temperature, severe radiation, metal ion stress, nutrient limitation, and oxidative stress, while biotic stresses come from pathogens and pests (Hong et al., 2016). The latter further include for example inter-specific competition (neighbor plants), herbivory (above-ground and below-ground herbivores) (Gianoli et al., 2007) and soil microorganisms. Several articles studied the effect of these different 'stressing' factors on secondary metabolism of plants and other factors, like the effect of plants grown in *in vitro* cultures, the use of growth regulating agents, salt stress, plant nutrition, UV elicitation, mechanical wounding (Banchio et al., 2005) and so on. These studies are frequently endowed with the intention of selection of the most suited method and factor(s) that allow to define a plan of production of biomass with reduced costs. Thus, with the purpose of the selection of plants that are resistant or tolerant to these factors while maintaining or improving the demanded commercial traits. Indeed, added value to a product may be reached through different cultivation and processing methods.

In the present days demands from consumers and companies for environmentally friendly methods of production and/or products are increasing. There are already procedures, as GAP (Good Agriculture Practices), Good Harvesting Practices (GHP), and Good Manufacturing Practices (GMP) that can be incorporated as those type of methods. GAP (Fig.3) can be defined as a set of "practices that address environmental, economic and social sustainability for agricultural processes, that result in food and non-food quality and safety of agricultural products" (Baser et al., 2015). In the cosmetics and pharmaceutical industries, it is highly desirable that raw materials from cultivated plants are produced using GAP (Lubbe et al., 2011). It is currently not a legislative requirement, i.e. neither national laws nor EU law states that GAP should be used. However, most companies using raw plant materials will not accept cultivated material not produced in this way. The cultivation of plants according to GAP means using established standard operating procedures (SOPs) that will ensure good quality and safety, and thoroughly documenting all actions and procedures to ensure complete traceability (Lubbe et al., 2011). Traceability in the natural products field is important since understanding how the material was processed previously helps to make succinct correlations when examining the chemical profiles.

The complete traceability that GAP and regimented practices postulate is necessary when applying biotic and abiotic factors. The different practices mentioned permit a type of 'life cycle' perspective of the chain of production which is relevant also due to the benefit of coinciding with international standardized norms in a predictable way. The latter norms are relevant since it allows the natural product or raw material to enter the public market. The last-mentioned is pertinent since in medicinal plant research there is a gap in terms of analyzing the value chains which exist for herbal medical products, which very often are harvested from the wild or produced in small scale agricultural practices (Booker et al., 2012). In that sense, adulteration and contamination is commonplace and so the supply of good quality raw materials is limited. This limitation on good quality raw materials has stunted the industry's growth (Booker et al., 2012).

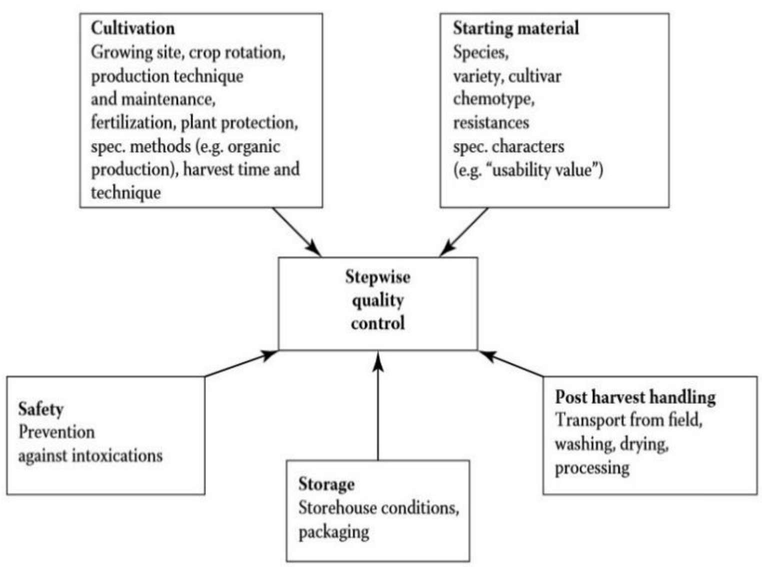



Fig.3: Main items of "good agricultural practices" (GAP) (Baser et al., 2015).

The lack of standardized/good quality supply has also to do with the level of productivity, impacted by the changes in content and spectrum of secondary metabolites (with age, time of the year, watering pattern, or location that modulate the chemical profile), and the biological and therapeutic effects associated with that plant sample that change in a non-predictable, nonreproducible manner (Cordell et al., 2012). The variety of factors affecting plants (biotic and abiotic) make some aromatic species to have a cultivation restricted to edaphoclimatic reasons, to certain parts of the globe.

In the case of Mediterranean climates, where plants are subjected to hydric stress (which seems to be associated with the raise of essential oil production in certain species), about 38% of the species are essential oil-bearing plants, whereas in tropical climates, that number decreases to 11% (Figueiredo et al., 2008). However, Portugal as one of the countries that has a Mediterranean climate, the number of essential oils producers is low. In a document released by the portuguese government in 2012, it is possible to find the following information: “Seven producers were recorded in the survey which, after harvesting the vegetative material, transformed their production into essential oils. Some producers of MAP that produce oils also import raw material and/or pick spontaneous plants, they are collectors.” (Retrieved from ‘Plantas Aromáticas e Condimentares’ in gov.pt, 2012). In this context, GAP could be compelling for the production of the plant material since it offers guidelines that are widely accepted by the cosmetic and pharmaceutical industry and besides that, there are several advantages over wild harvest/collection and/or importation to produce essential oils as: a) Avoidance of admixtures and adulterations by reliable botanical identification, b) Better control of harvested volumes, c) Selection of genotypes with desirable traits, especially quality and d) Controlled influence on the history of the plant material and on postharvest handling (information from ISSC-MAP guidelines). Apart from these factors it offers a non-dependence from wild resources, which are of major concern since in Europe 90% of the medicinal plants used are harvested from wild resources (McChesney et al., 2007). In addition, when a plant is cultivated in controlled conditions different procedures may be applied in order to have an established product and possibly reduce inputs and increase outputs.

Addressing the factors mentioned and essential oil-bearing plants producers, pilot models or micro enterprises could be acknowledged in order to encourage the sustainable use of biodiversity and implement more effective and targeted crop development, whereas for retailers better marketing and delivery strategies should be sought (Jokar et al., 2017). Furthermore, studying main markets and postulate from the beginning what will be the application of the product is key. Abiotic and biotic factors use, short-termed or prolonged, may be chosen based on those *a priori* postulations.

Considering the genus *Mentha* and genus *Ocimum*, it is possible to find multiple studies which used an arbuscular mycorrhizae fungus, water deficit, and in a smaller proportion studies which examined the interaction of both biotic and abiotic factors. For example, a study which used mint species collected from different mountain areas asserted that AMF provokes an increase of biomass and that mint mycorrhizal plants had relatively high levels of Limonene,1,8-Cineole, Carvone, Eugenol and(E)-Methylcinnamate (Karagiannidis et al., 2010). Hence, with *Mentha arvensis* L. subjected to several irrigation levels it has been reported that hydric stress has a detrimental effect on herb yield (Shormin et al., 2009; Misra et al., 2000) and that menthol and menthone content increased under severe water stress in comparison with unstressed plants and that menthyl acetate was significantly reduced (Misra et al., 2000). Regarding the genus *Ocimum*, there is studies with this genus and with different varieties/cultivars within the genus, for example there is a study with *O. basilicum* L. var Genovese inoculated with three AMF fungus which reports that AMF stimulates growth and that at the end of the experiments it was clear that *Gi. rosea* significantly increased the concentration of camphor, αterpineol, and the total amount of essential oils (also in relation to the increased number of leaves), while plants treated with *Gi. margarita* had significantly decreased eucalyptol, linalool, eugenol content, and the total concentration of essential oils (Copetta et al., 2006). Furthermore, a study with *Ocimum gratissimum* L. subjected to hydric stress and an AMF reported that the analyses show that AMF increases the yield of oils with a maximum recorded in stressed mycorrhizal plants (0.33%) and the lowest in non-stressed non-mycorrhizal plants (0.22%). The contents of total phenolic compounds increased in non-mycorrhizal plants under stress (104% in leaves and 97% in the roots) unlike the mycorrhiza which did not stimulate the synthesis of these compounds (Hazzoumi et al., 2015).

To conclude, there are different normative systems and challenges associated with plant production since plants produce a tailored response to specific multiple stress conditions. Both positive and negative interactions between biotic and abiotic factors have been observed to be dependent on the timing, nature, and severity of each stress (Atkinson et al., 2012). If well understood, biotic and abiotic factors are possible tools to address challenges of production while being in accordance with the norms since these tools may allow the standardization of the natural product if in controlled conditions and simultaneously assuring environmental sustainability which is an increasingly important goal of product development; both growing consumer concerns and increasing regulation are forcing companies to consider how their products impact the environment. However, a challenge associated with the use of these factors which is important to emphasize is that an increasing volume of evidence from field, laboratory, and molecular studies suggests that plants respond to a specific combination of stresses in a non-additive manner, producing effects that could not have been predicted from the study of either stress individually (Mittler, 2006; Rizhsky et al., 2004). The application of these factors can be said to be based on biomimicry which can be defined as the technical emulation of biological processes and systems (Kennedy et al., 2016). Nevertheless, the emulation of biological processes that happen in nature entails the occurrence of simultaneous biotic and abiotic stresses which present an added degree of complexity, as the responses to these are largely controlled by different hormone signaling pathways that may interact and inhibit one another (Anderson et al., 2004; Asselbergh et al., 2008). In the industry of natural products, it will be a problem if the action of a substance differs even though it has been cultivated under the same circumstances. The regulatory control is—among others—the most difficult aspect. In short, to better dissect the plant metabolic regulatory networks and their functions in the responses to complex abiotic and biotic stresses, integrated multiple-omics analysis is required (Hong et al., 2016).

## 1.4 Case study: Mint and Basil

In regard of species for marketing in green, in Portugal, coriander is the dominant species of the 21 listed, and by far the most important in terms of area. In fourth place is *Ocimum basilicum* L. and *Mentha* spp. in the seventh. The extraction of oils from these plants in comparison with the sales of their dry or fresh material is low (Retrieved from 'Plantas Aromáticas e Condimentares' in gov.pt, 2012); that may be related to the fact that there is lack of infrastructure/capacity building for quality control of essential oils which is a requirement for their respective sales. Nevertheless, the genus *Mentha* and *Ocimum* are known genus in the industry of essential oils, and as stated these plants are in the list of Portuguese most planted species per ha (Retrieved from 'Plantas Aromáticas e Condimentares' in gov.pt, 2012).

The **genus *Mentha*** (Fig.4) is one of the most important of the Lamiaceae family. This genus comprises 25 to 30 species grown in different parts of the world (Kostik et al., 2015). Some of these species are *Mentha arvensis* L., *Mentha* x *piperita* L., *M. longifolia* (L.) Huds. and *M.spicata* L., which are commonly known as menthol mint, peppermint, wild mint and spearmint, respectively. These are frequently cultivated in many countries of East Asia, Europe, America and Australia to produce essential oils (Kostik et al., 2015). The most known concerning the production of essential oils is *Mentha* x *piperita* L. (peppermint), which is a sterile hybrid between *Mentha spicata* L. and water mint *Mentha aquatica* L., generated over 250 years ago (Murray et al., 2011). This is a species distributed widely in temperate and sub-temperate climatic regions. Generally, the plants from this genus are perennial, propagate by stolons and are usually characterized by violet stems, opposite leaves oval-sharp and jagged, inflorescences in tight spikes of flowers, bilabiate with purple corolla (Kostik et al., 2015).

The essential oils and extracts from mint species have been used since ancient times for the treatment of many digestive track diseases and in cuisines (Kostik et al., 2015). Nowadays, the essential oils of mint species are not only used for the latter purposes but also may be used for the preservation of processed foods as well as for pharmaceutical and natural therapies for the treatment of infectious diseases in humans and plants (Salehi et al., 2018). However, it is important to emphasize that previous studies concerning the antimicrobial properties of mint essential oils yielded results that are difficult to reconcile (Sivropoulou et al., 1995). This is mainly due to the great variation found in the chemical composition of mint essential oils and to a lesser extent to differences in the experimental techniques applied (Fancello et al., 2017).

Fig.4: *Mentha* sp. from the experiment with 92 days of culture. Plants treated with hydric stress plus mycorrhizae.

The **genus *Ocimum*** (Fig.5) also from Lamiaceae family, collectively known as basil has long been acclaimed for the genetic diversity of the species within the genus, which comprises at least 65 species, but more than 150 species according to some sources, of herbs and shrubs (Paton et al., 1999). This genus is native to Asia and can be observed in tropical and sub-tropical regions (Paton et al., 1999). *Ocimum basilicum* L. or sweet basil, is a widely grown aromatic perennial crop. This plant is cultivated either for production of essential oil, dry leaves for the fresh market, or as an ornamental. Nevertheless, basil is known mainly as a spice (herb for sauces; pesto) and as a source of essential oils. This genus is extensively grown in the Mediterranean region and in the islands of the Indian Ocean. In the 1600's, the English used basil as a food flavoring and insecticide (Paton et al., 1999). Now the latter use is validated since it is proven that the essential oil from this plant possesses a range of biological properties which include action as an insect repellent and nematocidal (Deshpande et al., 1997). Several aroma compounds can be found in different basil chemotypes, such as citral, eugenol, linalool, methylchavicol, and methyl cinnamate, which are traded in the international essential oil market (Vieira et al., 2000). As such, different species and forms of *Ocimum* spp. vary in growth habit, color and aromatic composition, making the true botanical identity of basil difficult (sometimes due to interspecific hybridization and polyploidy) (Kintzios et al., 2008). However, recently, the classification of different *O. basilicum* L. cultivars has been achieved by means of DNA genotyping (Labra et al., 2004). The European basil (yield 0.2-1%), is considered to have the highest quality aroma, containing linalool and methylchavicol as the major constituents (Simon et al., 1999). The basil leaf is traditionally used orally or as a co-adjuvant for the treatment of digestive functional disorders. Furthermore, the essential oils of basil also have potent antioxidant properties. According to an investigation by Leea et al (2005) eugenol, thymol, carvacrol and 4-allylphenol had stronger antioxidant activities than other volatile components of basil and these antioxidant activities were comparable to those of the known antioxidants, alfa-tocopherol and butylated hydroxy toluene (BHT) (Leea et al., 2005).

The essential oil of the chemotype with methylchavicol (methylchavicol> 75%) is intended for high-quality perfumery while the chemotypes with linalool (linalool 45-62%), eugenol (2-15%), cineole (2-9%)) is intended for food preparations and for "secondary" quality perfumery (Bruneton et al., 1991).

Specifically, the cultivar "Genovese" is characterized by upright growth habit, can exceed 40-45cm of height, with "spoon-like" oval shape leaves which are crinkly and intensely green, with clearly visible veins (Flamini et al., 2006). The quality traded in Europe, Western Asia, and North America is characterized by 1,8-cineole and linalool, plus smaller amounts of estragole (methylchavicol) and eugenol (Simon et al., 1999).

Fig.5: *Ocimum basilicum* L. cv. Genovese Gigante from the experiment, with 91 days of culture. Plant in control condition.

To sum up, these plant secondary metabolites from these two species are sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals. A resume of the characteristics from the both genus of the plant’s species used in this experiment are described on Table 1.

Table 1: Some characteristics of the genus *Mentha* and *Ocimum*.

| Botanical Name (Genus) | Habitat | Plant description | Flowering and fruiting | Uses | References |
|---|---|---|---|---|---|
| *Mentha* | Streambanks, lake shores, moist fields and roadsides. Mostly cultivated in North America and eastern Asia. | While most species are either uniformly prostrate or upright, some species, such as *M. pulegium* L., assume an upright stance only at the time of flowering. Most species of this genus produce long thin rhizomes, commonly called stolons. | Perennial plant, rarely annual. Flowering from May to August. Seeds appear in the end of flowering stage. | Widely used in the flavor industry, mainly in confection, alcoholic beverages, and tobacco industry. Also, widely used in the fragrance industry in personal care and oral care products and, to a lesser extent, in fine fragrances. Use also found in the pharmaceutical industry, particularly in nonprescription products. A more recent use and lesser-known is in aromatherapy. | Lawrence (2006) |
| *Ocimum* | Can be observed growing wild in tropical and sub-tropical regions. | Great genetic diversity of the species within the genus. Most commonly grown type is 'Sweet basil'. Other type 'Genovese Gigante' which also as an erect habit, dark green leaves up to 2 cm long and white flowers. This plant can reach 1 meter tall and has "spoon-like" oval shape leaves, crinkly, with clearly visible veins. | Perennial plant. Flowering from May to August. Seeds appear in the end of flowering stage. | The most common use is for culinary purposes. In addition, the cosmetic industry uses basil oil in lotions, shampoos, perfumes and soaps. Extracts of the plant have been reported to contain biologically active constituents that are insecticidal, nematocidal, fungistatic, and antimicrobial. | Kintzios et al (2008) |

## 1.5 Biotic factor

### 1.5.1 Mycorrhizae

Bacteria and fungi are organisms capable of invading host organisms and altering genetic and metabolic processes along the way (French, 2017). Indeed, to diminish or raise certain compounds of interest, there are different biological inducers (similar to what is done in bioreactors) that may be used to modulate the metabolism and, in this case, the chemical profiles of essential oils (Copetta et al., 2006). One example of an inducer is symbiotic fungi, which elicit a response by the plant, altering the proportion of certain compounds. This might be useful since different fungi can modulate the yield and content of essential oils differently in the same plant (Karagiannidis et al., 2012; Copetta et al., 2006). An interesting fungus for that application is mycorrhizae; the term mycorrhiza is derived from the Greek words for ‘fungus’ and ‘root’ (Kirk et al., 2001).

Arbuscular mycorrhizal fungi (AMF) can be defined as ubiquitous soil inhabitants forming the largest group symbiotically associated with agricultural groups (Smith et al., 1997) and the oldest symbiosis known to science; earliest evidence for the existence of Glomeromycetes comes from spores and hyphae observed in Ordovician fossils, dating back some 460 million years ago (Redecker et al., 2000). AMF are obligate symbionts and can be called ‘generalists’ (present in many soil types; different from ‘specialists’ that are almost exclusively found in specific soil types and/or under specific land use intensities) which include *Glomus diaphanum*, *Glomus fasciculatum*, *Glomus intraradices*, *Glomus etunicatum*, ‘*Gi. occultum*’ group, *Archaeospora trappei* and *Scutellospora calospora* (Oehl et al., 2010). A key role that the AMF symbiosis can play as an ecosystem service (benefits that humans freely gain from the natural environment and from properly-functioning ecosystems) is to be a provider of guaranteed plant productivity and quality in emerging systems of sustainable agriculture (Gianinazzi et al., 2010). Besides that, AMF offers a suitable system to investigate the specificity of ‘phytometabolome’ responses (Schweiger et al., 2014). Indeed, AMF symbiosis causes both global (species-independent) and local (species-specific) changes in plant metabolism (French, 2017). Global changes include increased production of amino acids (glutamic, aspartic, and asparagine acid), fatty acids (palmitic and oleic), secondary metabolites (phenyl alcohols, linolenic acid, apocarotenoids, isoflavonoids), plant hormones (oxylipin, cytokinins and jasmonic acid), activation of the oxylipin pathway and increased sugar metabolism (Fernández et al., 2014; Gaude et al., 2015). In contrast, levels of specific secondary compounds increase according to plant species identity (French, 2017).

These changes may be related to the evolutionary history of land plants which is closely entwined with the evolution of AMF as seen above. This co-evolution might explain the clear physiological diversity of AMF species/isolates (Gianinazzi-Pearson, 1996; Munkvold et al., 2004). However, the selection or breeding for plant varieties under high-nutrient conditions (which causes unneeded symbiotic activity) is leading to the generation of plant genotypes which are less or non-receptive to mycorrhiza (Hetrick et al., 1993; Hartmann, 2007). Likewise, that may happen to mycorrhizae, which can be called 'cheaters' (for example in highly administered soils, or in adverse environments, such as very high temperatures) which instead of determining a higher plant biomass and flowering, the mycorrhiza reduces the growth of the host plant (Johnson et al., 1997).

Nevertheless, in controlled experiments, it is becoming evident through literature examination that the AMF symbiosis can stimulate the synthesis of plant secondary metabolites, which are important for increased plant tolerance to abiotic and biotic stresses or beneficial to human health (Gianinazzi et al., 2010). The raise in synthesis of plant secondary metabolites may be due to a defense response to AMF colonization (Chaudhary et al., 2008). The interaction between plant and fungus is in the presence of a compatible host; in that context, fungus and plant exchange several secreted signals that initiate the symbiotic cellular program (Bonfante et al., 2010, 2011). This mutual long-lasting beneficial association is possible because AMF do not elicit massive plant defense responses. Nevertheless, during an early phase of the symbiosis, the plant reacts to the colonization of AMF by inducing transient defense and stress responses (Liu et al., 2007). After the early contact, AM fungus, like *Glomus intraradices,* secretes effector proteins that play a role in managing an accommodation process of the fungus within the plant roots (Kloppholz et al., 2011). Furthermore, other explanation for the enhanced production of terpenoids in mycorrhizal plants may be due to increased uptake of nutrients since synthesis of terpenoids is resource-demanding and is dependent on the availability of nutrients (Kapoor et al., 2016). So, the early defense responses induced in the plant, and increase in nutrient availability in the soil for root uptake, are some of the primary reasons known for the terpenoids increased production due to mycorrhization.

The latter mentioned prospective role of arbuscular mycorrhiza symbiosis in improving the accumulation of secondary metabolites has gained recognition over the past two decades. Indeed, several studies demonstrate that the associations of PGPR (plant growth-promoting rhizobacteria) and AMF with aromatic and medicinal plants positively affect the quantitative and/or qualitative profile of the secondary metabolites (e.g., essential oils and alkaloids), in addition to improved growth parameters, biomass and yield (Arora et al., 2016).

A difficulty may reside in the fact that most of the studies published in this area were made in controlled environments (in greenhouses) which can bring doubts about the applicability of AMF in field conditions. Notwithstanding, Lekberg et al (2005) examined 290 field and greenhouse experiments and found that in general, increased root colonization resulted in yield increase of 23% across all management practices (Lekberg et al., 2005). However, the application of such management methods in field conditions should include a change of the breeding strategies that select plants adapted to high fertilizer and biocide usage to the selection of plants with increased capacities to exploit AMF attributes. The latter would be beneficial since as mentioned before AM fungi are involved in the production of health-related biomolecules by fruits, vegetables or medicinal plants that have pharmaceutical properties (Copetta et al., 2007) and also allows the resistance of the plants to some unpredictable environmental fluctuations.

Nonetheless, it is important to consider that the number of AMF species used in the described experiments is rather small even considering the limited number of Glomeromycota (or Glomeromycetes). Widening the range of AM species tests could only be positive for a better understanding of the effects induced in plants. Also, the number of plant species studied is not extremely large (about 30). This most likely is related to the overwhelming importance of a relatively small number of plant species for agriculture, human and animal nutrition, and industry (Arora et al., 2016).

Despite these limitations, some facts do emerge: (a) Soil microbes and symbiotic fungi can affect the quality of crops (often increasing yield as well), (b) Even if most experiments have been carried under controlled conditions, and on a reduced scale, the relatively rare tests run in the field show that a practical exploitation is feasible and can result in better products in real life agriculture and (c) combination of different microorganisms can give different results on the same plant species (for instance, promoting the accumulation of a specific secondary metabolite). This opens the possibility of using selected isolates or strains according to the environmental conditions or according to the kind of result as product that is expected (Arora et al., 2016).

Furthermore, the effects AMF induces have to be endowed with a certain ‘know-how’ of the interaction between the plant species and the AMF species. For example, certain soil conditions are not conducive to mycorrhiza formation. Thus, in certain habitats mycorrhizas may be less beneficial because plant productivity is very low, and periods of root activity are brief. Also, it is important to consider the timings, mainly when are the plant roots placed in contact with the fungus and when should the roots be analyzed. The latter is pertinent and important since mycorrhizas require synchronized plant-fungus development for ongoing nutrient exchange and hyphae normally only colonize young roots in mutualistic associations (Brundrett, 2009). This happens because plants control mycorrhizal associations by growing new roots, by the digestion of old interface hyphae in plant cells or altering the root system form (Brundrett, 2009). Furthermore, just as plant taxa vary in mycorrhizal dependency, fungal taxa and isolates vary in mycorrhizal effectiveness (Johnson et al., 1997) which is an important detail to consider.

The species used in this study is *Glomus intraradices* since it is one of the most commonly studied AMF as it colonizes host plants rapidly (Martin et al., 2008). Also, is one of the most used species of *Glomus* among studies, the second most used (Jayne et al., 2014). Furthermore, several factors have led to *G. intraradices* being chosen for the first genome sequencing of an AM fungus (Martin et al., 2008). One of them, besides the rapid colonization is that it is a widespread fungal species since it is present in different ecosystems throughout the world, including temperate and tropical locations (Smith et al., 2008). Fungi that are rapid colonist appear to generate the greatest growth benefits in low-P-fertility soils, and the greatest growth depressions in highly fertilized soils (Abbott et al., 1985).

All the information mentioned clearly justifies the comparison of mycorrhiza to a ‘health insurance’ for plants (Smith et al., 1988). The challenge though, is to increase total yield while maintaining or improving quality assurance (Zeng et al., 2013) of the plant material and respective natural product.

To finalize, usually, by living together, plants and mycorrhizal fungi improve each other’s probability for survival and reproductive success. However, sometimes, generally due to anthropomorphic reasons, plant ‘interests’ conflict with those of fungi. The ‘interests’ of plants and mycorrhizal fungi are likely to diverge in highly managed agricultural systems, where fertilization eliminates shortages of soil nutrients (Johnson et al., 1997).

## 1.6 Abiotic factor

### 1.6.1 Hydric Stress

Moisture and water deficit are a significant challenge to the future crop production but if controlled and measured, applying water stress can be an efficient way to help the sustainability of water resources, and at the same time enriching secondary metabolites production (like raising the content of phenolic compounds according to Luna et al (2015)). Hence, water management is interesting in agricultural practices related to essential oil-bearing plants since mild water stress has been described to increase essential oil percentage and main constituents of the essential oil of basil (Khalid, 2006) and other plants. Several metabolic alterations in response to water deficit can occur on the following three levels: i) disturbance of metabolic pathways leading to an accumulation or loss of metabolites; ii) alterations in enzyme activities, and iii) changes in the patterns of protein synthesis. This metabolic background of the stress-induced enhancement of natural products can be clarified as follows: because of the stomata closure due to the incipient water deficiency, the uptake of $CO_2$ markedly decreases (Selmar et al., 2013). As result, the consumption of reduction equivalents ($NADPH+H^+$) for the $CO_2$-fixation via Calvin cycle declines considerably, generating a massive oversupply of $NADPH+H^+$. As consequence, metabolic processes are pushed toward the synthesis of highly reduced compounds, like isoprenoids, phenols or alkaloids (Selmar et al., 2013) in order to reestablish homeostasis.

Applying hydric stress is compelling since several studies have reported that medicinal plants grown under semi-arid conditions reveal much higher concentrations of relevant natural products than the equivalent plants but cultivated in moderate climates (Selmar et al., 2013). Thus, especially in arid and semi-arid areas, water use control and efficiency could be applied as a simple and inexpensive technique for increasing essential oil components, for example in basil (Mandoulakani et al., 2017), if the variety has susceptibility to the abiotic stress. Some studies (Farahani et al., 2009; Akula et al., 2011) affirm that depending upon the plant species and plant genotype, drought stress can increase, decrease or have no effect on the level of metabolites. If it decreases the biomass, which is common, growth regulators, like salicylic acid (or AMF) can ameliorate the negative effects on plant growth due to drought stress (Kordi et al., 2013). In addition, drought duration and intensity also affect differently the same species plant response; in short, there are different plant responses to different time scales of stress. However, most of the studies concerning drought-related plant responses are short-termed studies (Zhou et al., 2016).

The hydric level used in this experiment is considered mild water stress, at 60% Field Capacity (whereas severe water stress is at 20% Field Capacity) (Samarah, 2005; Jeshni et al., 2017). Most studies compare well-watered, mild and severe water stress. However, the last-two mentioned irrigation regimes are the ones who appear to reflect the best outcomes in terms of essential oil quality. As such, mild water stress was chosen in order to guarantee that these plants, mint and basil, would resist to the physiological damages provoked by the water stress and permit the posterior analysis of the chemical profiles revealed by the essential oils. Furthermore, this irrigation regime, specifically between 50%-60% Field Capacity, is found in many articles concerning essential oil-bearing plants.

Concluding, it is a well-known fact that water is one of the important factors affecting plant growth and yield. On the other hand, water resources need to be used efficiently because of the increasing competition of the limited water resources between domestic, industrial and agricultural consumptions, and because of global warming (Ekren et al., 2012). Thus, there is no doubt that the application of drought stress frequently enhances the concentration of secondary plant products. However, it must be considered that drought stress also reduces the growth of most plants. Accordingly, very often it is stated that under drought stress – in principle – the same amounts of natural products are synthesized and accumulated by the plants as under well-watered conditions, but – due to reduction in biomass – their concentration simply is enhanced. In literature, the overall content of the natural products on a whole plant basis was not really in the center of the focus (Selmar et al., 2013) and it should be, to make comparisons. Reaching the right drought stress regime and precise time of application to have the quality outcome desired is important for all the reasons already mentioned.

## 1.7 Interaction of factors

### 1.7.1 Mycorrhizae and Hydric Stress

Mycorrhizae permit the plant to uptake and access water in a more efficient way (Giri et al., 2017). In that sense applying water stress with a bioinoculant might be interesting to minimize physiological damages of the hydric stress provoked in the plant. It has been reported that the highest water use efficiency was among AMF-inoculated plants under drought conditions compared to well-watered plants while at the same time decreasing the phosphorous requirement (Farahani et al., 2008). However, drought stress tolerance can differ with the arbuscular-mycorrhizal fungal isolate with which the plants are associated (Ruiz-Lozano et al., 1995a,1995b).

Still, while the role of hydric stress in the yield and quality of essential oils has a lot of research, and the role of AMF in alleviation of abiotic stresses and effect on essential oil quality is also well documented as seen above, the impact of AMF inoculation in conjunction with an abiotic stress on terpenoid fluxes has received relatively little attention (Lermen et al., 2015). There are few studies analyzing the interaction between mycorrhizae and hydric stress on essential oil yield and composition. A study conducted in 2015 with basil, applying hydric stress and mycorrhizae, demonstrated that the essential oil content is very influenced by the mycorrhization since it was noted a higher synthesis in both cases: stress and irrigation (Hazzoumi et al., 2017). In this scenario questions that arise are: Are certain species of *Glomus* more effective at improving plant–water relations? As already mentioned, there aren’t so many published studies of the intersection of an abiotic (water stress) and biotic (mycorrhiza) factor. A group of researchers performed a meta-analysis by selecting eligible published articles which were based on the criteria that the analysis had to be a manipulative study comparing water stressed mycorrhizal and non-mycorrhizal plants. Citing Jayne et al (2014): “After reading the abstracts of the 285 articles, 221 papers were rejected based on these criteria and the list was refined to 64 eligible studies. Still more were excluded because data were only reported graphically, and authors were unresponsive to requests for raw data, ultimately resulting in 54 eligible studies.” (Jayne et al., 2014).

Even though there are few studies the overall effects from the published ones is that under water deficit conditions, mycorrhizal plants outperform non-mycorrhizal plants in most measures of growth and yield. Whole plant measures usually show the most improvement from mycorrhiza. Both perennial and annual plants respond favorably to the symbiosis; however, perennial species show more growth overall (Jayne et al., 2014). This meta-analysis concluded that “the analysis made is the first to quantitatively affirm the view that the arbuscular mycorrhizal symbiosis benefits plants in terms of morphological growth when exposed to low-water conditions and reveal variations in those effects within differing contexts. AMF can provide a range of benefits to their hosts, and it is worth noting that other factors, such as improved P uptake, may have interacting effects on plant growth when less water is available.” The latter supports the assertion that mycorrhizal plants show better growth and reproductive response under water deficit than non-mycorrhizal plants do (Jayne et al., 2014).

Highlighting once again, AMF have a broad host range and are consequently the most credible group of soil microflora for enhancing the production of secondary metabolites (Kapoor et al., 2016). Since these symbiotic organisms in nature play their part in the presence of many other factors, abiotic factors might be chosen in order to increase the efficiency of AMF. To sum up, this symbiotic species affects the plasticity of the responses of plants to abiotic factors, and abiotic factors may be carefully selected to obtain the quality and quantity of the natural product desired, mostly in controlled environments, since the predictability of the outcome is higher and permits the removal of other factors that bluster the examinations.

## 1.8 Post-harvest Methods

### 1.8.1 Drying

The post-harvesting process of medicinal plants has great importance in the production chain because of its direct influence on the quality and quantity of the active ingredients in the product sold (Rocha et al., 2011). The first step in many postharvest operations is the removal of water that is, drying. Drying is basically defined as the decreasing of plant moisture content, aimed at preventing enzymatic and microbial activity, and consequently preserving the product for extended shelf life (Rocha et al., 2011). Drying may also contribute to a regular supply and facilitate the marketing of plants, because drying results in reduction of weight and volume of the plant with positive consequences for transport and storage (Calitxo, 2000).

During the process of drying and depending on the temperature, air velocity and other factors, many compounds which are dragged to the leaf surface by the evaporating water may be lost (Moyler, 1994). However, it is important to emphasize that losses of compounds, for example, are not in all cases continuously increasing with temperature. The reaction of the various components to drying air temperature is not univocal but as general tendency components with high retention time show an increasing share in the total essential oil at higher temperatures, whereas components with lower retention time show a decrease. That might not be a problem since for certain applications distinct compounds might be more important than the total essential oil content (Muller, 2007). After the drying process, the packing method is also an important factor in the quality conservation of the product during storage (Martinazzo et al., 2009).

The sensitivity of the substances with interest to maintain is the parameter that determines the temperature of drying process because the plant temperature is increased during the drying and high temperatures may promote loss by volatilization or degradation of the active principles (Venskutonis et al., 1996) as last-mentioned. Also, when choosing the drying method an important parameter to consider is in what part of the plant are the essential oil's secretory glands. For example, *Cymbopogon citratus* L. stores the essential oils in the vascular tissue whereas *Mentha* and *Ocimum* genus stores them on the surface of the leaves, in trichomes. Different methods related research has been published considering drying treatments where plants were dried in the sun, in the laboratory at room temperature conditions and plants dried in the oven at 40°C. Also, some articles studied different air velocities of ovens used for drying. The examination of literature concerning drying procedures reveals a great range and diversity of results affecting the quantity and quality of the essential oils. The values recommended in literature and those used in practice are often far apart, confirming that there is an urgent need for research on the topic.

Nevertheless, drying air temperatures between 50 and 60°C appear to be feasible for drying large number of medicinal plants (Rocha et al., 2011). However, due to the high heterogeneity among medicinal plant species, these global recommendations can only serve as a rough indication (Muller, 2007).

## 1.9 Chemical variation throughout the plant development

To achieve better oil yields and more profitable economics of cultivation under diverse agroclimatic, seasonal and soil conditions, research efforts have been focused, not only on development of better cultivars, chemotypes and ecotypes, but also on discerning physiological modulations of essential oil production. The synthesis of essential oils depends on the tissue differentiation (secretory cells and excretion cavities, as last-mentioned) which is related to the ontogenetic phase of the respective plant. The knowledge of the latter is necessary to harvest the correct plant parts at the right time. Plants from Lamiaceae family in resume have two different stages of development: vegetative and flowering stage. The flowering stage include: early, full and late bloom (Leather, 2010). The differential genetic expression that underlies these different stages has to do with the genetic makeup of the plant species and with photoperiod. The genetic sequences that have interest in this study are the ones encoding the protein domain belonging to the terpene synthase family; its role is to synthesize terpenes; the main compounds present in essential oils. Indeed, many terpenes are direct products of terpene synthases, but others are formed through transformation of the initial products by oxidation, dehydrogenation, acylation and other reaction types (Dudareva et al., 2004). According to some researchers posttranscriptional or posttranslational regulation of terpene synthases were obtained in studies of sesquiterpene volatile biosynthesis in flowers of *Arabidopsis* ecotypes (Tholl, 2006). In addition, analysis of the seasonal variation of isoprene formation in poplar leaves suggested posttranslational modification of isoprene synthase activity (Mayrhofer et al., 2005). This differential expression of genes encoding terpene synthases due to photoperiod/season related stimuli incites finding the optimal developmental stage of the plant material to harvest. Thus, plant material collected at different times of the year may contain different novel compounds with other bioactivities. The latter highlights the fact that the production of essential oils not only depends upon the metabolic state and preset developmental differentiation program of the synthesizing tissue, but also is highly integrated with the physiology of the whole plant (Sangwan et al., 2001).

Concluding, the evolution of terpene synthases in multi-gene families, their ability to form multiple products, and their differential expression that is mediated by developmental and stress-related programs, together drive the complexity and plasticity in terpene production (Fang et al., 1996; Tholl, 2006).

## 2. Research Goals

The aim of the thesis is to understand if different factors applied to plant species (*Ocimum basilicum* L. cv. Genovese Gigante and *Mentha* sp.), can increase the yield of essential oils and obtain different, and interesting commercially, chemical profiles.

The central research question is if the application of two isolated factors (mycorrhizae and hydric stress), the interaction of both and post-harvest techniques like drying, affect relevantly or not the essential oil content from these both species. Specifically, it was asked in the present experiment whether:

(i) AMF affects the yield and composition of essential oils,

(ii) Hydric stress affects the yield and composition of essential oils,

(iii) In drought stress conditions the AMF permits the maintenance of biomass,

(iv) Dried plants essential oils don't differ from the ones from fresh material.

# 3. Methodology

## 3.1 Plant material (germination and culture)

Plant Material: Commercial seeds of *Ocimum basilicum* L. cv. Genovese Gigante and *Mentha* sp. were used for the development of this research. It is only possible to mention the genus of the mint used in this experiment since the identification of the species was not achievable. The seed lot of mint seeds was incorrectly identified and in this experiment the mint plants didn't reach the reproductive stage which difficulted the identification. However, the chemical profile allowed to assert that the species used is not *Mentha* x *piperita* L. (which was the species identified in the seed lot).

The experiment was organized according to the Randomized Complete Block Design (RCBD) (Table 2) which is a standard design for agricultural experiments. For basil it was applied 6 replicas of each treatment, and for mint 4 replicas. The treatments were: the use of a mycorrhizae fungus (M), drought stress (HS), mycorrhizae fungus plus drought stress (HS+M) and control (without mycorrhizae and without drought). This assay was performed in an experimental area of Centro Hispano-Luso de Investigaciones Agrárias (CIALE) in the province of Salamanca, Spain (Fig.6).

Table 2: *Mentha* sp. and *Ocimum basilicum* L. cv. Genovese Gigante experimental design. The two harvest dates were in winter and late spring respectively.

| *Mentha* | 1st Harvest | 2nd Harvest |
|---|---|---|
| Control (C) | 4 plants | 4 plants |
| Hydric Stress (HS) | 4 plants | 4 plants |
| Mycorrhizae (M) | 4 plants | 4 plants |
| Hydric Stress + Mycorrhizae (HS+M) | 4 plants | 4 plants |

| *Ocimum* | 1st Harvest | 2nd Harvest |
|---|---|---|
| Control (C) | 6 plants | 6 plants |
| Hydric Stress (HS) | 6 plants | 6 plants |
| Mycorrhizae (M) | 6 plants | 6 plants |
| Hydric Stress + Mycorrhizae (HS+M) | 6 plants | 6 plants |

Fig.6: Glasshouse from CIALE, Salamanca, Spain; Pictures of *Ocimum basilicum* L. cv. Genovese Gigante.

Before germination, the seeds were prepared in a sterile laminar flow chamber. A hypochlorite solution (10%) was added to the seeds and left acting for 5 minutes. After the 5 minutes the solution was removed, and distilled water was added. Distilled water was removed and re-added several times until there was no smell from the solution. Posteriorly, all the water was removed, and seeds were put on agar-agar petri dishes. Then, each petri dish was pulverized 2 to 3 times with CAPTAN fungicide at a concentration of 0.3% (Bacchetta et al., 2008).

In addition, besides sterilizing seeds, it was also applied a treatment without the sterilization with hypochlorite solution to analyze if it affected the germination rate. The seeds were placed in the fridge at 25ºC in petri dishes with 16-h light/8-h dark period. In total there was 10 seeds per petri dish and 10 petri dishes which amounts in total to 100 seeds per species and per treatment (sterilized and not sterilized). Daily it was noted how many seeds had germinated. After 10 days the seeds were taken to analyze the seedlings and transplant the number needed for the experiment. Posteriorly, seeds were sown in commercial nursery trays at 2 of November 2017 under controlled conditions (25-30ºC) in the laboratory.

The percentage of germination was calculated for each replica and by the given formula bellow:

$$\frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds - empty\ seeds}\ x\ 100$$

To clarify, empty seeds are seeds that may have no embryo at all or seeds that do not have functional complete embryos; these seeds didn't germinate. The final percentage of the test is calculated by making the average between all the replicas subjected to the same conditions of germination (Bacchetta et al., 2008). In parallel, the mean germination time (MGT) measured in days was also calculated which allows to know the average time of germination of the seeds analyzed (Tompsett et al., 1998; Bacchetta et al., 2008). This value is calculated by determining the number of seeds germinated each day, considering the total of germinated seeds:

$$MGT = \frac{n_i d_i}{N}$$

where: $\boldsymbol{n_i}$: number of seeds germinated on day d; $\boldsymbol{d_i}$: number of days since the beginning of the germination test; **N**: total number of seeds germinated at the end of the test.

After 10 days in commercial nursery trays, the plantlets were transferred to the glasshouse and onto pots (3.5L) filled with peat moss which contained macro and micronutrients (Attachment I). There was a total of 80 pots, 48 pots with basil and 32 with mint as mentioned before. Furthermore, the pots position on the greenhouse bench was shifted weekly to control for any differences in light conditions.

The plants were grown in a greenhouse with controlled temperature (26ºC) and measurements of radiation per minute through all the experience were taken using a sensor connected to 'ECHO2 utility' software (Attachment II).

When the plants were transferred to the 3.5L pots, the roots of basil and mint plants were put in contact with 1g of material consisting of hyphae and spores of *Glomus intraradices* (100 000 spores per gram) and soil. The latter fungus inoculum was obtained and multiplied by pot culture with maize. This mixture of hyphae and spores was added in the planting hole of basil plants when these plants were placed on the pots. However, with mint plants it was after 27 days of the transplanting moment since the plants hadn't fully developed yet and parasitic mycorrhizal associations may occur at certain stages of development. This was in accordance with the information that the formation of arbuscular mycorrhizas can depress seedling growth in the first few weeks following germination (Johnson et al., 1997). The plants were irrigated with water in each plot without creating any water stress until the plants adapted to the soil conditions and reached an average of 28-30cm (Ekren et al., 2012). After 90 days (16/01/2018) and 97 days (22/01/2018) of transplanting to the bigger pots, for basil and mint respectively, the plants reached an average of 28-30cm of height and it was applied the different water regimes, control and mild water deficit.

## 3.2 Applying hydric stress

In the Instituto de recursos naturales y agrobiología of Salamanca (IRNASA) the method suggested to use in order to apply hydric stress was the gravimetric weight method. Furthermore, the irrigation levels chosen were: a level at 80% Field Capacity as control (since plants in the seasons studied don't lose great quantities of water and 100% Field Capacity would not be adequate) and a level at 60% Field Capacity as treatment, respectively.

First, to reach the values needed, it was determined the soil moisture at 100% field capacity (Fig. 7a). For that purpose, 10 pots (equal to the ones used in the experience) with soil (taken out from the soil bag) were used. These pots were subjected to several irrigations till water started draining out of the pot. After 24h, 10 soil samples from the pots were selected. Then, these samples were weighed and placed under 120ºC in a oven for 24h. Then the weight was measured once again. Then, soil moisture (volume of water in a given volume of soil) of 100% field capacity was determined by the following formula:

$$\Theta = \frac{Moist\ soil\ weight - Dry\ soil\ weight}{Dry\ soil\ weight}$$

Posteriorly, it was selected soil samples from another 10 pots, without irrigation till draining. These were used for the determination of the soil moisture that the soil already presents without irrigation. All the samples were weighted. Furthermore, one of the pots besides the weighting was placed under 120ºC in an oven for 24h. With the previous and latter data, it was possible to determine the 100% Field Capacity, 80% Field Capacity and 60% Field Capacity. To control the irrigation levels, and according to the method selected, an electrical balance was used (Fig. 7b), from the beginning of the application of the hydric stress till the end of the experiment.

In short, the gravimetric method involves weighting soil samples, drying them in an oven, weighing them again, and using the difference in weight to calculate the amount of water in the soil. While too time consuming to be used for day-to-day management decisions, this highly accurate and low-cost method is often used (Morris, 2006). This method allowed to maintain the control condition and the mild stress condition through the weighting of the pots daily.

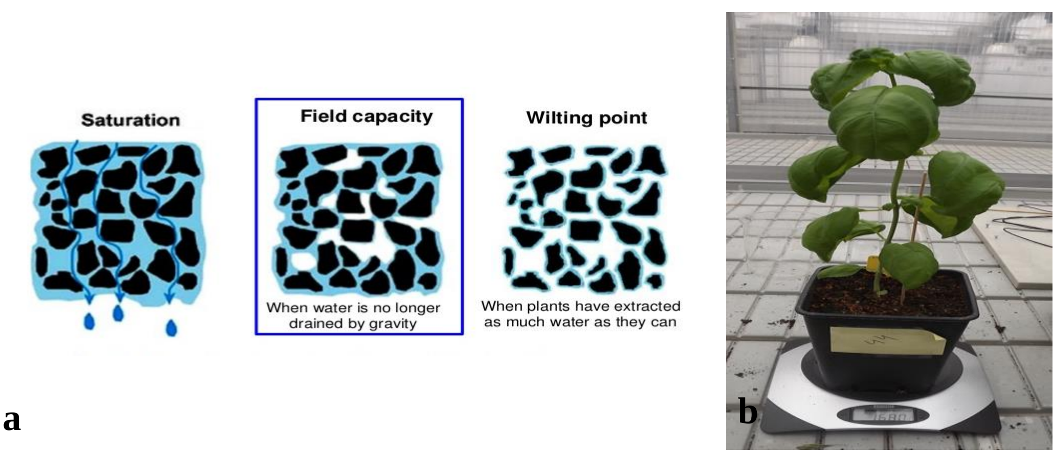



Fig.7: (a) Field capacity resumed explanation scheme (Source: slideshare.net "Understanding Soil Water"). (b) Gravimetric weight method.

## 3.3 Mycorrhizal root infection and root systems

The identification of mycorrhizal root colonization was with the aid of optical microscopy and a technique of staining with trypan blue (TB) described by Brundrett et al (1996), revealing the set of fungal biomasses. Besides that, descriptive tables were used for the determination of precise timings for analyzing the roots.

After one month of the plants being in contact with the mycorrhizae (on the day 2/12/17 and 28/12/17 for basil and mint respectively) the root samples, less than 1g, taken at random from each treatment and control, were removed from the plants. Posteriorly, these roots were thoroughly rinsed with distillated water to remove adhering substrate. Then, the root fragments were digested in a solution of potassium hydroxide (KOH) at 10% for 1 hour at 60ºC in a water bath (Precisbat, Grupo Selecta) to empty the cells of their cytoplasmic contents which facilitates their coloring. After the water bath the roots were thoroughly rinsed with distilled water and placed in a solution of lactoglycerol (20mL acid lactic, 40mL glycerol and 40mL distilled water) with trypan blue (0.05%). To accelerate the process, the sample can be placed again in the water bath, for 30 mins (this time depends on the pigmentation of the roots). Posteriorly, the covers with the tainted root fragments were observed at the microscope.

For the analysis of the root systems and following Bohm (1979) the method selected for the determination of root biomass was washing roots by hand. This is the most economic technique of separating roots from soil, which is performed with a jet or spray of water aided by hand manipulation. The soil-root sample is suspended in water and poured overfine-mesh sieves where the roots are retained and collected for further cleaning. The mesh size used was $0.5mm^2$ (Bohm,1979). Each pot was irrigated several times and after transferred to a pail. The pail is then placed on the washing table and the soil-root-water mixture is stirred by hand until it is a homogeneous suspension. When the soil-root-water mixture is fully dispersed, the stirring is interrupted for a few seconds to allow the heavy soil particles to settle down. The roots tend to float in this suspension.

Then the suspension, without the settled soil particles, is poured on to the sieve. The roots remain on the sieve and the fine suspended soil particles pass through. The operation is aided by a hand sprinkler so the roots on the sieve are immediately freed from adhering soil suspension. Then the pail with the remaining soil is half filled again with water by means of the hand sprinkler and the process of suspension and decantation is repeated. This is done until all roots are transferred to the sieve by the decantation process. Depending on the amount of roots and soil in the sample the procedure must be repeated three to eight times. During the manual technique of stirring and suspending, some roots are broken, but this does not influence the results if root weight or root length are to be determined (Bohm, 1979).

## 3.4 Processing of harvested material, extraction and analysis of essential oils

After one month of drought treatment (in the days 16/2/18 and 29/2/18) it was executed the first harvest of 24 plants and 16 plants of basil and mint respectively, corresponding to the three treatments and control. Each plant is divided in two, vertically and uniformly, for fresh and dry material. The latter process guaranteed that each part of the material (for fresh distillation and for drying) had the same amount of old and young leaves. The plants that would be distillated in fresh were freeze-dried at -18ºC until the plant material was distilled in Instituto Politécnico de Bragança. The first harvest was for the analysis of winter season chemical profiles. The second harvest, of 24 plants and 16 plants of basil and mint, was for the analysis of late spring season, which was after 2 months of hydric stress, specifically in the dates 14/4/18 and 29/4/18, for basil and mint respectively.

The necessary quantities for the extraction were always collected from the aerial part of the plants. Also, in the reproductive phase, which basil reached, the flowering tops were not distilled separately. Both species were harvested at 8h a.m for fresh material and for dried material (45ºC for basil and 30ºC for mint in an memmert® oven air drier for 24h) (Muller, 2007) distillations. The dried material was then transferred to a dark chamber with temperatures between 4ºC and 7ºC. The plants were stored 1 month (Baritaux et al., 1992) in brown paper bags in this chamber until the time of distillation.

In the hydro-distillation process, the plant material is completely immersed in boiling water. The characteristic feature of this process is that there is direct contact between boiling water and the raw material. Following the parameters of European Pharmacopeia, each distillation took 3 hours with a Clevenger type apparatus (Fig.8). Furthermore, the essential oil was collected with the aid of solvent pentane. Posteriorly, the essential oil samples were stored at 4ºC until analysis. The yield was considered using the following formula:

$$YEO\ (mL/100g\ Fm) = (V/Fm \times 100)$$

Where YEO represents the essential oil yield of fresh/dry matter, V the volume of essential oils collected (mL) and Fm represent the fresh/dry plant mass (g).

The quantity of fresh weight distilled in the 1st harvest of basil and mint was in average 122g and 63g, respectively. In the 2nd harvest, the quantity distilled for basil and mint was 271.5g and 91.7g respectively. As mentioned before, each plant was vertically divided in each harvest and one of the parts were subjected to drying. The quantity of dry weight distilled in the 1st harvest was for basil and mint in average 13g and 21.3g. Relatively to the 2nd harvest the dry weight was for basil and mint in average 62g and 46.5g.

Fig.8: One of the Clevenger type apparatus used in IPB and essential oil of one of the samples.

After extraction, all the essential oil samples were analyzed by Gas-Liquid Chromatography (GC) on a Perkin Elmer 8700 gas chromatograph, equipped with two flame ionization detectors (DIC), a data processing (Perkin Elmer TurboChromTM Workstation software) and two columns of fused silica of different polarity with high resolution. The Gas-Liquid Chromatography performed has the following characteristics:

-DB-1, methylsilicone immobilized phase (30m x 0.25mm d, 0.25µm film thickness, J & W Scientific Inc.),

-DB-17HT, phenylmethylsilicone immobilized phase (30m x 0.25mm d, 0.15µm film thickness, J & W Scientific Inc.).

The analyzes were performed under the following conditions:

- oven temperature programmed at 45ºC with increments of 3ºC.min$^{-1}$ until reaching 175ºC, and later with increments of 15ºC.min$^{-1}$ up to 300ºC,
- injector temperature programmed at 280 ° C and the two detectors at 290 ° C,
- carrier gas, hydrogen, adjusted to a linear velocity of 30cm.s$^{-1}$,
- sample injection volumes on the order of 0.2 µl, with a flow distribution of 1:50.

The percentage composition of each of the essential oil constituent compounds was determined by the normalization method, integrating the areas of the peaks without the use of correction factors. The values given are the average value of two injections per sample.

Subsequently, analysis was carried out through Gas-Liquid Chromatography / Mass Spectrometry (GC-MS).

The latter was performed on a Perkin Elmer Clarus 600 chromatograph equipped with a DB-1 fused silica column (30m x 0.25mm dm, 0.25µm film thickness, J & W Scientific Inc.) attached to a Perkin Elmer Turbomass Clarus 600T mass spectrometer. The oven temperature was programmed as previously described. The injector was programmed to a temperature of 300ºC, the transfer line to 280ºC and the ionization chamber to 220ºC. Helium was used as the carrier gas, adjusted for a linear velocity of 30cm.s$^{-1}$. Samples were injected with a 1:40 flow distribution using an ionization energy of 70eV, an ionization current of 60µA and a range of masses, 40-300u. The scanning time was 1s, and volumes on the order of 0.1µl were injected. The identification of the compounds was determined by comparing their retention indices and mass spectra with reference compounds present in the laboratory and by comparison with a library of mass spectra developed in the laboratory respectively. The mass spectra obtained were then used to identify the chromatograms and the latter were employed as references to identify the compounds of the chromatograms from the other samples.

## 3.5 Plant Functional Traits

Regarding plant functional traits, the following parameters were determined: plant height (cm) (the plants were measured from the soil surface till the top during the experiment and before each harvest, using a ruler) (Cornelissen et al., 2003), root fresh weight (g), above-ground biomass (g) (fresh weight), seed germination rate and mean germination time.

Besides the latter, it was determined the essential oil yield (mL/g) and essential oil relative percentage (%).

## 3.6 Descriptive statistics

In this study the dimension of the samples didn't permit multivariate analysis, as such, it was used mainly box-and-whisker plots (univariate analysis). Box-and-whisker plots which, as an abbreviation, are called boxplots, are powerful graphical representations that give an overview and a numerical summary of a data set (Ferreira et al., 2016). In these graphs it is possible to examine horizontal lines of the boxes which show the 25, 50 and 75th percentile, while the outer limits of the whiskers show the min. and max. values; solid circles indicate the average value. Furthermore, the outliers permit to examine potential uncommon observations.

Basically, boxplot graphs permit an initial exploratory analysis of profiles (Parks et al., 2014), in this case, essential oils chemical profiles. These graphs have been widely used in food chemistry for example to identify the foods that stand out as being a rich source of potassium and how the contents of amino acids are distributed in egg white and yolk. Other examples of possibilities for the use of boxplots include many comparisons: fatty acid composition of different plant oils (sunflower, palm, olive, and others); vitamin content of fruits and vegetables; caloric value of some food products; phenols in white and red wine (Ferreira et al., 2016) and essential oils (Geleta et al., 2011; Kittler et al., 2017). If the dimension of the sample of this study was bigger PCA (Principal Components Analysis) would be the method used.

Descriptive statistics was carried out with program R 5.3.1.

# 4. Results and Discussion

## 4.1 Seed germination, Mycorrhization and Functional Traits

In terms of the germination rate (%G) and the mean germination time (MGT), the results obtained were in accordance with the literature. It was compared the latter two parameters of seeds sterilized (E) and non-sterilized (NE) (Table 3). Furthermore, the examination of these parameters was executed with the aid of a Leica magnifier (Leica M205 FA) (Fig.9). The genus *Ocimum* germination rate and the mean germination time are corroborated by Zhou (2012) which reported that for cultivars like the 'Genovese' the MGT was within 2 days, in temperatures superior to 27ºC and that the final maximum percentages of germination were greater than 90% (Zhou, 2012; Mijani et al., 2013). For basil it is a good result since for this species the germination rate of seeds should be between 80-95%, and seeds should not be planted if the germination percentage is less than 70% (Simon et al., 1999). The treatments didn't affect in a relevant manner the rate of germination and mean germination time.

Table 3: Germination rate (% G) and mean germination time (MGT) of seeds sterilized (E) and not sterilized (NE) of basil and mint, respectively.

| | Basil | | Mint | |
|---|---|---|---|---|
| Treatment | % G | MGT | % G | MGT |
| E | 94 | 4.4 | 51 | 8.8 |
| NE | 93 | 5.3 | 57 | 9.8 |

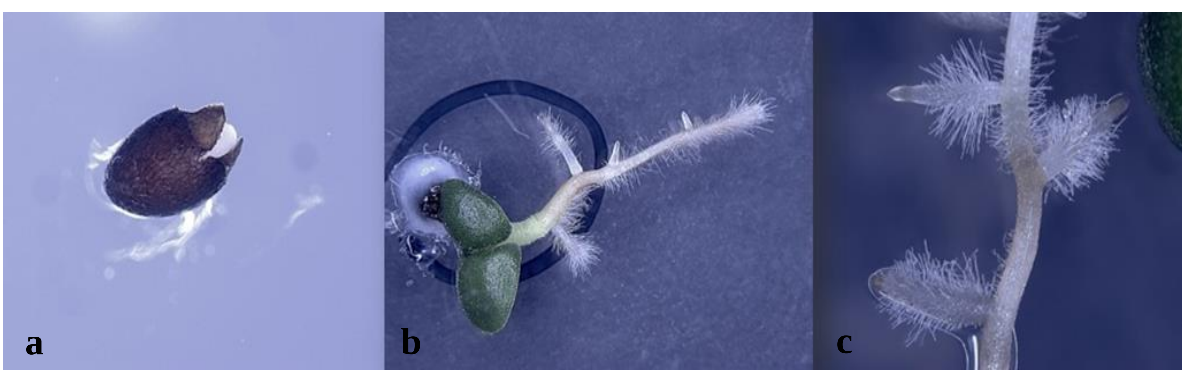



Fig. 9: (a) Seed of *Mentha* sp. (20x) (b) seedling of *Ocimum basilicum* L. cv. Genovese Gigante (10x) (c) *Ocimum basilicum* L. cv. Genovese Gigante roots (15x).

In the case of *Mentha* sp. the results were not so favorable which is in accordance with the literature. It is well known that this genus is better to be propagated vegetatively since seed production and germination are often poor, the seeds are very small and difficult to handle, and development is slow leading to delay in yield production (El-Keltawi et al., 1986). These seeds took an average of 9 days to germinate and the germination percentage was between 50-60% which isn't a good seed trait and indeed vegetative propagation might be more successful. In addition, and like *Ocimum basilicum* L. cv. Genovese Gigante, the sterilization and without sterilization treatments didn't affect relevantly the germination rate and the mean germination time of mint.

These tests were done since successful seedling establishment is the first critical step for crop production and determines the success or failure of the future harvest. Furthermore, high seed quality is essential for crop production to be both sustainable and profitable and is therefore widely accepted as a critically important agronomic trait (Finch-Savage et al., 2015).

Regarding the mycorrhizal colonization, one species of AMF was selected to be inoculated with the plants from this experiment. The species, *Glomus intraradices*, was obtained from the department of Plant Physiology of the Faculty of Sciences of the University of Lisbon and inoculated into basil and mint roots to examine their effect on plant growth and essential oil quality. Following the suggestion of researchers from this faculty the colonization percentage was not needed and microscopical photographs would be enough to discriminate the control from the treatment.

It is possible to infer by the Fig. 10 that no AMF colonization was detected in control roots. The roots were analyzed after 1 month in contact with the fungus since it is expected that the fungus normally grows to the central wall in the first 4 weeks and crosses the wall with a 1 or 2-week delay. When at least one hypha crosses into the rootless side, the mycelium starts to grow very vigorously, forming a conspicuous hyphal front and colonizing the entire compartment within 2 weeks. The colonization continues to increase for 3-4 months (Arnaud et al., 1996). Examining the Fig.10 bellow it is possible to postulate that the hyphae crossed the rootless side and started forming vesicles (the round dark blue circles) which are lipid-rich propagules.

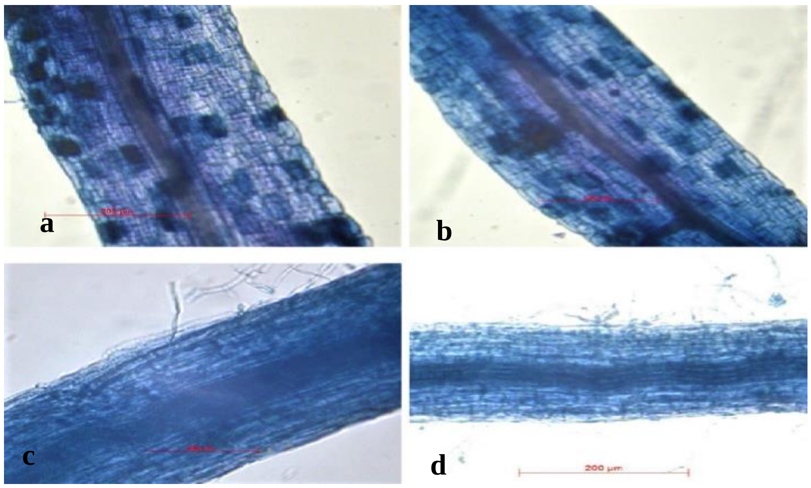
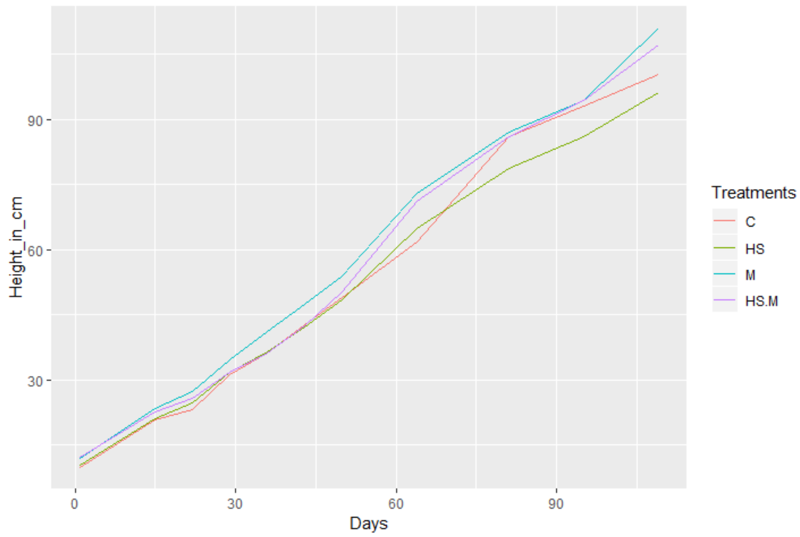



Fig.10: Roots of *Ocimum basilicum* L. cv. Genovese Gigante: **a**: *Glomus intraradices* treatment, with vesicles (40x). **c**: Control (40x). Roots of *Mentha* sp.: **b**: *Glomus intraradices* treatment, with vesicles (40x). **d**: Control (40x).

In terms of the functional traits, as mentioned in the methodology, one of the parameters measured was the plants height (cm). The height was measured weekly till the end of the experiment as mentioned in the methodology.

In a period of 5 months after inoculation the basil plants were measured. The latter allowed to verify an increase in plant growth in all the mycorrhizal plants (M and HS+M) compared with nonmycorrhizal plants (control and HS). These differences were maintained until the end of the experiment (Graph 1) (Fig.11). Analyzing the data collected is possible to suggest that *O. basilicum* L. cv. Genovese Gigante plants responded positively to the inoculation of AMF. Observing the graph 1 we can infer that by decreasing order of height it was M treatment > HS+M treatment > C > HS treatment. The highest height, obtained with the M treatment, is in accordance with the general assumption by several articles of the performance of mycorrhizae as a biofertilizer and bioprotectant (Dwivedi et al., 2015; Berruti et al., 2016). Indeed, mycorrhizae (depending on the host plant species, the soil nutrients, mycorrhizal rate of colonization and other factors) cause root and shoot biomass increase, yield and plant nutrition improvement, and plant resistance to a given pathogen (Berruti et al., 2016).




Graph 1: Plant growth patterns of *Ocimum basilicum* L. cv. Genovese Gigante plants measured as shoot length (cm) during 5 months under greenhouse conditions till the last harvest. Graph data is the mean of 48 replicates. C: Control; HS: Hydric Stress; M: Mycorrhizae; HS+M: Hydric Stress + Mycorrhizae.


Fig.11: Height difference of *Ocimum basilicum* L. cv. Genovese Gigante without mycorrhizae and with mycorrhizae, respectively. (a) Vegetative stage. (b) Flowering stage.

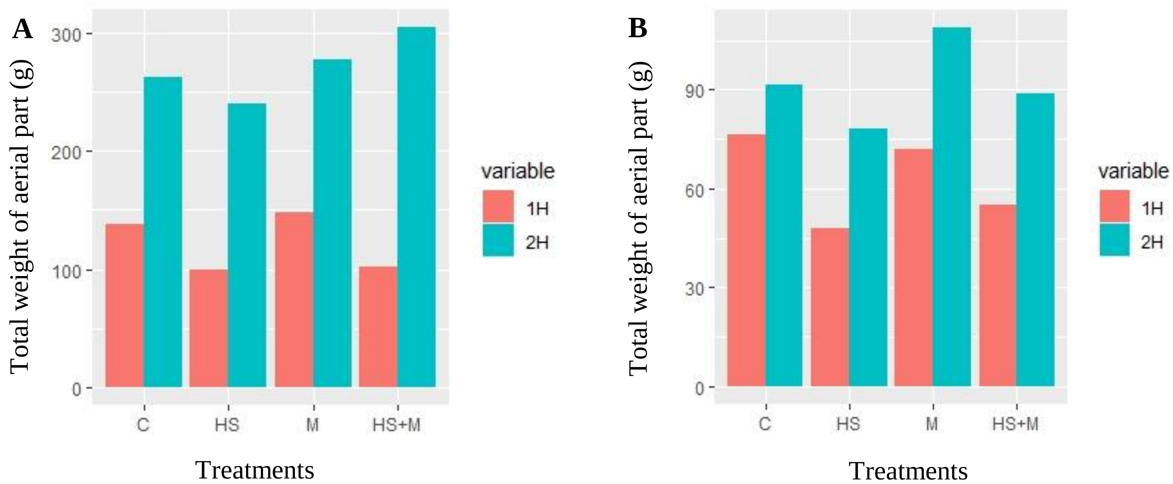
Increased growth and development in AMF plants, compared to nonmycorrhizal ones, was reported for many different species (reviewed in Smith et al., 1997). The results of the present work concerning *O. basilicum* L. cv. Genovese Gigante agree with such reports. Furthermore, all mycorrhizal plants (without drought stress and with drought stress) showed a higher degree of root branching in comparison with the control, which is consistent with previous literature (Smith et al., 1997; Copetta et al., 2007) (Fig.12).


Fig.12: Root branching of basil with mycorrhizae treatment (a) and control (b) after 193 days of the inoculation with the symbiotic fungi.

Concerning the second treatment with highest height, HS+M, it is possible to infer that mycorrhizae helped the plants to cope or ameliorate the effects of the adverse condition of mild hydric stress. AMF stimulates growth and drives the water status in plants to an optimal level, thus confirming the role of mycorrhizal symbiosis in plant defense against abiotic stresses. In short, the water supply deficit was offset in stressed mycorrhizal plants generating growth at the root and aerial part (Hazzoumi et al., 2017). The two treatments examined above are in accordance with the statement that elongation of basil plants is largely driven by mycorrhiza with the maximum growth recorded in mycorrhizae non-stressed plants (Hazzoumi et al., 2017). Regarding the HS treatment, it is possible to infer that the hydric stress inhibited the growth of the aerial part mainly in non-mycorrhizal plants (HS treatment). This has been confirmed by some researchers that water stress leads to growth reduction, which is reflected in plant height, leaf area, dry weight, and other growth functions (Boutraa et al., 2010). Concerning the control plants, not stressed and without mycorrhizae, it is possible to assert that it presented a higher height in comparison with HS but still had a smaller height compared with the plants non-stressed with mycorrhizae and stressed with mycorrhizae.  In accordance to these results is a study with *Ocimum gratissimum* L. (Hazzoumi et al., 2017) that reported that the water content and growth was in the decreasing order as the values obtained in the latter graph.

The height of the *Mentha* sp. was not measured since it is a plant with multiple stems so the method for measuring the height was difficult to apply and thus plant height of mint has been suggested to be negatively correlated with oil content (Sharma et al., 1992), so this parameter was not considered for mint. However, for the both species, other functional traits were registered, notably the total weight of aerial part (Graph 2) and the fresh root weight (Graph 3) in the 1$^{st}$ and 2$^{nd}$ harvest.

Examining the Graph 2 we can see that for basil in the first harvest in decreasing order of the total weight of aerial part we had M>C>HS+M>HS whereas in the second harvest it was HS+M>M>C>HS. Regarding the effects of the three treatments applied to *Ocimum basilicum* L. cv. Genovese Gigante it is suggested that mycorrhizae treatment raises the total weight of aerial part in this species. A research which examined the impact of three AMF species on "Genovese" basil growth, distribution of glandular hairs, and essential oil production concluded that different AMF species have different effects on several parameters but in general mycorrhizae significantly increases biomass, root branching and length (Copetta et al, 2006). Analyzing the first harvest, the total weight of aerial part between M treatment and Control were not relevantly different. The latter might have to do with the transient defense response induced by mycorrhizae at an early stage which may stunt growth in favor of defense mechanisms. Indeed, defense activation generally comes at the expense of plant growth (Huot et al., 2014). According to Bompadre et al (2014) olive plants inoculated with two strains of mycorrhizae had an additional energetic expense in growth due to an adaptative response to the mycorrhizal colonization at the first transplant. However, at the second transplant the inoculation improved the olive plants growth and protected the plant against oxidative stress (Bompadre et al., 2014). The last-mentioned may explain the similarity of control and mycorrhizae in the 1$^{st}$ harvest. Furthermore, the treatments HS+M and HS revealed the lowest values. Since this is the first harvest the adjustment of the plant to the abiotic stress might explain these values. Dave et al (2012) harvested water-stressed mycorrhizal plants at 45, 90, 180 and 270 days of growth stages. They reported that mycorrhizal plant materials show higher drought tolerance by enhancing antioxidant enzyme activities and that these activities were enhanced at 180 days of crop harvest (Dave et al., 2012).



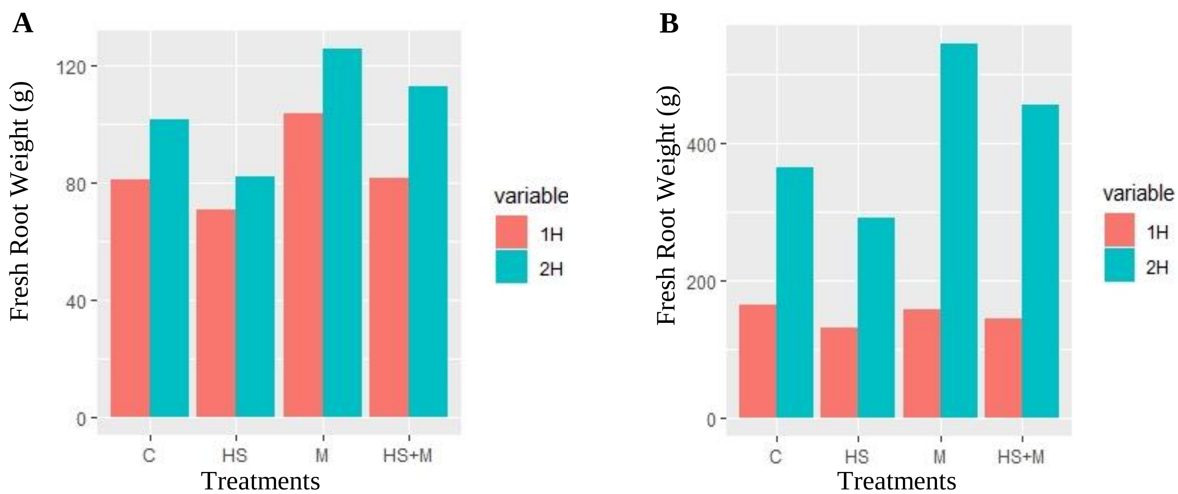

Graph 2: Average of total weight of aerial part (g) in the 1st harvest (1H) and 2nd harvest (2H) of basil (A) and mint (B). **SD**: σ (A): 1H: 21.3; 2H: 23.6. σ (B): 1H: 11.7; 2H: 11.

It seems that there is a temporal-related influence on the response of the species to the inoculation of mycorrhizae and acclimatization to drought stress. This highlights the importance of including multiple harvests to assess responses that have the capacity to change over time, especially plant and fungal growth (Augé, 2000). In the 2nd harvest the highest total weight of aerial part was with the HS+M treatment followed by M treatment, control and HS treatment. This is accordance with a study with basil inoculated with the fungus used in this study which showed better growth than non-inoculated plants, both in terms of well-watered conditions and in conditions of water stress (Hazzoumi et al., 2017). Still, in this study latter mentioned, the shoot weight was higher in mycorrhizal water stressed plants than in control. However, the results from this study don't mention how many days of culture the plants had when they were harvested. Furthermore, the results of the 2nd harvest which were HS+M with the highest total weight of aerial part, might be explained by the fact that by this stage of harvesting the days are longer, and it has been reported that drought accelerates flowering under long days but delays flowering under short days (Kazan et al., 2016). The last-mentioned facts plus mycorrhizae acting as a biofertilizer may explain the highest total weight of aerial part of basil subjected to HS+M.

In regard to mint, in the first harvest the highest total weight of aerial part was in decreasing order: C>M>HS+M>HS, while in the second harvest the M treatment exceeded the control in this parameter since the values were M>C>HS+M>HS. An important fact about mint that possibly is important to note is that this is a plant that has smaller leaves compared with basil, so the photosynthetic capacity and carbon uptake might be lesser than in basil. Following the latter assumption, it is probable that these results might be related to developmental stage of the plant and the physiology of mint species. The last-mentioned characteristics might influence the type of interaction the fungus has with the plant. Indeed, plant-mycorrhizal interaction may shift between mutualistic and parasitic depending on the life stage of the plant (Bethlenfalvay et al., 1982; Koide, 1985). Also, the relative cost-benefit of the association may also shift seasonally (Lapointe et al., 1997). It might be that the carbon uptake by mint was still not aligned with the demands of the fungi which produced a decrease of biomass (in the M treatment) in comparison with the control. However, after a longer term ($2^{nd}$ harvest), mint species might have reached an advantageous stage of cost-benefit relationship (possibly due to development and leaf expansion) with the symbiotic fungus and the results in terms of total weight of aerial part changed, in the second harvest, to the treatment of M as the more relevant in this parameter. This is in accordance with Cabello et al (2005) which concluded that the maximum growth of *Mentha piperita* L. inoculated and non-inoculated with *G. mosseae* and with RP (vermiculite-perlite substrate with rock phosphate) was observed after the first harvest and that mycorrhizal plants had the highest biomass production after their second harvest. They suggest this is relevant because the mint is a perennial plant and it may be harvested four times during its life cycle (Cabello et al., 2005). Moreover, these interaction-related shifts of AMF with the plants emphasize the importance of longer-term experiments that incorporate all stages of the plant life cycle.

In regard to hydric stress treatment (HS) which presented the lowest total weight of aerial part in both harvests this is in accordance with general assumption that water stress reduces plant growth through inhibition of various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, nutrient metabolism and hormones (Bahreininejad et al., 2013). Regarding HS+M treatment (which presented in both harvests a lower total weight of aerial part than control) it is important to consider that depending on the plant species, mycorrhizae strain, level of water stress applied and how much time the latter is applied, different responses might take shape. Some studies indicate that for example moderate water deficit (50% FC) appears to be the best condition for the AMF inoculum to promote nutrient (particularly P) uptake in the host plants (Bakr et al., 2017). Same results were obtained with berseem clover which in contrast to the well-watered conditions, under water stress conditions, the AM symbiosis enhanced aboveground biomass production (Saia et al., 2014). The results obtained in this experiment are consistent with the findings of other authors who found that in pot-based studies there is a beneficial effect of AMF symbiosis under water restriction for non-legume plants (Saia et al., 2014; Ruiz-Lozano et al., 1995a, 1995b). Nevertheless, even though mycorrhizae fungi contribution to plant drought tolerance is well documented the underlying mechanisms are still unclear (Saia et al., 2014). Even though in mint the treatment HS+M didn't surpass the control regarding the parameter discussed, for basil it was observed that in the last harvest the treatments with mycorrhizae (with drought or without drought) presented the highest weights.

Now concerning the Graph 3 it is possible to examine that in the first harvest of basil in decreasing order of fresh root weight the results were M>C ≈ HS+M>HS whereas in the second harvest it was M>HS+M>C>HS. The similarity between control and HS+M in the 1$^{st}$ harvest possibly can be explained by the fact that mycorrhizal plants constrained to the same soil volumes as non-mycorrhizal plants can thus be expected to deplete the available soil water more quickly than non-mycorrhizal plants (Augé, 2000). In this pot experiment, probably the root weight was not so extensive in HS+M treatment and had a similar weight to control since it may have depleted available water faster than in control plants, plus the fact that less water was available (drought stress). Still, M treatment had the highest fresh root weight in both harvests, which is in accordance with other studies who tested mycorrhizae without other abiotic factors and concluded that for basil moreover, all mycorrhizal plants showed a higher degree of root branching whereas root and shoot weight were lower in control plants (Rasouli-Sadaghiani et al., 2010). Nevertheless, in the second harvest, HS+M exceeded the Control. This might have to do with fluctuations related to the adjustment of the root system to the confined pot.




Graph 3: Average of fresh root weight (g) in the 1st harvest (1H) and 2nd harvest (2H) of basil (A) and mint (B). **SD**: σ (A): 1H: 12.1; 2H: 16. σ (B): 1H: 13.2; 2H: 95.6.

Regarding mint fresh root weight, it is assumed that in the 1st harvest the highest fresh root weight was in decreasing order C≈M>HS+M>HS. In the 2nd harvest M and HS+M treatment exceed in fresh root weight the control condition. Still, in the last-mentioned stage of harvest, HS presented the lowest weight. Based on these results it is possible to infer that mycorrhizae colonization can change specific root length, root architecture and root/shoot ratio (Fig.13) (Berta et al., 1993). In most conditions, root weight tends to increase in mycorrhizal plants. This greater root development might account for the higher P accumulation observed in mycorrhizal than non-mycorrhizal plants grown under stress conditions as seen in other studies (Morte et al., 2000). A study with *Mentha piperita* L. noted that 37 days after inoculation with arbuscular mycorrhizal fungi (AMF) the plantlets showed a tendency to have a larger number of leaves per plant and a larger root system than uninoculated control plantlets. However, in relation to root dry matter there was no significant difference between inoculated and uninoculated plants (Silveira et al., 2006) which is in accordance with the results obtained for the root weight from the first harvest of mint, i.e., the similarity between control and mycorrhizae weight.  Zou et al (2013) with *Poncirus trifoliata* inferred that the AMF treatment significantly increased root volume, irrespective of water status (Zou et al., 2013), which corroborates the results with M treatment for mint and basil where the root weight of basil and mint in the 2nd harvest were significantly higher than the other three treatments by the end of the experiment. As mentioned before longer-term experiments would permit to investigate better the shifts AMF has when in contact with different species of plants and the effect of the prolonged water stress.

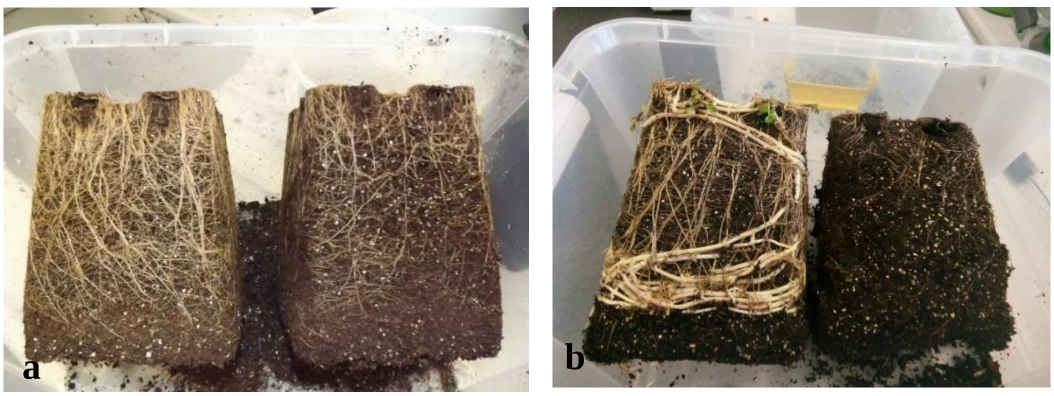



Fig.13: Roots of basil and mint from the 2nd harvest respectively. a: Treatment of *Ocimum basilicum* L. cv. Genovese Gigante with mycorrhizae and without mycorrhizae, respectively. After 193 days of the inoculation with the fungus b: Treatment of *Mentha* sp. with mycorrhizae and without mycorrhizae, respectively. After 209 days of the inoculation with the fungus.

## 4.2 Essential oils composition and yield

The totality of identified compounds of basil and mint were 24 and 18 compounds respectively (Table 4; Table 5, respectively) which were revealed by the analysis of retention times reflected in the chromatograms of basil and mint (Fig.14; Fig.15, respectively) and with spectra from the mass spectrometry equipment. Detailed information can be found in Attachment III and IV.

The major dominant compounds of *O. basilicum* L. cv. Genovese Gigante essential oils were Linalool and Eugenol, and the major dominant compound of *Mentha* sp. essential oils was Piperitenone oxide.

Examining the percentage of the compounds identified in basil samples it is possible to state that this cultivar is Genovese Gigante. A study of several cultivars of basil (which included the Genovese Gigante) asserted that eugenol is prevalent in almost all analyzed cultivars, except in the case of one accession (Basilico a foglia lattuga), where the percentage was considerably reduced. Cineole, terpineol and farnesene were found in all the cultivars. Cineole can be considered the third main component (except in the case of accession mentioned before) (Labra et al., 2004).

Furthermore, a study of the cultivar Genovese stated that eugenol was the most abundant component, followed by linalool and eucalyptol (1,8-cineole). Caryophyllene, α-pinene, limonene, and camphor were present in smaller amounts (Copetta et al., 2006). Accordingly, our sample also has as main components Eugenol, Linalool, 1,8-Cineole. Alike Copetta et al (2006) estragol (methylchavicol), and skatol were not detected in this cultivar. Also, this plant reached in the late flowering stage an average of 90 cm which characterizes this cultivar, whereas Genovese cultivar can reach 40-45 cm tall (Flamini et al., 2006).

Table 4: Compounds identified from EO's samples of *Ocimum basilicum* L. cv. Genovese Gigante. Legend: CC – Classes of Compounds; MH – Monoterpene hydrocarbons; OM – Oxygenated Monoterpenes; OTHS – Others; SH – Sesquiterpene hydrocarbons; OS – Oxygenated Sesquiterpenes.

| Compounds | CC | Retention Index | Compounds | CC | Retention Index |
|---|---|---|---|---|---|
| Sabinene | MH | 958 | Eugenol | OTHS | 1327 |
| β-Pinene | MH | 963 | Methyleugenol | OTHS | 1377 |
| β-Myrcene | MH | 975 | β-Elemene | SH | 1388 |
| 1,8-Cineole | OM | 1005 | *trans*-α-Bergamotene | SH | 1434 |
| *trans*-β-Ocimene | MH | 1027 | α-Humulene | SH | 1447 |
| Terpinolene | MH | 1064 | t*rans*-β-Farnesene | SH | 1455 |
| Linalool | OM | 1074 | Germacrene-D | SH | 1474 |
| Camphor | OM | 1102 | γ-Cadinene | SH | 1500 |
| Borneol | OM | 1132 | β-Sesquiphellandrene | SH | 1508 |
| α-Terpineol | OM | 1134 | τ-Cadinol | OS | 1616 |
| Octanol acetate | OTHS | 1189 | 1-*epi*-Cubenol | OS | 1624 |
| Bornyl acetate | OM | 1265 | α-Cadinol | OS | 1626 |

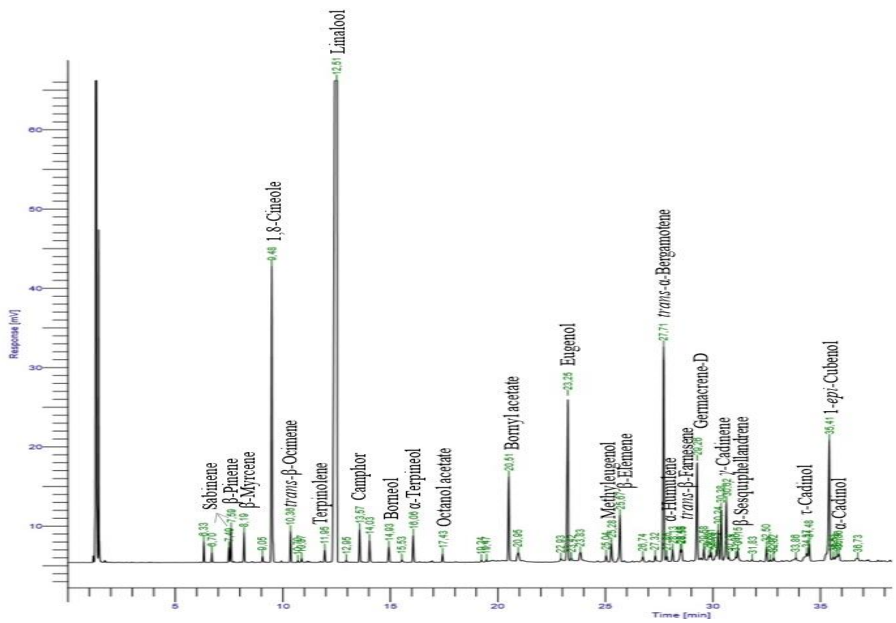



Fig.14: Chromatogram of *Ocimum basilicum* L. cv. Genovese Gigante. Sample from the 2nd harvest (dry material distillation).

Considering mint, the results of the analysis of the essential oil samples suggest that the seeds bought on a commercial seed shop had the identification incorrect since *Mentha piperita* L. is characterized by the presence of (+)-menthol (38.06%), menthol (35.64%), neomenthol (6.73%) and cineole (3.62%) (Kizil et al., 2010). This situation emphasized the importance of the science of chemotaxonomy or chemical taxonomy which is used for the classification of plants based on their chemical constituents (Singh, 2016). Chemotaxonomy is important in the natural products industry mainly because within a genus different species may exhibit different levels of intraspecific chemical variability.

The main compounds in decreasing order of mint essential oils from this experiment were Piperitenone oxide > Germacrene-D > *cis*-Piperitone oxide > β-Myrcene > 1,8-cineole. *Mentha piperita* L. is characterized always by the presence of menthol and *Mentha pulegium* L. contains almost always pulegone as the main compound or one of the main compounds (Lawrence, 2007; Baser et al., 2015). From this information it is possible to eliminate the possibility of our plant being *Mentha piperita* L. or *Mentha pulegium* L. since the samples didn't present neither menthone, menthol or pulegone respectively.

The presence of piperitenone oxide (PO) in high percentages has been reported by some researchers for the oil composition of *Mentha suaveolens* Ehrh. (Baser et al., 2015). Focusing on the latter, some researchers asserted that the overall chromatographic profile of the oil sample was dominated by the oxygenated constituents and by ketones among which carvone (50.59%) was a major constituent in the oil (Kashoury, et al., 2014). Furthermore, another study differentiates three chemotypes of *Mentha suaveolens* Ehrh. According to Bozovic et al (2015) three profiles of EOMS (‘essential oils of *Mentha suaveolens*’) have been described: the first profile is rich in pulegone, the second in PO and the third one contains similar quantities of PO and piperitone oxide (Bozovic et al., 2015).

In addition to the notes of Bozovic et al (2015), other study that made analysis of the essential oil of *Mentha suaveolens* Ehrh. obtained from plants grown in the Tarquinia forests (Italy) showed a predominance of PO (>90%), with limonene and 1,8-cineole among minor constituents (Avao et al., 1995).

Table 5: Compounds identified from EO’s samples of *Mentha* sp. Legend: CC – Classes of Compounds; MH – Monoterpene hydrocarbons; OM – Oxygenated Monoterpenes; SH – Sesquiterpene hydrocarbons; OS – Oxygenated Sesquiterpenes.

| Compounds | CC | Retention Index | Compounds | CC | Retention Index |
|---|---|---|---|---|---|
| α-Pinene | MH | 930 | γ-Terpinene | MH | 1035 |
| Sabinene | MH | 958 | Linalool | OM | 1074 |
| β-Pinene | MH | 963 | cis-Piperitone oxide | OM | 1211 |
| β-Myrcene | MH | 975 | Thymol | OM | 1275 |
| p-Cyme ne | MH | 1003 | Piperitenone oxide | OM | 1330 |
| 1,8-Cineole | OM | 1005 | β-Caryophyllene | SH | 1414 |
| Limonene | MH | 1009 | Germacrene-D | SH | 1474 |
| *cis*-β-Ocimene | MH | 1017 | 1-*epi*-Cubenol | OS | 1624 |
| *trans*-β-Ocimene | MH | 1027 | α-Cadinol | OS | 1508 |

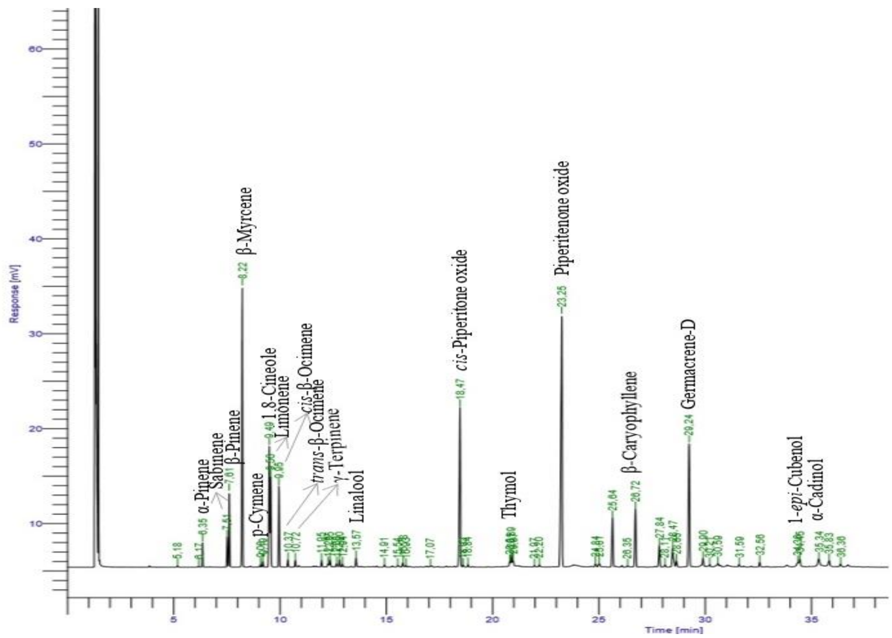


Fig.15: Chromatogram of *Mentha* sp. Sample from the 2nd harvest (fresh material distillation).

In short and furthermore, piperitenone oxide accompanied or not by low quantities of *cis* and *trans*-piperitone oxide has been found in high amounts in the essential oils of (1) *M. longifolia* (L.) Huds. plants from the Netherlands, India, Italy, and Syria (2) *M. suaveolens* Ehrh. plants from the United Kingdom, Germany, Greece, the Netherlands and Italy and (3) *M. spicata* L. plants from Greece, Belgium, Denmark, Sweden, Czech Republic, India and Italy (Linskens et al., 2012). This emphasizes the great of variability within this genus which is also due to artificial cross breeding.

Since the plants of mint from this experiment didn't reached the reproductive stage which would give an additional trait to identify the species it is difficult to make certain assumptions. Nevertheless, the data obtained suggests that our species is *Mentha suaveolens* Ehrh. since according to Bozovic et al (2015) our sample fits the second chemotype described inasmuch as it had a relative percentage of piperitenone oxide between 22-90.65% and piperitone oxide with < 16%. However, some published articles of *M. spicata* L. and *M. longifolia* (L.) Huds. chemical profiles also look similar to our samples. These dissimilar values in published articles don't permit to identify this species based only on chemical analysis, highlighting the need of classical taxonomy besides chemotaxonomy. Furthermore, this problem emphasized the importance of acquiring certified seeds.

Regarding the yield, the measurements from the 1st harvest of basil and mint were difficult to determine since the quantity was very low (Fig.16). However, in the 2nd harvest it was possible to determine (Table 6) even though it was still a small amount.

Fig.16: Example of the quantity of oil from one of the distillations from the 1st harvest.

Examining Table 6 it is possible to infer that both plants, had an enhanced essential oil yield in the dry samples. Furthermore, plants subjected to HS treatment and posteriorly dried present an even more enhanced yield. The latter has to do with the reduction in overall plant biomass due to drying and also the reduced overall plant biomass due to hydric stress. Furthermore, it has been reported that water stress increases essential oil yield due to the reduction in leaf area (Simon et al., 1992). Besides the latter, the differences between treatments weren't striking.

According to Tarraf et al (2015) several studies on many species of Lamiaceae family, such as *Mentha arvensis* (Freitas et al., 2004), *Ocimum basilicum* L. (Copetta et al., 2006) and *Origanum* sp. (Khaosaad et al., 2006) described consistent findings about increasing the oil quantity in favor of inoculation by AM fungi (Tarraf et al., 2015). However, when examining each of these studies one doesn't find a mention to yield (mL/g) and yes, a mention to the percentage of main compounds. Many of these studies refer to the 'yield' of a particular compound mainly like Copetta et al (2006), which asserted (with *O. basilicum* L. cv. Genovese) that plants colonized by *Gi. margarita* showed a significant increase in the yield of methyleugenol in comparison with all the other treatments, while those colonized by *Gi. rosea* increased bornyl acetate content (Copetta et al., 2006). The same is visible in a study with *Mentha arvensis* L. where it is discussed the yield in terms of menthol (Freitas et al., 2004). It seems that the effect of mycorrhizae on yield (mL/g) is disregarded and that the most important influence is related to the content and proportion of certain compounds.

Regarding the hydric stress, it is important to emphasize that the effect it might have depends on the species or cultivars. For example, Petropoulos et al (2007) with parsley inferred that the effect of water deficit stress on the yield of essential oils was higher in the leaves of plants of plain-leafed and curly-leafed parsley subjected to water stress, but not in the leaves of the turnip-rooted cultivar (Petropoulos et al., 2007). Furthermore, in many studies the yield increase is mentioned without referring the weight of the biomass distilled. Notwithstanding, the yield of fresh samples didn't differ relevantly between treatments and control, and in dry samples also, except for HS which has to do with the reduction of weight due to drying plus the natural occurring weight decrease due to drought, as latter mentioned.

Table 6: Essential oil yield (%) from the 2nd harvest of basil and mint depending on fresh and dry weight. In bold are marked the highest weights.

| Basil | | | | | Mint | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatments | Fresh Weight (g) | EO yield (%) | Dry Weight (g) | EO yield (%) | Treatments | Fresh Weight (g) | EO yield (%) | Dry Weight (g) | EO yield (%) |
| C | 263 | 0.03 | 63.7 | 0.19 | C | 91.3 | 0.05 | 47.2 | 0.10 |
| HS | 240 | 0.02 | 51.3 | 0.29 | HS | 78.2 | 0.06 | 47 | 0.16 |
| M | 278 | 0.03 | **80.4** | 0.12 | M | **108.9** | 0.07 | **47.4** | 0.10 |
| HS+M | **305** | 0.03 | 52.2 | 0.19 | HS+M | 89 | 0.06 | 44.4 | 0.11 |

## 4.3 Quality variation in response to the biotic and abiotic factor

### 4.3.1 Mint

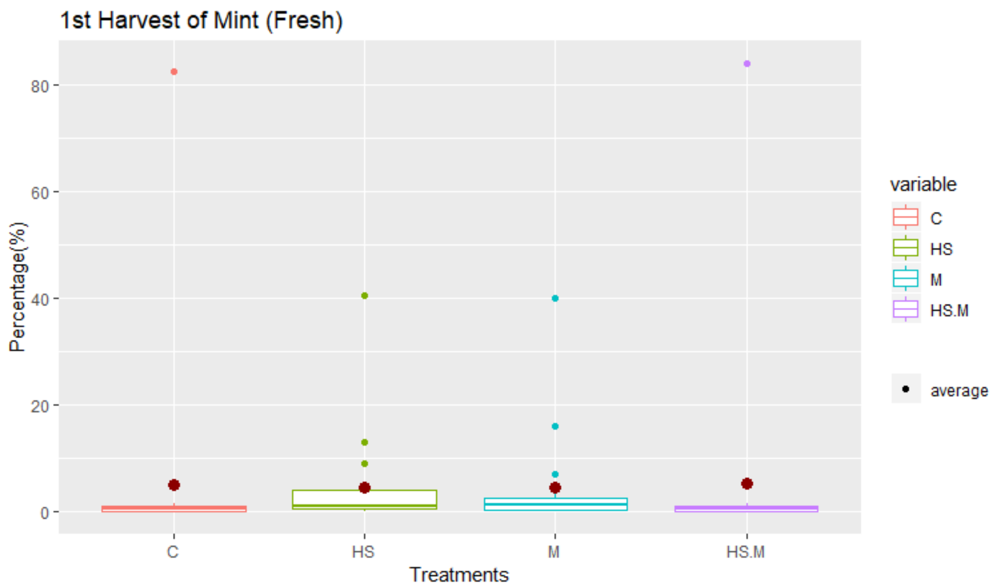
#### A. 1st Harvest and 2nd Harvest of Mint (Fresh)

The variation of the relative percentage of identified compounds of Mint in the 1st harvest in response to the treatments is presented in the **Graph 4**. Examining the Graph 4, it is possible to infer by the average that the content between treatments and control didn't vary in a relevant manner. Nevertheless, notable differences can be observed, in terms of proportion, of certain compounds (outliers). In Control and in HS+M the outlier was Piperitenone oxide with 83% and 84.1% respectively. In HS the outliers were Piperitenone oxide (41%), Germacrene-D (13%) and β-Myrcene (9.1%) whereas in M the outliers were Piperitenone oxide (40%), Germacrene-D (16.2%) and β-Caryophyllene (7%).

It is possible to infer that the relative percentage of Piperitenone oxide is higher, the double, in the treatments of HS+M (84.1%) and Control (83%) compared with HS (41%) and M (40%) treatments. This compound is part of the class of the oxygenated monoterpenes along with 1,8-cineole, linalool, *cis*-piperitone oxide and thymol (present in the sample). A study in accordance with the last-mentioned values of HS and M treatment is a study with *O. gratissimum* L. which didn't find high qualitative diversity in this class like our results indicate, since the authors concluded that contents of oxygenated monoterpenes changed between 87% in non-mycorrhizal stressed plants (HS) and a maximum reached 97% in mycorrhizal non-stressed plants (M) (Hazzoumi et al., 2017). The latter study is in accordance to our results in regard to the similarity between HS and M in terms of the class of compounds of oxygenated monoterpenes (which change considerably with variation of the percentage of piperitenone oxide). Furthermore, another study, with peppermint inoculated with AMF, stated that a large part of the balanced equilibrium of contents could depend on the chemical control exerted by terpenoids on fungal growth. They documented the general increase of terpene volatilization from leaves is after fungal colonization (Mucciarelli et al., 2007). Concerning control, a study of different water regimes with *Mentha piperita* L. came to results that the highest values of growth parameters and essential oil percent and yield were observed under 100% field capacity (control) (Khorasaninejad et al., 2011). Indeed, the average (which is related to the content) was slightly higher in control and HS+M. Thus, the effect on the proportion of this compound by isolated treatments doesn't have to be obligatorily additive when examining the two treatments together (HS+M). In fact, HS+M might resemble the control condition since it has been reported that mycorrhizal plants show greater stomatal conductance in all soil moisture conditions, but the effect of mycorrhizal association is more pronounced in moderate drought (Augé et al, 2014).

Other compound that was an outlier is the compound Germacrene-D which presented for the samples of HS and M the relative percentage of 13% and 16.2% respectively, in comparison with the control (1.6%) and HS+M (0.5%). Germacrene-D has been compared to an 'indicator of a defense response' (Frost et al., 2008). In this 1$^{st}$ harvest, the values for this compound in the HS and M treatments may be related to the plant response to the commencement of drought stress and the initial stages of root colonization by AMF which are accompanied by transient induction of selected plant defense (Cameron et al., 2013). Contrarily to our results, with *M. viridis* L., the germacrene-D decreased with inoculation of two strains of *Glomus*. In mycorrhizal plants the relative percentage of this compound was 1.22% and in non-inoculated plants it was 2.17% (Karagiannidis et al., 2011). Nevertheless, it was a different mint species and a different *Glomus* species. Similarly, to what occurred with the compound Piperitenone oxide, it might be that the understanding of plant responses to multiple simultaneous stresses is of limited use since when stresses occur in combination certain genes are activated that are not induced by either stress individually (Rizhsky et al., 2004; Mittler, 2006).

In addition, in the HS and M treatment other compounds highlighted in Graph 4 were β-Myrcene (9.1%) and β-Caryophyllene (7%) respectively. The higher relative percentage of β-Myrcene obtained in HS samples (Control samples presented 0.6%) is in accordance with a study with spearmint that came to the results that in well-watered plants (100% FC) the values obtained for this compound were 293.3∓4.5 (μg g-1 DW) while in moderately water-stressed plants (50% FC) it was 421.5∓7.1 (μg $g^{-1}$ DW) (Delfine et al., 2005). Regarding β-caryophyllene, the M samples presented for the latter the relative percentage of 7% whereas control samples presented 0.5%. Similarly, a study with coriander have also reported a significant increase of this compound when the plants were inoculated with *Glomus* fungi (Kapoor et al., 2002).




Graph 4: Variation of the relative percentage (%) of the totality of the 18 compounds from the 1st Harvest of Mint (fresh).

Examining the **Table 7**, it is possible to affirm that oxygenated monoterpenes constituted the principal class of compounds in Mint, followed by sesquiterpene hydrocarbons, monoterpene hydrocarbons and oxygenated sesquiterpenes.

The relative percentage of oxygenated monoterpenes was fairly influenced by the presence of the compound Piperitenone oxide which was the double in HS+M treatment and Control than the treatments HS and M. In the case of monoterpene hydrocarbons and sesquiterpene hydrocarbons HS and M treatment presented a higher relative percentage in comparison with Control and HS+M treatment. Possibly the loss in oxygenated monoterpenes compensated in an increase of hydro carbonated classes of compounds in HS and M treatments.

Harley (2013) reported that under moderate drought stress, non-oxygenated terpenes are not influenced, while oxygenated ones can be drastically reduced (Harley, 2013). A study with *Salvia officinalis* L. under hydric stress reported that, concerning monoterpenes hydrocarbons, the values were 41.91∓0.6 (control), 462.88∓0.63 (mild water deficit), 274.75∓0.84(severe water deficit) and for sesquiterpenes hydrocarbons it were 27.61∓0.43 (control) 91.20∓0.37(mild water deficit) and 36.34∓0.58 (severe water stress) (µg g-1 DW) (Bettaieb et al., 2009), which might explain these values for this class (MH) in HS since peltate and capitate glandular trichomes of *Salvia officinalis* L. are also found in mint. The latter is also in accordance with the values obtained for SH in HS treatment. The same was observed in the samples from M treatment for both classes MH and SH. This might be explained by the fact that, according to Venkateshwaran et al (2014), the MVA pathway is among other processes necessary for the earliest responses of plants to symbiotic signals produced by nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (Venkateshwaran et al 2014). The last-mentioned involvement of MVA in the earliest responses of plants to symbiotic signals possibly explains the higher relative percentage of SH in M treatment. In addition, the MH increased percentage in M treatment may be related to the fact that mycorrhizal fungi and fungal endophytes infection seems also to result in specific-enhancement of the MEP pathway metabolic flux in plants (Zhi-lin et al., 2007). The specific enhancement in this case of the M treatment was in terms of the MH and SH in detriment of the OM, like in the HS treatment as above mentioned. Furthermore, the latter is in accordance with a study with *Artemisia drancuncalus* L. inoculated with *Glomus intraradices* which reported a reduced ratio of oxygenated monoterpenes to sesquiterpenes which is possibly caused by changes in cell bioenergetics under environmental stress (Lamian et al., 2017). In addition, the percentage of oxygenated sesquiterpenes was rather similar between all treatments which can be explained by the example of the chemical profile of *Mentha* x *piperita* L. which is composed primarily of monoterpenes with less than 2% oxygenated sesquiterpenes (Croteau et al., 1973). Besides, this class of compounds in our mint samples was based in only two components, α-cadinol and 1-*epi*-cubenol.

Table 7: Relative percentage of chemical classes of the essential oil from the 1st harvest of Mint (fresh). In bold are the highest relative percentages (%).

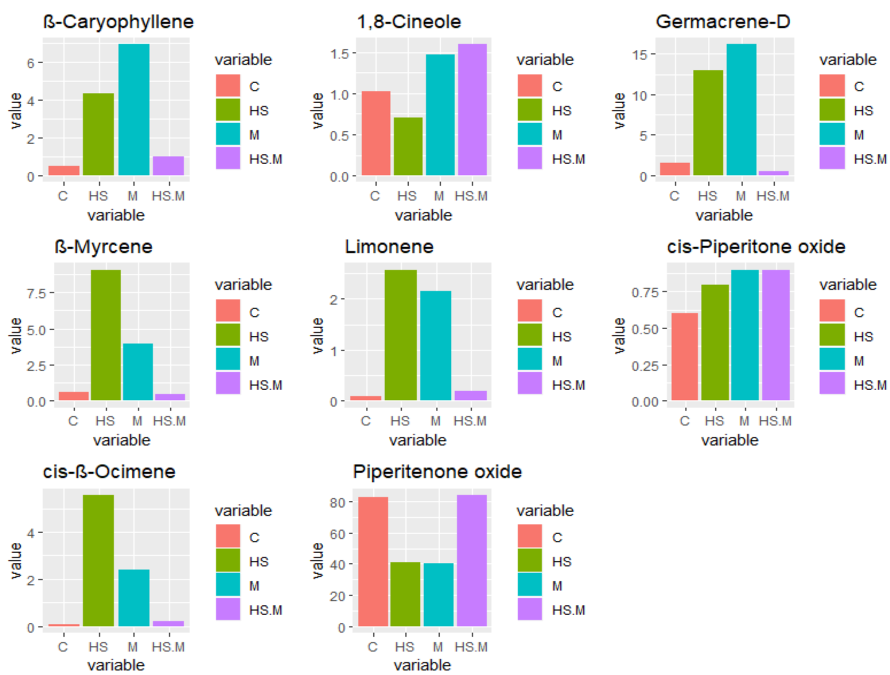
| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | 2.3 | **19.8** | 10.4 | 1.3 | 8.5 |
| Oxygenated monoterpenes | 85.4 | 43.9 | 45.7 | **87.2** | 65.6 |
| Sesquiterpene hydrocarbons | 2.1 | 17.3 | **23.1** | 1.5 | 11 |
| Oxygenated sesquiterpenes | 2.2 | 2.4 | 2.5 | **3.0** | 2.5 |
| Sum | 92 | 83.4 | 81.7 | **93** | |

In regard of the individual relative percentage of prevalent compounds (> 4.5%) (**Graph 5**) it is possible to infer that for β-Myrcene, Limonene and *cis*-β-Ocimene the best treatment was HS, for Piperitenone oxide, *cis*-piperitone oxide and 1,8-cineole it was HS+M and for β-Caryophyllene and Germacrene-D it was the M treatment.

The latter compounds, β-Caryophyllene and Germacrene-D prevalence in the M treatment are also described in a study with *Citrus Jambhiri* Lush inoculated with different *Glomus* species. They observed that β-Caryophyllene had a relative higher proportion in mycorrhizal plants (Nemec et al, 1990). Also, Rapparini et al (2006) described an induce production of β-Caryophyllene in *A. annua* L. in response to a fungal elicitor (Rapparini et al., 2006). Germacrene-D high relative percentage might be due, like mentioned before, to a transient induction of selected plant defenses. Furthermore, β-Myrcene, Limonene, *cis*-β-Ocimene which are monoterpene hydrocarbons were prevalent in HS treatment. This in accordance with a study with *Mentha pulegium* L. subjected to drought which reported that the percentage of the monoterpene hydrocarbons and sesquiterpene classes increased under drought stress (Hassanpour et al., 2014).

Piperitenone oxide and *cis*-piperitone oxide are also monoterpenes but oxygenated which had the highest relative percentage in HS+M treatment.  This might be related to, according to other studies, that the production of isoprenoids by leaves is favored by AM symbiosis under water stress (Asensio et al., 2012). This has been attributed to more carbon demand by AM fungi which affect the amount of carbon allocation and carbon partitioning among various classes of isoprenoids.

Regarding 1,8-cineole, this compound presented in the essential oil of mint treated with HS+M and M treatment the highest relative percentages respectively. According to Geneva et al (2010) with *Salvia officinalis* L. there was also a raise, with *Glomus intraradices*, from 2.94% (control) to 6.30% (AMF) (Geneva et al., 2010). It seems like mycorrhizae has more influence concerning this compound since in HS it presented the lowest relative percentage.




Graph 5: Relative percentage of individual chemical compounds of the 1st harvest of Mint

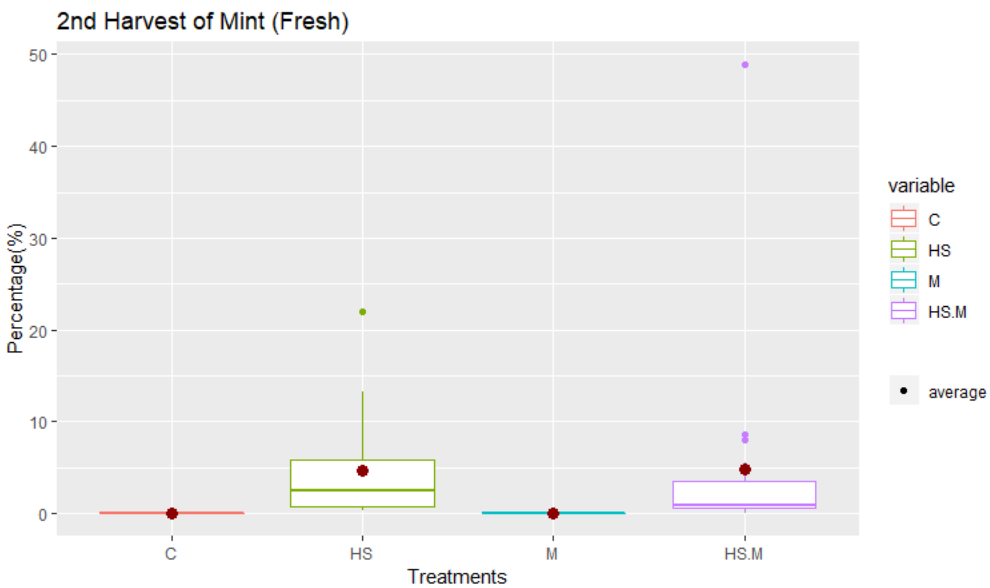
Considering the 2nd harvest and due to a problem with the utilization of the distillation equipment it is only possible to analyze the HS and HS+M treatment because the essential oil from control and from M treatment was lost. The variation of the relative percentage of the identified compounds of Mint are presented in the **Graph 6**. Also, and like the former graph, it is possible to verify that in terms of average there is a similarity between treatments. Furthermore, in both treatments the notable difference observed is also in regard to the relative percentage of the compound piperitenone oxide. The relative percentage of this compound in HS was 22.0% and in HS+M it was 48.9%. Thus, HS+M samples had as outliers

Germacrene-D and β-Myrcene with 8.5% and 8.1% respectively. Furthermore, HS had other compounds with high relative percentage, mainly the compound *cis*-piperitone oxide with 11.4% and β-Myrcene with 13.3% among others, which explains the larger boxplot.

Concerning the *cis*-piperitone oxide, an oxygenated monoterpene, prevalent in HS treatment, a possible explanation may reside in ontogeny (such as growth and leaf development stage) or due to the abiotic stress. As mentioned above under moderate drought stress, non-oxygenated terpenes are not influenced, while oxygenated ones can be drastically reduced (Harley, 2013). However, contrarily to the latter, in a study with *R. officinalis* L. subjected to progressive drought, the oxygenated monoterpenes seemed not to be affected by stomatal conductance (Nogués et al., 2015). Indeed, it is assumed that a monoterpene inside a leaf containing less water (low RWC) will tend to reach faster the gas–liquid phase equilibrium and, consequently stomata will have less opportunity to exert control (Harley, 2013). Notwithstanding, it may be that these oxygenated monoterpenes, *cis*-piperitone oxide and piperitenone oxide aren't correlated with the prolonged water stress which describes the stress subjected by the time of this harvest since in *R. officinalis* L. the oxygenated monoterpenes didn't appear to differ with the lower stomal conductance provoked by prolonged drought. So, the higher amount of *cis*-piperitone oxide in HS may be correlated with the lower amount of Piperitenone oxide, which in HS+M was higher and *cis*-piperitone oxide had trace amounts (0.47%). Similarly, HS+M maintained a higher amount of Piperitenone oxide and HS a lower amount in the 1$^{st}$ harvest. The explanation may reside in the conversion of (+)-piperitenone oxide to (-)-*cis*-piperitone oxide which is controlled by three different genes (Lawrence,1978). The decrease from the 1$^{st}$ to the 2$^{nd}$ harvest in both treatments possibly further highlights an influence more related to phenological factors than drought.

Regarding β-Myrcene in both samples (HS and HS+M) it is possible to infer that there was an increase from the 1$^{st}$ to the 2$^{nd}$ harvest, more accentuated in HS+M treatment. This might be related with ontogeny since it has been reported with peppermint that the relative percentage of this compound increases with leaf age (Gershenzon et al., 2000).

Concerning Germacrene-D, this compound increased 8% in HS+M samples from this 2nd harvest in comparison with the 1st harvest whereas in HS it decreased 4%. In fact, the results for HS samples it validated by a report with *O. vulgare* L. ssp. *virens* subjected to prolonged mild water stress that this compound decreased (Morshedloo et al., 2016). However, in the treatment of HS+M the last-mentioned is not seen which may indicate an effect of the mycorrhizae.




Graph 6: Variation of the relative percentage (%) of the totality of the 18 compounds from the 2nd Harvest of Mint (fresh). <u>Samples from Control and Mycorrhizae treatment not in the graph</u>.

Regarding the **Table 8** it possible to examine the classes of compounds. It is possible to infer that the sum of the relative percentage of the classes of compounds of monoterpenes (hydrocarbons plus oxygenated) for HS was 69.8% and for HS+M was 73.8% while in the 1st harvest it was 63.7% and 88.5% respectively. So, presumably, there was a decrease and raise of monoterpenes from the 1st to 2nd harvest in HS+M and HS respectively.

Following the latter and since these results are from the 2nd harvest of mint, the values obtained in the samples from HS+M treatment might be explained by the fact that the temporarily defense responses by the plant in contact with the fungus changed by this stage of harvest. Indeed, the outcome of foliar metabolic changes mediated by the AM establishment in the plant probably strongly depends on the developmental stage of the mutualism, as, from the plant's 'point of view', both the costs (in terms of carbohydrates delivered to the AMF) and the benefits (in terms of P gained via the AMF) change over the progress of interaction (Schweiger et al., 2014). Nevertheless, these changes might also be related with constitutive production. In fact, monoterpene content of peppermint leaves rises rapidly between 12 and 20 d of age, levels off as full expansion is reached, and then remains stable for the remainder of leaf life (Gershenzon et al., 2000). The latter may explain the decrease of the class of monoterpenes in HS+M treatment. In addition, and since the monoterpenes in the 1$^{st}$ harvest of HS+M were similar to control it might be that by this stage rather alike values would be obtained and are related to ontogeny.

Concerning HS, and contrarily to HS+M, there was a raise of the relative percentage of monoterpenes from the 1$^{st}$ to the 2$^{nd}$ harvest. Specifically, MH raised 10% whereas OM was rather maintained (a decrease of 2%). In accordance, a study with *O. vulgare* ssp. *virens* and prolonged water stress, noticed that in comparison with control there was an increase of MH (3%) and a decrease of OM (4%) with prolonged mild water stress (Morshedloo et al., 2017). In the case of HS+M, MH raised (16%) and OM decreased substantially (31.07%). As above mentioned this might be related with ontogeny-related regulation.

Concerning the sum of the relative percentage of sesquiterpenes (hydrocarbons and oxygenated), there was a decrease and increase in HS and HS+M respectively. According to Ormeno et al (2007), sesquiterpenes are probably replaced by monoterpenes when drought is prolonged, because drought could impede cyclization of sesquiterpene precursors (Ormeno et al., 2007). Indeed, in HS while the totality of sesquiterpenes (hydrocarbons and oxygenated) decreased, the MH increased considerably, and OM slightly decreased. However, the values obtained in HS+M show an increase in the totality of the relative percentage of sesquiterpenes and also an increase of MH. The latter possibly is related to the ameliorating effects mycorrhizae has to plants subjected to drought stress.

Comparing the two harvests (Attachment V) and considering only the HS and HS+M treatments, it is possible to examine that HS+M treatment seems to be in accordance with the assumption that the rate of monoterpene declines as full leaf expansion is reached (Gershenzon et al., 1999). The latter is particularly visible with the class OM. However, the same didn't happened with HS. In the latter it is observed a rather maintenance from the 1st to the 2nd harvest, since in the 1st harvest both MH and OM accounted for 64% and in the 2nd harvest these classes of compounds accounted for 70% which may have to with the prolonged water stress. This raise has mainly to do with what was mentioned previously that when drought is prolonged, drought could impede cyclization of sesquiterpene precursors (Ormeno et al., 2007). Indeed, there was a decrease of SH and OS from HS samples in this 2nd harvest.

In addition, the values obtained for OM in drought stress may be related with the assertion that in drought stress condition the stomatal conductance (Gv) decreases. The latter provokes a rise in the internal concentration of monoterpenoids which balances the decrease in $G_V$, and the same flux as before the changes in $G_V$ is maintained at a lower stomatal aperture. This means that when the volatile build-up does not affect its synthesis rate, stomata may affect the VOC emission. It is assumed that strong stomatal effects are on the emission of monoterpenes that preferably partition to aqueous phase, as oxygenated monoterpenes, and that there are no stomatal effects for compounds that primarily partition to gas phase, as hydrocarbon monoterpenes (Niinemets et al., 2004). The latter may explain the values obtained of OM in drought stress plants since drought provokes stomata closure and a raise in internal concentration (due to reduced emission). Possibly in HS+M the conductance is higher due to the symbiotic fungi and bursts occurred, which might explain the drop in OM. Previous studies have described that oxygenated monoterpenes as linalool exhibit a large overshoot of the emission after a moderate increase in stomatal openness (Niinemets et al., 2004).

Table 8: Relative percentage of chemical classes of the essential oil from the 2nd harvest of Mint (fresh). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | - | **28.8** | - | 17.7 | 23.3 |
| Oxygenated monoterpenes | - | 41.0 | - | **56.1** | 48.6 |
| Sesquiterpene hydrocarbons | - | **13.6** | - | 12.3 | 13 |
| Oxygenated sesquiterpenes | - | 1.4 | - | 1.4 | 1.4 |
| Sum | - | 84.8 | - | **87.5** | |

Now regarding the **Graph 7** it is possible to examine individually the variation of prevalent compounds (>4.5%) in response to the treatments.

Concerning β-Caryophyllene and Germacrene-D we can affirm that the relative percentage is practically the same in both HS and HS+M treatment in this 2nd harvest. In HS from the first to the second harvest there was a rather maintenance in the terms of the relative percentage of these both compounds whereas in HS+M a raise of 2.5% for β-Caryophyllene and 8% for Germacrene-D respectively. These compounds are sesquiterpene hydrocarbons. It is stated that only leaf monoterpene emissions may be favored or maintained against prolonged water withholding periods. Furthermore, since sesquiterpene emissions are considered to be mainly involved in aerosol formation, which may contribute to negative precipitation anomalies at a local scale (Chou, 2005) this is a possible explanation to why plants drastically stop sesquiterpene release under severe drought conditions according to Ormeno et al (2007). The latter may explain the rather maintenance of these latter compounds in HS and raise in HS+M. Possibly, the emission was stunted, and internal concentrations were maintained or raised on the leaves due to the decreased emission in response to prolonged water stress.

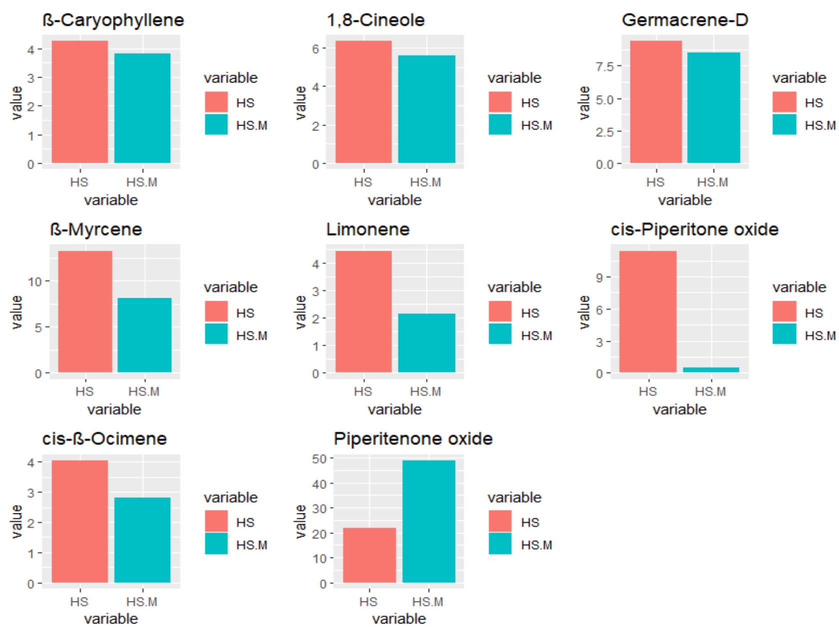
Concerning piperitone oxide, HS+M had more than the double of relative percentage in comparison to HS like in the 1st harvest. Notwithstanding, in this harvest *cis*-piperitone oxide had a higher relative percentage in HS samples than in HS+M. However, the relative percentage of piperitenone oxide had a half decrease from the first to the second harvest in both these treatments as mentioned before. This may be due to development or with prolonged drought since only monoterpenes provide an additional barrier to plant damage during severe lack of water resources (Ormeno et al., 2007) in comparison with other classes of compounds. Furthermore, when the leaves expand the synthesis of monoterpenes in mint decrease.

The compound *cis*-β-Ocimene was slightly higher in HS treatment in comparison with HS+M. The relative percentage of this compound was rather maintained from the first to the second harvest in HS and a slight increase in HS+M. The latter is in accordance with the general assumption that non-oxygenated monoterpenes are unaffected by moderate drought (Harley, 2013; Niinemets et al., 2002).

Limonene was the double in HS in comparison with HS+M treatment. Thus, from the 1$^{st}$ to the 2$^{nd}$ harvest there was a slight raise of 2% for both treatments. Niinemets et al (2004) reported that limonene and *trans*-β-ocimene may be more closely controlled by the rate of photosynthetic electron transport, i.e. the availability of NADPH and ATP in chloroplasts than stomatal conductance decrease (Niinemets et al., 2004). Following the latter, this increase might be more related to the increase of photosynthetic rate due to the prolonged photoperiod in April, the month by which these plants were harvested.

In addition, β-Myrcene was also higher in HS. However, for both treatments, HS and HS+M, there was an increase of the relative percentage of this compound from the 1$^{st}$ to the 2$^{nd}$ harvest. The latter is in accordance with a study with *Satureja hortensis* L. which presented an increase of this compound with mild water stress and decreased with severe water stress (Baher et al., 2001). The same was observed in the 1$^{st}$ harvest of mint where these latter three compounds mentioned where higher in HS treatment. Possibly the effect of amelioration by AMF of the water status in the plant subjected to drought stress didn't reflected such relevant changes like the ones seen in HS.

Regarding 1,8-cineole, in this second harvest, this compound presented a higher relative percentage in HS than HS+M. Thus, from the 1st to the 2nd harvest there was an increase of the relative percentage of the latter compound for both treatments. This might be related with the prolonged water stress and the stage of development. A study with *Artemisia annua* L. found that mild water stress in the vegetative stage presented 5.36% of 1,8-cineole whereas the drought stress in the end of the vegetative stage, beginning of the reproductive stage increased the relative percentage of this compound to 16% (Yadav et al., 2014). Indeed, from the 1st to the 2nd harvest in both HS and HS+M the relative percentage of this compound increased which might be due to a joint effect of the stage of development and prolonged water stress.



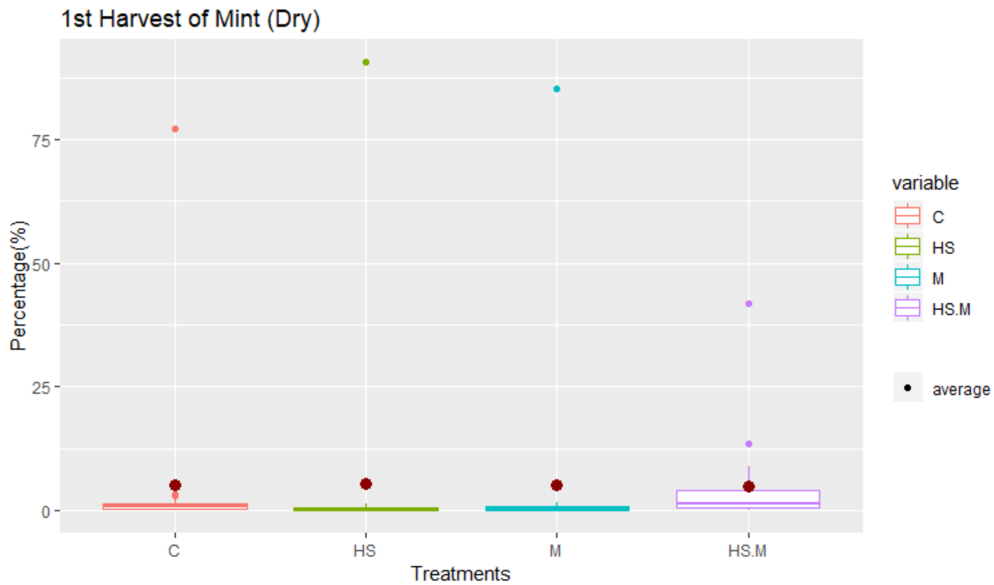

Graph 7: Relative percentage of individual chemical compounds of the 2nd harvest of Mint (fresh).

### B. 1st Harvest and 2nd Harvest of Mint (Dry)

In this type of variable, drying the material, it was expected that no significant changes would be seen in terms of the content.  This step of the production chain might be interesting since the plant can be preserved and distilled later in time. It is possible to examine by the **Graph 8** that the average of the compounds identified was rather equal between treatments, however the proportion of certain compounds was altered. The outlier compound seen in the graph in all treatments and control is piperitenone oxide which in decreasing order of highest percentage was HS (90.7%)>M (85.2%)>Control (77.2%)>HS+M (41.7%). Other outliers are β-Myrcene with 3% and 14% and 1,8-cineole with 3% and 9% in Control and HS+M respectively.

The rather similarity between the totality of identified compounds seen by the average in the samples of control and the three treatments is in accordance with a study which implied drying *Salvia officinalis* L. at 60ºC. The latter study asserted that up to a temperature of 50ºC no losses in essential oil occurred – even in the case of over-drying to equilibrium moisture content (Muller, 2007). Similarly, with rosemary dried at ambient temperature the essential oil content was similar to the fresh plant (Szumny et al., 2010). Same is seen in these results in relation to the average (which reflects the content) and of the main compound which was quite similar between control in fresh plant and control in dry plant (83% and 77.2%). Furthermore, the samples from control fresh and control dry in the 1st harvest presented 92.03% and 93.61% of content respectively, which is the sum of the relative percentage of the identified compounds.

β-Myrcene, a monoterpene hydrocarbon, presented an increase of 13% from HS+M fresh samples to HS+M dry samples. Similarly, comparing control fresh and control dry there was an increase, however in M and HS there was a decrease. A study by Beigi et al (2017) which dried peppermint leaves noticed also an increase of this compound in all the treatments (hot air dried, microwave dried and shade dried) in comparison with control fresh except for microwave drying at 800 W (Beigi et al., 2017). The reasons by which this increase is not seen in M and HS are difficult to reach. Furthermore, in the same latter study, there was a great increase of the compound 1,8-cineole in hot air and shade dried in comparison with fresh plants (Beigi et al., 2017). Similarly, in this study in all the treatments and control it is observed an increase of the relative percentage of 1,8-cineole in the samples from dried mint plants where the highest relative percentage was obtained from samples of plants treated with HS+M treatment.




Graph 8: Variation of the relative percentage (%) of the totality of the 18 compounds from the 1st Harvest of Mint (dry).

Examining **Table 9** it is possible to examine in an appropriate manner the classes of compounds. Regarding the classes of compounds OM, it is possible to infer that in general there was a maintenance of the relative percentage of OM when comparing control fresh (85%) and control dry (81%) whereas in HS and M treatment there was an increase. However, in HS+M there was a great decrease. Thus, while in control fresh versus control dry there was a rather maintenance of SH (a slight increase of 1.6%), in HS+M there was a higher increase (6%) and in HS and M there was a great decrease (16% and 22%, respectively).

It is possible that the values obtained for OM in the three treatments and control are related with SH. Where it is seen a slight decrease and great decrease in this class of compounds (OM) in Control and HS+M respectively it is also seen an increase of SH. Furthermore, where there was a great increase in OM, in HS and M, there was a great decrease of SH. In fact, as mentioned in the introduction it has been reported recently that the plastidic MEP pathway (related to the synthesis of monoterpenes) ‘cross-talks’ with cytosolic MVA pathway (were sesquiterpenes are mainly synthetized). Furthermore, drying might affected differently the plants subjected to the different treatments since the relative water content (RWC) is certainly not the same between treatments and control. Thus, a satisfactory basis for relating cellular water status to metabolism is RWC (Lawlor et al., 2002).

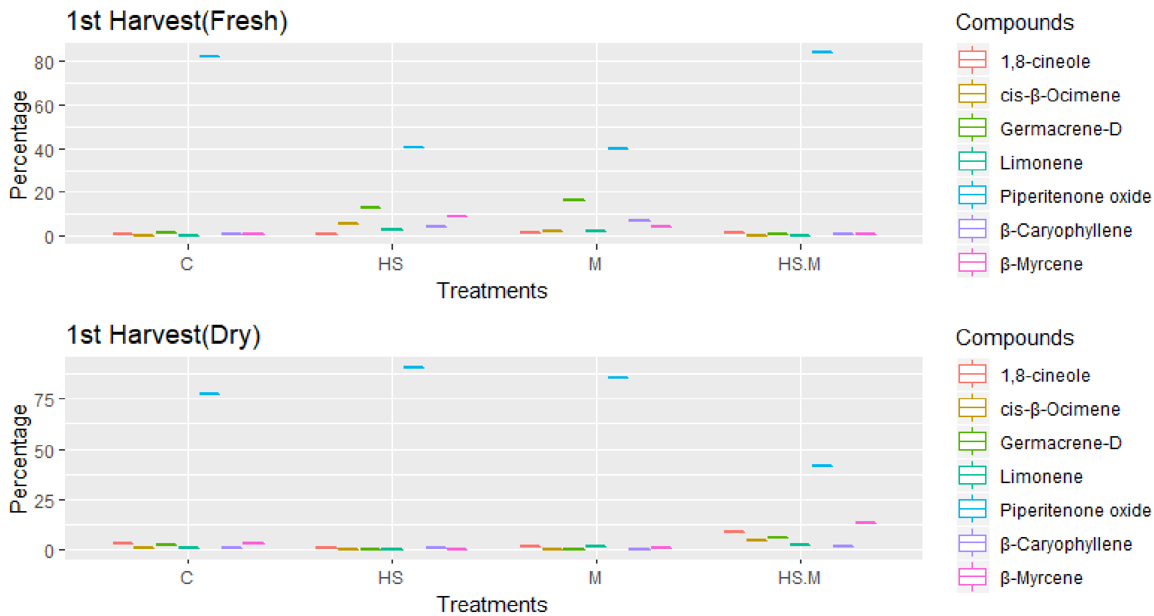
Regarding MH, there was a rather maintenance and increase when comparing the 1st harvest fresh with 1st harvest dry in control and in HS+M treatment respectively, whereas in HS and M a decrease, similarly to what happened with the class of compound SH. The effects of drying on plants that have generally an optimal RWC (M) and a reduced RWC (HS) might be different because the partitioning of the compounds in gas/liquid phase of leaf has been described to be distinct when the stomatal conductance is variable and reduced respectively. The drying procedure possibly changed the Henry's law constant (equilibrium gas/liquid-phase partition coefficient) and plus the fact that this equilibrium is already altered by the procedures applied to the plants previously to drying, the latter may explain these values.

Table 9: Relative percentage of chemical classes of the essential oil from the 1st harvest of Mint (dry). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | 6.7 | 1.0 | 3.5 | **26.9** | 9.5 |
| Oxygenated monoterpenes | 81.0 | **93.5** | 87.8 | 52.5 | 78.7 |
| Sesquiterpene hydrocarbons | 3.7 | 1.4 | 0.5 | **7.7** | 3.3 |
| Oxygenated sesquiterpenes | **2.2** | 0.4 | 1.6 | 1.2 | 1.4 |
| Sum | 93.6 | **96.2** | 93.4 | 88.3 | |

The **Graph 9** presents a comparison between prevalent compounds (>4.5%) from fresh material and dry material from the 1st harvest. The drying procedure didn't affect in a relevant manner the majority of the main compounds of the EO. However, there was a few exceptions. The difference that attracts attention the most is relative to the compound piperitenone oxide. The dried samples of the plants treated with HS and M presented a higher relative percentage of the latter compound. Furthermore, in HS+M there was a decrease whereas in control there was a rather maintenance when comparing the values obtained in samples distillated in fresh.

It is stated that lipophilic compounds, such as non-oxygenated terpenes, can be stored in significant quantities in the lipid phase of the leaf, which generally comprises 1–3% of total leaf dry mass while hydrophilic compounds, such as oxygenated monoterpenes, by contrast, are found in high concentrations in the aqueous phase of the leaf (Niinemets et al., 2004). It might be that the lipid phase of the leaf composition, which store non-oxygenated terpenes was less affected by drying than the aqueous phase which stores oxygenated monoterpenes. Furthermore, this may be a plausible explanation for the raise of piperitenone oxide, an oxygenated monoterpene, in the dry HS, which presented the highest relative percentage (90.6%) between all treatments and control. In addition, a study that tested different methods of drying (shade, sun, oven at 40ºC and 80ºC, Microwave 600 W and 1000 W) of *M. longifolia* (L.) Huds. reported that the amount of piperitenone oxide was in all treatments higher than in fresh samples (Saeidi et al., 2016). The researchers argue that the latter might have happened since it is likely that hot drying temperatures had an effect on the biosynthesis and accumulation of those compounds. Furthermore, HS plants usually present a decreased leaf water potential, which declines further with increasing temperature. The last-mentioned may explain the enhanced concentration of this compound with the drying treatment in HS. Concerning M, it has been reported that the relative water content (RWC) of leaves is higher in inoculated plants with AMF (Barzana et al., 2012). Since oxygenated monoterpenes as piperitenone oxide are found in high concentrations in the aqueous phase of the leaf it might be that drying had counteracting effects with the higher relative water content in the leaves of mycorrhizal plants and the relative concentration of the latter was affected (an increase of 8% in comparison with control dry samples). Concerning the HS+M treatment, there was a halved decrease of piperitenone oxide in comparison with fresh HS+M. The value for this compound was even less than dry control; 35% lesser. A study with hydric stress and two strains of mycorrhiza applied to soybean plants demonstrated that 60% FC water conditions with *Glomus* present the highest value of RWC (relative water content) in comparison with non-water stressed mycorrhizal plants (Aliasgharzad et al., 2006). Thus, isoprene drops to values below those measured (in normal conditions) in fully hydrated leaves (Beckett et al., 2012). The latter may explain the decrease of the compound in question in HS+M treatment and also due to counteracting effects related to drying.



Graph 9: Comparison of the relative percentage of prevalent compounds (>4.5%) of Mint samples in fresh weight (upper graph) and dry weight (lower graph) of the 1st harvest.

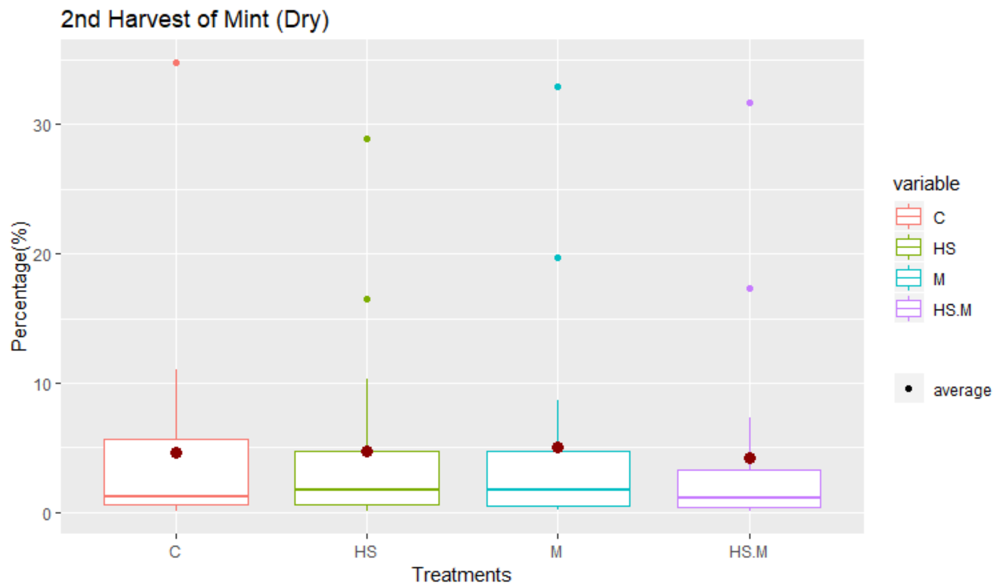
Concerning now the 2nd harvest of dry Mint, we can see in **Graph 10** that in all treatments and control the average is rather the same. The compound that is an outlier in the treatments and control is Piperitenone oxide, where we had for Control, HS, M and HS+M treatments 34.8%, 28.9%, 33% and 32% respectively. Furthermore, the three treatments had other compounds emphasized by the graph. For HS it was *cis*-piperitone oxide, 16.5%, which control had 1.3%, for M it was β-Myrcene, 19.71%, which control had 8% and for HS+M it was Germacrene-D, 17.4%, which control had 11%.

As mentioned above in the 2nd harvest in fresh the results obtained related to *cis*-piperitone oxide might be more related to constitutive production than induced or '*de novo*' production and also seems to be affected by drying. From the samples of control dry from this 2nd harvest it is inferred that there was an increase of this compound in comparison with control fresh (from the 1st harvest). Biogenetic data have demonstrated that the dominant *gene* A is responsible for the reduction of piperitenone to pulegone, whereas the recessive *gene* a, preventing this reduction is responsible for the accumulation of piperitone. Lawrence (1978) suggested that (1) the recessive *gene* o is responsible for the formation of Piperitenone oxide and *trans*-piperitone oxide from Piperitenone and piperitone respectively, and (2) the reduction of Piperitenone to (+)-piperitone and the conversion of (+)-piperitenone oxide to (-)-*cis*-piperitone oxide are further controlled by the dominant *gene* Ps and *gene* Pr, respectively. Since in the HS treatment piperitenone oxide has a lower relative percentage than control, and *cis*-piperitone oxide increased we might assume that these values are more related to internal regulating factors than the external treatment. However, the drying and storing enhanced even more *cis*-piperitone oxide concentration in these samples than what was seen in the 2$^{nd}$ harvest in fresh. The relative percentage of *cis*-piperitone oxide in HS treatment was higher in this 2$^{nd}$ harvest dry distillation (16.5%) than in the HS samples from the 2$^{nd}$ harvest fresh distillation (11.4%) which possibly demonstrates an effect of drying and storing. Similarly, *Lavandula angustifolia* L. dried and stored presented an increase of oxygenated monoterpenes of 6.7 % in comparison with control (Smigielski et al., 2011).

Regarding β-Myrcene, in M treatment, there was a double increase of the relative percentage in comparison to dry control, from 8% to 19.71%. A study with *Cymbopogon citratus* L. reported a trace amount of this compound without AMF in 100 ml/kg-1 of Pb which increased with AMF at the same amount of Pb to 3.36% (Lermen et al., 2015). This is in accordance with the statement that arbuscular mycorrhiza formation leads to enhanced levels of monoterpenes and sesquiterpenes, including monoterpenes such as myrcene (Sharma et al., 2015). In fact, in fresh, the M treatment also presented highest relative percentage of this compound in comparison with control. Plus, a study with *Satureja bachtiarica* Bunge subject to different drying methods (sun, shade, oven and freeze drying) revealed an increase amount of this compound in all the treatments in comparison with fresh samples (Pirbalouti et al., 2013).

Concerning germacrene-D, this was a compound that was an outlier in the HS+M treatment which presented a relative percentage of 17% in comparison with 11% in control. Germacrene-D is a sesquiterpene; sesquiterpenes are hydrophobic compounds which are stored in specialized tissues to isolate them from largely aqueous mesophyll tissues where photosynthesis is taking place (Delatte et al., 2017). Thus, it has been reported that the drying of the peppermint leaves resulted in significant increments ($p \leq 0.05$ and/or $p \leq 0.01$) in certain volatiles such as menthyl acetate, β-caryophyllene, germacrene-D, and others (Beigi et al., 2017). Furthermore, this compound is involved in defense responses induced in the plant for example when the plant is in contact with a symbiotic fungus. However, since this is the second harvest the latter are not pertinent. Nevertheless, in longer experiments, it has been stated that the accumulation of specific sesquiterpenes in leaves is altered in mycorrhizal plants compared to control plants (Rapparini et al., 2006). Furthermore, plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi (Akiyama et al., 2005) which is a behavior occurring synchronized with the continuously plant growth, after the early contact. The latter mentioned, plus the fact that this compound is part of a class that is characterized to be hydrophobic, may possibly explain these values after drying, since drying might have enhanced the concentration of this compound in the leaves of the mycorrhizal water stressed plants.

Furthermore, the boxplot or box-and-whisker plots presented for this harvest larger plots than the previous 1$^{st}$ harvest in dry samples. Similar happen in the 2$^{nd}$ harvest in fresh in comparison with 1$^{st}$ harvest in fresh (which, as already discussed, it has to do with the biosynthesis rate characterized by the developmental stage of the plant). Even though the average of identified compounds didn't vary relevantly, since in the 1$^{st}$ harvest in fresh the sum of the totality of the identified compounds in control and in the treatments ranged from 81.67%-93.08% and in the 1$^{st}$ harvest in dry it varied from 88.3%-96.24%, the individual compounds did vary. Furthermore, in the 2$^{nd}$ harvest fresh, 25% of the data presented a higher relative percentage in comparison with the samples from the 1$^{st}$ harvest fresh, which is also seen in this 2$^{nd}$ harvest dry samples.




Graph 10: Variation of the relative percentage (%) of the totality of the 18 compounds from the 2nd Harvest of Mint (dry).

Regarding now the **Table 10** it is possible to infer that oxygenated monoterpenes are the dominant class followed by monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. To analyze these values, we may compare theses classes between the 2nd harvest in fresh with the 2nd harvest in dry of HS and HS+M treatment. In short, there was an increase of OM and a decrease of SH in HS treatment whereas in HS+M treatment there was a decrease of OM and an increase of SH. Sellami et al (2011) reported that drying *Laurus nobilis* L. increased the contents of sesquiterpene hydrocarbons which passed from 1.26% in fresh leaves to 11.45% in air dried leaves (Sellami et al., 2011). An increase was also stated with oven dried leaves but to a lesser extent. As for the oxygenated sesquiterpenes, they were reduced significantly under air drying (Sellami et al., 2011). The increase of SH and decrease of OM concerning the latter article is true for HS+M in comparison with the 2nd harvest in fresh. The latter might indicate that these results were due to an effect of the drying procedure. However, in HS the reverse occurred.

As mentioned previously, oxygenated monoterpenes are hydrophilic compounds which are found in high concentrations in the aqueous phase of the leaf. It might be that this decrease of OM in HS+M whereas in HS there was an increase in comparison with the 2nd harvest in fresh, might be based on the relative water content found in the leaves of HS+M, which as above asserted, is the highest (even more than in control condition) when a species of *Glomus* has as host a plant subjected to 60% FC. Possibly in HS the water loss might haven't been so detrimental since the leaves didn't had *a priori* a high relative water content, which probably explains the increase in OM. Nevertheless, the sum of the relative percentage of the identified compounds in the 2nd harvest in fresh and in the 2nd harvest in dry was for HS 85% and 84.5% respectively which meets the main purpose of the drying procedure. However, in HS+M the sum of the relative percentage of the identified compounds in 2nd harvest in fresh and in the 2nd harvest in dry was for HS+M 87% and 76.4% which is related with the decrease of OM in this treatment.

The differences between SO weren't relevant indicating that drying didn't had an effect. Regarding MH, there was a rather similarity between Control and HS+M, whereas in HS and M there was a 4.9% and 21.5% increase respectively in comparison with dry control. Certainly, these values have to do with the effects these treatments have previously in the plants, and drying enhanced, maintained or decreased those values depending on the actual concentration of the compounds, their solubility, and availability of water on the leaves.

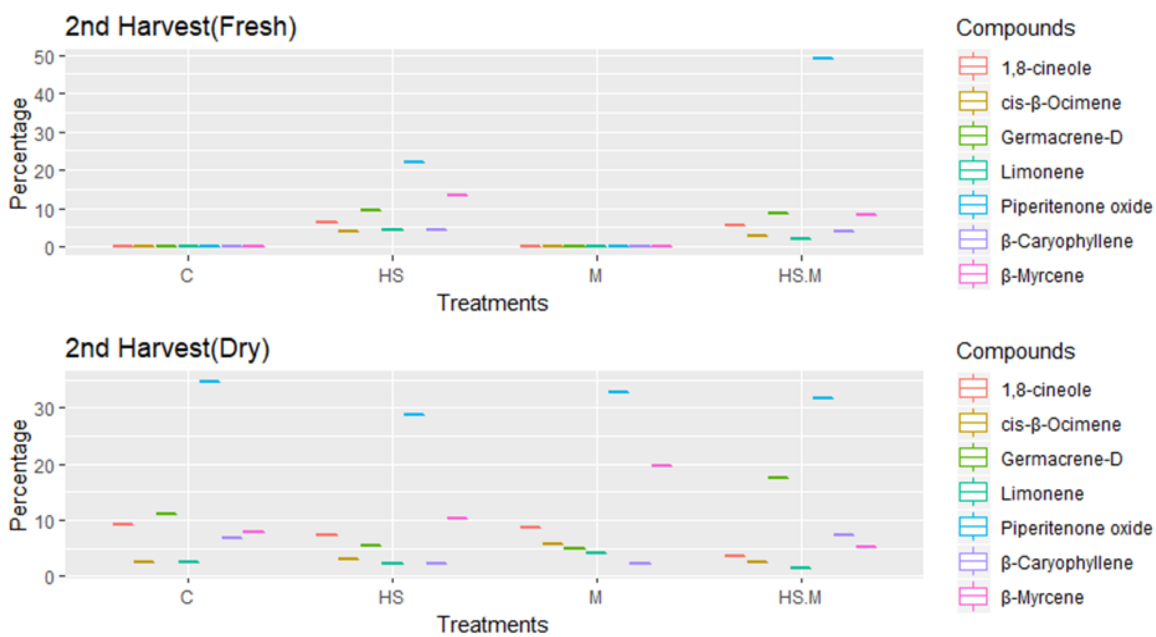
Table 10: Relative percentage of chemical classes of the essential oil from the 2nd harvest of Mint (dry). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | 16.8 | 21.7 | **38.3** | 11.0 | 22 |
| Oxygenated monoterpenes | 46.9 | **53.8** | 44.5 | 37.7 | 46 |
| Sesquiterpene hydrocarbons | 17.8 | 7.6 | 7.0 | **24.7** | 14.3 |
| Oxygenated sesquiterpenes | 1,5 | 1.4 | 0.9 | **3.0** | 1.7 |
| Sum | 83 | 84.5 | **90.7** | 76.4 | |

Now concerning prevalent compounds (>4.5%), **Graph 11**, we can see that the relative percentage between the fresh and dry HS and HS+M treatment didn't vary greatly except for Piperitenone oxide and Germacrene-D.

Nevertheless, and overall, in the treatments that is possible to compare, the sum of the identified compounds was similar, and the proportion of the majority of the prevalent compounds didn't differ relevantly. Drying seemed, for *Mentha* species, an interesting procedure to apply to drought stressed plants if the compounds aimed for are oxygenated.

Regarding Germacrene-D, which raised from 8.5% to 17.4% with the drying procedure in the samples from HS+M treatment this might have to do, as above mentioned, with the report that the drying of the peppermint leaves results in significant increments of this compound which possibly explains the value obtained for this treatment. However, the relative percentage of this compound decreased 4% in HS. Furthermore, Piperitenone oxide increased 7% in these samples from the 2$^{nd}$ harvest in dry in the HS treatment in comparison with the samples from the 2$^{nd}$ harvest in fresh whereas in HS+M the relative percentage decreased 17% in comparison with the samples from the fresh plants of the 2$^{nd}$ harvest. The latter two compounds, a sesquiterpene hydrocarbon and an oxygenated monoterpene had discerning values regarding the both treatments which indicate that besides drying other factors might be involved.




Graph 11: Comparison of the relative percentage of prevalent compounds (>4.5%) of Mint samples in fresh weight (upper graph) and dry weight (lower graph) of the 2$^{nd}$ harvest.

### 4.3.2 Basil

#### A. 1st Harvest and 2nd Harvest of Basil (Fresh)

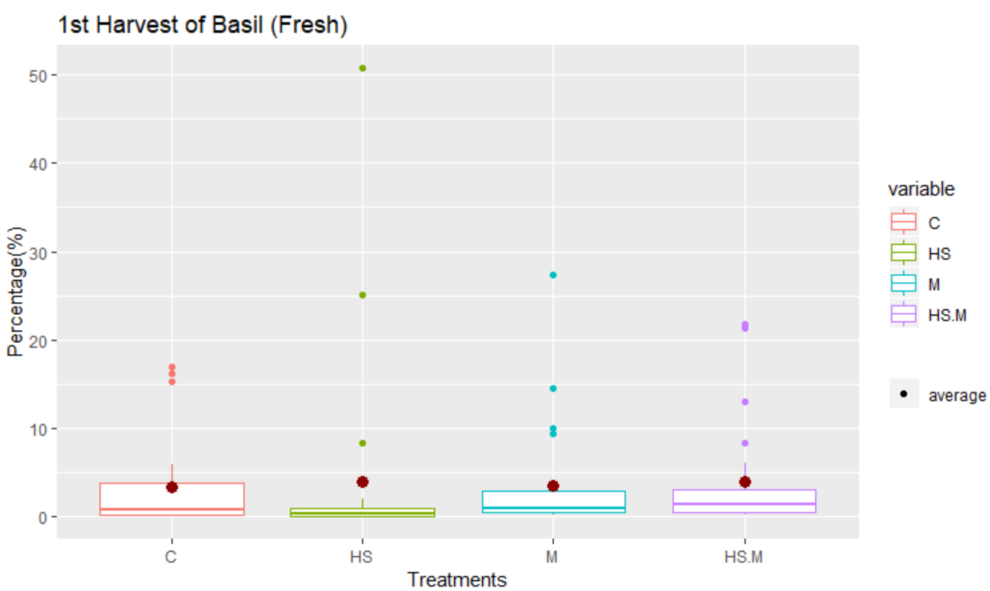
Examining the **Graph 12** it is suggested that basil responded differently to each treatment in terms of the proportion of compounds. Notwithstanding, the average of identified compounds was similar between the treatments and control. Nevertheless, HS treatment revealed 25% of the data with lower values (seen by the upper quartile) in comparison with control and the other treatments. Furthermore, the graph reveals some outliers in each of the treatments and control. In control it is Linalool (17%), Eugenol (16.2%) and *trans*-α-bergamotene (15.4%). In HS treatment it is Eugenol (50.8%), Linalool (25.1%), Methyleugenol (8.38%) and 1,8-Cineole (4%). In M treatment it is Linalool (27.3%), Eugenol (14.5%), Methyleugenol (9.3%) and *trans*-α-bergamotene (10%). Finally, in HS+M the outlier compounds are Linalool (21.8%), Eugenol (21.3%), Methyleugenol (13.1%) and *trans*-α-bergamotene (8.3%).

Regarding the control, the values latter mentioned are in accordance with Miele et al (2001) study of this cultivar which reported that *Ocimum basilicum* L. cv. Genovese Gigante presents linalool as the main component (Miele et al., 2001). However, the latter researches further assert that it is important to note that at the sites with a mild climate the plants had eugenol as prevalent in the essential oil and that on the contrary, plants grown in northern localities were rich in methyleugenol. The latter is interesting since one of the characteristics that defines these climates is the amount of water. The results of HS suggest that eugenol is the most prevalent compound instead of Linalool. In addition, previous studies with basil (*Ocimum basilicum* L.) showed that drought stress increased the amount of methylchavicol, methyleugenol, β-myrcene and α-bergamotene, whereas the maximum relative amount of these compounds was observed at 50% field capacity (Mandoulakani et al., 2017). Similarly, HS had the relative percentage of 8.38% of methyleugenol whereas control had 6%. The control treatment had the lowest amount of methyleugenol in comparison with the other three treatments whereas HS+M (13.1%) had the highest amount followed by M and HS. It is suggested that methyleugenol derives from eugenol by a specific methylation involving an S-adenosylmethionine dependent O-methyltransferase (EOMT). The strict correlation between methyleugenol and eugenol found in basil has been further described in the sense that the activity of the invoked enzyme decreases when plants grow (Miele et al., 2001). Nevertheless, in initial stages of growth (1st harvest) it was expected that methyleugenol wouldn't reveal trace amounts. The higher amounts of methyleugenol in HS+M and HS may be related to, according to Mandoulakani et al (2017), that drought stress probably increases the methylchavicol and methyleugenol content, in part, through increasing the expression levels of EOMT (Mandoulakani et al., 2017). Still, M also presented a high relative percentage of methyleugenol in comparison with control. A study by Copetta et al (2007) reported that in *Ocimum basilicum* L. plants inoculated with *Gi.margarita* the activity of EOMT did not decrease (Copetta et al., 2007). This information and the latter about hydric stress may explain the high amount of methyleugenol in HS+M followed by M and HS treatment in comparison with the control, with lowest amounts. However, eugenol, which has a strict correlation, metabolically, with methyleugenol, didn't present the highest amounts in the reverse order of methyleugenol, it was in decreasing order HS>HS+M>C>M which further indicate that some of these values are not related solely on constitutive production. In drought stress there is an increase of EOMT activity as above mentioned, explaining the high amount of methyleugenol, however eugenol didn't decrease, it presented a high relative amount, particularly in HS treatment. The latter may have to do with the prevalence of the latter compound in mild climates and also, depending on the chemotype, O-methyltransferase activity does not accept eugenol as a substrate (Lewinsohn et al., 2000). Also, the high amount of eugenol and the high amount of methyleugenol in HS+M might be related to the joint effect of mycorrhizae and drought stress. As above mentioned the enzymatic activity with mycorrhizae doesn't decrease and is maintained while it is reported that it increases with drought stress.

Focusing on *trans*-α-bergamotene, another study that tested three different *Glomus* species, and NM (non-mycorrhizae) effect on essential oil profile of basil showed that *Glomus intraradices* treatment had the lowest relative percentage of this compound in comparison with the other treatments (Rasouli-Sadaghiani et al., 2010) which is in accordance with the results obtained since this compound had the highest relative percentage in the control condition (15.4%) in comparison with M (10%) and HS+M (8.3%). However, HS had even lower amounts of *trans*-α-bergamotene than control and the two treatments. This is contrary to the results obtained by Mandoulakani et al (2017) as mentioned above, who reported an increase of this compound with drought. However, according to Palmer-Young et al (2015) the sesquiterpenes, (E)-ß-farnesene and (E)-α-bergamotene, may function instead against biotic stresses since emission is more responsive to herbivory than to light, temperature, and humidity (Palmer-Young et al., 2015) which may indicate that the trigger of synthesis of these compounds is more responsive to biotic factors than drought which possibly explains the lower relative percentage of *trans*-α-Bergamotene in HS treatment.

Linalool had the highest relative percentage with mycorrhizae treatment, which in decreasing order of relative percentage it was M>HS>HS+M>C. This is accordance with a study with coriander that came to the results that linalool (which enhance the essence quality), increased in inoculated plants in comparison to non-inoculated plants in non-sterile soil. They reported a relative percentage difference for this compound of 364.40±24.40 ($\mu g\ g^{-1}$) in control and 871.36±159.95 ($\mu g\ g^{-1}$) in inoculated coriander (Rydlová et al., 2015). Furthermore, and contrary to the results of this experiment, other study with *Ocimum basilicum* L. came to the results that linalool profiles were 66.30% (in non-mycorrhizal plants), 65.26% (with *Glomus intraradices*) and 66.74% (with *Glomus etunicatum*) (Rasouli-Sadaghiani et al., 2010). The latter study is contrary to our results since the inoculation with *Glomus intraradices* in this cultivar, raised the amount of Linalool from 17% (control) to 27.3% (M treatment). However, in accordance with our results is a study by Aslani et al (2014) which stated that the concentration of Linalool increased by 10 and 8% on inoculation with *G. mosseae* and *G. intraradices* of basil respectively as compared to control in non-water stressed conditions. Furthermore, Aslani et al (2014) added that in the mild stress condition the concentration of Linalool increased by 13 and 15% on inoculation with *G. mosseae* and *G. intraradices* respectively as compared to control (Aslani et al., 2014). Alike results were obtained in this study, where HS+M treatment had, in comparison with control, a relative percentage of Linalool 5% higher. Hence, also HS had 8.1% higher relative percentage of Linalool compared with control.

Another compound that is an outlier is 1,8-cineole. This compound revealed its highest relative percentage in HS, 4%, and HS+M, 2.5%, treatments while in control and mycorrhizae treatment it had low amounts, 0.42% and 0.69% respectively. A study with *Salvia officinalis* L. came to the results that moderate water deficit increased the content of main volatile constituents – camphor, α-thujone and 1,8-cineole (Bettaieb et al., 2009). The latter was validated by other researcher that tested salvia, lemon catmint and lemon balm under moderate water stress (Manukyan, 2011). Furthermore, the latter researchers reported that cineole synthase and bornyl-pyrophosphate-synthase were strongly up regulated under drought stress, revealing a time course similar to that of the dehydrin (DHN) up-regulation (DHN is a multi-family of proteins present in plants that is produced in response to cold and drought stress according to Puhakainen et al (2004)). It seems that drought has a great relevance for the upregulation of these synthases, possibly explaining the results obtained for 1,8-cineole in HS and HS+M.




Graph 12: Variation of the relative percentage (%) of the totality of the 24 compounds from the 1st harvest of Basil (fresh).

Now examining the **Table 11**, we have a set of classes of compounds called 'Others' which include in these samples of basil the two compounds eugenol and methyleugenol, which are phenylpropanoids. It is possible to assert that Others is the dominant class of compounds followed by OM, SH, OS and MH. It is inferred that MH had the highest relative percentage in HS+M treatment, OM in M treatment, SH in control, OS in HS+M and Others in HS.

Results obtained so far by other researchers suggest that the AM fungal-mediated increase in concentration of terpenoids is due to enhanced production of IPP/DMAPP derived from the MEP pathway (Mandal et al., 2015). Thus, Venkateshwaran et al (2014) also reported that mevalonic acid (MVA) is crucial for the transduction of symbiotic signals produced by AM fungi to induce symbiotic gene expression in plants (Venkateshwaran et al., 2014). The latter may explain the relatively high values of the class of oxygenated monoterpenes and monoterpene hydrocarbons and the class of oxygenated sesquiterpenes in the treatments of M and HS+M. However, SH had the highest relative percentage in control (32.6%) followed by M (22.5%), HS+M (17.9%) and by HS treatment with trace amounts. The latter decreasing order of relative percentage might indicate a detrimental effect of drought. Indeed, a study by Caser et al (2017) with *Salvia sinaloensis* L. asserted that oxygenated sesquiterpenes increased with 50% FC while the sesquiterpene hydrocarbons decreased (-28.5%) (Caser et al., 2017). This may explain the values obtained with HS+M treatment, the second treatment with lowest relative percentage of SH and HS treatment. Furthermore, AMF has also been proved to decrease the percentage of SH independently of the stage of inoculation (early or late). The latter might indicate that hydrocarbon sesquiterpenes (mainly synthetized through the MVA pathway) were the most pivotal for the transduction of symbiotic signals produced by AM fungi. According to Babikova et al (2014) AM fungi negatively affects production of sesquiterpenes particularly the sesquiterpenes (E)-caryophyllene and (E)-β-farnesene (Babikova et al., 2014) which are sesquiterpenes hydrocarbons.

Concerning the phenylpropanoids, the highest relative percentage was in HS treatment. Gershenzon (1984) asserted that water stress could lead to increased levels of phenolic compounds by reducing the rate of growth leading to a buildup of lignin precursors, which are then metabolically diverted to various phenolics (Gershenzon, 1984). The latter is corroborated by other authors that stated that stress-induced increase in the activity of phenylalanine ammonia-lyase (PAL) may be regarded as the beginning of the acclimation of cells facing water stress (Hazzoumi et al., 2017; Luna et al., 2015). PAL is the entry-point enzyme of the general phenylpropanoid pathway, which channels L-Phe from the primary metabolic pool to the synthesis of trans-cinnamic acid (t-CA). The t-CA produced is then further transformed into many phenolic compounds (Zhang et al., 2015). The latter assertions may explain the values of phenylpropanoids which were in decreasing order of relative percentage HS>HS+M>M>C since the impact of drought stress in PAL activity have been described to be notorious. Furthermore, the values of control and M treatment didn't differ significantly which may indicate that HS is the main treatment affecting the biosynthesis of this class of compounds. Nevertheless, members of different classes of plants defense genes, including phenylpropanoid metabolism enzymes have also been detected in plant cells containing arbuscules (García-Garrido et al., 2002).

Monoterpene hydrocarbons didn't vary relevantly between treatments. Still, HS+M presented the highest relative percentage followed by M, HS and control. Monoterpene hydrocarbons have been shown to ameliorate abiotic stresses in a number of plant species via two proposed mechanisms: membrane stabilization and direct antioxidant effects (Palmer-Young et al., 2015). The latter possibly explains the slightly higher relative percentage of this class of compounds in this 1$^{st}$ harvest in comparison with the control, since drought is an abiotic stress and mycorrhizae also induces the plant transient defense system in the early contact which possibly further enhanced the synthesis of this class of compounds.

Table 11: Relative percentage of chemical classes of the essential oil from the 1$^{st}$ harvest of Basil (fresh). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | 0.5 | 1.4 | 2.6 | **3.8** | 2.1 |
| Oxygenated monoterpenes | 20.3 | 30.6 | **31.8** | 28.9 | 28 |
| Sesquiterpene hydrocarbons | **32.6** | 0.4 | 22.5 | 17.9 | 18.4 |
| Oxygenated sesquiterpenes | 3.2 | 4.1 | 3.0 | **8.4** | 4.7 |
| Others | 22.3 | **59.2** | 24.0 | 34.6 | 35 |
| Sum | 78.9 | **95.7** | 83.9 | 93.6 | |

Examining now **Graph 13** it is possible to examine the prevalent compounds (>4.5%) from the samples.

Regarding 1,8-cineole, an oxygenated monoterpene, it is possible to infer that the highest relative percentage was in HS samples in comparison with the other two treatments and control. As mentioned above cineole synthase is strongly up regulated in drought conditions. Furthermore, a study with *Thymus vulgaris* L. by Llorens-Molina et al (2006) noted that the main component (1,8-cineole) rate showed a high and significant increase during the drought period, the same way the oxygenated monoterpenes in whole (Llorens-Molina et al., 2006). The latter is in accordance with the results obtained, for 1,8-cineole and for the class of compounds.

Regarding Linalool, the relative percentage was higher in M treatment. Even though AMF–plant symbiosis appears to increase the transcription of the MEP pathway (related to the synthesis of monoterpenes), the exact processes are not fully understood (Walter et al., 2000). A study by Zolfaghari et al (2013) with *O. basilicum* L. came to the results that Linalool with *G.intraradices* and control was 31.05% and 21.75% respectively (Zolfaghari et al., 2013). Similarly, in this study there was an increase of the relative percentage of Linalool of 10% from control to mycorrhizae treatment which may reflect an influence of the fungus. Furthermore, a study by Prasad et al (2011) tested the effect of the application of metals and AMF and reported that the content of linalool in sweet basil oil was increased by the inoculation with AM fungi without the application of metals (Prasad et al., 2011).

Concerning Eugenol, this compound had a higher relative percentage in HS. It is important to emphasize that while the metabolic energy spent on growth decreases when plants are subjected to drought stress, the PAL activity increases. PAL is an enzyme with an active role on the biosynthesis of the main fragrance phenylpropanoids compounds in glandular peltate trichomes of *Ocimum basilicum* L. (Xie et al., 2008). Furthermore, the raise in relative percentage of the main constituents and also the relative percentage of the sum of the identified compounds under drought stress have been reported in both *O. basilicum* L. and

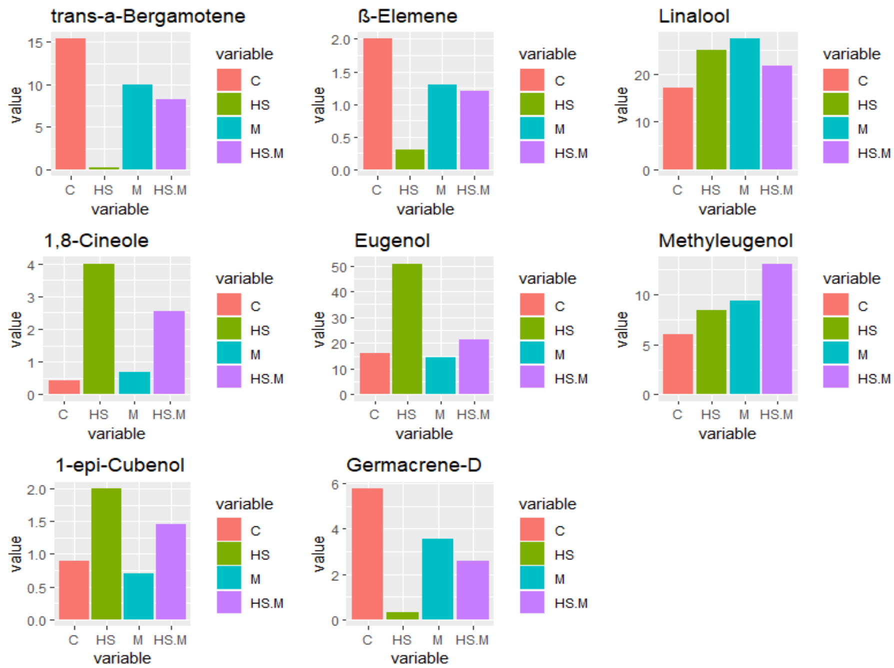
*O. americanum* L. (Khalid, 2006). In fact, the sum of the classes of compounds was higher in HS, 95.7%, whereas control presented 78.9%.

Methyleugenol graph reveals a rather interesting effect, maybe additive, since it is possible to assert that the relative percentage of this compound in increasing order was C(6%)>HS(8%)>M(9%)>HS+M(13%). Prasad et al (2011) asserted that the AM fungal inoculation maintained the level of methyl chavicol and methyl eugenol in sweet basil oil, which were either increased or decreased by the application of metals (Prasad et al., 2011). This is in accordance with our results in the aspect that methyleugenol relative percentage was rather similar between the control and M treatment (an increase of 3%). HS and HS+M also presented an increased relative percentage of this compound in comparison with control, of 2% and 7% respectively. As already mentioned, drought stress probably increases the methylchavicol and methyleugenol content, in part, through increasing the expression levels of CVOMT and EOMT (Mandoulakani et al., 2017) and PAL. Indeed, phenylalanine ammonia-lyase activity is a key early enzyme in this pathway whereas the last biosynthetic step is dependent on O-methyltransferase activity (Lewinsohn et al., 2000). HS+M possibly revealed a higher amount of methyleugenol since M maintains and or/slightly increases the activity of the enzymes and drought increases the activity of these enzymes, which may have led to this additive effect.

Concerning *trans*-α-bergamotene, this compound had the highest relative percentage in control. Copetta et al (2007) who tested the influence of arbuscular mycorrhizal fungi on growth and essential oil composition in *Ocimum basilicum* L. cv. Genovese came to the results that the compound α-bergamotene in control had the highest relative percentage in comparison with *Glomus mosseae*, with *Gigaspora margarita* and with *Gigaspora rosea* (Copetta et al., 2007). This is in accordance with our study since the relative percentage of this compound in the M treatment was lower than in control. Thus, HS+M even had lowest amounts. Still, drought stress has also been reported to be deleterious to sesquiterpene hydrocarbons synthesis as above mentioned.

Regarding Germacrene-D, this compound also presented the highest relative percentage in the samples of control. According to Caser et al (2017) drought stress applied to *Salvia sinaloensis* L. revealed a very sharp decrease (4.4 times) for Germacrene D (from 22.0 to 5.0% in full irrigation and no irrigation, respectively) (Caser et al., 2017). Aziz et al (2008) also reported a decline in p-cymene, germacrene and caryophyllene in *Thymus vulgaris* L. affected by 4 different irrigation levels (Aziz et al., 2008). Thus, a study with *A. annua* L. asserted that in control, in AMG (mycorrhizal plants inoculated with a single species of *Glomus* fungus) and AMM (mycorrhizal plants inoculated with a mixture of different *Glomus* species and bacteria) the values of Germacrene-D were 0.68 ± 0.17, 0.50 ± 0.10 and 0.50 ± 0.13 (%) respectively (Rapparini et al., 2006). The last-mentioned possibly explains the trace amounts in HS treatment and lower amounts of this compound in HS+M and M in comparison with control.

Concerning β-Elemene, the latter compound had the highest relative percentage in Control followed by M, HS+M and HS. The values obtained for HS are in accordance with a study with turnip-rooted parsley which was subjected to a deficit irrigation level (45-60% FC) similar to the one applied in this experiment and it was noted a decrease of β-Elemene from 0.66% in control to 0.3% in the water deficit condition (Petropoulos et al., 2007). Also, a study with *Piper nigrum* L. inoculated with AMF reported a decrease from 1.2% in control to 0.2% in inoculated plants and also a decrease of the class of compounds SH from 25.0% in control to 10.2% in inoculated plants. The latter is in accordance with the values obtained for this compound and the values obtained in this class of compounds, a decrease (in comparison to control) of 10% in the M treatment. In regard to 1-*epi*-cubenol the highest relative percentage was in HS. Similarly, a study with salvia came to the results that under moderate water stress (50%) the values obtained for oxygenated sesquiterpenes were 90.52 ± 1.63(μg g−1 DW) in control and 900.74 ± 2.74 (μg g−1 DW) (Bettaieb et al 2009) which possibly explains the highest values for this compound in HS followed by HS+M. In addition, the class of compounds OS was also higher in the latter two treatments.



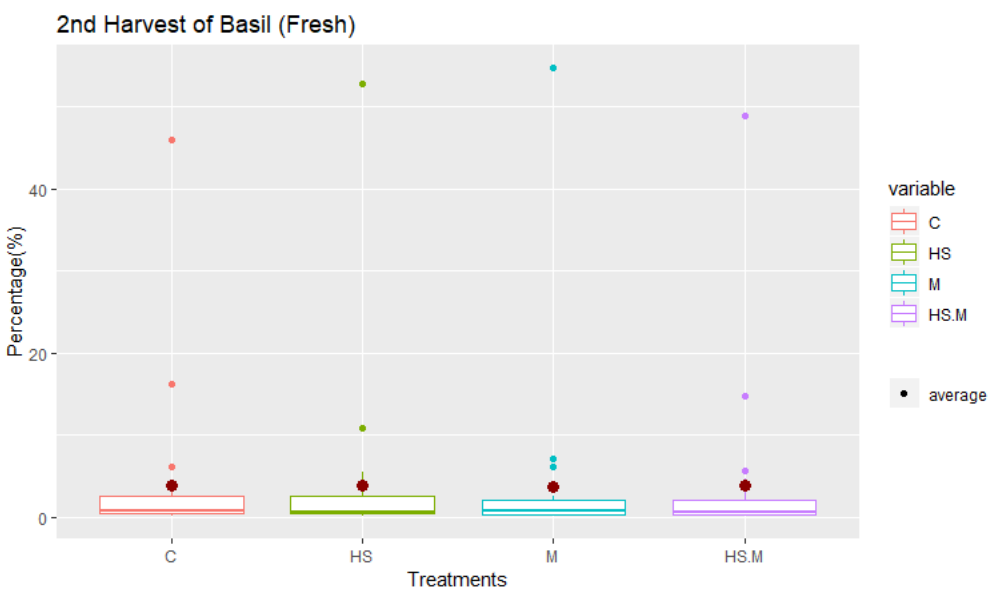

Graph 13: Relative percentage of individual chemical compounds of the 1st harvest of Basil (fresh).

Considering the 2nd harvest, in fresh, it is possible to infer by the **Graph 14** that the average was the same between control and treatments. In addition, it is possible to assert that from 1st to the 2nd harvest there was a decrease of essential oil content of 25% of the data, seen by the upper quartile, of Control, M and HS+M treatment, except for HS. The latter might be related to the assumption that young leaves display much higher levels of enzyme activity than more developed leaves in basil (Deschamps et al., 2010). Also, it has been reported with basil that total terpene content is negatively correlated with total phenylpropene content and PAL activity (Iijima et al., 2004) which possibly explains the boxplot size of HS treatment in this stage. Furthermore, the compounds that are outliers in Control are Eugenol (16.2%), Linalool (46%) and 1,8-cineole (6.2%). In HS is Eugenol (10.9%) and Linalool (52.8%). In M treatment it is 1,8-cineole (6.1%), Eugenol (7.1%), and Linalool (54.7%). Finally, in HS+M treatment it is 1,8-cineole (5.6%), Eugenol (14.7%) and Linalool (48.9%).

Regarding Linalool it is possible to assert that the treatment that had highest relative percentage of Linalool is the M treatment with 54.7% whereas control had 46%. In addition, HS had 52.8% and HS+M 48.9%. However, in all the treatments there was a raise of the relative percentage of this compound from the first to second harvest and the decreasing order of higher relative percentage of this compound was the same in both harvests. This in accordance with other studies who concluded that maximum linalool content is obtained from the inflorescence at the commencement of flowering in basil (Nurzyńska-Wierdak et al., 2012).

Considering Eugenol, in comparison with control there was a decrease of the percentage in all the treatments. Control condition presented the highest relative percentage of this compound in this 2$^{nd}$ harvest which presented an equal relative percentage in the 1$^{st}$ harvest. Investigations of EOMT enzyme activities in the phenylpropene pathway in basil suggest that this pathway may be developmentally regulated (Lewinsohn et al., 2000; Gang et al., 2002). The young developing tissues appear to be the primary sites of essential oil biosynthesis, as young leaves display much higher levels of enzyme activity than more developed leaves, where the activity is negligible (Deschamps et al., 2010) as previously mentioned. This is in accordance with the results obtained in the second harvest since methyleugenol decreased to trace amounts, which indicates a decrease in the activity of EOMT; also, eugenol decreased for all treatments in comparison with the first harvest which possibly reflects the developmental regulation. However, Eugenol in the samples from HS, M and HS+M samples had a substantially reduced relative amount of this compound in comparison with control which presented a minor decrease (-0.07%). The values obtained with HS might be related to the prolongation of the abiotic stress. It is stated that the activity of PAL significantly increased in the leaves of *Trifolium repens* L. under the early stages of drought stress but decreased gradually as the period of stress was extended (Lee et al., 2007; Wang et al., 2016). Since these are samples from the 2$^{nd}$ harvest this up and down regulation of the enzyme activity may explain the values obtained for this compound in the HS treatment and possibly the HS+M treatment. However, M even presented a lower amount of Eugenol. Phenylpropanoids as mentioned, are fundamental regulating factors during the early events of AM symbiosis. Nevertheless, it has been reported by Volpin et al (1994) that after reaching maximum levels of PAL, CHI, and chitinase activity the activities decline rapidly. The authors conclude it is reasonable to suggest that following the fungal colonization, a nonspecific defense response is induced, which is subsequently specifically depressed (Volpin et al., 1994).

Furthermore, in M, HS+M and Control, 1,8-cineole appeared as an outlier in the box-and-whisker plot. However, there was a raise from the 1st to the 2nd harvest in all the treatments and control where values ranged from 5.5-6.2%. Possibly, cineole synthase has more activity further in the development stage in basil. Indeed, according to Lee et al (2016) the concentration of the compounds such as 1,8-cineole, camphene, camphor, *cis*-thujone, limonene and *trans*-thujone were the lowest during early stages of development in other essential oil-bearing plants (Lee et al., 2016). Since the differences by this stage of harvest weren't striking between treatments it is possible that in fact these values obtained for this compound are more related to phenological factors.




Graph 14: Variation of the relative percentage (%) of the totality of the 24 compounds from the 2nd Harvest of Basil (fresh).

Regarding the values related to the classes of compounds (**Table 12**) it is possible to state that the dominant class was OM, followed by Others, SH, OS and MH. Furthermore, in control the classes SH and Others presented the highest relative percentage. Also, MH relative percentage was higher in control, however, it was rather similar to the three treatments. In M treatment the OM had the highest relative percentage and OS had the highest relative percentage in HS and HS+M.

Concerning the sum of the classes of monoterpenes (oxygenated plus hydrocarbon) it is inferred that in decreasing order the values were: M treatment(66.5%)>HS treatment(65.8%)>HS+M treatment(61.5%)>Control (58.9%). This is in accordance with results obtained in a study with *A. annua* L. where the total amount of monoterpenes emitted by mycorrhizal plants was moderately higher than that detected in non-mycorrhizal plants (Rapparini et al., 2006) which implies an enhanced biosynthesis. Concerning the sesquiterpenes, the sum of this class (oxygenated plus hydrocarbon) in decreasing order of relative percentage was Control(17.3%)>HS(15.9%)>HS+M(15.6%)>M(14%). Regarding Others, it was expected to see a decrease in all treatments and control in comparison with the first harvest since it is well known that methyleugenol decreases to trace amounts with the increasing height of the plant and decreasing activity of EOMT and PAL as described before. Accordingly, from the first to the second harvest in all treatments there was a decrease of the relative percentage of this class of compounds. However, this decrease was less accentuated in control. As mentioned above, the PAL activity is increased under the early stages of drought stress but decreases gradually as the period of stress is extended. A study by Chakraborty et al (2002) of the water stress response of various tea plants came to the results that PAL activity is initially increased after which the activity declined steadily (Chakraborty et al., 2002). It is possible that for HS the values obtained might have to do with prolonged water stress, possibly revealing in this way the pronounced decrease from the 1$^{st}$ to the 2$^{nd}$ harvest in comparison with control. Concerning the M treatment, it is known that in the mycorrhizae infectious process, plant cells usually respond by increasing the level of pre-existing antifungal phenols at the infection site, after an elicited increased activity of the key enzymes (PAL and chalcone synthase) of the biosynthetic pathway (Lattanzio et al., 2006). In fact, PAL is the first enzyme in a pathway leading to multiple products besides lignin and is inducible by wounding, light and developmental signals in addition to fungal infection (Allen et al., 1992). The accentuated decrease from the 1$^{st}$ to 2$^{nd}$ harvest in this class of compounds might be related to the fact that after the early contact the plants possibly adjust to the symbiotic fungi, possibly reflecting a change of PAL activity and subsequent EOMT activity. In fact, after reaching a maximum, activities of all enzymes (PAL and others) decline to those of uninoculated roots (Volpin et al., 1994). This might explain the values for M treatment since like in the drought stress, when the contact with mycorrhizae is prolonged there is no more recognition process needed between plant and the fungus which possibly mirrors the values obtained in the class of compounds Others.

Furthermore, comparing the classes of compounds from both harvests (Attachment VI) it is possible to postulate that from the 1$^{st}$ to the 2$^{nd}$ harvest of basil in control and the three treatments there was an increase of oxygenated monoterpenes in detriment of the class of compounds 'Others'. Furthermore, except for HS treatment, from the first to the second harvest, there was a decrease in sesquiterpene hydrocarbons. The results obtained in HS treatment could be explained by the fact that when drought is prolonged plants drastically stop sesquiterpene release (Ormeno et al., 2007). Nevertheless, in general, the values obtained in this second harvest are in accordance with the statement by Lemberkovics et al (1993) which studied the relationship between essential oil and flavonoid biosynthesis in sweet basil. The latter researchers assert that at early stages of flowering, linalool and sesquiterpenes constituted 40–60% and 5–20% respectively (Lemberkovics et al., 1993). Inspecting control condition, the latter assertions about the early stages of flowering are in accordance with our results since the totality of sesquiterpenes decreased from the 1$^{st}$ (36%) to the 2$^{nd}$ harvest (17%) and linalool increased from the 1$^{st}$ (17%) to the 2$^{nd}$ harvest (46%).

Furthermore, the decrease of the class of compounds Others due to ontogeny which is related to the decrease of PAL activity is related to the increase of other classes of compounds since biosynthetic enzymes in the terpene pathways, coupled with lower levels of PAL activity, tend to restrict the phenylpropene pathway and increase the flux in the terpene pathway (Iijima et al., 2004).

Table 12: Relative percentage of the chemical classes of the essential oil from the 2nd harvest of Basil (fresh). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | **3.5** | 3.1 | 2.5 | 2.9 | 3 |
| Oxygenated monoterpenes | 55.4 | 62.7 | **64.0** | 58.6 | 60.2 |
| Sesquiterpene hydrocarbons | **12.6** | 10.9 | 11.6 | 10.6 | 11.4 |
| Oxygenated sesquiterpenes | 4.7 | **5.0** | 2.4 | **5.0** | 4.3 |
| Others | **16.5** | 11.6 | 7.4 | 15.0 | 12.6 |
| Sum | 92.7 | **93.3** | 87.9 | 92.1 | |

Now in the **Graph 15** it is possible to examine prevalent compounds (>4.5%) individually.

Regarding 1,8-cineole there is no great variation between treatments since it is in the range of 5.5%-6.2%. However, there was a raise in all the treatments in comparison with the 1st harvest which has more to do with developmental regulation since this compound relative percentage is characterized to be low at early stages of development as mentioned above.

Linalool also didn't reveal great variation; the range was between 46%-55%. Still, M treatment had the highest relative percentage. Same was obtained in other study with *O. basilicum* L. where the range of Linalool in control and with three AMF species varied from 63.85%-66.74% with the highest relative percentage obtained with inoculated plants (Rasouli-Sadaghiani et al., 2010).

Eugenol had the highest relative percentage in Control and the lowest in M. This as mentioned previously may have to do with the activity of phenylalanine ammonia-lyase (PAL). This key enzyme in phenylpropanoid metabolism, was increased in tomato roots inoculated with *G. mosseae* (Dehne et al., 1979) and in roots of *Medicago sativa* inoculated with *G. intraradices*, but only at the early stage of infection; the increase in PAL activity coincided with the time of colonization (Volpin et al., 1994). Similarly, in HS as mentioned before PAL activity is initially increased after which the activity declines steadily when drought is prolonged. Interestingly, the level of phenylalanine ammonia-lyase was found to decrease with mild to moderate water stress and to recover readily with rewatering (Bardzik et al., 1971) indicating an acclimation of the plants to the abiotic factor. Since these values are from the $2^{nd}$ harvest, not coinciding with the time of colonization and the water stress is considered prolonged by this stage, the decrease of eugenol in the treatment of HS+M may be explainable by the latter mentioned, nevertheless, the individual treatments (HS and M) presented an even lower amount of this compound, eugenol.

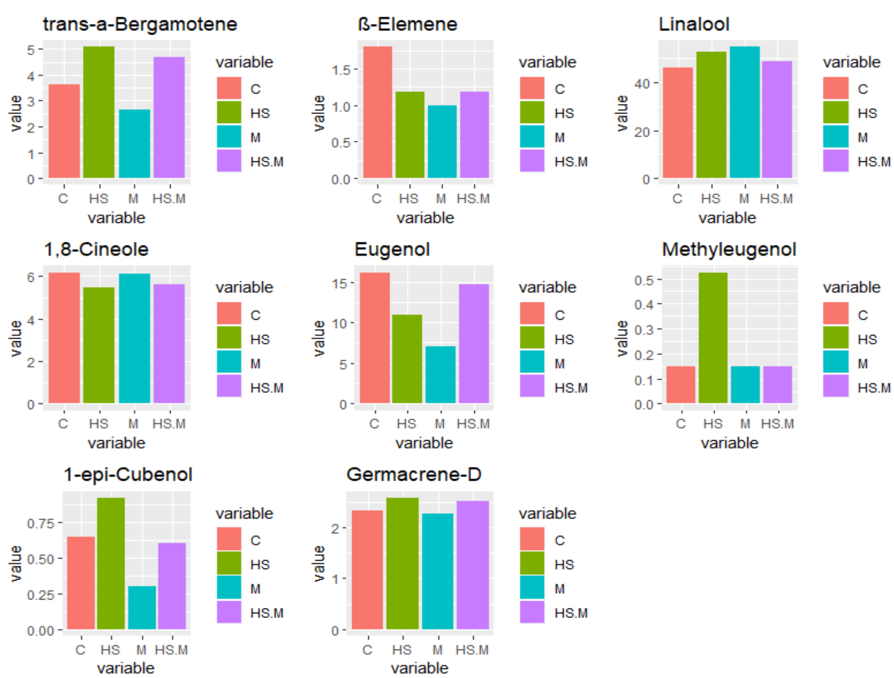
Regarding methyleugenol, the range of relative percentage between treatments and control was 0.15-0.53%. These values seem to be more related with ontogeny. According to Miele et al (2001) *O. basilicum* L. cv. Genovese Gigante plants 3.5-6.5 cm high contain methyleugenol as prevalent whereas in taller plants eugenol is largely predominant. The latter researchers concluded that basil taller than 16 cm presents eugenol as the main compound and methyleugenol is greatly reduced (Miele et al., 2001). By the time of this harvest the plants from this experiment had already surpassed the 40 cm which explain the values obtained for methyleugenol in all the treatments and control.

Considering *trans*-α-bergamotene the values indicate that in decreasing order of higher percentage of this compound was in HS, followed by HS+M, control and M. In accordance with these results is a study with oregano which revealed that in mild water stress the EO samples revealed a higher relative percentage of this compound ($0.81 \pm 0.08\%$) than control ($0.53 \pm 0.39\%$) (Morshedloo et al., 2017). Similarly, in other study with basil, the plants which were water supplied with 55% FC had 6.94% relative percentage of *trans*-α-bergamotene whereas in control this compound presented 4.4% (Omidbaigi et al., 2003). M treatment had the lowest relative percentage of this compound which is in accordance with a study by Copetta et al (2006) that inferred that in control, with *Glomus mossea*, with *Gigaspora margarita* and *Gigaspora rosea* the values from this compound were respectively $17.25 \pm 5.01$, $17.05 \pm 3.89$, $13.56 \pm 2.52$, and $13.99 \pm 6.91$(%) (Copetta et al., 2007).

Furthermore, germacrene-D ranged from 2.26%-2.58% in this $2^{nd}$ harvest, so no significant changes between treatments were observed and a rather maintenance is seen from the $1^{st}$ to the $2^{nd}$ harvest.

Regarding β-Elemene it is possible to infer that the highest relative percentage was obtained in control, followed by HS+M and HS (with similar values) and M. It is possible to note that from the 1st to the 2nd harvest there was an increase of the relative percentage of this compound in HS samples of 1% whereas in control, M and HS+M it is seen a decrease of 0.2%, 0.3% and 0.02% respectively. These minor changes possibly are more related with ontogeny since the values for this compound in other report ranged from 1.2-1.4% in the vegetative stage, budding stage and beginning of flowering stage in sweet basil (Nurzyńska-Wierdak et al., 2012).

Finally, the compound 1-*epi*-cubenol had the highest relative percentage in HS treatment samples, similar to what was observed in the first harvest. This has to do with the above mentioned that when drought is prolonged the sesquiterpenes emission is drastically reduced, possibly explaining a higher internal concentration of the latter.




Graph 15: Relative percentage of individual chemical compounds of the 2nd harvest of Basil (fresh).

Concerning both the relation between eugenol and methyleugenol it is possible to infer that to a higher extent these compounds are very influenced by ontogeny and subsequent enzymatic activity. Regarding eugenol, authors like Fischer et al (2011) indicate that in basil the levels of eugenol ranged from 9.3% in the older leaf at position 2 to a maximum of 53.6% in the younger leaf at position 8 (Fischer et al., 2011) which explains the decline in control and the three treatments of this compound in the 2nd harvest. In accordance with the last-mentioned study, Renu et al (2014) reported that there is a higher level of EOMT transcripts during juvenile and pre-flowering stages and conversely, a remarkable decrease of EOMT transcripts at post-flowering stages of several chemotypes indicating an extensive transcriptional reprogramming associated with decreased accumulation of the metabolites (Renu et al., 2014). Similarly, in the flowering stage (Fig.17), when the 2nd harvest was performed, the essential oil samples presented very low amounts of methyleugenol and eugenol, which decreased from the 1st to the 2nd harvest. Also, the decrease of eugenol might be associated with a rapid turnover for eugenol, which might be used by the plant for lignin biosynthesis (Manitto et al., 1974). It has been proposed that monolignol biosynthesis in phenylpropene-emitting tissues is shared with the lignin biosynthetic pathway. Nevertheless, the scent biosynthesis is prioritized over lignin formation in petals (Muhlemann et al., 2014).

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Fig.17: Reproductive stage of basil by the time of the 2nd harvest.

### B. 1st Harvest and 2nd Harvest of Basil (Dry)

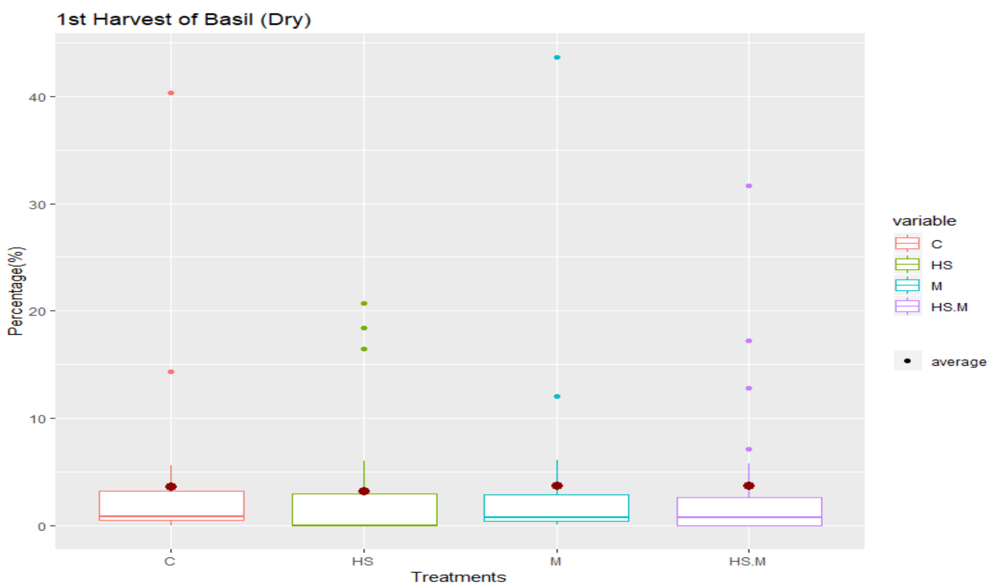
Concerning the first harvest of basil, but this time subjected to drying and then distillated it is possible to infer by the **Graph 16** that there are some differences between treatments. Nevertheless, the average is similar between control and the treatments. In control the outliers are *trans*-α-bergamotene with 14.4% and Linalool with 40.3%. In HS treatment it is 1-*epi*-cubenol with 16.5%, Linalool with 18.4% and 1,8-cineole with 20.7%. In M treatment it is *trans*-α-bergamotene with 12.1% and Linalool with 43.6%. In HS+M it is Eugenol with 7.14%, 1,8-cineole with 12.8%, β-elemene with 17.2% and Linalool with 31.6%.

Analyzing the compounds which where emphasized by the graph that weren't present in this higher amounts in all treatments is the 1-*epi*-cubenol in HS with 16.5% (whereas control had 5.6%), and β-elemene in HS+M with 17.2% (whereas control had 0.4%). The relative percentage of 1-*epi*-cubenol in HS possibly is in accordance with a study of *Ocimum basilicum* L. subjected to different irrigation regimes who had cubenol at 100% field capacity in the percentage of 0.56% and at 55% field capacity had 0.75% (Omidbaigi et al., 2003). No information about drying effect on this compound was found. Concerning β-elemene, a study of the effect of AMF on *Leptospermum scoparium* essential oil content revealed that no significant differences were observed in terms of the concentrations of maltol, β-elemene, trans-calamenene or grandiflorone between the treatments (Wicaksono et al., 2017). However, M treatment was the treatment followed by HS+M with a higher relative percentage of this compound which possibly indicates an influence of the symbiotic fungus. Notwithstanding, possibly drought had a counteracting effect. A study with parsley subjected to different irrigation regimes was applied at the following levels: 30–45% (level 1) and 45–60% (level 2). The values of β-elemene were in control, level 1 and level 2, 1.05%, 1.1% and 1.5%, respectively (Petropoulos et al., 2007). Also, the effects of drying sage increased the relative percentage of this compound in comparison with fresh material (Sellami et al., 2012). It is difficult to infer why this compound had this value in this treatment, but it looks like the drying and water deficit, following the latter articles possibly had a greater influence in the relative amount of this compound.

Linalool had differing values in this samples from dried basil. In decreasing order of relative percentage, it was obtained M(43.6%)>C(40.3%)>HS+M(31.6%)>HS(18.4%). Contrarily to these results is a study with basil which compared fresh basil with convective dried basil and they reported for linalool a decrease but not significant in comparison with fresh (Calín-Sánchez et al., 2012). The values presented by the analysis in this experiment show an increase of Linalool in Control from 17% ($1^{st}$ harvest fresh) to 40.3% ($1^{st}$ harvest dry). Another experiment dried basil leaves and stems for one week at low temperature (38°C) and previously this basil had been subjected to water stress. Decreases of linalool, eugenol and sesquiterpenes were accompanied by an increase in methyl chavicol as water stress increased (Simon et al., 1992). In terms of the relative percentage of Linalool this is in accordance for the HS treatment when comparing with the values obtained in HS fresh from the $1^{st}$ harvest that suggest a decrease due to drying. Furthermore, Baritaux et al (1992) studied the essential oil of *Ocimum basilicum* L. dried at 45°C for 12 hours and stored. The content of methylchavicol and eugenol decreased drastically after drying and storage, while that of linalool and 1,8-cineole increased over the same period (Baritaux et al., 1992). The latter is true, regarding Linalool, to control, HS+M and M treatment. Furthermore, and in accordance, eugenol decreased, and 1,8-cineole increased in all the treatments and control when comparing dried samples and fresh samples from the $1^{st}$ harvest. Baritaux et al (1992) study is in accordance with the values obtained, which indicates that the drying and storing had an effect.

Regarding *trans*-α-bergamotene, in decreasing order of relative percentage it was C(14.4%)>M(12.1%)>HS+M(2.8%)>HS(trace amount). Still in the study of Baritaux et al (1992) the values for this compound were raised due to drying and storing in comparison with fresh. This was true for Control and M. However, considering the values of the treatments that had hydric stress, HS and HS+M, this compound decreased. Possibly, drying affected this sesquiterpene (a class of compounds known to be very sensitive to temperature) since it has been previously reported that the volatility of sesquiterpenes is expected to increase more with temperature than the volatility of oxygenated monoterpenes (Copolovici et al., 2015). The latter might explain the values obtained for HS+M and HS due the characteristics above mentioned.

Regarding eugenol, in decreasing order of relative percentage it was HS+M(7.14%)>HS(6%)>C (5.6%)>M(1.2%). No relevant differences are seen between treatments and control, except for M treatment. It may be that the increased PAL activity induced by fungi in the early contact and afterwards (before the activity declines as seen in the 2nd harvest) had a feedback effect with the up and down activity of PAL during storage as reported by other authors (Benkeblia, 2000), revealing this rather different percentage of eugenol in the M treatment in comparison with the other treatments and control.




Graph 16: Variation of the relative percentage (%) of the totality of the 24 compounds from the 1st Harvest of Basil (dry).

Now regarding **Table 13** it is possible to deduce that OM are the dominant class, followed by SH, OS, Others and MH. Comparing the control from the 1st harvest fresh to the control from the 1st harvest dry it is possible to state that there was an increase of MH of 2%, an increase of OM of about 20%, a decrease of SH of 8%, an increase of OS of 4% and a decrease of Others of 6%. Similar to control, in HS, M and HS+M treatments there was, with the drying, a raise of OM, OS and a decrease of Others in comparison with the respective treatments in fresh. However, MH in HS, M and HS+M treatment decreased 1%, 0.5% and

3% respectively (in comparison with the treatments in fresh) whereas in control dry there was a raise of 2% (in comparison with control in fresh). Furthermore, alike control, HS presented a decrease of SH whereas M and HS+M had an increase of SH of 0.2% and 2.6% respectively.

The raise of OM in all the treatments and control is substantiated by a study with basil which reported that in dried and frozen basil there was a decrease in sesquiterpene hydrocarbons and monoterpene hydrocarbons which was accompanied by an increase in oxygenated products (Klimánková et al., 2007). However, the decrease of MH is only observed in HS, M and HS+M, and the decrease of SH is only observed in Control and HS. The raise in OS in all the treatments and control is in accordance with a study with *Melissa officinalis* L. which compared sun, shade and oven drying in two different harvests, and in all the treatments there was a relevant raise of this class of compounds in comparison with fresh (Khalid et al., 2008). Regarding Others, a decrease of compounds from this class of compounds due to drying have also been described. According to Baritaux et al (1992) the content of methylchavicol and eugenol (phenylpropanoids) decreased during drying and storage whereas the content of linalool and 1,8-cineole increased. The latter is in accordance, since eugenol relative percentage decreased in control and the three treatments (and also methyleugenol) which explains the decrease of Others, whereas 1,8-cineol increased for all treatments and control and also linalool (except for HS where it slightly decreased) which explains the increase of OM. The small differences in terms of percentage related to the decreases and increases of MH and SH in comparison with respective fresh treatments and fresh control is minor.

Table 13: Relative percentage of chemical classes of the essential oil from the 1st harvest of Basil (dry). In bold are the highest relative percentages (%).

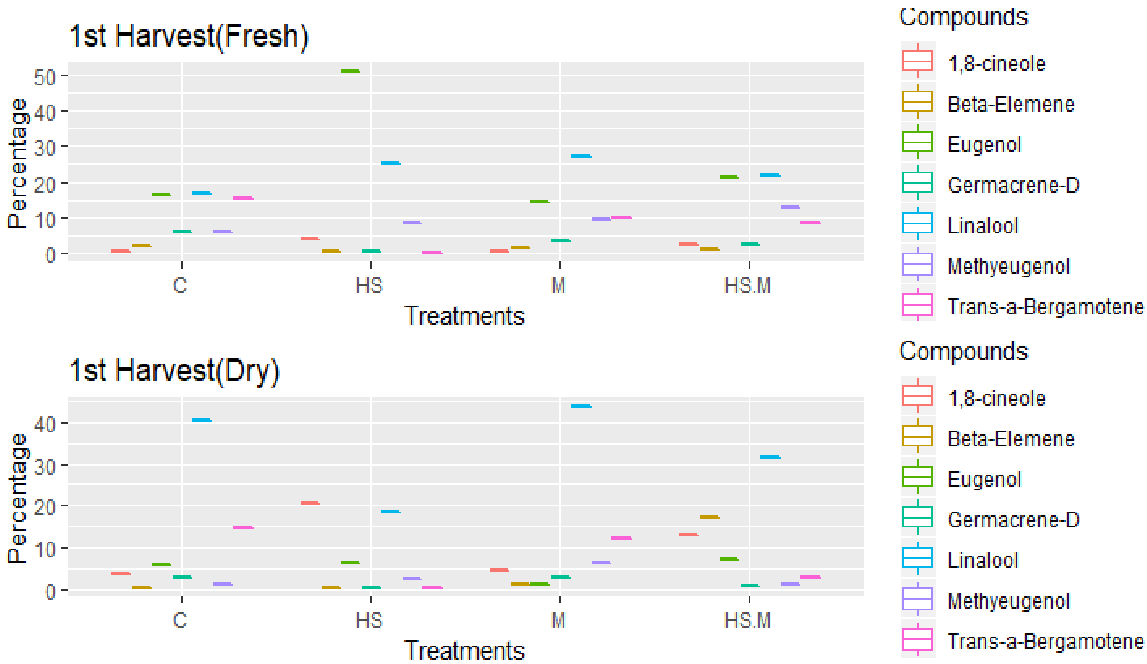
| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | **2.2** | **0** | 2.1 | 0 | 1.1 |
| Oxygenated monoterpenes | 48.4 | 41.7 | 52.4 | **49.0** | 47.9 |
| Sesquiterpene hydrocarbons | 24.7 | 0 | **22.6** | **22.6** | 17.5 |
| Oxygenated sesquiterpenes | 7.2 | **27.1** | 5.7 | 10.5 | 12.6 |
| Others | 6.5 | **8.5** | 7.6 | 8.0 | 7.7 |
| Sum | 89 | 77.3 | **90.4** | 90.1 | |

Examining now **Graph 17** it is possible to observe a comparison between the composition of prevalent compounds (>4.5%) from the samples of the 1st harvest in fresh and the 1st harvest in dry. The most notable difference is in terms of the compound Linalool. In control in fresh the relative percentage of this compound was in decreasing order M (27.3%) > HS (25%) > HS+M (21.8%) > C (17%) whereas after drying it was M (43.6%) > C (40.3%) > HS+M (31.6%) > HS (18.4%). For control, for M and HS+M there was a raise, except for HS. Bowes et al (2003) tested the effect of drying on different cultivars of basil and the relative proportion of linalool in fresh and air-dried was 50.8% and 53.8%, so a slight increase in linalool relative percentage is seen in the air-dried method (Bowes et al., 2003). In this case we see a raise in proportion of Linalool in all the samples except for HS as already mentioned. Unfortunately, there are no published literature about how drought stressed plants can have interacting effects in the moment of drying the plant matter.

Examining the compound 1,8-cineole, it is possible to infer that drying raised the relative percentage of this compound in all the treatments and control; in control it is observed a raise from 0.42% to 3.4%. 1,8-cineole is an oxygenated monoterpene which is in the same class of compounds as linalool, however, this compound didn't reveal a decrease due to drying in the samples from drought stressed plants. These values possibly have to do with solubility and respective domains. As mentioned previously same was obtained by Baritaux et al (1992) since while the content of methylchavicol and eugenol decreased during drying and storage, the content of linalool and 1,8-cineole increased. The latter is in accordance with what was obtained, however instead of methylchavicol, which isn't present in these samples, methyleugenol decreased. Furthermore, the last-mentioned is validated with the assertion by Di Cesare et al (2003) that lower retentions of methyleugenol and eugenol were observed in basil leaves dried by traditional methods, namely air-drying and air and freeze- drying after blanching (Di Cesare et al., 2003). Drying seems to be an interesting method to decrease methyleugenol if the plants are harvested soon (because 'naturally' the plants reduce to trace amounts this compound with development) since it is proven that this compound is toxic (Johnson et al., 2000) and deteriorates the quality of the plant material.

Another compound, *trans*-α-bergamotene also decreased after drying (except for M treatment). Contrarily, Baritaux et al (1992) noticed increases in β-elemene, β-caryophyllene, *trans*-α-bergamotene, germacrene-D and γ-cadinene, and these changes were also observed by Nykanen et al (1987). Also, and contrarily to these latter mentioned literature, germacrene-D diminished the relative percentage from fresh to dry in all the treatments and control. In addition, β-elemene also decreased in the treatments and control except for HS+M. The latter three compounds, sesquiterpene hydrocarbons, didn't reveal a relative percentage in accordance with the literature related to the effect of drying in the essential oil quality.

Nevertheless, the average of the identified compounds between treatments and control didn't varied greatly except for HS, which has to do, to great extent, to the percentage of Linalool.



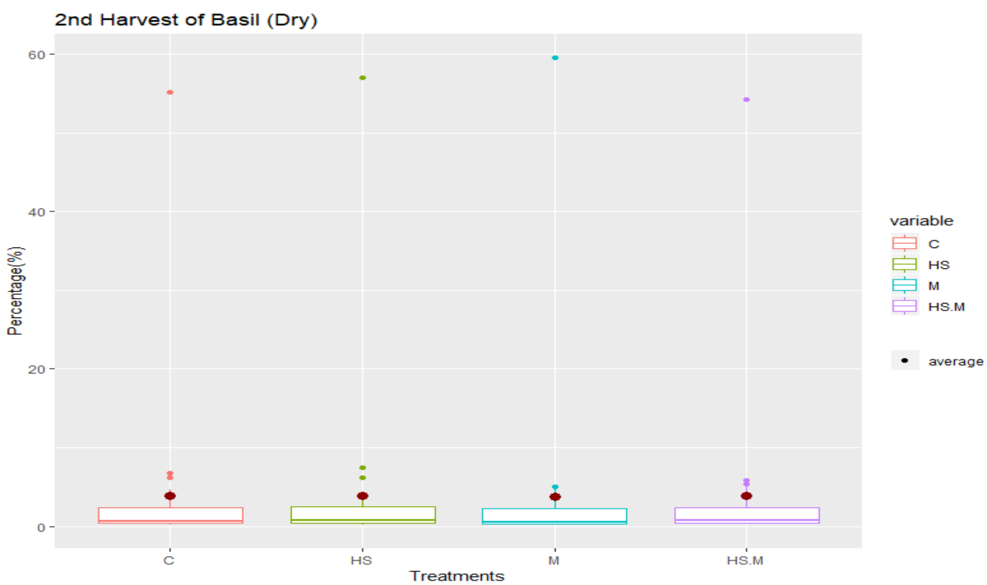

Graph 17: Comparison of the relative percentage of prevalent compounds (>4.5%) of Basil samples in fresh weight (upper graph) and dry weight (lower graph) of the 1st harvest.

Concerning the 2nd harvest in dry, it is possible to infer by the **Graph 18** that the average is similar between control and the treatments. Nevertheless, there are some outliers. In Control it is *trans*-α-bergamotene (6.2%), 1,8-cineole (6.8%) and Linalool (55.2%). In HS it is *trans*-α-bergamotene (6.2%), 1,8-cineole (7.5%) and Linalool (57%). In M it is 1,8-cineole (5.1%) and Linalool (60%). In HS+M it is 1,8-cineole (5.8%), *trans*-α-bergamotene (5.42%) and Linalool (54%).

Regarding Linalool, the highest percentage was in M treatment, similarly to the second harvest in fresh but with an increase of 5%. In terms of 1,8-cineole the highest value was in HS with 7.5% followed by Control with 6.8% whereas HS+M and M had 5.84% and 5.1% respectively. Concerning *trans*-α-bergamotene, this compound had the highest relative percentage in HS and Control which was 6.2%.

Similarly, to what happened with the samples from the 1st harvest in fresh to the 1st harvest in dry there was an increase in the relative percentage of Linalool with drying in control and in the three treatments where the highest relative percentage was obtained with M (60%).

However, in this 2nd harvest contrarily to what was obtained in the 1st harvest in dry the Linalool also increased in HS. This is accordance with an above-mentioned study which stated that drying and storing provoked in basil a drastically decrease of the content of methylchavicol and eugenol while that of linalool and 1,8-cineole increased over the same period (Baritaux et al., 1992). Similarly, the eugenol decreased in control and the three treatments, and methyleugenol content didn't varied or decreased slightly. Also, 1,8-cineole increased (2% in HS) or was maintained (C, HS+M and M). *Trans*-α-bergamotene increased in control and the three treatments in comparison with the 2nd harvest in fresh. The same was observed by Baritaux et al (1992), however, contrarily to the 1st harvest in dry, in this stage there was an increase of this compound that was extended to control and the three treatments.




Graph 18: Variation of the relative percentage (%) of the totality of the 24 compounds from the 2nd Harvest of Basil (dry).

Regarding now the **Table 14** it is possible to deduce that oxygenated monoterpenes are the dominant class, followed by SH, OS, Others and MH. Comparing the control from the 2nd harvest fresh with the 2nd harvest dry it is possible to state that there was a decrease of MH of 1%, an increase of OM of about 10%, an increase of SH of 1%, and increase of OS of 0.1% and a decrease of Others of 9%. Similar to control, in HS, M and HS+M treatments there was, with the drying, a raise of OM, OS, SH and a decrease of Others in comparison with the respective treatments in fresh. However, MH increased in HS (0.1%) (in comparison with the treatment in fresh) whereas in control, M and HS+M dry there was a decrease of 1%, 0.3% and 0.3% respectively (in comparison with control and the treatments in fresh). Since the latter are minor changes it may be asserted that MH values didn't differed relevantly between control and the treatments due to drying.

The raise of SH in control and the three treatments is in accordance with a study which reported that the highest percentages of sesquiterpene hydrocarbons compounds resulted from the treatment of oven-dried herb at 40°C (Khalid et al., 2008). In addition, there was an increased proportion of OS (which was also observed in the samples from the 1st harvest in dry control and in the three treatments). The latter two observed relative increases (of SH and OS) might be related with the higher retention time of the compounds from these classes since as a general tendency compounds with high retention time show an increasing share in the total essential oil at higher temperatures whereas compounds with lower retention time show a decrease (Muller, 2007). Furthermore, the increased proportion of OM, which was also observed in the 1st harvest dry samples, is in accordance with a study with *Salvia officinalis* L. which reported that the values for this class of compounds in fresh, air dried (shade under ambient temperature) and oven (45ºC) were $83.24 \pm 2.03$, $88.65 \pm 2.25$ and $91.52 \pm 2.38$ (%) (Sellami et al., 2011).

The decrease of Others, which was also observed in the 1st harvest dry samples, is in accordance with other articles related to the effects of drying in the essential oil composition of basil. In fact, a study by Pirbalouti et al (2013) reported that the percentage of phenylpropanoids decreased significantly in samples dried in an oven at 60ºC or microwaved and that in contrast, the percentages of sesquiterpene hydrocarbons and oxygenated sesquiterpenes significantly increased when aerial parts of both landraces were dried in an oven at 60ºC or microwaved. Similarly, the percentages of SH and OS increased (except for OS in HS treatment which decreased 2 %) and Others relative percentage proportion decreased.

In addition, the sum of the identified compounds, the content, wasn't altered or presented a higher percentage since in the second harvest in fresh control had 93%, HS had 93%, M had 88% and HS+M had 92% whereas in dry control had 92%, HS had 93%, M had 91% and HS+M 92%.

An interesting fact to add related to drying is that Lawrence (1992) has criticized chemotype classification in cases when cultivars contain at least two major components. The reason for this criticism is the fact that essential oil composition of basil oils can be different before and after drying as shown in many studies. The study of Baritaux et al (1992) shows that the fresh plants represented a clear-cut eugenol chemotype, whereas in dried and stored plants represented a linalool chemotype (Hiltunen et al., 2005). The same was inferred from the results obtained in this experiment.

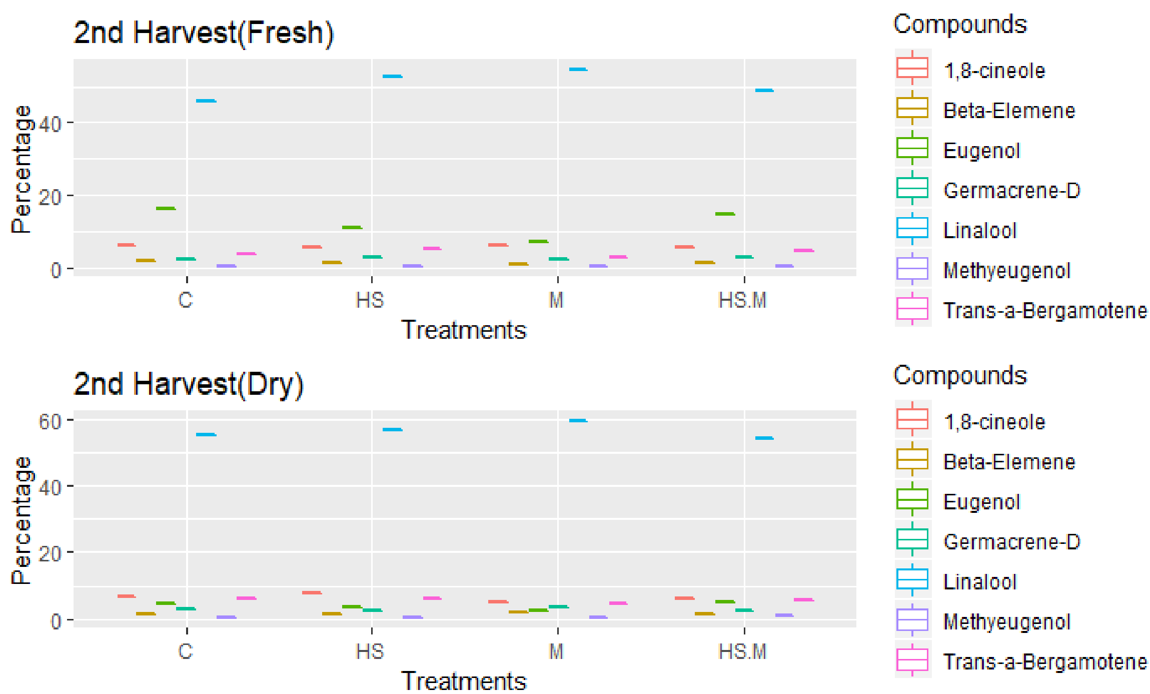
Table 14: Relative percentage of chemical classes of the essential oil from the 2nd harvest of Basil (dry). In bold are the highest relative percentages (%).

| Classes of compounds | C | HS | M | HS+M | $\bar{x}$ |
|---|---|---|---|---|---|
| Monoterpene hydrocarbons | 2.6 | **3.1** | 2.1 | 2.6 | 2.6 |
| Oxygenated monoterpenes | 66.2 | **69.1** | 68.2 | 64.6 | 67 |
| Sesquiterpene hydrocarbons | **13.4** | 12.1 | 12.7 | 12.2 | 12.6 |
| Oxygenated sesquiterpenes | 4.8 | 3.8 | 5.0 | **6.2** | 5 |
| Others | 5.1 | 4.4 | 3.1 | **6.4** | 4.8 |
| Sum | 92.1 | **92.5** | 91.1 | 92 | |

Examining now **Graph 19** it is possible to observe a comparison between the composition of the prevalent compounds (>4.5%) from the samples of the 2nd harvest in fresh and the 2nd harvest in dry. The most notable difference is in terms of the compound Linalool which presented an enhanced relative percentage due to drying in control and the three treatments samples. However, eugenol decreased (in control fresh it was 16% whereas in dry it was 5%) and methyleugenol maintained the trace amounts that the plants present in fresh by the stage of the 2nd harvest.

The drying didn't affect relevantly 1,8-cineole proportion since in all the treatments and control it is observed a maintenance of the relative percentage or a slight increase in comparison with control and with the three treatments in fresh.

Furthermore, *trans*-α-bergamotene increased in control and in the three treatments whereas germacrene-D and β-elemene, like 1,8-cineole, weren't affected by drying, it is observed a rather maintenance of the relative percentage. The drying of the plants in this 2$^{nd}$ harvest allowed to obtain a stable essential oil in comparison with fresh control with an enhanced quality (higher percentage of Linalool). Furthermore, as mentioned above, the content, was not affected in a relevant manner by drying.




Graph 19: Comparison of the relative percentage of prevalent compounds (>4.5%) of Basil samples in fresh weight (upper graph) and dry weight (lower graph) of the 2$^{nd}$ harvest.

## 4.4 Treatments effect on essential oil compounds with commercial interest

The results described below are based on the commercial interest of the compounds reported for this samples. Regarding mint, which is praised for the essential oil, it is possible to state that Piperitenone oxide was the main component in the samples. This compound was tested against several microorganisms where pulegone was the most active against all bacteria, followed by Piperitenone oxide; the activity of piperitone oxide seems to be two-fold lower than that of Piperitenone oxide (Ouzmil et al., 2002). In addition, β-Myrcene, is a monoterpene that has been reported to exert analgesia (Rao et al., 1990) and it is present in cosmetic formulations for the prevention and therapy of hair loss (patent number: USOO6270752B1). Finally, 1,8-cineole, have been reported to have anti-inflammatory, anti-microbial, and antioxidant activity (Brown et al., 2017).

Examining the data related to the latter mentioned three compounds (Attachment VII) it is possible to infer that in the 1$^{st}$ and 2$^{nd}$ harvest in fresh the treatment that revealed higher amounts of Piperitenone oxide was HS+M (however in the 2$^{nd}$ harvest it decreased to half). 1,8-cineole in the 1$^{st}$ harvest had the highest relative percentage also in HS+M where in the 2$^{nd}$ harvest the relative percentage of this compound was similar between HS and HS+M (however in the 2$^{nd}$ harvest for both treatments it increased six times the relative percentage). Regarding β-Myrcene for both harvests in fresh the best treatment was HS (where in the second harvest there was an increase of 4.2%). So, an essential oil rich in Piperitenone oxide can be obtained in the 1$^{st}$ harvest in fresh, with Control and HS+M treatment. An essential with higher amount of 1,8-cineole, but 30% less amount of Piperitenone oxide can be obtained in the 2$^{nd}$ harvest in fresh with HS treatment. Similarly, a higher amount of β-Myrcene can be obtained in the 2$^{nd}$ harvest in fresh with HS treatment.

In the 1$^{st}$ and 2$^{nd}$ harvest in dry the latter relative proportions differed. In the 1$^{st}$ harvest dry HS presented the highest amounts of Piperitenone oxide (90.7%) in comparison with control (77.2%) (in the 2$^{nd}$ harvest it decreased to half in control and the three treatments). 1,8-Cineole in the samples from 1$^{st}$ harvest dry had the highest relative percentage in HS+M, where in the 2$^{nd}$ harvest the highest relative percentage was in control (the relative percentage obtained in control in this harvest was the same as HS+M in the 1$^{st}$ harvest dry). Regarding β-Myrcene in the 1$^{st}$ harvest the treatment that presented highest relative percentage was HS+M (13.51%) and in the 2$^{nd}$ harvest the M treatment (19.71%).

Considering the stability of the plants in terms of development the suggested stage as to yield and biomass is the 2$^{nd}$ harvest. Thus, in this stage, an essential oil rich in Piperitenone oxide can be obtained from both dry and fresh material, in control and the treatments except for HS. However, in fresh the relative percentage is 20% higher. Similarly, in the 2nd harvest, an essential oil with high relative percentage of 1,8-cineole, but with much lesser amount of Piperitenone oxide can be obtained in this stage in dry with control (9.13%) or with M treatment (8.68%). In addition, a higher amount of β-Myrcene can be obtained in the 2nd harvest in dry with M treatment (19.71%). In short, for a higher yield and biomass and for an essential oil with high amount of Piperitenone oxide the 2nd harvest in fresh is suggested with HS+M treatment (49%) whereas in dry it is suggested using control or M treatment (35% and 33% respectively). For essential oils with high amount of 1,8-cineole and β-Myrcene in the 2nd harvest it suggested drying the plants subjected previously to control condition (for 1,8-cineole) and M treatment (for β-Myrcene) respectively.

Now regarding basil, the main compounds with reported interest in regard to its essential oil are Linalool, Eugenol and 1,8-cineole. Besides, this plant is highly praised for cuisine purposes. For the latter a high-quality plant matter has to have a low relative percentage of methyleugenol. Regarding the compound 1,8-cineole, as above mentioned, an interest in its anti-inflammatory, anti-microbial, and antioxidant activity has been reported. Furthermore, Linalool was evaluated for its psychopharmacological activity in mice, revealing marked dose-dependent sedative effects on the central nervous system (CNS) (Buchbauer et al., 1991) which might be interesting for aromatherapy purposes. In addition, Eugenol has been reported to have anti-inflammatory and antioxidant activities in *in vitro* and animal models (Fujisawa et al., 2016).

Examining the data related to the three compounds last-mentioned (Attachment VII), in the 1st and 2nd harvest, it is possible to infer that in fresh the treatment that revealed higher relative percentage of Linalool was M (however in the 2nd harvest there was a double increase). Regarding Eugenol, this compound presented in the 1st harvest the highest relative percentage in HS whereas in the 2nd harvest the values for this compound remarkedly decreased for all the treatments and control. The compound 1,8-cineole presented in the 2nd harvest the highest relative percentage with control which had similar values in the M treatment.

In the 1st and 2nd harvest in dry the samples revealed different results in comparison with fresh samples. In the 2nd harvest M presented the highest amount of Linalool. Hence, in the 1st harvest Eugenol revealed the highest relative percentage in HS+M, where in the 2nd harvest the relative percentage decreased slightly. Regarding 1,8-Cineole in the 1st harvest the treatment that presented the highest relative percentage was HS whereas in the 2nd harvest it presented a halved decrease.

In short, and similarly to mint, considering the stability of the plants in terms of development and biomass the suggested stage as to yield and biomass is the 2nd harvest. In this stage an essential oil rich in Linalool can be obtained in dry, with the M treatment. Furthermore, an essential oil with higher amount of Eugenol can be obtained in fresh with control or HS+M treatment. Thus, a higher amount of 1,8-cineole can be obtained with either dry or fresh material with any treatment and control. For an essential oil with high amount of Linalool the 2nd harvest in dry with M is suggested whereas oils with a higher amount of Eugenol and 1,8-cineole were presented in the 1st harvest with HS+M and HS treatment respectively. Nevertheless, for a stable production the 2nd harvest is recommended. In this case, Eugenol and 1,8-cineole presented the highest amounts with fresh plants subjected to the control condition and the dry plants previously subjected to hydric stress treatment respectively. Regarding the plants for consumption (for example as pesto), the 2nd harvest in fresh is suggested, in control or any of the treatments since methyleugenol presented trace amounts in all the samples. Also, in the 2nd harvest in dry the amount of methyleugenol maintained the trace amount relative percentage (or slightly decrease). Furthermore, if the plants have to be harvested sooner (in the 1st harvest), drying is advisable since it decreased relative percentage of methyleugenol than the amount revealed in the samples from the 1st harvest in fresh.

Basically, a specific treatment or processing may be chosen for the essential oil quality wished for. This might allow that within the same species one has different products depending on the treatments applied to the plant previously to extraction.

Concerning the yield, the results from this experiment in terms of this parameter were that the variation between treatments and control was not very striking; the treatments in comparison with control neither increased nor decreased in a relevant manner the yield of mint and basil species. However, in dried samples and in dried water stressed plants it was observed an enhanced yield since drying (and drought and drying), due to the reduction of weight augment the yield values. On the other hand, the timings by which the samples were distillated didn't seem to be the most adequate since in the 1$^{st}$ harvest for both species the amount of oil was negligible, didn't permit comparisons and difficulted the chemical analysis posteriorly.

Nevertheless, a few studies report the increase of yield of essential oil (mL/g) with mycorrhizae. However, most of the studies report the importance of AMF in changing the proportion of major compounds. Thus, the fungal symbionts have acquired a great reputation in the biofertilization and crop production since the close contact between plant roots and fungus hyphae creates bidirectional nutrient transfer between plant-fungus (Smith et al., 2004) and opens the possibility to minimize agrochemical input and as consequence reduce the production costs.

## 5. Conclusions and work limitations

The research goals defined assumed that biotic and/or abiotic factors applied previously and during the development of plants can posteriorly rise the yield from of essential oil-bearing species and obtain different, and interesting commercially, chemical profiles. Specifically, it was asked in the present experiment whether (i) AMF affects the yield and composition of essential oils, whether (ii) Hydric stress affects the yield and composition of essential oils, if (iii) in hydric stress conditions the AMF allows the maintenance of biomass, and if (iv) the dried plants essential oils didn't differ relevantly from the fresh material.

Concerning the first point (i) and (ii), we may say that neither AMF and drought stress affected the yield regarding the quantity of replicas used in this experiment, however, these treatments affected the quality of the essential oils, some different from the control, others resembling the control. In those cases, of resemblance, it was a good result since there is less use of water and/or the use of an arbuscular mycorrhiza which protects the plant from several stresses and permits a certain 'guarantee' of production. Thus, it was proven by this study that in fact AMF ameliorates the consequences of hydric stress, in terms of biomass while also presenting differing essential oil profiles, which answers the third point (iii). The chemical analysis of the essential oils from mint and basil, in control conditions and subjected to the three treatments, revealed for both harvests, whether fresh or dried, that the main compound of mint, Piperitenone oxide, varied between 22%-90.7%, and that the main compounds of basil, Linalool and Eugenol, varied between 17%-60% and 1.2%-50.8%, respectively.

To answer the point one (i) and two (ii) in terms of the quality, fresh distillated material may be examined. In fresh distillated mint from the 1$^{st}$ harvest, control presented for the main compound, Piperitenone oxide a relative percentage of 83% whereas the treatment with highest percentage was hydric stress plus mycorrhizae treatment with 84.1%. In the 2$^{nd}$ harvest, no conclusions can be made since there is no control to compare. In fresh distillated basil from the 1$^{st}$ harvest, control presented for the main compounds, Linalool and Eugenol, 17% and 16.2% whereas the treatment with highest percentage was mycorrhizae for Linalool, 27.3% and hydric stress for Eugenol, 50.8%. In the 2$^{nd}$ harvest, control presented for the main compounds, Linalool and Eugenol, 46% and 16.2% whereas the treatment with highest percentage was mycorrhizae for Linalool, 54.7%, and for Eugenol the control condition, 16.2% (the control condition maintained from the 1$^{st}$ to the 2$^{nd}$ harvest the relative percentage of this compound). So, in fact, mycorrhizae, hydric stress and mycorrhizae plus hydric stress expressed, to a certain manner, an effect on the composition of the essential oils in both species.

Concerning the point four (iv), it is important to mention that due to timings limited to do the work the tracing of the moisture percentage present on the leaves, stems and flowers couldn't be followed. However, a way of homogenizing this parameter was treating both species in the same manner, 24 hours of oven and 1 month of storing. This was followed by the assertion that like many other crops, medicinal plants have to be dried before storage (Muller, 2007).

Nevertheless, not many studies published used this type of procedure. In this experiment it was used this procedure since it was expected that it didn't alter the content of the essential oil in a relevant manner, it is non-costly and efficient in time. When the plants were dried and then distillated, it was possible to observe that for the mint, in the 1st harvest, the main component Piperitenone oxide was maintained and/or raised except for HS+M treatment. Regarding the 2nd harvest it is difficult to make certain assumptions as already mentioned previously. However, in the 1st harvest of mint, distillated in fresh and dry, the sum of the identified compounds revealed the range of 81.7%-93.1% whereas in dry it was 88.3%-96.2%. Regarding basil, when dried, the main compound Linalool was maintained and/or raised in both harvests where the highest relative amount was with dried material in the 2nd harvest, revealing a relative percentage of 60% in the M treatment. However, Eugenol, had a lower relative percentage in the material dried from both harvests. Even in the control condition. Yet, the sum of the identified compounds in basil reveals for the 1st and 2nd harvest in fresh the range of 78.9%-95.7% and 87.9%-93.3% whereas in dry it was 77.3%-90.4% and 91.1%-92% for control and the three treatments. We may postulate that drying affected individual compounds but didn't affect in a relevant manner the total content. Furthermore, examining samples from dried material was to a certain extent intriguing since possibly plants drought stressed, or drought stressed symbiotic plants possibly have different metabolic turnovers in the process of drying.

The matters discussed above suggest that the production of essential oils from aromatics plants is under diverse physiological, biochemical, metabolic and genetic regulation. The physiological regulation, besides being exerted in a development-specific fashion, is highly susceptible to modulation through environmental regulation (Millar et al., 2016). To understand these complex metabolic modulations, it is necessary long experiments (in time), more replicas and less variables applied. In this experiment in particular, the latter mentioned is pertinent since there are shifts in the type of ecological behavior plant has with the fungi which is aligned with the plant development stages. Furthermore, drought stress also has a different impact when it is applied in a short period of time and in a prolonged period of time (also applying drought stress and rewatering has particular outcomes metabolically). Besides these temporal related responses, the individual susceptibility to these factors may vary. Different species or different cultivars have different affinities to symbiotic fungi, just like different symbiotic fungi have different affinities to different plants species. In short, the degree of influence of different AM fungi on the same medicinal plant or of the same AM fungus on different medicinal plants can vary (Zeng et al., 2013). Thus, different species or different cultivars have different responses to drought stress. Some plant species under drought may be more responsive where others may be more unsusceptible. Indeed, the same water deficit in a sensitive species and a resistant species may not trigger the same response (Bray, 1997). Besides these differential responses, the heterogeneity between plant individuals cannot be dismissed as a possible explanation for plant terpenoid variability which once again leads to the need of more replicas. Propagating plants from cuttings or using cloned plants could contribute to exclude the contribution of plant genetic variability to mycorrhizae-associated or drought-associated plant terpenoid accumulation.

These multiple-level controlled mechanisms affecting plants interacting with more than one variable require a more holistic approach to understand this phenomenon. Even though working in controlled conditions, in a glasshouse, facilitates the process by which one might infer about the effect of each factor, there is an *a priori* knowledge needed, for example about the compounds that are synthetized due to ontogeny (epistatic) and the ones that are vulnerable to change due to external factors (hypostatic). In short, plants produce, in normal conditions ('healthy state') a set of secondary metabolites, which is considered as constitutive production. When subjected to any traumatic stimulus, there may be *de novo* production as mentioned in the introduction. In the majority of cases, *de novo* production is characterized by compounds which are produced in greater quantity and/or in a different ratio (Figueiredo et al., 2008).

As mentioned above a holistic approach is needed in order to draw the line between constitutive (epistatic) and *de novo* production (hypostatic) of natural products when in contact with diverse external factors. Nevertheless, this study demonstrated that essential oil composition of mint and basil species can be conditioned by water stress and/or symbiotic fungi. Apparently, the results expressed by the essential oil constituents in the two species were directly affected by changing environmental conditions.

In conclusion, the results of this study might be useful to improve the productivity, cultivation management and quality control of mint and basil in warmer countries and with less input of agrochemicals.

Regarding further studies, there is a need to deal with the limitations of this study mentioned above. Besides, it would be interesting to examine the bioactivity and toxicity of these essential oils which vary according to the composition which is decided by many interdependent factors, some factors which, in this experiment were traced.

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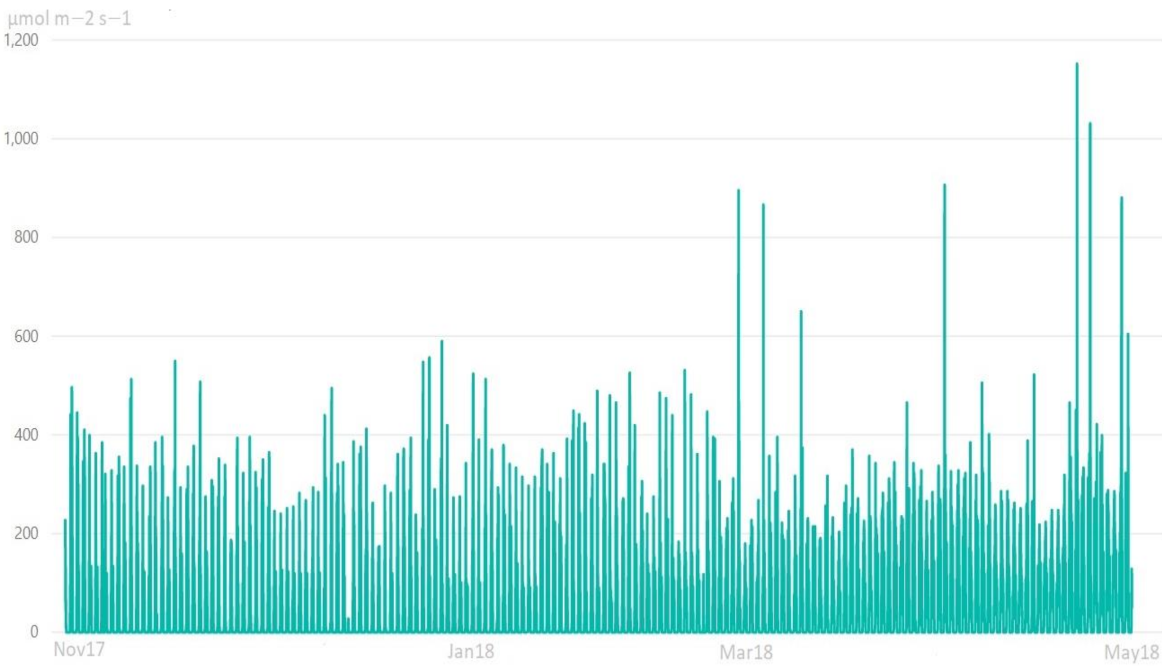
# Attachments

**Attachment I** – Physical and chemical properties of soil used.

| Boron (B) (mg/kg) | Copper (Cu) (mg/kg) | Iron (Fe) (mg/kg) | Zinc (Zn) (mg/kg) | Manganese (Mn) (mg/kg) |
|---|---|---|---|---|
| 10.62 | 2.65 | 52.14 | 46.24 | 28.19 |

| | |
|---|---|
| Carbon (C) (%) | 40.87 |
| Nitrogen (N) (%) | 1.13 |
| Phosphor (P) (%) | 0.16 |
| Potassium (K) (%) | 0.52 |
| Calcium (Ca) (%) | 0.56 |
| Magnesium (Mg) (%) | 0.10 |
| pH | 6,20 |
| Conductivity (μs) | 267 |

**Attachment II** - Radiation measured during the experiment in µmol $m^{-2}$ $s^{-1}$



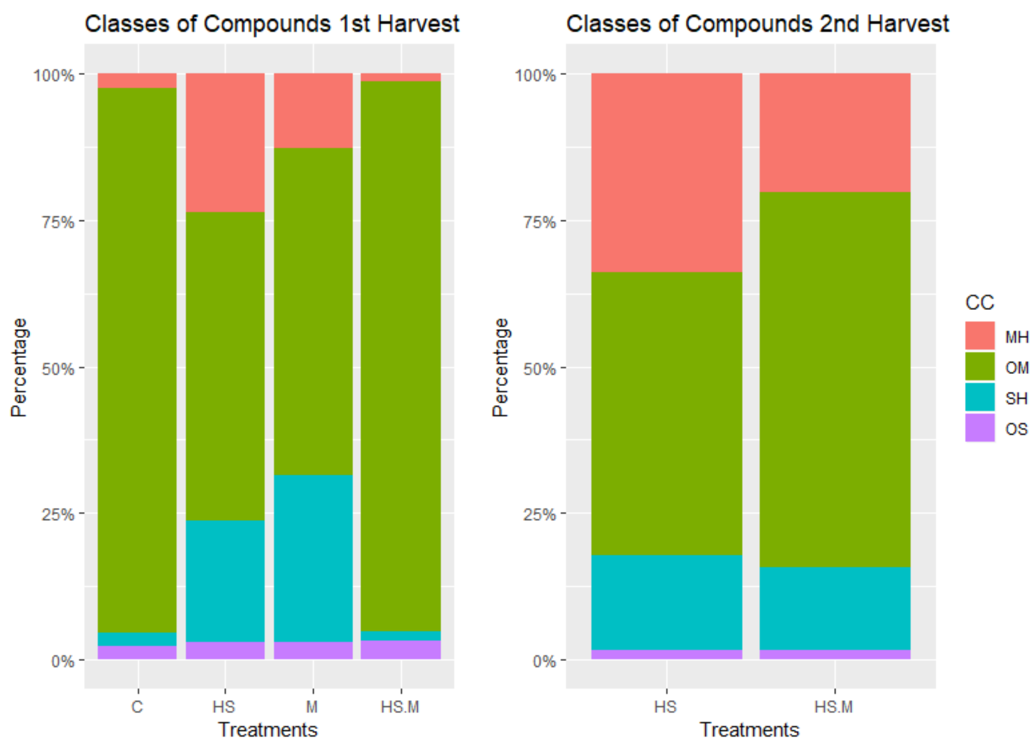
**Attachment III** - Identified compounds and respective percentage of *Ocimum basilicum* L. cv. Genovese Gigante (each harvest value is the average of two injections); IR - Index of classification for the series of alkanes $C_9$-$C_{17}$.

| | | | CONTROL | | | | HYDRIC STRESS | | | | MYCORRHIZAE | | | | HYDRIC STRESS&MYCORRHIZAE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FRESH | | DRY | | FRESH | | DRY | | FRESH | | DRY | | FRESH | | DRY | |
| **Compounds** | **CC** | **IR** | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH |
| Sabinene | MH | 958 | 0.045 | 0.245 | 0.24 | 0.295 | 0.01 | 0.19 | 0.01 | 0.365 | 0.14 | 0.685 | 0.22 | 0.24 | 0.175 | 0.295 | 0.01 | 0.285 |
| β-Pinene | MH | 963 | 0.175 | 0.81 | 0.54 | 0.685 | 0.665 | 0.575 | 0.01 | 0.805 | 0.59 | 0.2 | 0.48 | 0.54 | 0.725 | 0.63 | 0.03 | 0.665 |
| β-Myrcene | MH | 975 | 0.075 | 0.6 | 0.825 | 0.62 | 0.01 | 0.59 | 0.01 | 0.78 | 0.5 | 0.715 | 0.695 | 0.445 | 0.69 | 0.65 | 0.02 | 0.685 |
| 1,8-Cineole | MO | 1005 | 0.42 | 6.185 | 3.425 | 6.765 | 4 | 5.475 | 20.69 | 7.45 | 0.69 | 6.1 | 4.625 | 5.05 | 2.545 | 5.625 | 12.79 | 5.84 |
| *trans* -β-Ocimene | MH | 1027 | 0.16 | 1.475 | 0.57 | 0.68 | 0.635 | 1.285 | 0.02 | 0.775 | 1.135 | 0.53 | 0.62 | 0.555 | 1.89 | 1.215 | 0.01 | 0.675 |
| Terpinolene | MH | 1064 | 0.05 | 0.325 | 0.03 | 0.3 | 0.14 | 0.455 | 0.01 | 0.38 | 0.26 | 0.365 | 0.13 | 0.32 | 0.345 | 0.15 | 0.03 | 0.3 |
| Linalol | MO | 1074 | 17.015 | 45.95 | 40.275 | 55.2 | 25.06 | 52.84 | 18.4 | 56.955 | 27.295 | 54.745 | 43.63 | 59.555 | 21.75 | 48.89 | 31.62 | 54.185 |
| Camphor | MO | 1102 | 0.25 | 0.61 | 0.605 | 0.78 | 0.28 | 0.49 | 0.625 | 0.6 | 0.455 | 0.47 | 0.75 | 0.595 | 0.38 | 0.935 | 0.76 | 1.15 |
| Borneol | MO | 1132 | 0.325 | 0.445 | 0.555 | 0.43 | 0.73 | 0.595 | 0.02 | 0.565 | 0.275 | 0.48 | 0.255 | 0.315 | 0.47 | 0.465 | 1.15 | 0.38 |
| α-Terpineol | MO | 1134 | 0.43 | 0.655 | 0.25 | 0.7 | 0.515 | 0.67 | 2 | 0.745 | 0.46 | 0.615 | 0.425 | 0.56 | 0.49 | 0.655 | 2.13 | 0.745 |
| Octanol acetate | Others | 1189 | 0.13 | 0.15 | 0.01 | 0.205 | 0.02 | 0.18 | 0.03 | 0.195 | 0.205 | 0.1 | 0.24 | 0.16 | 0.21 | 0.165 | 0.01 | 0.25 |
| Bornyl acetate | MO | 1265 | 1.835 | 1.58 | 3.245 | 2.275 | 0.02 | 2.595 | 0.01 | 2.82 | 2.605 | 1.575 | 2.74 | 2.075 | 3.245 | 1.98 | 0.575 | 2.3 |
| Eugenol | Others | 1327 | 16.22 | 16.155 | 5.555 | 4.74 | 50.795 | 10.87 | 6.045 | 3.665 | 14.455 | 7.105 | 1.215 | 2.675 | 21.295 | 14.725 | 7.14 | 5.165 |
| Methyleugenol | Others | 1377 | 5.97 | 0.15 | 0.985 | 0.19 | 8.38 | 0.525 | 2.41 | 0.53 | 9.335 | 0.15 | 6.095 | 0.22 | 13.055 | 0.15 | 0.875 | 0.97 |
| β-Elemene | SH | 1544 | 1.925 | 1.76 | 0.37 | 1.46 | 0.02 | 1.18 | 0.02 | 1.18 | 1.31 | 1 | 0.965 | 1.705 | 1.205 | 1.185 | 17.235 | 1.45 |
| *trans* -α-Bergamoten | SH | 1434 | 15.35 | 3.61 | 14.39 | 6.18 | 0.13 | 5.08 | 0.01 | 6.245 | 9.935 | 2.63 | 12.08 | 4.68 | 8.3 | 4.69 | 2.75 | 5.42 |
| α-Humulene | SH | 1447 | 1.145 | 3.295 | 0.84 | 0.345 | 0.01 | 0.29 | 0.02 | 0.31 | 0.885 | 0.23 | 0.81 | 0.41 | 0.59 | 0.3 | 0.01 | 0.365 |
| *trans* -β-Farnesene | SH | 1455 | 3.6 | 0.17 | 2.125 | 0.38 | 0.01 | 0.29 | 0.01 | 0.325 | 3.42 | 2.63 | 3.525 | 0.35 | 2.895 | 0.34 | 0.76 | 0.37 |
| Germacrene-D | SH | 1474 | 5.77 | 2.34 | 2.94 | 2.89 | 0.01 | 2.58 | 0.01 | 2.39 | 3.53 | 2.26 | 2.75 | 3.33 | 2.6 | 2.525 | 0.575 | 2.575 |
| γ-Cadinene | SH | 1500 | 4.51 | 1.16 | 3.31 | 1.76 | 0.25 | 1.19 | 0.02 | 1.3 | 2.81 | 1.96 | 1.9 | 1.86 | 1.71 | 1.36 | 1.275 | 1.66 |
| β-Sesquiphellandren | SH | 1508 | 0.285 | 0.295 | 0.685 | 0.39 | 0.01 | 0.33 | 0.01 | 0.355 | 0.6 | 0.91 | 0.575 | 0.32 | 0.56 | 0.22 | 0 | 0.33 |
| τ -Cadinol | SO | 1616 | 1.635 | 3.6 | 0.96 | 4.02 | 1.46 | 3.635 | 5.935 | 2.915 | 1.345 | 1.89 | 0.72 | 3.79 | 6.01 | 3.9 | 5.775 | 4.93 |
| 1-*epi* -Cubenol | SO | 1624 | 0.905 | 0.65 | 5.64 | 0.505 | 1.965 | 0.92 | 16.465 | 0.545 | 0.665 | 0.255 | 4.535 | 0.68 | 1.45 | 0.62 | 2.635 | 0.83 |
| α-Cadinol | SO | 1626 | 0.69 | 0.4 | 0.47 | 0.25 | 0.675 | 0.485 | 4.68 | 0.36 | 1.005 | 0.24 | 0.45 | 0.49 | 0.915 | 0.5 | 2.095 | 0.475 |
| Total | | | 78.915 | 92.615 | 88.84 | 92.045 | 95.8 | 93.315 | 77.47 | 92.555 | 83.905 | 87.84 | 90.43 | 90.92 | 93.5 | 92.17 | 90.26 | 92 |

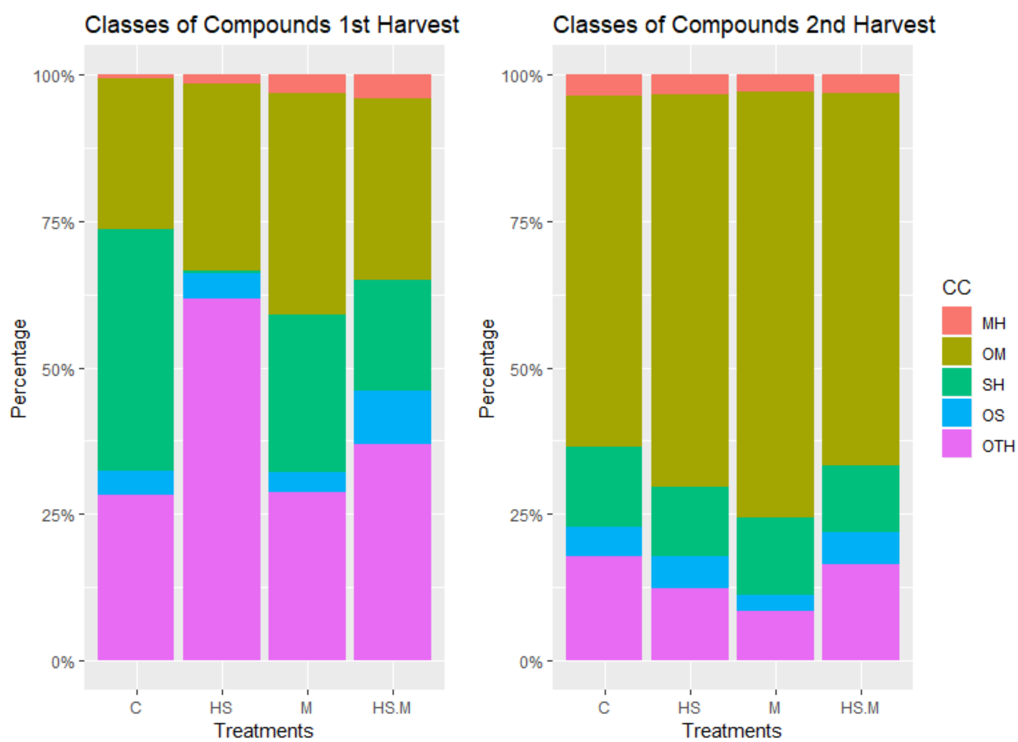
**Attachment IV** - Identified compounds and respective percentage of *Mentha* sp. (each harvest value is the average of two injections). IR - Index of classification for the series of alkanes $C_9$-$C_{17}$.

| | | | CONTROL | | | | HYDRIC STRESS | | | | MYCORRHIZAE | | | | HYDRIC STRESS&MYCORRIZHAE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FRESH | | DRY | | FRESH | | DRY | | FRESH | | DRY | | FRESH | | DRY | |
| **Compounds** | **CC** | **IR** | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH | 1ªH | 2ªH |
| α-Pinene | MH | 930 | 0.01 | | 0.26 | 0.53 | 0.165 | 1.09 | 0.015 | 1.15 | 0.035 | | 0.045 | 1.575 | 0.06 | 0.79 | 1.175 | 0.165 |
| Sabinene | MH | 958 | 0.02 | | 0.27 | 0.84 | 0.455 | 1.385 | 0 | 1.22 | 0.14 | | 0.02 | 1.875 | 0.49 | 0.96 | 1.345 | 0.36 |
| β-Pinene | MH | 963 | 1.24 | | 0.89 | 1.975 | 1.335 | 3.535 | 0.355 | 3.055 | 1.045 | | 0.01 | 4.175 | 0.235 | 2.475 | 3.2 | 1.01 |
| β-Myrcene | MH | 975 | 0.645 | | 3.29 | 7.95 | 9.055 | 13.255 | 0.41 | 10.35 | 3.965 | | 0.865 | 19.71 | 0.5 | 8.07 | 13.505 | 5.17 |
| p-Cymene | MH | 1003 | 0.01 | | 0.025 | 0.08 | 0.105 | 0.295 | 0.01 | 0.095 | 0.22 | | 0.35 | 0.15 | 0.01 | 0.035 | 0.18 | 0.085 |
| 1,8-Cineole | MO | 1005 | 1.02 | | 2.95 | 9.125 | 0.7 | 6.34 | 0.95 | 7.425 | 1.475 | | 1.5975 | 8.68 | 1.595 | 5.59 | 8.945 | 3.605 |
| Limonene | MH | 1009 | 0.085 | | 0.885 | 2.56 | 2.57 | 4.445 | 0.015 | 2.295 | 2.145 | | 1.5975 | 4.2 | 0.02 | 2.165 | 2.475 | 1.28 |
| *cis* -β-Ocimene | MH | 1017 | 0.07 | | 0.665 | 2.42 | 5.565 | 4.04 | 0.01 | 3.07 | 2.39 | | 0.38 | 5.71 | 0.02 | 2.825 | 4.54 | 2.51 |
| *trans* -β-Ocimene | MH | 1027 | 0.15 | | 0.18 | 0.235 | 0.51 | 0.375 | 0.02 | 0.27 | 0.24 | | 0.015 | 0.485 | 0.01 | 0.26 | 0.375 | 0.245 |
| γ-Terpinene | MH | 1035 | 0.155 | | 0.26 | 0.225 | 0.065 | 0.38 | 0.17 | 0.19 | 0.245 | | 0.235 | 0.465 | 0.055 | 0.115 | 0.15 | 0.165 |
| Linalol | MO | 1074 | 0.69 | | 0.27 | 0.645 | 1.205 | 0.44 | 1.025 | 0.405 | 0.59 | | 0.36 | 0.555 | 0.12 | 0.59 | 0.935 | 0.685 |
| *cis* -Piperitone oxide | MO | 1211 | 0.585 | | 0.25 | 1.345 | 0.835 | 11.445 | 0.2 | 16.495 | 0.885 | | 0.4 | 1.715 | 0.855 | 0.47 | 0.33 | 0.96 |
| Thymol | MO | 1275 | 0.625 | | 0.37 | 1.045 | 0.5 | 0.805 | 0.65 | 0.57 | 2.72 | | 0.26 | 0.56 | 0.57 | 0.575 | 0.56 | 0.79 |
| Piperitenone oxide | MO | 1330 | 82.5 | | 77.165 | 34.785 | 40.665 | 22.005 | 90.65 | 28.89 | 40.005 | | 85.165 | 32.945 | 84.095 | 48.905 | 41.725 | 31.695 |
| β-Caryophyllene | SH | 1414 | 0.515 | | 1.335 | 6.75 | 4.365 | 4.245 | 1.33 | 2.245 | 6.93 | | 0.495 | 2.11 | 1.02 | 3.78 | 1.655 | 7.29 |
| Germacrene-D | SH | 1474 | 1.585 | | 2.38 | 11.035 | 12.965 | 9.39 | 0.025 | 5.32 | 16.155 | | 0 | 4.865 | 0.495 | 8.535 | 6.03 | 17.375 |
| 1-epi-Cubenol | SO | 1624 | 1.16 | | 0.835 | 0.865 | 1.41 | 0.74 | 0.235 | 0.675 | 1.44 | | 0.835 | 0.48 | 1.45 | 0.54 | 0.69 | 1.335 |
| α-Cadinol | SO | 1626 | 1.005 | | 1.33 | 0.675 | 0.95 | 0.705 | 0.2 | 0.74 | 1.045 | | 0.72 | 0.38 | 1.54 | 0.86 | 0.485 | 1.7 |
| Total | | | 92.07 | 0 | 93.61 | 83.085 | 83.42 | 84.915 | 96.27 | 84.46 | 81.67 | 0 | 93.35 | 90.635 | 93.14 | 87.54 | 88.3 | 76.425 |

**Attachment V** - Totality of the relative percentage of the identified classes of compounds of the 1st and 2nd harvest of Mint respectively. MH – Monoterpenes hydrocarbon; OM – Oxygenated monoterpenes; SH – Sesquiterpenes hydrocarbon; OS – Oxygenated sesquiterpenes.

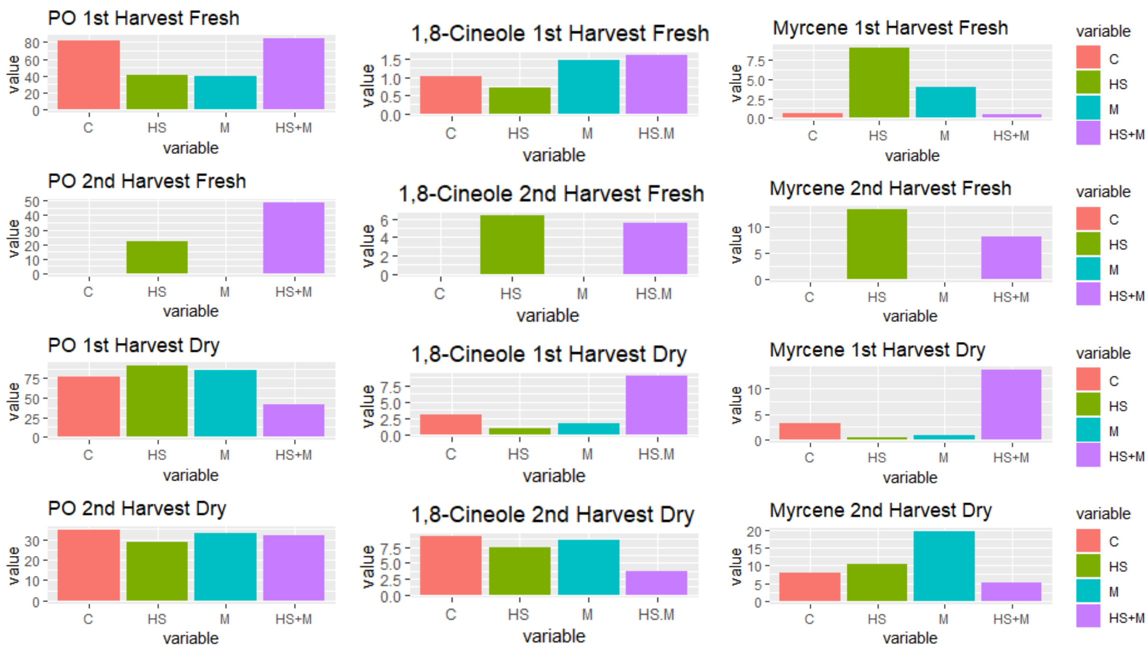


**Attachment VI** - Totality of the relative percentage of the identified classes of compounds of the 1st and 2nd harvest of Basil respectively. MH – Monoterpenes hydrocarbon; OM – Oxygenated monoterpenes; SH – Sesquiterpenes hydrocarbon; OS – Oxygenated sesquiterpenes; OTH – Others.



**Attachment VII** – (A) Variation of compounds reported to have commercial interest from mint samples. (B) Variation of compounds reported to have commercial interest from basil samples.

**A**




**B**