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The effects at ecosystem level of recurrent environmental stresses in Mediterranean Climate regions

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To myself

The real voyage of discovery consists

not in seeking new landscapes,

but in having new eyes

[Marcel Proust]

We do not inherit the earth from our ancestors,

we borrow it from our children

[Native America Proverb]

Abstract

The Mediterranean regions are a climate hotspot where the effects of anthropogenic climate change are expected to appear more rapidly and impact the areas more harshly compared with other regions. These regions are particularly affected by increasing temperatures and decreasing precipitations. Summer heatwaves, combined with severe drought spells and high solar irradiance, have become more frequent because of climate change. These events are threatening both the diversity and the survival of Mediterranean forests, causing a loss in biodiversity, through a modification in the composition of native plant communities and facilitating biotic invasion of exotic species, which are more competitive in these new habitats. Furthermore, drought stress, high temperature stress and the intraspecific competition between plants could lead to a variation in Biogenic Volatile Organic Compound (BVOCs) emissions, secondary metabolites that play different roles in plants. Firstly, these compounds have a key antioxidant function (quenching Reactive Oxygen Species), thus improving ozone and drought tolerance, while also acting as pollinator attractors and repellents for dangerous herbivorous insects (contributing to the taste and odour of different plant tissues). In addition, these compounds could modify the ozone and the Secondary Organic Aerosols (SOAs) contents, playing an important role in the air quality. Lastly, some of these compounds, in particular some terpenes, may also provide multiple health-promoting benefits for humans, thanks to their use in different types of industries, such as pharmaceutical, nutraceutical, and cosmetic industries. In this work the first aim was to develop a fast and easy-to-handle analytical methodology to sample BVOCs using solid-phase microextraction (SPME) fibres at the canopy level. An improvement of BVOCs adsorption from SPME fibres was obtained by coupling the fibres with fans to create a dynamic sampling system (DBSS) and the results obtained showed high efficiency and sensitivity of SPME fibres, reducing sampling time. Afterwards, these DBSS devices were used for three years to seasonally monitor the changes in BVOC emission of a *Quercus ilex* (holm oak) coastal forest in two stands characterized by two levels of tree mortality estimated by crown defoliation assessment. In addition, another study was conducted in south-eastern Australia and focused on the invasiveness of an aggressive invasive native species: *Pittosporum undulatum*. Our results, in both studies, showed the efficiency of this new device. In addition, in the study regarding the high mortality of holm oak in Tuscany the results suggested that terpene emissions from Mediterranean forests would be modified by an increase in *Q. ilex* dieback, with important consequences for the atmospheric chemistry and functioning of this forest ecosystem. In the study, regarding the alterations on composition of Eucalypt forests due to the invader *Pittosporum undulatum,* our results suggested that the invasiveness of *P. undulatum* might be linked to the biosynthesis of compounds that play a protective role against abiotic stresses. In conclusion, the DBSS technique shows great potential applications in several conditions and studies, both in natural environments (such as those presented in this thesis), as well as in urban environments, to monitor air quality and plant emissions. However, it is necessary to mention that the next step in this field of research would be to develop a reliable quantification procedure, required to further improve the DBSS sampling strategy, which will then provide quantitative data on BVOCs emitted in the environment.

Acknowledgments

With this thesis, three years full of emotions and events are coming to an end. Three years of Ph.D.; three years of learning to live alone in another city and country; three years away from my love; three years of trials and challenges; including two years with a global pandemic. Three unforgettable years.

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List of published works

I. **Pasquini, D.**, Gori, A., Ferrini, F., Brunetti, C., 2021. An Improvement of SPME-Based Sampling Technique to Collect Volatile Organic Compounds from *Quercus ilex* at the Environmental Level. *Metabolites* 11, 388. https://doi.org/10.3390/metabo11060388

II. **Pasquini, D.**, Detti, C., Ferrini, F., Brunetti, C., Gori, A., 2021. Polyphenols and terpenes in Mediterranean plants: an overview of their roles and possible applications. *Italus Hortus* 28, 3. https://doi.org/10.26353/j.itahort/2021.1.0331

In addition, during the current study, I have also authored and co-authored a number of works (papers and conference presentations) that were not included in this thesis. These works are listed below.

Published works:

I. Brunetti, C., Alderotti, F., **Pasquini, D.**, Stella, C., Gori, A., Ferrini, F., Righele, M., Centritto, M., 2021. On-line monitoring of plant water status: validation of a novel sensor based on photon attenuation of radiation through the lead. *Science of the Total Environment*. https://doi.org/10.1016/j.scitotenv.2021.152881

Conference presentations:

1. **Conference: Biodiversity 2021, 7-9 September 2021** (Talk)

   Pasquini D., Gori A., Ferrini F., Brunetti C.: An innovative SPME-based sampling technique to collect volatile organic compounds at environmental level.

2. **Congress: Plant Biology Europe (PBE), 28 June – 1 July 2021** (Poster)

   Pasquini D., Brunetti C., Ferrini F., Gleadow R.: Chemical responses of *Pittosporum undulatum* in Eucalypt forest: new insights into the defence mechanisms against abiotic stresses.

3. **Conference: XIII scientific day of Italian Ortoflorofrutticoltura Society (SOI), 22-23 June 2021** (Talk)

Pasquini D., Balestrini R., Lumini E., Ferrini F., Brunetti C.: Polyphenolic profiles of *Rosmarinus officinalis* and *Lavandula angustifolia* inoculated with arbuscular mycorrhizal fungi.

4. **Conference: 1st International Electronic Conference on Plant Science (IECPS 2020), 1-15 December 2020** (Poster)

   Pasquini D., Brunetti C., Ferrini F., Gleadow R.: Identifying allelopathic compounds emitted by *Pittosporum undulatum* in *Eucalyptus* forests.

List of original publications and manuscripts

This thesis is based on the following publications and manuscripts:

- **Appendix A - Published:** An Improvement of SPME-Based Sampling Technique to Collect Volatile Organic Compounds from *Quercus ilex* at the Environmental Level. Authors: **Pasquini, D.**, Gori, A., Ferrini, F., Brunetti, C.

  Dalila Pasquini and Cecilia Brunetti contributed to the study conception, investigation, and design. Material preparation, data collection, software and laboratory analysis were performed by Dalila Pasquini. The first draft of the manuscript was written by Dalila Pasquini and all authors contributed to the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

- **Appendix B – Ready for submission:** Effects of Drought-Induced Holm oak die-back on BVOC emissions in a Mediterranean forest. Authors: **Pasquini, D.**, Gori A., Pollastrini M., Alderotti F., Ferrini F., Centritto M., Brunetti C.

  Dalila Pasquini, Cecilia Brunetti and Antonella Gori contributed to the study conception, investigation and design. Material preparation, software and laboratory analysis were performed by Dalila Pasquini. Data collection were conducted by Dalila Pasquini and Martina Pollastrini. The first draft of the manuscript was written by Dalila Pasquini and all authors contributed to the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

- **Appendix C - Submitted:** Chemical responses of *Pittosporum undulatum* in eucalypt forest show stronger defence mechanisms to abiotic stresses. Authors: **Pasquini D.**, Dos Santos Nascimento L.B., Brunetti C., Ferrini F., Gleadow R.

  Dalila Pasquini, Cecilia Brunetti and Roslyn Gleadow contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dalila Pasquini and Luana Beatriz Dos Santos Nascimento. The first draft of the manuscript was written by Dalila Pasquini and all authors contributed to the final version of the manuscript. All authors have read and approved the final manuscript.

- **Appendix D - Published:** Polyphenols and terpenes in Mediterranean plants: an overview of their roles and possible applications. Authors: **Pasquini D.**, Detti C., Ferrini F., Brunetti C., Gori A.

  All authors contributed to the study conception. Dara investigation and collection was made by Dalila Pasquini and Cassandra Detti. The original draft preparation was written by Dalila Pasquini and Cassandra Detti. All authors contributed to the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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1. Introduction

1.1 Mediterranean climate regions

Mediterranean climate regions represent one of the rarest and more complex areas of the planet in terms of biodiversity (Cox and Underwood, 2011). There are five regions with Mediterranean climate around the world (di Castri, 1981; Myers, 1990): the Mediterranean basin, the tip of South Africa, South-west Australia, central Chile and California (Figure 1).

Figure 1. In light-blue are shown the five Mediterranean-climate regions of the world.

The name of this climate region originates from the Mediterranean basin which extends between latitudes 31-45° N and covers an area of 2,300,000 km^2 (Medail and Quezel, 1997). The climate is characterised by hot, dry summers and cool, wet winters (Köppen and Geiger, 1936). The Mediterranean basin presents a high variety of vascular plant species, about 25,000 species (48,250 species if considering all five Mediterranean climate regions) with more than half of them endemic (Cowling et al., 1996). Thus, the Mediterranean basin, despite covering less than 2% of the world's surface, hosts around 20% of its global floristic diversity (Medail and Quezel, 1997). For this reason, the Mediterranean area is defined as a "hotspot", that is a *reservoir* of plant biodiversity (Myers, 1990). The term hotspot indicates an area with high levels of biodiversity and a

high concentration of endemic species; thus, resulting in complex and unique ecosystems, particularly endangered by the ongoing global change (Caldecott et al., 1996).

1.2 Plants morphological and physiological strategies against abiotic stresses

Plants around the world have adapted to avoid and mitigate the effects of specific detrimental environmental factors by developing several mechanisms including escape, tolerance and avoidance (Wang and Huang, 2004). In the Mediterranean basin, which represents a transitional habitat between temperate and tropical regions, plants have evolved under low water (Sardans et al., 2011) and nutrient availability (Sardans et al., 2006), as the most important growth regulating factor consists of hot and dry summers. For this reason, in the arid regions characterised by Mediterranean climate, the vegetation is dominated by sclerophyll plants. Their leaves have a thick cuticle composed of hydrophobic compounds (*i. e.*, lipids impregnated with waxes) to avoid and limit dehydration. Other features of sclerophyll plants are the deep rooting pattern (Nardini et al., 2014); short internodes, which limit the drying action of the wind (Pereira and Chaves, 1995) and promote leaf self-shading to reduce leaf temperatures and water stress (Valladares and Pearcy, 1999); small plant size, facilitating the movement of water to the apex (Fernández-Marín et al., 2017; Pignatti, 1978); and small xylem conduits are less vulnerable to embolism events during dry periods (Lo Gullo and Salleo, 1993; Robert et al., 2017). However, in spite of these adaptations, maintaining the foliage physiologically active during summer (Pignatti, 1978), requires some adaptive strategies to limit water losses and tissue dehydration. Indeed, plants present two main strategies, introduced by Berger-Landefeldt (1936), classified in "isohydric" and "anisohydric". The first class of plants, during dry conditions, are able to maintain a constant daily water potential by reducing stomatal conductance. On the other hand, anisohydric species show fluctuations of water potential responding to the reduction of water availability (Nardini et al., 2014). However, despite this classification is largely used, there are not

clear distinctions between these two strategies (Hochberg et al., 2018). In fact, plants do not conform clearly to this classification, but merely show a tendency to present one strategy over the other; even plants belonging to the same species manifest differences in their isohydric strategy (Hochberg et al., 2018).

For example, a typical Mediterranean plant, the *Quercus ilex* L. is an evergreen oak, with leaves characterised by a thick cuticle. Its shrubby habitus is typical of arid areas, while in more favourable zones it can be found as a tree of up to 20 m. *Q. ilex* has a slow growth rate with a deep rooting system, which allows it to have a high tolerance against drought (Pignatti, 1978). As a strategy to limit water loss, *Q. ilex* it was mainly classified as belonging of the isohydric class (Garcia-Forner et al., 2017; Nardini et al., 2014; Trifilò et al., 2015), but sometimes, under particular environment conditions, *Q. ilex* shows an anisohydric behaviour (Aguadé et al., 2015). In addition to these morpho-anatomical traits, there are also other strategies that confer drought tolerance, such as a partial abscission of leaves during summer (Barbeta and Peñuelas, 2016), or the summer dormancy, when plants slow down their metabolism until a complete stop, to survive the drier months (Gillespie and Volaire, 2017; Kozlowski, 1976). Furthermore, plants may utilize multiple defence mechanisms to face against drought periods, such as the biosynthesis of secondary metabolites.

1.3 Secondary metabolism

The interaction between plants and the environment is a natural phenomenon, necessary to develop defence mechanisms and strategies for plants to acclimate with the extreme environmental conditions in which they grow. One of the main defence strategies utilised by plants is the production of different compounds, through their secondary metabolism. A few primary metabolites are the main building blocks for synthesising the many known secondary metabolites. These precursors of primary metabolites are represented by: the acetate unit (*i.e.*, C2 for the synthesis of polyketides and fatty acid); the phenylalanine/tyrosine derived unit (*i.e.*, C9 for the synthesis of phenylpropanoids); and the isomeric isopentenyl diphosphate and dimethylallyl

diphosphate units (*i.e.*, C5 for the synthesis of the terpenoids) (Delgoda and Murray, 2017). Mediterranean plants synthesise a large number of secondary metabolites (Ormeño et al., 2007a; Sardans and Peñuelas, 2013). These compounds are synthetised to increase the plant tolerance to abiotic and biotic stresses: (i) photo-protection against UV-radiation and excessive light conditions (Gil et al., 2013, 2012); (ii) drought stress tolerance (Babaei et al., 2021); (iii) ozone and heat tolerance (Loreto and Schnitzler, 2010; Sharkey and Singsaas, 1995); and (iv) protection from reactive oxygen species (ROS) (Bonn et al., 2019; Parveen et al., 2019). The environmental stressor UV-B (280-315nm) induces alterations in gene transcription and translation, as well as causing damages in the photosynthesis process. Phenolic, terpenoids and alkaloids represent intermediate compounds of complex biochemical interaction against UV-B radiation (Ncube et al., 2012). Drought stress reduces plants growth, since it causes their stomata to close and the photosynthesis to stop (Tattini et al., 2015). Secondary metabolites, especially phenolic components, accumulated in plants tissues, play the role of antidesiccant (Horner, 1990). Heat stress, with temperatures higher than 35 °C, induces physiological, biochemical and molecular alterations in plants, such as damages to membrane integrity or protein denaturation. In response to these conditions, plants may activate oxygen species-scavenging enzymes (*e.g.*, superoxide dismutase, catalase, peroxidase, *etc*…) as the first line of defence (Gill and Tuteja, 2010; Griesser et al., 2015) and increase the biosynthesis of Volatile Organic Compounds (VOCs), then released in the atmosphere (Sharkey and Loreto, 1993; Sharkey and Yeh, 2001; Zobayed et al., 2005). Furthermore, among secondary metabolites, terpenes can also provide protection against herbivores, insects and various microorganisms as well as attracting pollinators (Holopainen et al., 2013). Lastly, through allelopathic actions, they improve plant competitiveness for habitat resources such as space, light, water and nutrients within plant communities (Holopainen et al., 2013; Polechońska et al., 2019).

1.3.1 Terpenes

The emission of terpenes shows daily and seasonal trends, which could be explained by their protective role against UV and thermal stress. Indeed, their biosynthesis is stimulated by high changes in temperature and irradiance, and for the most volatile monoterpenes the maximum emission rate happens during the warmest months in the year and the warmest hours of the day (Llusià and Peñuelas, 2000).

Terpenes are a large class of organic compounds synthesised by secondary metabolism, including more than 50,000 molecules (Ajikumar et al., 2008; Keasling and Eiben, 2019; Maimone and Baran, 2007; Mewalal et al., 2017). All terpenes are hydrocarbons derived from isoprene, a molecule with 5 atoms of carbon; for this reason, terpenes are classified according to isoprene units (in units of five-carbons each): *hemiterpenes* (C_5H_8), *monoterpenes* ($C_{10}H_{16}$), *sesquiterpenes* ($C_{15}H_{24}$), *diterpenes* ($C_{20}H_{32}$), *triterpenes* ($C_{30}H_{48}$), *polyterpenes* ($(C_5H_8)_n$ with $n > 8$) (Ashour et al., 2010; Mewalal et al., 2017; Tetali, 2019). Mono- and sesquiterpenes are volatile in nature, and for this reason they are usually referred to as Biogenic Volatile Organic Compounds (BVOCs) (Langenheim, 1994). Terpenes are synthesised from products deriving from primary metabolism through two biosynthetic pathways: (i) Mevalonic Acid pathway (MVA) starting from the fusion of 3 molecules of acetyl-CoA (Maffei et al., 2011), and (ii) Methylerythritol Phosphate pathway (MEP) starting from pyruvate and glyceraldeyde 3-phosphate (GA3P) condensation (Loreto and Schnitzler, 2010; Schuhr et al., 2003). The final step, in both pathways, is the synthesis of isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), in the MAV pathway with the help of 2 NADPH and 3 ATP (McGarvey and Croteau, 1995) and in the MEP pathway with the support of 1 NADPH, 1 CTP and 1 ATP (Lichtenthaler, 1999). The simplest of terpenoids is isoprene which is synthesised from DMAPP by a diphosphate elimination and a rearrangement of double bonds (Figure 2).

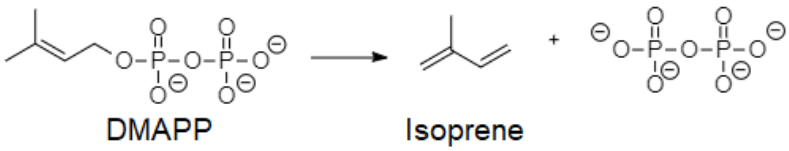

Figure 2. Diphosphate elimination and formation of isoprene.

This step is catalysed into chloroplasts (Silver and Fall, 1995) by the enzyme isoprene synthase which requires Mg^{2+} or Mn^{2+} to be activated (Kuzma and Fall, 1993). The other terpenoids have been synthesised from unions between one or more IPP and DMAPP molecules (Eisenreich et al., 2004). The MVA pathway occurs in eukaryotes (*i.e.*, animals and fungi) and in archaebacteria; the MEP pathway takes place in eubacteria (Eisenreich et al., 2004). Plants possess both pathways, the MVA's reactions take place in the cytosol while the MEP's reactions happen in the plastids (Oldfield and Lin, 2012; Singh and Sharma, 2015). Based on the pathway, different terpenes are synthesised: the sesquiterpenes and triterpenes are synthesised following the MVA pathway and the hemiterpenes, monoterpenes and diterpenes following the MEP pathway (Feng et al., 2019; Kesselmeier and Staudt, 1999; Loreto and Schnitzler, 2010; Oldfield and Lin, 2012). Finally, Sallaud et al. (2009) reported that sesquiterpenes may be also derived from MEP pathway. It should be mentioned that the two metabolic pathways are not strictly separated, with some intermediate metabolites able to pass through the plastidic membranes (Eisenreich et al., 2004).

1.4 Sampling techniques to analyse volatile terpenes: a brief overview

Given the increasing global interest on volatile organic compounds and the related important information they can provide on the status of plants (considered as proxies for stress signals), it is useful to list and provide a review of the main sampling techniques developed in the last twenty years to analyse *in-vivo* these volatile compounds. The

traditional sampling is destructive and focuses only on some elements of the plant (*e.g.*, roots, stems, leaves, flowers, fruits) and not on the entire individual. In this case, the sampling strategy could result in unwarranted stress and mechanical damages modifying the BVOCs biosynthesis and emission (Manzo et al., 2014). Furthermore, this provides measures only on a small group of individuals within a given community and does not allow for a clear representation of large areas. Additionally, these traditional methods require sample preparation through solvent extraction and often distillation. In many instances, the alterations that these samples are subjected to through these processes, would lead to the formation of artefacts (Zhu et al., 2013). For these reasons, a different *in-vivo* sampling, such as the use of solid-phase microextraction (SPME) techniques, should be preferred (Risticevic et al., 2016). SPME was introduced in 1989 to provide a rapid sample preparation technique, and it is based on a small amount of extracting phase dispersed on a support. The SPME fibre is exposed for a well-defined period of time, so as to reach an equilibrium between the sample matrix and the extraction phase (Risticevic et al., 2016). The most common use of SPME to collect BVOCs *in-vivo* is by using the headspace (HS) sampling (*i.e.*, expose the SPME fibre to the gaseous phase of sample - Tholl et al., 2006). The HS technique allows for a non-invasive and non-destructive sampling strategy and it can be either static (S-HS) or dynamic (D-HS) (Pawliszyn, 2012). In both cases (*i.e.*, S-HS and D-HS), the whole plant or a singular living part of it is sealed in a closed system to concentrate the volatile components. The S-HS strategy of BVOCs collection presents some evident disadvantages, as there is a micro-climate alteration into the closed system inside which the living plant (or part of it) is located: the CO_2 concentration decreases, while the O_2 concentration and the humidity level increase, especially under illumination. All these factors result in a change of biosynthesis and emission of BVOCs. For these reasons, this method of sampling is commonly used for qualitative analyses of BVOCs (Tholl et al., 2006). On the other hand, the D-HS method, for plant BVOCs analysis, was introduced in the 1960s (Wahlroos and Nikkilä, 1966). This method is nowadays one of the most used approaches in this research area providing more accurate measurements (Niinemets et

al., 2011). D-HS consists in an inert gas flowing through a trapping system where the plant is positioned (Tholl et al., 2006). In all cases, the SPME fibres must be desorptioned to ensure full analyte recovery, which requires the use of a Gas Chromatographic-Mass Spectrometry system (GC-MS). Thus, to obtain a correct set-up for this strategy and a reliable *in-vivo* sampling, a relatively complex equipment and the standardisation of several parameters is needed (Pawliszyn, 2012). Both systems (S-HS and D-HS) have the limitation of analysing only a small number of individuals and do not provide a signal for the whole ecosystem.

A novel, easy-to-use, fast, portable, and sensitive sampling method to investigate and determine BVOCs profile of plants in the atmosphere is fundamental to the correct characterisation of plants metabolism and of their relationships with the ecosystem, particularly considering the ongoing climate changes and its related consequences.

1.5 Climate change and its effects on forest ecosystems

Global climate presents continuous phases of changing, which can be influenced by external factors. Some of the most important causes having a big impact on the recent and fast rate of climate change are human activities, indicated by IPCC (Intergovernmental Panel on Climate Change) as the principal cause of global warming (Field et al., 2014). Indeed, high anthropogenic activities, including changes in land-uses, habitat fragmentation, fossil fuel consumption have played a main detrimental role in ecological communities (Fahrig, 2003; Newbold et al., 2020). Nowadays, global temperatures have registered an increase with a range of 0.8-1.2 °C with respect to the preindustrial levels, and the carbon dioxide concentration in the atmosphere has almost doubled in the past three centuries (from 280 ppm to 420 ppm). The IPCC, with its special report of 2018, has announced that with high probability global warming will increase to 1.5 °C by 2050 entailing irreversible implications for the world's climate and for the habitats of the Earth (Masson-Delmotte et al., 2018). Climate change is a great danger to biodiversity and to human health and well-being in the near future. Plants are also highly affected by this, since they are not able to rapidly adapt to weather changes

(Ramakrishna and Ravishankar, 2011). The high level of CO_2 in the atmosphere and the related global warming can cause changes in the chemical composition of plants secondary metabolites (Idso and Idso, 1989; Lommen et al., 2008; Ncube et al., 2012), and the consequences of this are still unclear to date. Several studies investigated these alterations on plant secondary metabolism, but results are often contrasting because they are species-specific (Hartley et al., 2000; Loreto et al., 2006; Ramakrishna and Ravishankar, 2011; Williams et al., 1994).

Mediterranean biomes are identified as a climate hotspots, thus they are among the most exposed to the potentially harmful effects of ongoing climate change (Gualdi et al., 2013). In the Mediterranean climate regions, climate change is leading to an increase in temperature, with more warmer days, a disruption in the precipitations patterns during all seasons, resulting in a decrease in available water and nutrients in the growing seasons. In fact, in the last decades, anomalous drought and heat waves episodes have already shown the potentially large impacts of modification of ecosystems on Mediterranean vegetation (Cowling et al., 1996; Martínez-Vilalta et al., 2002; Matusick et al., 2013; Pereira and Chaves, 1995). Thus, a modification of biomes and of habitats will be likely inevitable, involving high levels of mortality for some native species as the environmental conditions will not be favourable for them and might involve the dispersal of new, exotic species.

1.5.1 Dieback after drought

Anomalous events of drought and heat are becoming more frequent in Mediterranean regions and their impacts on forest communities are more intense, causing partial to total crown defoliation, and increasing the tree mortality (Pollastrini et al., 2019). Drought stress can cause tree mortality by two main processes: hydraulic failure and carbon starvation (Colangelo et al., 2017; McDowell et al., 2008). Hydraulic failure describes a lethal level of embolism, occurring during severe and prolonged droughts (Anderegg and Anderegg, 2013; Mitchell et al., 2013). The carbon starvation phenomenon is usually defined as the (total or partial) depletion of non-structural

carbohydrates leading to plant's distress and death (McDowell and Sevanto, 2010). Carbon starvation is a drought-induced mechanism, since the lack of photosynthetic sugars is linked to stomatal closure and decreases in carbon assimilation (Hartmann, 2015). Evergreen sclerophyllous species, typical of the Mediterranean regions, are supposed to be better adapted to respond and survive to drought events (Bussotti et al., 2014). However, increasingly intense and prolonged drought events and heat stress induce ongoing forests decline and mortality, as it has been reported in several parts of Mediterranean basin (Camarero et al., 2015; Peñuelas et al., 2018) and in Italy (Colangelo et al., 2017; Gentilesca et al., 2017; Pollastrini et al., 2019). In addition to widespread leaf desiccation, crown defoliation and tree mortality, these impacts are linked to alterations in terpene emissions by plants (Ormeño et al., 2007b). Identifying and monitoring terpene emission is crucial for ecological purposes, as they have significant effects on atmospheric chemistry, physics as well as on other organisms (Peñuelas and Staudt, 2010). Additionally, information on terpenes concentration is important for wildfire monitoring and management, as their high concentrations have been linked to increases in plant flammability and the associated fire risk (Ormeño et al., 2011). Therefore, it is necessary to estimate the magnitude of plant emissions and the diversity of volatile compounds to monitor the status of forests.

1.5.2 How climate change impacts biodiversity

The intensity of current climate change has the potential to alter the structure, composition, and functionality of forest habitats. Indeed, the changing climate is leading to changes in the environment: more rapid and excessive monsoon-like rainfall, changes in nutrients and water availability, warmer summer days, less frequent and lower rainfall during the dry season, stronger storms with gusty winds (Field et al., 2014; Masson-Delmotte et al., 2018). Plants, particularly the endemic plant species of some ecosystems, are more vulnerable to climate change than other organisms (Larson et al., 2020; Sardans and Peñuelas, 2014; Thomas et al., 2001). These alterations could lead to both an increase of plants mortality, as mentioned above (see section 1.5.1), as well as

making these habitats more susceptible to the introduction of native and exotic invasive species (D'Antonio et al., 1999; MacDougall et al., 2013; Rose and Fairweather, 1997). Invasive species could find a favourable environment and spread easily due to the dieback of previously present species as well as the altered environmental characteristics could result in better growing/living conditions for them (Adair and Groves, 1998; Cronk and Fuller, 1995; Klepeis et al., 2009). Indeed, as cascading effects, this invasion will contribute to a further unbalancing of the habitat: establishing additional competition for nutrients and other factors necessary to life (O'Leary et al., 2018; Simberloff, 2011), and, modifying the ecosystem via the emission of allelopathic substances (Foy and Inderjit, 2001; Gris et al., 2019). Finally, the biodiversity change at vegetation level could result in a change in wildlife (*e.g.*, birds and insects) richness and abundance, since they are strongly affected by vegetation structure and floristic richness and modify seed germination and dispersal (O'Leary et al., 2021). Monitoring and studying biodiversity alterations in forests could be crucial to provide plants terpenic profile and not only ecological inventories. Indeed, variations of terpene emission patterns, especially those of monoterpenes, as well as being affected by environmental conditions (Niinemets et al., 2004) and by seasonality (Peñuelas and Llusià, 1999) could also reflect historical changes of species distributions and species-complexes (Hanover, 1992; Loreto et al., 2009).

1.6 Terpenes functions outside the plant world

It is important to monitor and study forest terpene profiles for ecological reasons, as previously stated (see sections 1.5.1 and 1.5.2). Furthermore, it is crucial to study the environmental conditions that can trigger the biosynthesis of secondary metabolites, given their potential beneficial functions in the production of several natural products at a wide scale.

1.6.1 Role of terpenes on air chemistry

Terpenes do not only play a fundamental role in forest ecosystems, but they also have a great impact on biosphere–atmosphere interactions and reactivity, altering aerosol growth processes and cloud formation, in particular in the urban environment (Ghirardo et al., 2016). Plants are well known for their ability to increase CO_2 uptake and allow the detoxification of ozone, NO_x and anthropogenic VOCs (AVOCs) (Nowak, 2013). On the other hand, plants emit VOCs (*i.e.*, BVOCs), which can impact air quality because of their high chemical reactivity (Ghirardo et al., 2016). Indeed, with the oxidation of these reactive organic compounds, less volatile products are formed giving rise to particles involved in the information of the secondary organic aerosol formation (SOA) (Slowik et al., 2010). In detail, the products of terpenes oxidation, in particular those of monoterpenes, are precursors of tropospheric ozone (O_3) (Goldstein and Galbally, 2007; Griffin et al., 1999), while sesquiterpenes play an important contribution to SOA formation, even when found at low concentrations in the atmosphere and when they derive from AVOCs (> 90%) (Ghirardo et al., 2016; Mentel et al., 2013). Thus, the volatile terpenes biosynthesised by plants influence the chemical lifetime of reactive gases in the atmosphere, altering air composition (Goldstein et al., 2009). For these reasons, it is necessary to include the contribution of BVOCs to SOA and pollution formation and consequently in the study of air quality. On this basis, the landscape planning of urban areas should consider plant species characterised by low emission of BVOCs to mitigate the VOC load in the atmosphere (Churkina, 2015).

1.6.2 Uses of terpenes in different industries.

Furthermore, the demand of plant secondary metabolites has steadily increased due to their multiple uses in different industries. Indeed, several terpenes are useful for the daily human life, acting as: sustainable source of energy by replacing fossil fuels (Mewalal et al., 2017; Tetali, 2019); additive flavours in food, beverages and in the perfume industry (Ashour et al., 2010); as natural insecticide (Isman, 2006); and by providing several benefits for human health (Pasquini et al., 2021a). In fact, the use of

terpene-based pharmaceuticals was estimated at 12 billion US $ in the pharmaceutical industry (Guimarães et al., 2014). Natural compounds and extracts have been used in medicine since ancient times, but recently terpenic compounds have received an increasing interest in the sector because they offer a wide range of possible applications (Pasquini et al., 2021a).Terpenes can been used in chemotherapy, since β-pinene, p-cymene, myrcene and D-limonene have been shown to act as DNA protectors and cancer cells inhibitors (Bakarnga-Via et al., 2014; Kris-Etherton et al., 2002; Silva et al., 2007). Their actions are multiple: in the initial stage they can prevent cancer cells to attack DNA; if this has already happened, they can inhibit cancer cells migration and development. Additionally, they promote tumour regression, allowing cancer cell apoptosis (Crowell, 1997; Lu, 2004). Several studies have shown the key role of terpenes against liver and pancreas tumours, lung and breast, leukaemia as well as against neuronal diseases (*e.g.*, borneol is an important neuroprotective against Alzheimer's disease) (Cheng et al., 2014; Hong et al., 2011; Legault and Pichette, 2007; Sobral et al., 2014). Moreover, menthol, 1,8-cyneol, α-terpineol and linalool present antimicrobial and antiparasitic functions (Rodrigues Goulart et al., 2004; Trombetta et al., 2005); thymol, 1,8-cyneol, limonene, α- and β-pinene provide analgesic actions and can improve the quality of life in patients subjected to several types of pain (*i.e.* headaches, arthritis, muscular aches…) (De Sousa, 2011; Guimarães et al., 2014). Finally, recent studies demonstrated that α-pinene, β-pinene, car-3-ene, borneol, verbenol and linalool, when inhaled, provide anti-depressive and anxiolytic functions (Linck et al., 2010; Souto-Maior et al., 2011; Woo and Lee, 2020). The latter one, is well reflected in an ancient practice called by the Japanese Forest Agency "s*hinrin-yoku"* (*i.e.*, Forest bathing) and consist in the "immersion of yourself in nature by mindfully using all five senses" (Tsunetsugu et al., 2010). Naturally, inhaling terpenes while walking in a forest does not provide constant benefits, as these effects depend on the environment, the climate conditions and individual characteristics. In any case, Peterfalvi *et al.*, (2019) in a recent review reported that by walking for 2 hours in the forest, the concentration of Natural Killer cells could be increased, thus promoting protection against cancer.

1.7 Research aims

This thesis broadly focuses on the effects of climate change at ecosystem level. In particular, were focused two aspects related to climate change: the effects of recurrent and prolonged drought conditions on a natural Mediterranean ecosystem and the mechanisms involved in the invasion of natural habitats by invasive plants. These are two important threats to biodiversity conservation and ecosystem stability at regional and global scale. In this thesis, these two aspects focusing on the emission of BVOCs chemical profiles at the atmospheric level have been investigated. To address this general aim, several research objectives have been defined as follows:

Objective I: to develop and improve a method for the collection and analysis of BVOCs at canopy level (Appendix A – published).

Objective II: to evaluate seasonal differences in the chemical profiles and in the amounts of BVOCs emitted by plants in a natural reserve characterised by high and low rates of *Quercus ilex* mortality, while also observing a wide spectrum of ecological community aspects (Appendix B – ready for submission).

Objective III: to study BVOCs chemical profiles at the atmospheric level and other secondary metabolites stored into leaves between pure *Eucalyptus* forests and *Eucalyptus* forests invaded by *Pittosporum undulatum* (Appendix C – submitted).

Objective IV: to provide an overview of BVOCs biosynthesis, their role in plant physiology and their applicability for human health (Appendix D: - published).

2. Materials and methods

2.1 Solid-phase microextraction technique

Solid-phase microextraction (SPME) is a sampling technique that was developed by Pawliszyn in 1989 (Belardi and Pawliszyn, 1989). Since 1993, the year in which SPME fibres were placed on the market, this methodology has greatly evolved (Dietz et al., 2006). The most common applications are with organic compounds, *i.e.*, volatile organic compounds (VOCs), the group of benzene, toluene, ethylbenzene, and xylenes (BTEX), polycyclic aromatic hydrocarbons (PAHs), pesticides, odours and flavours analysis. Furthermore, there are several fields of application, including environmental, clinical, forensic, food and botanical analysis (Dietz et al., 2006). The technique is based on creating an equilibrium between a solid support coated with a small amount of extracting phase and the sample matrix (Lord and Pawliszyn, 2000). After this step, the analytes undergo a subsequent desorption into the analytical instrument (Risticevic et al., 2009). Since the limits of this technique depend on the properties of the sorbent coating material (Dietz et al., 2006), it is fundamental to choose the right SPME coating for the purposes of the research among the various absorbent coating which can be commercially available. SPME fibres combine sampling, extraction and pre-concentration into an individual step and they provide repeatable results, while offering a solvent free sampling, being easy-to-handle, and durable (Shirey, 2012). Here, the type of fibre chosen was divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS-50/30 µm layer, Supelco, Sigma-Aldrich Co., Darmstadt, Germany), since this coating is considered the most appropriate for terpenoids acquisitions (Adam et al., 2005; Qian et al., 2019).

2.2 Dynamic BVOCs Sampling System

Each SPME fibre was held in the middle of a custom-made dynamic BVOCs sampling system (DBSS) (Pasquini et al., 2021b). This sampling system consists of a 17 cm long aluminium cylinder, with a smaller vertical cylinder 7 cm long perpendicularly

connected to it and acting as a support for the fibre (Appendix A). Since the chosen fibres were 2 cm long, the main cylinder was chosen of 4 cm to internal diameter to allow a proper and complete exposition of the fibre. In addition, one face of the main cylinder was closed by a metal net to protect the fibre from debris and to create a swirling flow, while on the other face was placed a small fan (Jamicon®, Kaimei Electronic Corp., New Taipei City, Taiwan, 12 V, 6200 rpm, 13 m^3/h, 40 × 40 × 20 mm) (Figure 3) powered by a lead–acid battery (Join®, Alpha Elettronica S.r.l., Collecchio, Italy, 12 V, 4.5 AH). All parts are linked together by screws instead of glue to allow the utilization under high-temperature conditions and to avoid any possible interferences during the sampling. The chosen material of the device is aluminium because it is light and resistant, thus allowing for an easier sampling and movement in the forest during fieldwork. After sampling, the fibres were positioned in a tray with Teflon cones to seal the needles, within a hermetic case then transported to the laboratory, and subsequently desorbed.

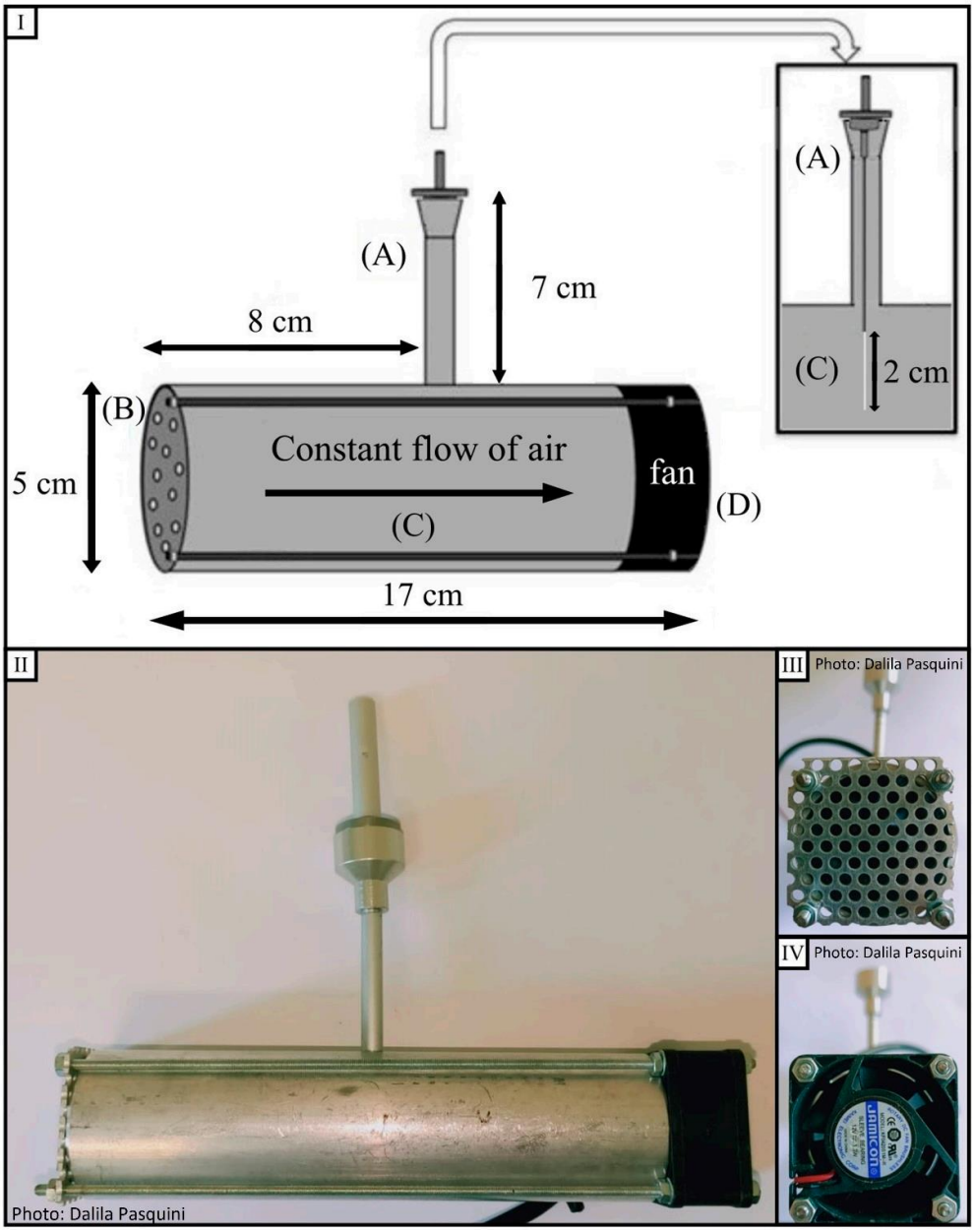

Figure 3. In panel I, are presented the schematics of the dynamic sampling system. The SPME fibres was firmly positioned in the appropriate vertical tube (A). A metal net (B) was installed at one end of the cylinder (C) to protect the fibre, while at the opposite end, a fan (D) was positioned to create a swirling flow. In panel II a picture of the whole device is reported, while in panel III and IV, the details of the net located in the front of the device and the fan set on the back are shown, respectively.

2.3 Collection and analysis of BVOCs

The collection of Biogenic Volatile Organic Compounds was performed, in all studies (Appendices A, B, C), during rainless days, characterised by the absence of gusty winds, to avoid possible damages to the SPME fibres. During all samplings days the air temperature and humidity, as well as wind direction and speed, were recorded using portable devices (Brift Hygrometer Bluetooth, SensorBlue, FC RoHS). The fibres, mounted on the DBSS instruments were left for 4 hours, from 12 pm to 4 pm, to collect BVOCs after switching on the external battery. This sampling time was selected because this is the time interval with the highest peak of emissions, as BVOC emissions are linked to high temperatures and solar radiation (Fuentes et al., 2007; Li et al., 2013; Strong et al., 2004). In addition, sampling duration was set to 4 h to allow for a long exposition of SPME fibres and to have a feasible sampling time in the field during every season. Indeed, longer acquisition times could be difficult to implement in winter and fall because of shorter daylight and of more frequent unsuitable weather conditions such as strong winds and rains. The DBSS devices were positioned at a height of 40-45 cm from the ground with a metal picket acting as support. This height was chosen to make the set up easier for the operator (*i.e.*, to expose and to retract the fibre; to turn on and off the fan), to limit possible wind action and since terpene concentrations are higher at heights from 0 to 4 m (Noe et al., 2012). In addition, the lead–acid batteries were positioned in a plastic open bag to avoid soil moisture deterioration in Autumn and with a sort of home-made coverage to avoid risk of fire from sunlight warming.

The desorption of SPME fibres was carried out with a gas chromatograph coupled with a mass spectrometer (more details of instruments used are provided in Appendices A – B and C) operating in EI ionization mode at 70 eV energy. In all studies the separation of analytes was carried out with a DB-Wax (60 m × 250 µm × 0.5 µm, Agilent J&W) column. The injector temperature was set to 260 °C, in splitless mode and the carrier flow (He) was set to 1.2 mL min^{-1}. The oven temperature program was initially set to 40 °C per one minute, then increased with 5 °C/min increments until a temperature

of 210 °C was reached, and then increased by 10 °C/min until 260 °C. Once this temperature was reached, it was held for 10 min, resulting in a total run time of 48 min. The mass spectra were acquired between the lower mass of 29 *m/z* and the higher mass of 350 *m*/*z* at three scans per second. Data were analysed using the Agilent Mass Hunter software package (Qualitative Analysis-Version B.06.00; Quantitative AnalysisVersion B.07.01/Build 7.1.524.0), with the resulting analytes being identified by matching their mass spectra and retention indices with those reported in NIST 11 spectral database library. In order to identify the compounds, information related to fragmentation patterns and retention times found in the literature was used (Goodner, 2008; Vezzola et al., 2019).

2.4 Sample collection and study area in Italy

2.4.1 Experimental fields in Sesto Fiorentino and BVOCs collection

One of the two areas used in the study of the development of the DBSS device (Appendix A) was located in Sesto Fiorentino (FI, Italy) at the experimental field of CNR (National Research Council of Italy, 55 m elevation, latitude 43°49'05" N, longitude 11°12'1" E) in the north-eastern part of Tuscany, Italy. The experimental field is a flat area equipped with small greenhouses and open areas (without overhead coverage); this whole area is fenced off and located next to the CNR laboratories. This site was chosen to test the capabilities of the new BVOCs sampling system at the environmental level, comparing it with the static technique which involved having the fibre exposed directly to the air. Having this experimental field near the CNR presented two advantages: (i) the possibility to analyse immediately the samples avoiding the risk of their deterioration and (ii) the presence of a nearby weather station (Consorzio LaMMa), allowing to monitor air temperature, humidity, wind speed, and direction. Lastly, we used *Quercus ilex* plants, a species of great study interest given its strong ongoing dieback occurring in maritime Tuscan forests. The potted plants (kindly supplied by Vivaio Matteini, Pistoia, Italy) were 3-year-old, with stem diameters of ca. 1 cm and heights of about 120 cm. BVOCs emissions were collected on the 6th of June

2019 (with 24 °C of air temperature, 45% humidity, 9-14 km/h wind speed, and W/SW wind direction) from 15 *Q. ilex* plants positioned in a circular pattern. Three DBSS devices coupled with as many SPME fibres and three additional fibres without the DBSS were located inside this circle at the same height from the ground and distance from the plants (Figure 4). These six fibres exposed with two different sampling techniques (*i.e.*, dynamic with DBSS and static), were used to compare the collection capacity of the BVOCs in the two selected strategies.

Figure 4. On the left-hand side is presented the blueprint of the sampling design of the proof of concept conducted in Sesto Fiorentino, at the experimental field of CNR, with the disposition of the 15 potted holm oaks and the 6 fibres inside of the circle (three set on the DBSS devices – red arrows; three in static condition – blue arrows). On the right-hand side is shown a picture of the two compared strategies.

2.4.2 Experimental fields in natural reserve in Tuscany and BVOCs collection

The first study (Appendix A) was continued in a second area, to observe the signals of BVOCs collected by DBSS compared to the static technique at an environmental level in a forest habitat. This site was in the Maremma Regional Reserve (Alberese, latitude 42°38'10" N, longitude 11°05'39" E, Grosseto, GR, Italy) near the San Rabano abbey, in

the south-western part of Tuscany, Italy (Figure 5). The Maremma Regional Reserve covers an area of about 18,000 ha, of which 8,902 ha have been designated as "protected area" since 1975. The habitats found here are multiple: marshes in the north, close to the estuary of the Ombrone river; next to the sea, on the western side of the Reserve there are costal dunes and steep cliffs; towards the centre, there are costal pine forests, Mediterranean maquis and holm oaks forests. The Reserve comprises the *Monti dell'Uccellina*, modest reliefs that reach 413 m above sea-level with *Poggio Lecci.* Here the forest vegetation is heterogeneous, and the dominant species is *Quercus ilex.* In *Poggio dell'Uccellina* is located the Benedictine abbey of San Rabano, near the top of *Poggio Lecci*, and within these *Q. ilex* forests the friars had gardens for their livelihood and for medical practices. This reserve and particularly the *Q. ilex* forests located between *Poggio dell'Uccellina* and *Oliveto del Collelungo* were chosen for the first study (Appendix A) to demonstrate the higher efficiency of the new BVOCs sampling system in a mature Mediterranean forest habitat devoid of potential interferences deriving from industries, cities or roads in its proximity. The BVOCs emissions were collected on two consecutive days: one day during summer, on the 20th of June 2019, and one in autumn, on the 1st of October 2019.

Additionally, the same area was chosen for the second study (Appendix B) because of the occurrence of a widespread dieback of *Q. ilex*. For this study, two zones have been selected: the first area (study area LD) was located near the San Rabano abbey and is characterized by a low rate of tree mortality, with only 7% of dead standing stems (latitude 42°38'05.73" N, longitude 11°05'43.90" E, 320 m above sea-level and facing S-SW); the second area (study area HD) was located about 1,200 m NW of the abbey, towards *Oliveto Collelungo* and presents a high rate of tree mortality, with 34% of all standing trees being dead of which 78% were *Q. ilex* (latitude 42°38'29.10" N, longitude 11°05'17.25" E, 311 m above sea-level and facing W-SW) (Figure 5).

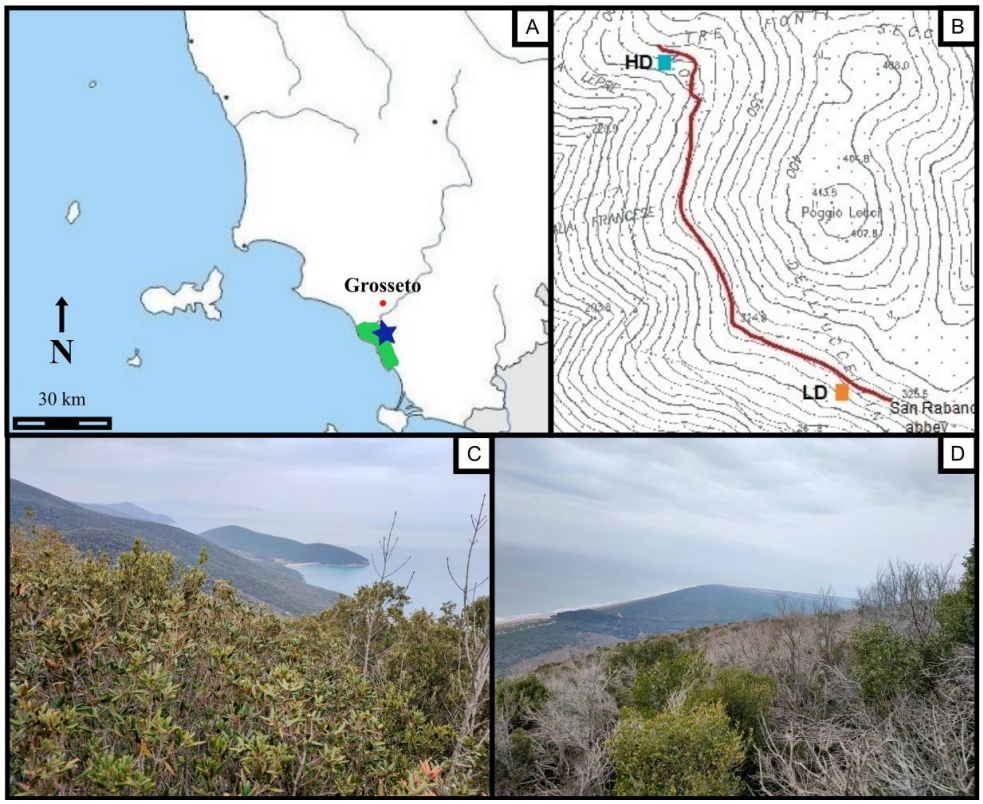

Figure 5. Maps of the study site: the map in panel A shows the location of the study areas (blue star) with respect to the Tuscany region (Italy), and to the city of Grosseto. In panel B, the orange rectangle (30 m × 30 m) represents the study area Low Dieback (LD), characterised by a low tree dieback rate. The light-blue rectangle (30 m × 30 m) represents the study area High Dieback (HD), characterised by a high tree dieback rate. The red line shows the path that links the two areas (ca. 1,200 m). Finally, the panels C and D represent the views of the two study areas LD and HD, respectively.

In both study areas, three plots of around 200 m^2 were established and designated by consecutive numbers following the letter of the area they belonged to (*i.e.*, LD1, LD2 and LD3; HD1, HD2 and HD3). In these plots some preliminary soil analyses were conducted: soil samples were collected in May 2019 to measure texture and soil pH. The forest presents a structure characterized by a single canopy layer, with a maximum tree height of 12 m and dominated by *Q. ilex*. No lichens or mosses were found on the forest floor, but several shrub species are present, especially *Cistus monspeliensis*, *Pistacia lentiscus* and *Rubus ulmifolius* (Table 1).

Table 1. List of the species identified in both areas (LD and HD) in all the seasons measured during the three years of the study (2019-2021). The species, the family and the vegetation *habitus* are reported.

Species	Family	Vegetation *habitus*	2019						2020						2021					
			Summer		Autumn		Winter		Summer		Autumn		Winter		Spring		Summer		Autumn	
			LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Acer campestre L.	*Sapindaceae*	Tree		√		√		√		√		√		√		√		√		√
Alliaria petiolata M. Bieb.	*Brassicaceae*	Herbaceous											√	√	√		√		√	
Arum italicum Mill.	*Araceae*	Herbaceous											√		√	√				
Asparagus acutifolius L.	*Asparagaceae*	Herbaceous															√		√	
Atropa belladonna L.	*Solanaceae*	Herbaceous	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Brachypodium rupestre Host.	*Poaceae*	Herbaceous			√						√		√			√				
Cistus monspeliensis L.	*Cistaceae*	Shrub	√	√	√	√	√	√		√	√	√	√		√	√	√	√	√	√
Clinopodium nepeta L.	*Lamiaceae*	Herbaceous																	√	
Cytinus hypocistis L.	*Cytinaceae*	Herbaceous														√				
Datura stramonium L.	*Solanaceae*	Herbaceous			√	√			√	√	√		√		√		√			
Dioscorea communis L.	*Dioscoreaceae*	Herbaceous													√					
Dittrichia viscosa L.	*Asteraceae*	Herbaceous	√	√	√	√			√	√	√	√	√	√	√	√	√	√	√	√
Erigeron canadensis L.	*Asteraceae*	Herbaceous															√	√	√	√
Euphorbia tinctoria L.	*Euphorbiaceae*	Herbaceous													√	√				
Ferula communis L.	*Apiaceae*	Herbaceous					√				√				√					

Table 1. (continued).

Species	Family	Vegetation *habitus*	2019						2020						2021					
			Summer		Autumn		Winter		Summer		Autumn		Winter		Spring		Summer		Autumn	
			LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Fraxinus ornus L.	*Oleaceae*	Tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Hedera helix L.	*Araliaceae*	Shrub	√		√		√		√		√		√	√	√		√		√	
Hyoscyamus albus L.	*Solanaceae*	Herbaceous							√		√		√	√	√		√			
Hypericum perforatum L.	*Hypericaceae*	Herbaceous			√	√					√	√	√	√	√	√	√	√	√	√
Juniperus communis L.	*Cupressaceae*	Shrub	√		√		√		√		√		√	√	√		√	√	√	√
Lamium bifidum Cirillo	*Lamiaceae*	Herbaceous											√							
Lathyrus tuberosus L.	*Fabaceae*	Herbaceous											√		√	√				
Lotus ornithopodioides L.	*Fabaceae*	Herbaceous													√	√				
Melissa officinalis L.	*Lamiaceae*	Herbaceous														√		√		
Parietaria judaica L.	*Urticaceae*	Herbaceous							√				√	√	√	√	√		√	
Phillyrea latifolia L.	*Oleaceae*	Tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Piptatherum miliaceum L.	*Poaceae*	Herbaceous							√		√		√	√		√	√	√	√	√
Pistacia lentiscus L.	*Anacardiaceae*	Shrub	√		√		√		√	√	√	√	√	√	√	√	√	√	√	√
Pyrola rotundifolia L.	*Ericaceae*	Herbaceous									√		√		√	√				
Quercus ilex L.	*Fagaceae*	Tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Quercus pubescens Willd.	*Fagaceae*	Tree	√		√		√		√		√		√		√		√		√	

Table 1. (continued).

Species	Family	Vegetation habitus	2019						2020						2021					
			Summer		Autumn		Winter		Summer		Autumn		Winter		Spring		Summer		Autumn	
			LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Rubus ulmifolius Schott.	*Rosaceae*	Shrub	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Ruscus aculeatus L.	*Asparagaceae*	Shrub																	√	
Senecio sylvaticus L.	*Asteraceae*	Herbaceous											√		√	√				
Sherardia arvensis L.	*Rubiaceae*	Herbaceous													√					
Smilax aspera L.	*Smilacaceae*	Herbaceous															√		√	
Sonchus oleraceus L.	*Asteraceae*	Herbaceous									√								√	
Trachynia distachya L.	*Poaceae*	Herbaceous							√				√		√		√		√	
Trifolium repens L.	*Fabaceae*	Herbaceous											√			√	√		√	
Urtica dioica L.	*Urticaceae*	Herbaceous					√						√		√	√				
Verbascum thapsus L.	*Scrophulariaceae*	Herbaceous			√	√			√	√	√	√			√	√		√	√	

In this site, a seasonal study of terpenic profiles (in the month of July for summer, and in October to autumn) was carried out over a three-year period. To evaluate possible differences in terpenic profiles and in the amounts of BVOCs emitted by *Q. ilex* in the two areas with different rates of tree mortality, 10 DBSS devices, coupled with the same number of SPME fibres, were utilised for two consecutive days (n = 20), following a similar protocol mentioned in section 2.3. The atmospheric temperatures and humidity of each sampling days were punctually recorded *in-loco* (Table 2). The two sampling seasons of terpenes collection were chosen as summer and autumn are the seasons with highest and lowest monoterpenes emissions, respectively (Holopainen et al., 2013; Llusià et al., 2013; Peñuelas et al., 2005; Peñuelas and Llusià, 1999).

Table 2. The table shows the sampling date in each season and the weather conditions: atmospheric temperature (°C), humidity (%) and wind speed (km/h).

Season	**Sampling Data** (dd/mm/yyyy)	**Air temperature** (°C)	**Air humidity** (%)	**Wind** (km/h)
Summer 2019	16/07/2019	31	50	12
	17/07/2019	30	55	10
Autumn 2019	30/09/2019	24	70	10
	01/10/2019	21	65	8
Summer 2020	14/07/2020	30	37	14
	15/07/2020	29	35	13
Autumn 2020	06/10/2020	20	80	8
	07/10/2020	22	83	10
Summer 2021	12/07/2021	30	53	12
	14/07/2021	29	50	12
Autumn 2021	25/10/2021	21	75	10
	26/10/2021	19	77	6

2.4.3 Defoliation status of the canopy and ecological inventory

To monitor the progress and the ongoing health of the holm oak forest in the Maremma Regional Reserve, visual assessments of crown status for each *Q. ilex* trees present in the three delineated plots for both areas and an inventory of every species were carried out seasonally for 3 consecutive years (2019-2021). These data were collected during the same sampling days in which the terpenic emissions were acquired

(for summer and autumn - Table 2) and in the other seasons when the terpenes were not collected (except for Spring 2020, when measurements are missing due to country-wide COVID-19 restrictions in Italy). To better describe the two study areas, and to make consecutive measurements easier, a hand-drawn map for each plot was prepared, displaying the position of all plants taller than 2 m, their code-name, height, and diameter at breast height (DBH). For all these recorded plants, a yellow tag was placed around the main stem.

For each plot, at every sampling time, an inventory of every species was carried out. Following identification, the species were divided into three main groups: (i) trees, including both overstory tree species (tree height over 2 m) and understory layer (tree height under 2 m), (ii) shrubs species, (iii) herbaceous species. Tree regeneration (presence of new seedling) was not considered in the present study due to negligible numbers. In addition, the surface (in cm^2) of soil covered in each plot was visually estimated for each species. Finally, the health status of *Q. ilex* crowns, was defined assigning one of four levels of defoliation: (1) low defoliation level (<25%); (2) low to medium defoliation level (25 - 59%); (3) medium to high defoliation level (60 - 99%); (4) high defoliation level (100%), in accordance with ICP Forests Manual (Part VII.1 Assessment of Ground Vegetation Version 04/2020).

2.5 Study area in Victoria (Australia)

2.5.1 Bunjil Reserve and Pittosporum undulatum in Victoria, Australia

The other study site was located in the Bunjil Reserve (north-east of Melbourne, Victoria, Australia, Figure 6), one of the seven Phantom Hill Bushland Reserves (Appendix C). The seven reserves extend from Smiths Gully, in the north, to Watsons Creek, in the south, spanning over an area of 140 hectares. The area is characterised by an annual average precipitation of 660 mm, with the most rain falling in November (73.3 mm), and the driest month being March (42.7 mm). Regarding the temperatures, the mean of warmest temperatures during summer and winter, are approximately 27.1 °C and 14.5 °C, respectively (according to the Australian Bureau of Meteorology Station

086068, in Viewbank, approximately 15 km from Bunjil Reserve). This site was chosen to study habitats alterations due to climate change in a different Mediterranean ecosystem. Particularly, the main objective was to investigate the chemical profiles of BVOC emissions and their possible role in explaining the invasiveness of a highly invasive species, such as *Pittosporum undulatum*. Thus, continuing ongoing work (Gleadow, 1982; Gleadow and Ashton, 1981; O'Leary et al., 2021), the study focused on the invasiveness of *P. undulatum* in forests of Central Victoria.

Pittosporum undulatum is a 5-15 m tall evergreen tree, native to eastern Australia, known for its invasive role in Australian forests as well as around the world (Gleadow and Walker, 2014; Gleadow and Ashton, 1981; Gleadow and Rowan, 1982; Nunes et al., 2014). *P. undulatum* caused a serious reduction in biodiversity and a decline of many native species in forests invaded by it (Rose and Fairweather, 1997). In particular, *P. undulatum* is considered an invader in Eucalypt forests, in fact, it preferentially establishes around the bases of these species, threatening the natural biodiversity of mixed eucalypt woodlands (Gleadow and Ashton 1981). *P. undulatum* has a strong ability to colonize different habitats thanks to several favourable traits, such as an high germination capacity, the high competitiveness of its seedlings and a dense crown (Bradstock et al., 1997; Gleadow et al., 1983; Gleadow, 1982; Gleadow and Ashton, 1981; Gleadow and Rowan, 1982; Goodland and Healey, 1997; Kentish et al., 1995). In addition to the traits above mentioned, another factor that could explain the invasiveness of *P. undulatum*, might be related to the synthesis and subsequent release of allelopathic compounds. Indeed, the roots and leaves of *P. undulatum* contain high concentrations of saponins (Faizal and Geelen, 2013; Gleadow and Ashton, 1981), that may lead to the inhibition of seed germination and growth of the seedlings of other plants (Chaudhuri and Ray, 2016; Li et al., 2010; Waller, 1989). Therefore, in addition to the saponin content, a characterisation of the secondary metabolites stored and emitted by leaves of this species was carried out in this study (Appendix C).

Bunjil Reserve contained both a zone invaded by *P. undulatum*, and one with high-quality native vegetation, which was ideal for observing possible environmental terpenic

differences in the two zones using the DBSS devices. Thus, two different areas were delineated: one invaded by *P. undulatum* (I) (37°38'31.94" S, 145°14'27.14" E), and one with high-quality remnant native vegetation (R) (37°38'49" S, 145°14'57.75" E) (Figure 6). In the I area, *P. undulatum* represented ca. 40% of woody species, while ca. 55% was dominated by *Eucalyptus* spp. (*E. goniocalyx, E. polyanthemos, E. ovata*), and *Acacia* spp. represented only the remaining 5%. On the other hand, the R area was characterised by mixed eucalypt woodland (95-98% of *Eucalyptus* spp.: *E. rubida, E. goniocalyx, E. polyanthemos, E. ova*ta) and only the 2-5% by *Acacia* spp.

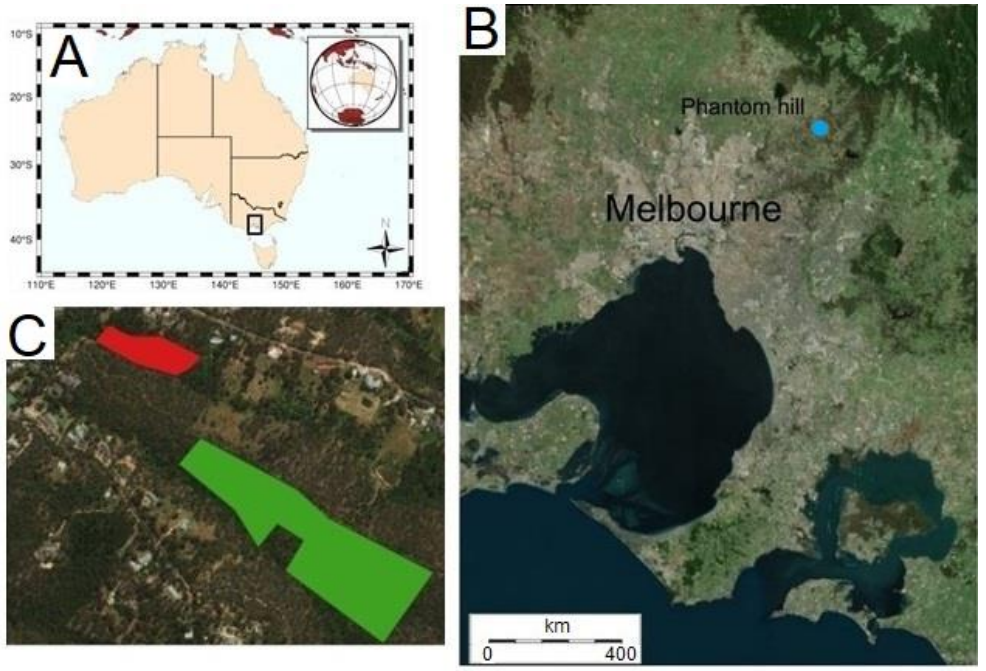

Figure 6. Location of the study sites. Panel A: location of Greater Melbourne Area within Australia. Panel B: location of the study area of Phantom Hill (light-blue dot) in respect to the city of Melbourne. Panel C: the red area represents the study site with the presence of P. undulatum (denominated I, invaded), and the green area represents the site characterised by the presence of *Eucalyptus* spp. and *Acacia* spp. (denominated R, remnant vegetation).

2.5.2 BVOCs collection in Victoria, Australia

The BVOCs collection was conducted during the Austral spring season (on the 20th of November 2019), in Phantom Hill, and five DBSS devices, coupled with an equal

number of Solid Phase Microextraction (SPME) fibres, were used at each sampling site at environmental level (Appendix C). This sampling was carried out to compare BVOCs emissions between the two areas with presence (I) and absence (R) of *P. undulatum*, to observe possible qualitative differences in terpene profiles and to identify possible compounds that could be linked to the strong invasiveness of *P. undulatum*. The mean of temperature during sampling was 37 °C, with 29% humidity, and 13 km/h wind speed with a NE wind direction. The characteristics of sampling (*e.g.,* height of DBSS, duration of sampling, sampling window time) followed the procedure presented in section 2.3.

Considering the high temperatures and the high risk of fire, special measures were taken regarding the lead–acid batteries. For this reason, the soil was cleaned of dry leaves, small deadwoods and woody debris, making sure not to move too much dust and a home-made cover was placed on top of each battery to shade it from the sun and thus avoid overheating (Figure 7).

Figure 7. Picture of DBSS device with SPME fibre during the BVOCs sampling in Bunjil reserve, Phantom Hill (Victoria, Australia).

2.5.3 Germination experiment

To assess the possible allelopathic and/or inhibitory action of *P. undulatum* towards eucalypt seed germination, a germination test was carried out. For this purpose, soil

samples were collected *in situ* in the two areas, to create nature-like germination conditions (*P. undulatum* soil from I; *Eucalyptus* soil in R). In addition, green leaves, and litter from *P. undulatum,* as well as litter from *Eucalyptus* spp. plants were sampled to prepare leachates to utilise to water the seeds during the germination experiment (the details of the single material amount sampled are reported in Appendix C). Since it has high concentration of allelopathic compounds compared with other plant parts (Abdelmigid and Morsi, 2017), only the litter was collected from *Eucalyptus* spp.

Three leachates were made (*i.e.*, *P. undulatum* green leaves, *P. undulatum* litter and *Eucalyptus* litter), placing the organic material in a volumetric flask with distilled water (to obtain a 5% aqueous extract) for 72 h at room temperature. Previously, all leaves had been washed with distilled water to eliminate possible external substances, placing extra care to not damage the tissues (Dorning and Cipollini, 2006; Parepa et al., 2012).

For the germination tests a factorial combination of three substrates (paper as control, *P. undulatum* soil, *Eucalyptus* soil), and four leachates (distilled water as control, *E.* spp. litter extract, *P. undulatum* green leaves extract and *P. undulatum* litter extract) was carried out. In addition, the test was done both on *Eucalyptus ovata* and *P. undulatum* seeds, to assess whether there were allelopathic effects of leachates on *P. undulatum* itself or on *Eucalyptus* spp. Thus, in conclusion, the experimental design consisted of 12 combinations per species and three replicates for each combination and each replicate consisted of a Petri dish (30 seeds of the same species per dish – details in Appendix C), for a total of 72 Petri dishes and 2,160 seeds (Figure 8). Seeds of *P. undulatum* were collected from a tree growing on the Clayton campus of Monash University (Melbourne, Victoria). Seeds of *E. ovata* were obtained from the Australian Tree Seed Centre (CSIRO, ACT, Australia, Seedlot 20808). The choice of *E. ovata* has test species was due to its distribution overlapping that of *P. undulatum* and, moreover, its seeds were readily available.

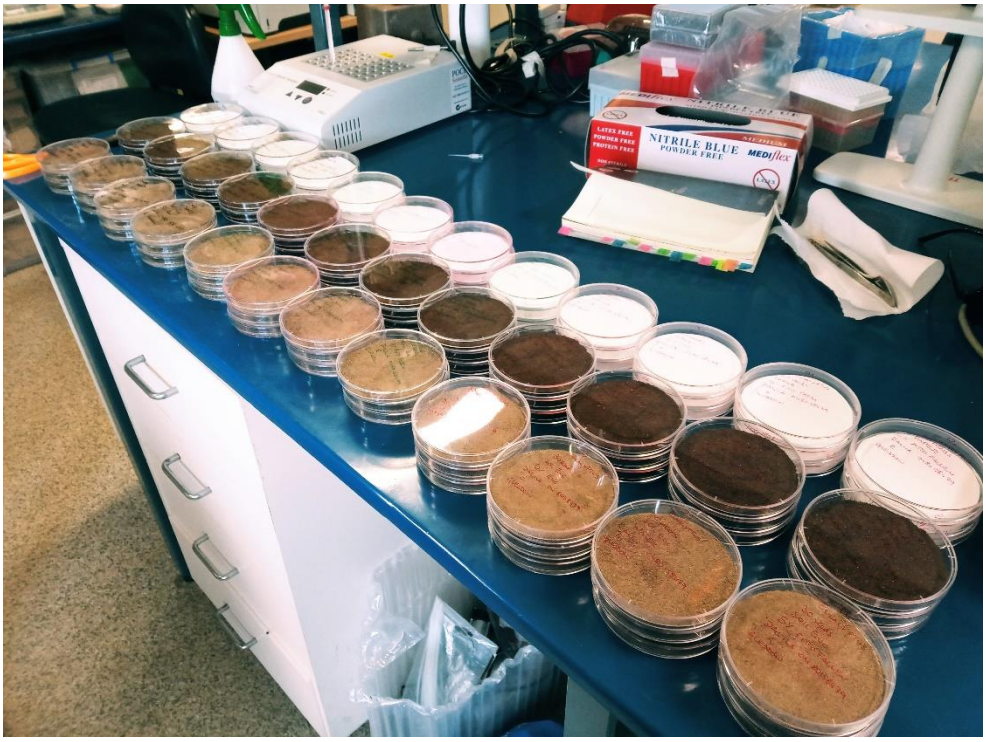

Figure 8. Picture showing all 72 Petri dishes with the different substrates before the start of the experiment (25/11/2019).

All seeds were sowed on the 25th of November, and daily visual inspections were carried out to count newly germinated seeds and to remove rotten ones. Additionally, notes on the status of cotyledons and seedlings tissues were taken regularly. At the end of the experiment (after 22 days for *E. ovata* and 38 days for *P. undulatum*) the length of hypocotyl, root and cotyledons for each seedling was noted. Seeds were considered to have germinated as soon as the embryo ruptured the seed coat (Battaglia, 1993) and the radicle became visible.

With the daily observations and data collected at the end of the experiment, germination curves were created for each species, using the package "germinationmetris" in R studio (Aravind et al., 2021). In addition, several indices were calculated to observe possible inhibition or damage to the tissues by the different leachates used during the experiment: Germination Percentage (GP), Mean Germination

Time (MGT), germination time, representing the time required to reach 50% of final germination (t_{50}) and the Vigor Index (VI) that employs the germination percentage and the lengths of the root and shoot (Farooq et al., 2005; ISTA, 2015; Kader, 2005; Ranal and Santana, 2006; Vashisth and Nagarajan, 2010) (for more details see Appendix C).

2.5.4 Biochemical analyses

To evaluate the possible allelopathic action of *P. undulatum*, chemical analyses were conducted on the leachates used in the germination experiment and the characterisation of polyphenols stored into green leaves and litter collected at the beginning of the experiment was carried out. Firstly, saponins and total condensed tannins were measured in all three leachates using UV/VIS spectrophotometer (Lambda 25, Perkinelmer) and all analyses, both for saponins and tannins, were conducted in triplicates. The Total Saponins Content (TSC) was measured following the procedure described by Le et al. (2018), and the TSC obtained was expressed as milligrams diosgenin equivalent (mg DE) per g of Dry Weight (DW) of plant material. Furthermore, the Total Condensed Tannin content (TCT) was measured following the protocol described by St-Pierre et al. (2019) and the results were expressed in catechin equivalent (mg CE) per g of DW (more details are provided in Appendix C). In addition to the green leaves and litter samples described above, to carry out a characterisation of polyphenols profiles, also approximately 50 g of green leaves of *Eucalyptus* spp. were collected during the same sampling day (20th November). Subsequently, few green leaves and litter of *P. undulatum* and *Eucalyptus* spp. were lyophilised and then powdered. After that, 150 mg of powder for each type were placed into test tubes and added with a total of 15 mL of a ethanol:water solution to extract polyphenols (totally protocol in Appendix C). 3 mL of these ethanolic extracts were analysed with spectrophotometer to obtained the content of saponins and tannins with the same protocols mentioned above; the remaining 12 mL were evaporated under vacuum and re-dissolved in 1 mL of methanol:water to be injected into Perkin® Elmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and coupled with a LC 200 diode array detector

(DAD) (Perkin Elmer®, Bradford®, CT, USA). The extracts were analysed in triplicates and the wavelengths used to quantify the compounds were 280 nm and 330 nm for *P. undulatum* and 280 nm and 350 nm for *E.* spp. (more details in Appendix C). The identification of each compound was conducted comparing the retention time and the spectra with authentic standards (gallic, caffeic, *p*-coumaric and ellagic acids, rutin and luteolin-7-O-glucoside) and with the literature. The quantitative results of polyphenols were reported as mg/g of DW.

2.6 Statistical analysis

All statistical analyses were carried out using R software (version 4.0.3 for Appendix A and version 4.1.0 for Appendix B and C).

Regarding the analysis of BVOC compounds, the relative amount of each monoterpene and monoterpenoids (MTs), expressed as the percentage of total monoterpene profile (TMTs) (Appendix A and Appendix B) and the relative amount of each MTs and sesquiterpene and sesquiterpenoids (SQTs) expressed as the percentage of total terpene profile (TMTs + TSQTs) (Appendix C), were calculated. Once the assumptions of normality and homoscedasticity were met, by carrying out Shapiro and Levene's tests (Gastwirth et al., 2009; Nordstokke and Zumbo, 2010; Shapiro and Wilk, 1965), respectively, terpene amounts were analysed by a one-way analysis of variance (ANOVA), selecting a 0.05 *p*-value threshold as cut-off value for significant differences. The one-way ANOVA was followed by a Tukey *post hoc* pairwise test to check for significant differences between different compounds.

To test possible temporal differences and changes in vegetation cover between the two studied areas (LD and HD) in the Maremma Regional Reserve in Tuscany (Appendix B), ecological data were analysed using linear mixed effects models (one for tree coverage and one for shrub coverage). These analysis were carried out using the library *nlme* (Pinheiro et al., 2016) (more details in Appendix B). To observe how the terpenic profile (vectors) changed with changes in canopy cover of holm oak, with respect to other more abundant woody species in the two areas (*Phillyrea latifolia* L.,

Pistacia lentiscus L., *Rubus ulmifolius* Schott.), a principal component analysis (PCA) was performed (Appendix B). The analysis was carried out using the FactoMineR package (Husson, 2011).

Additionally, to observe possible allelopathic and/or inhibitory effects on seeds of *Pittosporum undulatum* and *Eucalyptus ovata* from the different leachates tested, all index values calculated at the end of the germination experiment (Appendix C) were analysed with a one-way ANOVA, after the assumption of normality and homoscedasticity were met using Shapiro and Levene's tests, respectively.

Finally, for the Total Saponins Content (TSC), the Total Condensed Tannin content (TCT) and the total content of polyphenols of each class of identified metabolites from the different leachates and ethanolic extracts obtained from *P. undulatum* green leaves, *P. undulatum* litter and *E.* spp. litter a non-parametric one-way analysis of variance (Kruskal-Wallis Test) was conducted. This test was carried out, since the ANOVA's assumptions of normality and heteroscedasticity tested with Shapiro's and Levene's tests, respectively, were not met. After that, a Dunnett *post hoc* pairwise test was carried out.

3. Main results and discussion

3.1 Efficiency of the developed DBSS device

In the first study, the main objective was to test the efficiency of the developed Dinamyc BVOCs Sampling System device and compare its results with those obtained using the static technology to collect BVOCs at the environment level. Thus, terpene emissions were collected both using potted *Q. ilex* plants (denominated sampling point 1) and in a holm oak forest (denominated sampling point 2 and 3, respectively).

The analyses with Gas Chromatography and Mass Spectrometry (GC-MS) showed that the only terpenes collected were monoterpenes and monoterpenoids (MTs), while no other BVOCs were detected. This result was expected, since the *Q. ilex* terpenic profile is mainly characterised by monoterpenes (Bsaibes et al., 2020; Yassaa et al., 2010). In particular, the identified MTs during the three samplings were: acyclic monoterpene hydrocarbons (myrcene, β-cisOcimene, β-transOcimene), monocyclic monoterpene hydrocarbons (α-phellandrene, β-phellandrene, α-terpinene, d-limonene, γ-terpinene, *p*-cymene and terpinolene) and bicyclic monoterpene hydrocarbons (α-pinene, α-thujene, α-fenchene, camphene, β-pinene, sabinene, car-3-ene) (Table 3).

Table 3. List of the identified compounds by comparison with the NIST 11 library. Their names and retention times (RT) are presented. It is also reported the presence (y - yes) or the absence (n - no) of each compound in each of the different sampling points (sampling point 1 - SP 1, sampling point 2 - SP 2, sampling point 3 - SP 3) and with the two sampling techniques (dynamic with DBBS/static).

Compound Identified	**RT**	**Presence**		
	(min)	SP 1 (dynamic /static)	SP 2 (dynamic /static)	SP 3 (dynamic /static)
α-pinene	10.07 ± 0.02	y/y	y/y	y/y
α-thujene	10.15 ± 0.02	y/y	y/y	y/y
camphene	11.35 ± 0.05	y/y	y/y	y/y

β-pinene	12.61 ± 0.04	y/y	y/y	y/y
sabinene	12.76 ± 0.05	y/y	y/y	y/y
car-3-ene	12.97 ± 0.03	y/y	y/y	y/n
β-phellandrene	13.10 ± 0.03	n/n	y/y	n/n
myrcene	14.12 ± 0.02	n/n	y/y	y/n
α-phellandrene	14.35 ± 0.02	n/n	y/y	n/n
α-terpinene	14.83 ± 0.04	n/n	y/y	y/n
d-limonene	15.39 ± 0.02	y/y	y/y	y/y
1,8-cineole	15.68 ± 0.01	y/y	n/n	n/n
β-cis-ocimene	15.72 ± 0.02	n/n	y/y	y/y
β-trans-ocimene	13.99 ± 0.02	n/n	n/n	y/n
γ-terpinene	16.80 ± 0.01	n/n	y/y	y/n
p-cymene	17.62 ± 0.02	y/y	y/y	y/y
terpinolene	17.95 ± 0.01	n/n	y/y	y/n

In all sampling points the amount of each identified MTs were higher in samples collected by DBSS compared to the static technique (Figure 9).

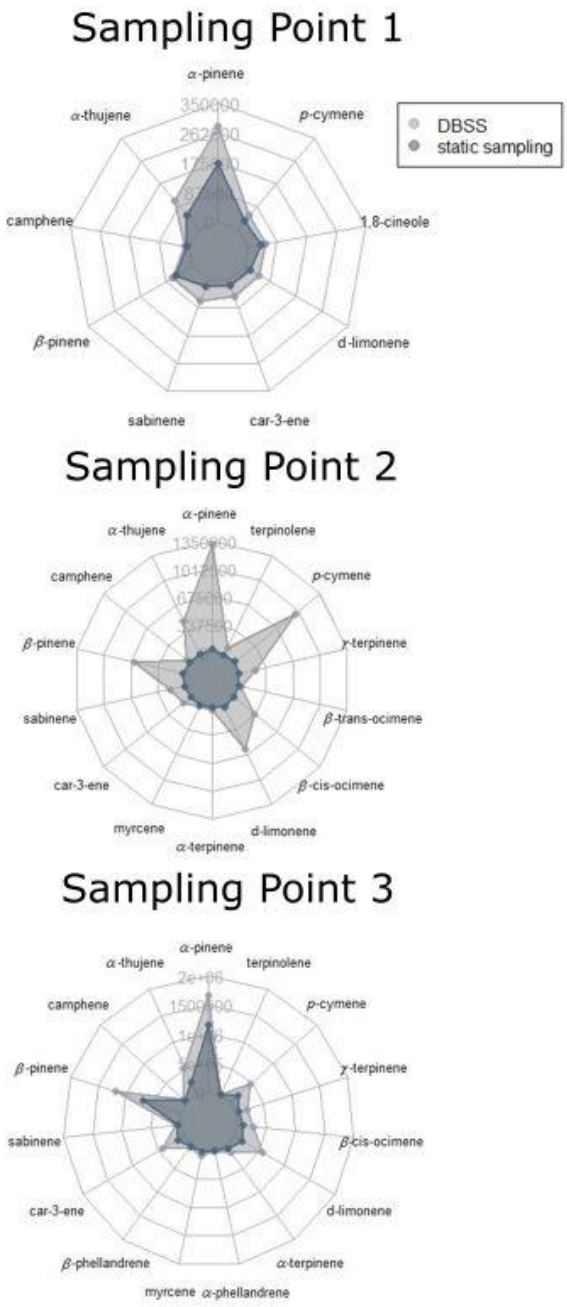

Figure 9. Radar-plots showing the results of the semi-quantitative analyses of BVOCs at sampling point 1; 2; 3 (n = 3), expressed as the peak area mean of each identified monoterpene.

Considering the total area of MTs in both experimental sites (Sesto Fiorentino and Maremma National Reserve), DBSS allowed the collection of a higher total amount of MTs compared to the static technique (Figure 10). In particular, during the sampling at the experimental field of CNR (Sesto Fiorentino – sampling point 1) and during the first sampling at the National Reserve (sampling point 2), the total amount of MTs obtained using static technic was around half compared to that of the DBSS (56% for sampling point 1 and 50% for sampling point 2). Furthermore, during the sampling conducted in October 2019 at the Maremma National Reserve (sampling point 3), the difference between the two techniques was considerably higher. Indeed, the amounts of TMTs collected using the static technique was only ~3% of the amounts obtained using the DBSS (Figure 10).

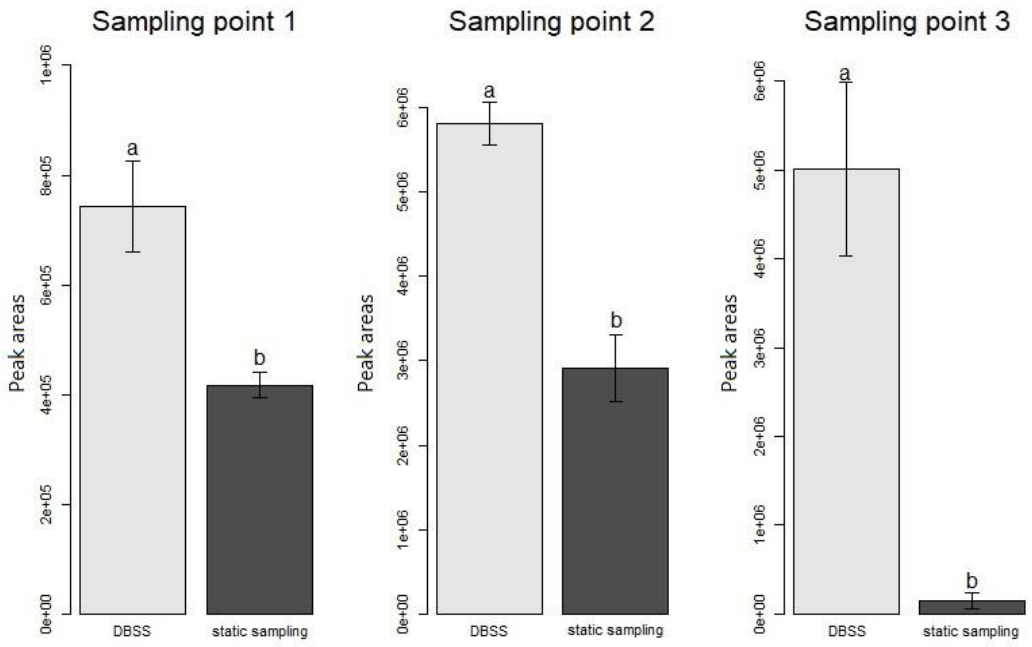

Figure 10. Amount of total MTs collected using DBSS (light grey) and static technique (dark grey) at sampling point 1, sampling point 2 and sampling point 3. Data are means ± standard deviation ($n = 3$). Data were analysed by one-way ANOVA test and the letters indicate statistical differences between the two sampling techniques in each sampling point obtained from Tukey *post-hoc* test ($p \leq 0.05$).

In addition, during the sampling point 3 some qualitative differences were also observed between the two techniques, with a higher number of MTs identified using the DBSS device compared to the static sampling method (14 compounds instead 8) (Table 3). These qualitative differences observed only at sampling point 3 may be explained when considering the lower air temperature and the potential sink effect played by humidity recorded in October compared to June. Indeed, BVOC emission is temperature-dependent, with an optimum around 25 °C. Furthermore, high air humidity could reduce BVOC adsorption in SPME fibres since these compounds are water soluble (Martos and Pawliszyn, 1997). Therefore, the collection of BVOCs using DBSS resulted particularly efficient compared with the static technique when environmental conditions are limiting BVOCs emissions (Fuentes et al., 2007; Holopainen et al., 2013; Peñuelas et al., 2005; Strong et al., 2004). Lastly, the DBSS could provide another advantage, namely the homogenization of the sample through the use of the fan. Indeed, the BVOCs could have heterogeneous concentrations under the forest canopy (Noe et al., 2012), and thus, the creation of a turbulent airflow by the combination of fan and net in front of the SPME fibre could result in a homogenization of the sample.

3.2 Effects of abiotic stresses on BVOCs profile in Q. ilex forest

The study conducted in the Maremma National Reserve in southern Tuscany (Italy) had the purpose of observing ecological changes and trends in BVOC emission induced by recent drought events related to climate change. The preliminary analysis of the soil showed that the two sites, characterised by different levels of *Q. ilex* mortality, had similar textural and pH values: the soil was predominantly loamy with rocks, while the pH was neutral (Figure 11 and Table 4). Thus, the two areas were found to be similar in terms of soil and climate since the areas are located approximately 1,200 m apart from each other and share the same altitude and exposition.

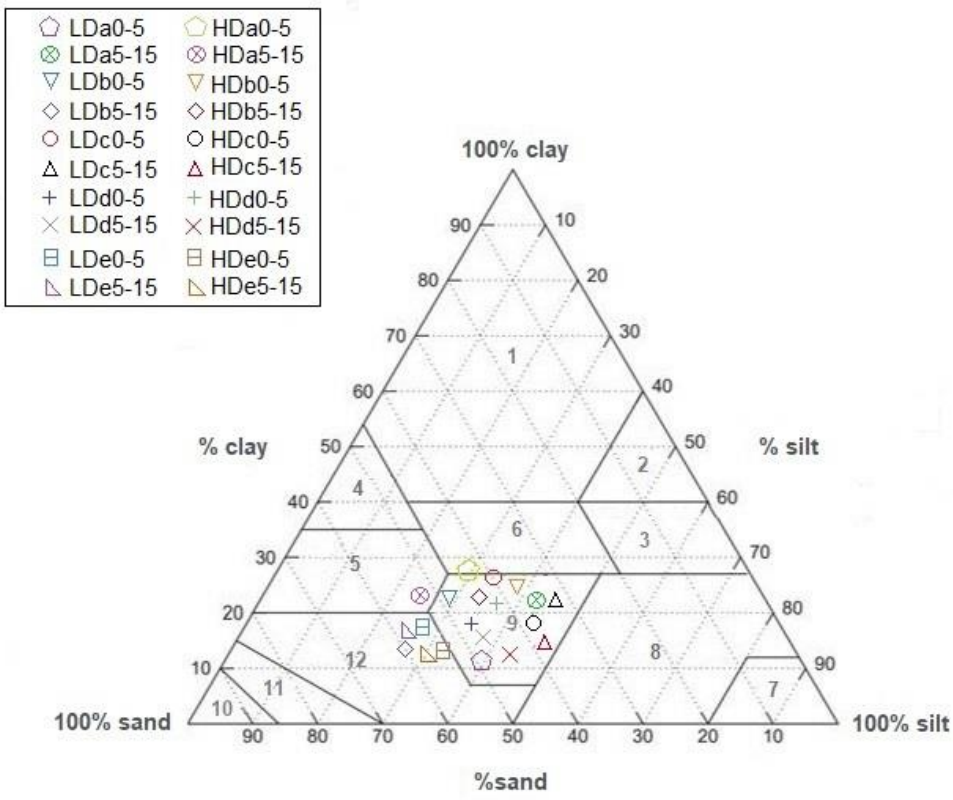

Figure 11. USDA Soil Textural Triangle with the 12 principal textural classes: 1) Clay, 2) Silt clay, 3) Silt clay loam, 4) Sandy clay, 5) Sandy clay loam, 6) Clay loam, 7) Silt, 8) Silt loam, 9) Loam, 10) Sand, 11) Loamy sand, and 12) Sandy loam. The coloured symbols represent the five samples (low letters a-e) of soil collected on May 2019 in the two studied areas (capital letters LD and HD) at two different depths: from 0 to 5 cm below-ground (0-5), and from 5 to 15 cm below-ground (5-15).

Table 4. pH values of 5 soil samples (low letters a-e) collected in the two studied areas (capital letters LD and HD) on two different depths: from 0 to 5 cm below-ground (0-5), from 5 to 15 cm below-ground (5-15). The pH value was calculated in H_2O and 1 mol L^{-1} KCl.

Sample	**0-5 cm**	**5-15 cm**	**Sample**	**0-5 cm**	**5-15cm**
LDa	6.87	6.97	**HDa**	6.54	6.69
LDb	6.75	6.93	**HDb**	6.94	7.09
LDc	7.02	7.10	**HDc**	6.77	7.04
LDd	7.01	7.12	**HDd**	6.80	7.12
LDe	6.74	6.94	**HDe**	7.04	7.17
mean	6.88	7.01	*mean*	6.82	7.02
sd	0.13	0.09	*sd*	0.19	0.19

The visual evaluations of the health status of the predominant species (*Q. ilex*) showed a seasonal decline in its canopy status, with a decrease along the three years of the relative frequency of level 1 of defoliation (0-25%) in both areas, and with a consequent increase of level 4 (100%) (Table 5). In addition, the results regarding the seasonal monitoring of vegetation cover, of all species present in the two studied area, showed that the canopy cover declined significantly in both areas (Figure 12 C-D). This was accompanied by an increment in the cover of the shrub layer (significant increment for area HD but not for area LD) (Figure 12 C-D). Furthermore, the changes at the canopy level went from a starting situation in which the areas were significantly different ($p = 0.015$) to a new situation in which the areas were not significantly different anymore ($p = 0.324$). The same trend was observed for the shrub layer, with the starting situation displaying a significant difference between the two areas ($p = 0.007$), becoming not significant at the end of the experimental campaign ($p = 0.953$).

Table 5. Relative frequencies of crown defoliation of *Q. ilex* (mean ± standard deviation, in %) in LD (Low Dieback) and HD (High Dieback) stands in the three years (2019, 2020, 2021) of the study. The defoliation level is reported as category correspond to: 1 - low defoliation level (<25%); 2 – low to medium defoliation level (25 - 59%); 3 – medium to high defoliation level (60 - 99%); 4 – high defoliation level (100%).

Defoliation	**Relative frequency 2019**		**Relative frequency 2020**		**Relative frequency 2021**	
level	**Area LD**	**Area HD**	**Area LD**	**Area HD**	**Area LD**	**Area HD**
1	12.82 ± 0.01	7.96 ± 1.15	12.96 ± 1.20	9.09 ± 0.23	8.38 ± 0.82	4.12 ± 1.02
2	25.64 ± 0.01	14.17 ± 2.67	27.88 ± 4.12	19.14 ± 4.35	22.79 ± 1.82	17.44 ± 4.18
3	46.15 ± 0.01	29.19 ± 0.88	42.02 ± 4.43	20.86 ± 3.32	48.24 ± 9.03	32.47 ± 8.20
4	15.38 ± 0.01	48.66 ± 0.64	16.76 ± 0.91	50.89 ± 1.25	20.58 ± 0.53	45.97 ± 4.72

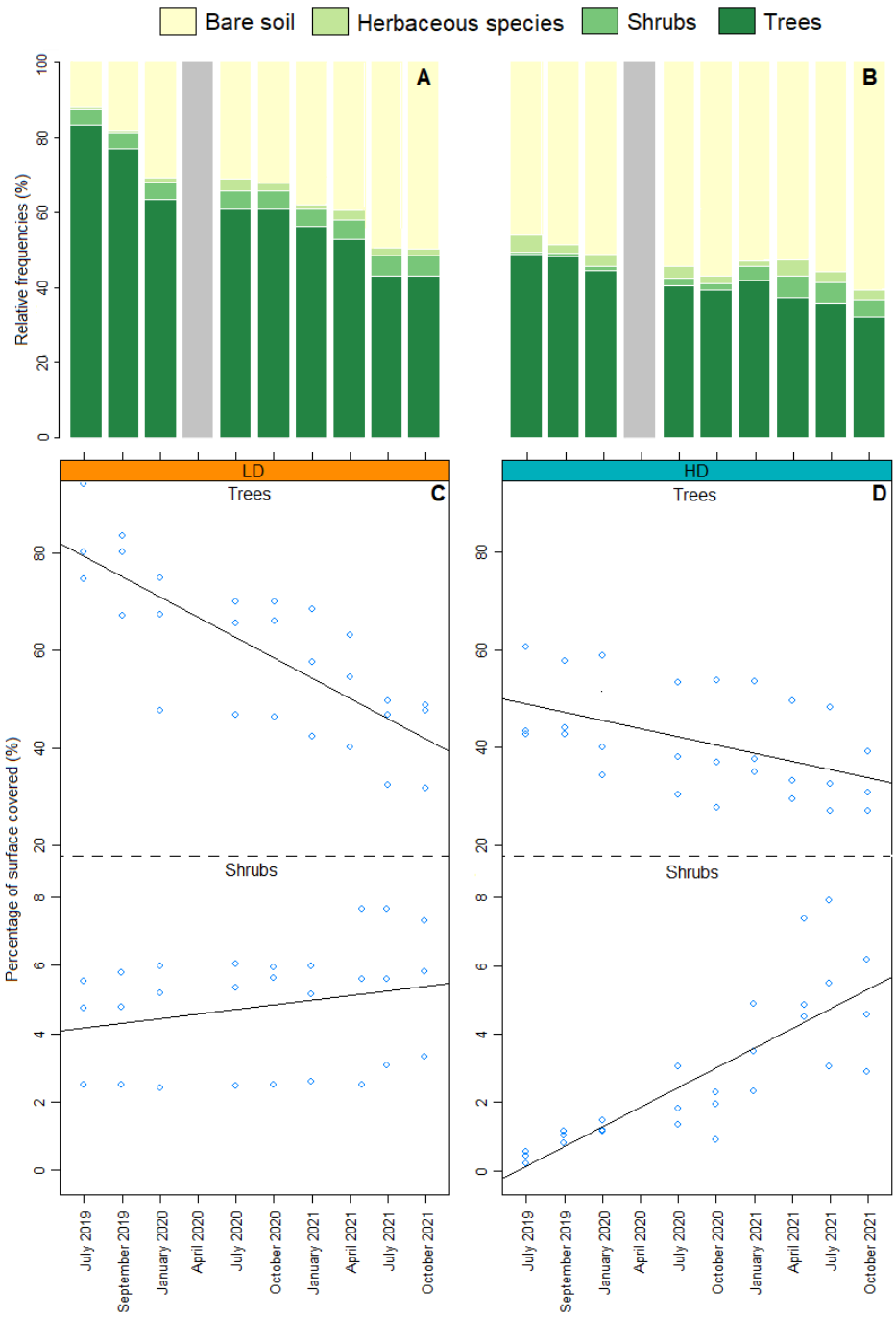

Figure 12. A-B) Relative frequencies of different classes of vegetation and bare soil. Different colours represent the different classes. C-D) Temporal progression of relative surface covered by

tree and shrub species (top row and bottom row, respectively) in the two studied areas LD (panel C) and HD (panel D). On the *x* axis are reported the sampling seasons. In each panel the blue dots represent the percentage of vegetation cover of the three plots (1, 2, 3). The black line represents the mean regression for each area.

This pronounced decline in the tree layer, especially in the canopy of *Q. ilex*, could be explained by the intensification of drought events in the Mediterranean region (Pollastrini et al., 2019). Additionally, the studied forest is characterised by a high tree density, tall trees with small basal areas, and it covers a sloping area with a rocky ground. All these characteristics likely reduce the possibility to store water, and thus increase the risk of dieback (Barbeta et al., 2013; Barbeta and Peñuelas, 2016; Carnicer et al., 2011). The decline in tree canopy cover, probably caused a change in microclimate conditions (*e.g.*, nutrients, light penetration, water viability), explaining the significant increase in understorey shrubs layer.

Finally, the herbaceous species cover was also estimated during the study period, but since these species are highly responsive to seasonal variations, the values obtained were too variable and the studied seasons were not enough to allow the implementation of a linear mixed effects model (Figure 13). To obtain any meaningful statistical results on the herbaceous layer further sampling seasons will be necessary.

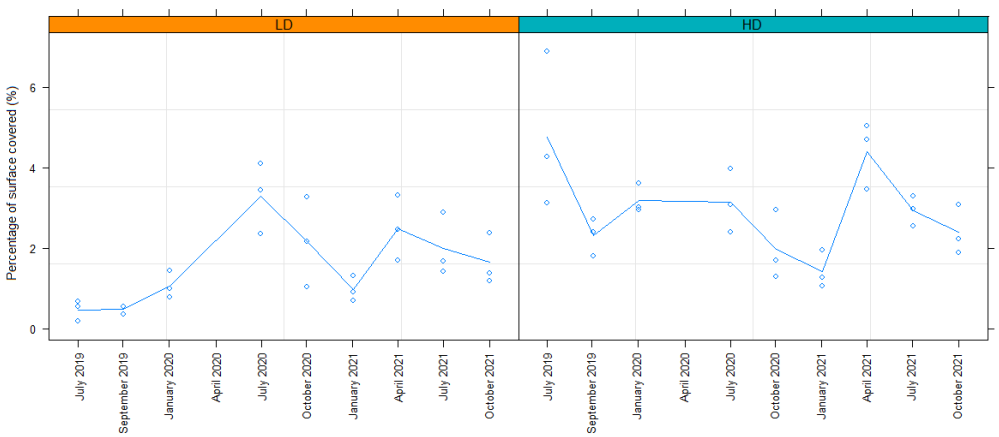

Figure 13. Temporal progression of relative surface covered by shrubs in the three plots (1, 2, 3) from area LD (top row) and HD (bottom row). On the *x* axis are reported the sampling seasons.

In each panel the blue line represents the values of percentage of surface covered by herbaceous species. A linear model was not possible to obtain due the high variability of the data.

During the studied period, a trend was identified in both areas, with BVOCs measurements conducted in summer richer in monoterpene emissions than those acquired in autumn (Figure 14). This trend coincides with the higher mean temperatures, since abiotic factors like temperature have been shown to have a direct effect on monoterpene emissions (Llusià and Peñuelas, 1999; Loreto and Schnitzler, 2010). In addition, using the DBSS technique it was possible to see a decreasing trend in emission rates of monoterpenes in both areas over the years (Figure 14). This could be explained by the intensification of drought over the last years. Indeed, drought events have a great impact on monoterpene emissions, as these could be reduced by a factor of up to ten (Loreto et al., 2001). Another factor influencing this decreasing trend found in our study could be the high degree of defoliation of the canopies and the dieback of the dominant species, *Q. ilex*. Indeed, the largest portion of monoterpenes emissions are likely deriving from *Q. ilex*, which is the predominant species in the forest and one of the major emitters in the Mediterranean basin, even greater than *Phillyrea latifolia* (Ait Said et al., 2011; Llusià and Peñuelas, 1998). Finally, the comparison the emission rates between the two studied areas showed that the two areas were significantly different at the beginning of study, but that this significant difference ceased to be present as time passed (Figure 14 - Summer 2019: $p < 0.001$; Autumn 2019: $p = 0.011$; Summer 2020: $p = 0.029$; Autumn 2020: $p = 0.012$; Summer 2021: $p = 0.999$; Autumn 2021: $p = 0.554$). The list of the sixteen detected compounds identified during the seasonal samplings is reported in Table 6, together with the average and standard deviation of their percentages.

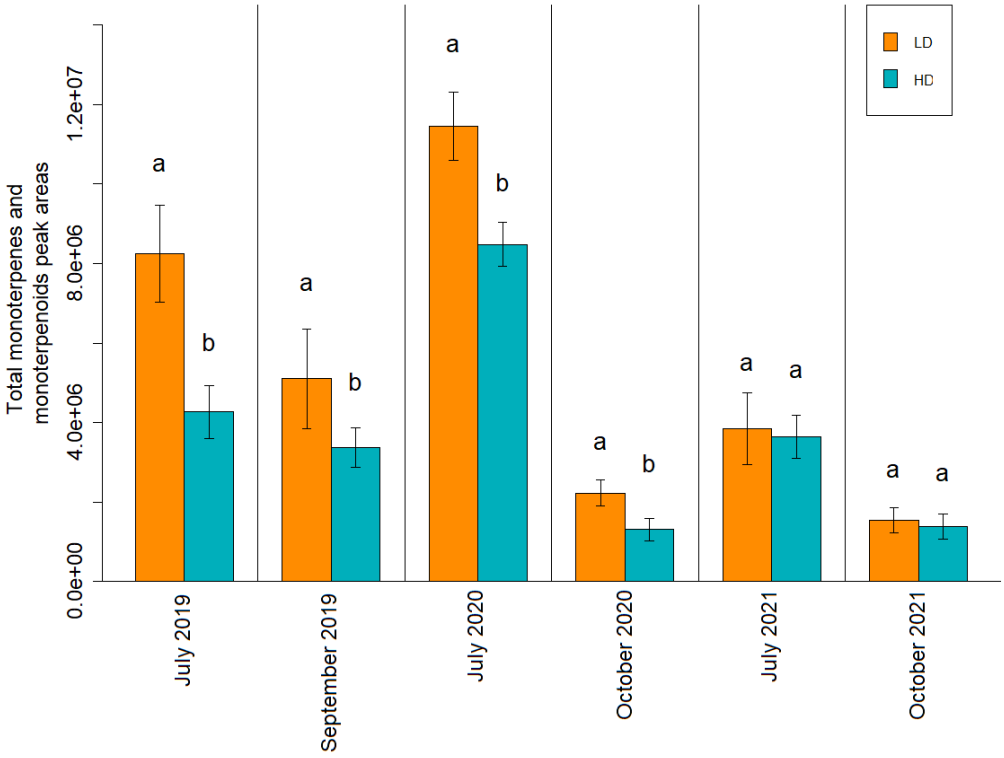

Figure 14. The amount of total MTs (Monoterpenes and Monoterpenoids) collected during the whole duration of the study period (2019-2021) (means ± standard deviations, $n = 10$). Data were analysed by one-way ANOVA for each month separately (ns - $p > 0.05$, * - $0.01 < p < 0.05$, ** - $0.001 < p < 0.01$, *** - $p < 0.001$).

In agreement with previous results, it was possible to notice a reduction in the number of the detected compounds (from 16 in summer 2019 to 11 in summer 2021). Furthermore, as time passed, the two areas tended to become more similar to each other and the significant differences between the single detected MTs disappeared, showing an overall high mortality trend of *Q. ilex* in both areas.

Table 6. List of detected MTs for every season sampled. Values of detected MTs are given as mean percent of the total MTs peak area for each season (n = 20). Sd represents standard deviation. *P*-values presented were obtained using a one-way ANOVA for each season (ns - $p > 0.05$, * - $0.01 < p < 0.05$, ** - $0.001 < p < 0.01$, *** - $p < 0.001$). – represents non detected compounds.

	Summer 2019			Autumn 2019			Summer 2020		
Terpenes	LD (% mean ± sd)	HD (% mean ± sd)	*p*	LD (% mean ± sd)	HD (% mean ± sd)	*P*	LD (% mean ± sd)	HD (% mean ± sd)	*p*
α-pinene	16.17 ± 5.95	4.51 ± 1.59	***	19.01 ± 13.00	6.23 ± 5.09	n.s.	14.22 ± 3.50	5.10 ± 1.84	**
α-thujene	15.40 ± 5.61	5.24 ± 2.79	**	9.30 ± 3.91	8.25 ± 2.03	n.s.	5.85 ± 3.85	3.59 ± 1.99	n.s.
α-fenchene	0.59 ± 0.27	0.35 ± 0.22	n.s.	-	-		-	-	
camphene	5.65 ± 2.52	4.25 ± 1.03	n.s.	0.75 ± 0.53	0.43 ± 0.24	n.s.	1.47 ± 0.43	0.70 ± 0.11	**
β-pinene	3.67 ± 1.00	1.60 ± 0.98	***	12.28 ± 6.87	10.04 ± 4.71	n.s.	8.17 ± 4.02	1.74 ± 1.17	**
sabinene	3.37 ± 2.13	2.06 ± 0.93	n.s.	5.92 ± 4.65	2.47 ± 1.98	*	0.72 ± 0.40	0.38 ± 0.27	n.s.
car-3-ene	0.88 ± 0.26	1.31 ± 0.28	n.s.	-	-		1.61 ± 0.61	0.87 ± 0.11	*
myrcene	4.02 ± 1.08	2.06 ± 1.27	*	0.89 ± 0.34	0.48 ± 0.35	*	3.01 ± 1.22	3.24 ± 0.68	n.s.
α-phellandrene	1.16 ± 0.34	0.50 ± 0.14	n.s.	2.28 ± 0.30	1.64 ± 0.26	***	0.38 ± 0.19	0.25 ± 0.06	*
α-terpinene	1.01 ± 0.52	0.28 ± 0.16	*	0.79 ± 0.35	0.50 ± 0.11	n.s.	-	-	
d-limonene	11.57 ± 3.02	7.91 ± 3.30	n.s.	8.14 ± 3.71	4.29 ± 1.25	n.s.	19.19 ± 5.36	22.77 ± 3.76	n.s.
β-cisOcimene	0.94 ± 0.21	0.33 ± 0.21	***	0.37 ± 0.07	0.27 ± 0.09	n.s.	0.47 ± 0.15	0.47 ± 0.10	n.s.
β-transOcimene	1.73 ± 1.07	0.78 ± 0.70	n.s.	-	-		-	-	
γ-terpinene	0.91 ± 0.37	0.39 ± 0.21	**	3.05 ± 1.13	1.39 ± 0.65	**	0.32 ± 0.14	0.50 ± 0.16	n.s.
p-cymene	0.45 ± 0.14	0.29 ± 0.15	n.s.	0.41 ± 0.19	0.22 ± 0.12	n.s.	0.29 ± 0.09	0.37 ± 0.10	n.s.
terpinolene	0.34 ± 0.23	0.20 ± 0.18	n.s.	0.46 ± 0.15	0.13 ± 0.06	***	2.04 ± 0.69	2.29 ± 0.32	n.s.

Table 6. (continued).

	Autumn 2020			Summer 2021			Autumn 2021		
Terpenes	LD (% mean ± sd)	HD (% mean ± sd)	*p*	LD (% mean ± sd)	HD (% mean ± sd)	*p*	LD (% mean ± sd)	HD (% mean ± sd)	*p*
α-pinene	21.61 ± 10.52	7.04 ± 2.28	**	11.27 ± 10.60	11.52 ± 9.04	n.s.	16.38 ± 9.11	14.67 ± 9.26	n.s.
α-thujene	8.92 ± 2.65	2.98 ± 2.21	**	8.88 ± 5.66	10.80 ± 3.54	n.s.	11.75 ± 4.44	11.12 ± 5.71	n.s.
α-fenchene	-	-		-	-		-	-	
camphene	1.22 ± 0.44	0.58 ± 0.11	*	0.71 ± 0.54	0.77 ± 0.45	n.s.	0.91 ± 0.51	0.98 ± 0.58	n.s.
β-pinene	10.81 ± 3.90	4.08 ± 1.75	**	9.07 ± 7.01	9.38 ± 5.64	n.s.	11.76 ± 5.39	9.59 ± 5.04	n.s.
sabinene	2.20 ± 1.20	1.32 ± 0.75	n.s.	1.69 ± 1.01	1.41 ± 0.39	n.s.	2.31 ± 1.23	1.28 ± 0.46	n.s.
car-3-ene	0.71 ± 0.12	0.69 ± 0.13	n.s	-	-		-	-	
myrcene	2.20 ± 0.79	2.87 ± 0.70	n.s.	4.31 ± 1.72	4.70 ± 1.44	n.s.	-	-	
α-phellandrene	-	-		0.43 ± 0.18	0.36 ± 0.12	n.s.	-	-	
α-terpinene	0.64 ± 0.34	0.45 ± 0.14	n.s.	0.41 ± 0.15	0.47 ± 0.10	n.s.	0.52 ± 0.21	0.52 ± 0.23	n.s.
d-limonene	15.89 ± 3.31	13.89 ± 1.18	n.s.	10.44 ± 4.79	9.94 ± 4.17	n.s.	7.41 ± 2.37	7.87 ± 2.08	n.s.
β-cisOcimene	-	-		-	-		-	-	
β-transOcimene	-	-		-	-		-	-	
γ-terpinene	0.97 ± 0.41	0.37 ± 0.09	*	0.86 ± 0.42	1.00 ± 0.21	n.s.	0.84 ± 0.52	0.78 ± 0.28	n.s.
p-cymene	0.63 ± 0.21	0.40 ± 0.13	n.s.	0.68 ± 0.25	0.59 ± 0.21	n.s.	0.48 ± 0.17	0.56 ± 0.19	n.s.
terpinolene	-	-		-	-		-	-	

Finally, the principal component analysis (PCA), performed to notice the changing of the terpenic profile due to the alteration of holm oak canopy cover, compared to other woody species highly present in the two areas (*Phillyrea latifolia* L., *Pistacia lentiscus* L., *Rubus ulmifolius* Schott.) is in agreement with what was previously reported. Indeed, considering the first two dimensions of the PCA (that represented cumulatively, over 70% of the variance), it could be possible to notice that the sampling seasons are separated along the first dimension (Figure 15). Hence, the first principal component dimension clearly separates the sampling seasons with a higher percentage of *Q. ilex* canopy cover from the sampling times with a lower percentage of *Q. ilex* canopy cover. Indeed, the first three sampling seasons (J_2019, O_2019 and J_2020) were characterised by a high percentage of *Q. ilex* canopy cover with respect to the last three sampling seasons (O_2020, J_2021, and O_2021). This and the results previously reported showing the lowest content of detected monoterpenes in both stands during the last sampling seasons (Figure 14), show that the *Q. ilex* species is the greater contributor to the terpenic profiles obtained. In addition, another interesting observation comparing Figure 14 with Figure 15, is that in both figures the two areas result initially significantly different and distant from each other, while over time they tend to get closer and become more similar. A further agreement between the two figures is that all the samplings carried out in the warmer seasons (J_2019, J_2020, and J_2021) have always a greater amount of terpenes detected than in their corresponding autumnal sampling (O_2019, O_2020, O_2021). Indeed, the summer samplings are located to the right of the corresponding sampling conducted during the autumn. On the other hand, the second dimension could be related to the variation of the compositional blend (Figure 15 panel A), and therefore it could be linked to *Q. ilex* health, with higher emissions of camphene, α-phellandrene and α-thujene, when *Q. ilex* is under less prolonged water stress.

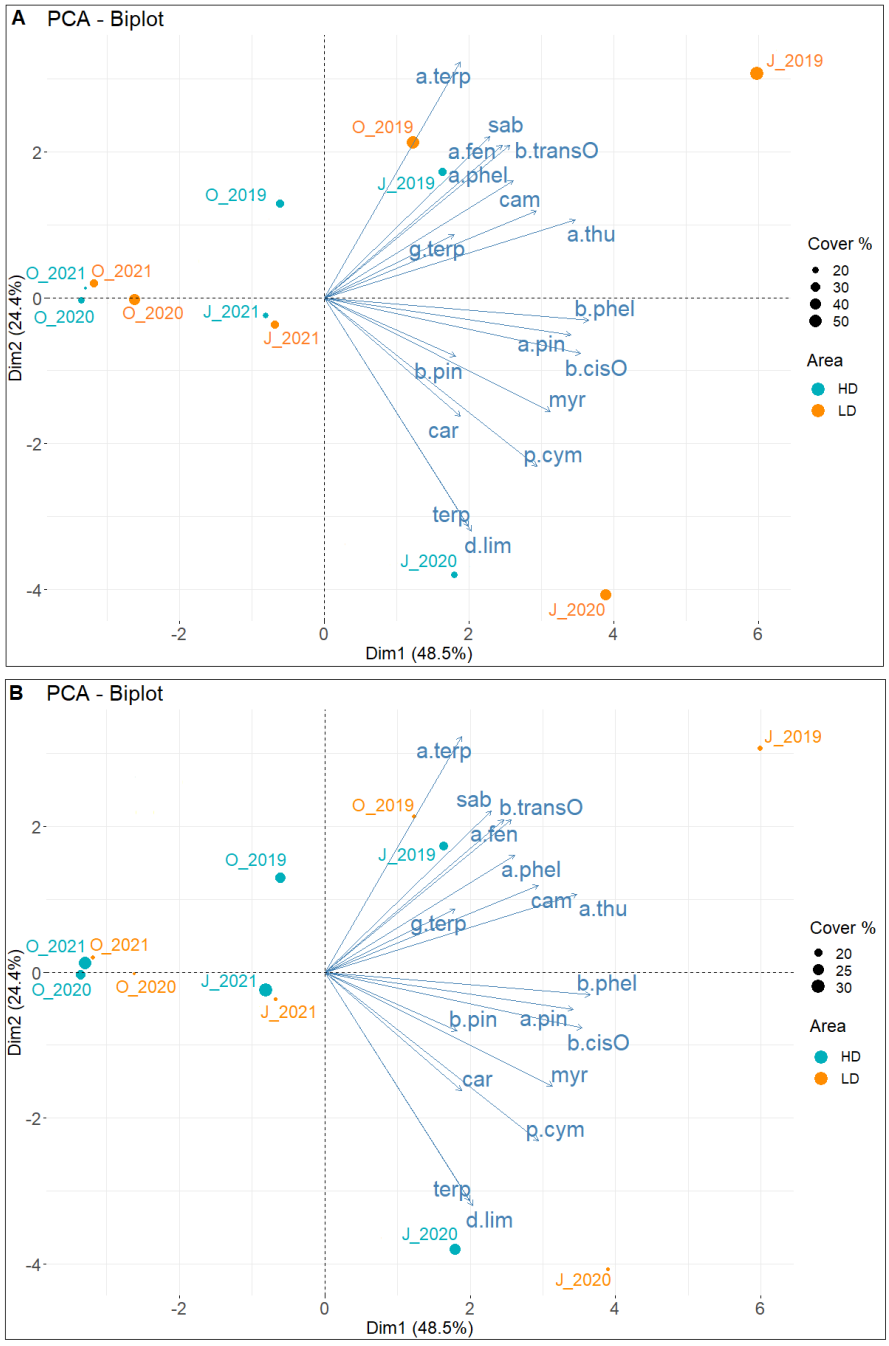

Figure 15. Principal component analysis biplots on monoterpene and monoterpenoid compounds. In all plots, loadings for each BVOCs are presented with arrows (a.pin: a-pinene,

a.thu: a-thujene, a.fen: a-fenchene, cam: camphene, b.pin: b-pinene, sab: sabinene, car: car-3-ene, myr: myrcene, a.phel: a-phellandrene, a.terp: a-terpinene, d.lim: d-limonene, b.cisO: b-cisOcimene, b.transO: b-transOcimene, g.terp: g-terpinene, p.cym: *p*-cymene, terp: terpinolene). The variance explained by each principal component (Dim) is shown in parentheses. (A) The individuals represented the values of percentage canopy cover (pointsize) of *Quercus ilex* L. in the two areas, LD and HD (colours) at the six sampling times: Summer 2019 (S_2019), Autumn 2019 (A_2019), Summer 2020 (S_2020), Autumn 2020 (A_2020), Summer 2021 (S_2021), Autumn 2021 (A_2021). (B) The individuals represented the values of percentage canopy cover (pointsize) of other main species (*Phillyrea latifolia* L., *Pistacia lentiscus* L., *Rubus ulmifolius* Schott.) in the two areas, LD and HD (colours) at the six sampling times: Summer 2019 (S_2019), Autumn 2019 (A_2019), Summer 2020 (S_2020), Autumn 2020 (A_2020), Summer 2021 (S_2021), Autumn 2021 (A_2021).

3.3 Study on Pittosporum undulatum invasiveness

This study was carried out in south-eastern Australia to investigate the possible biochemical bases of *P. undulatum* invasiveness in a eucalypt forest. The obtained results support the hypothesis that the high invasiveness of *P. undulatum* is not linked to any allelopathic actions, but rather it may be due to differences in the biosynthesis and emission of secondary metabolites. Indeed, under all combinations of treatments (soil and water treatments), no effects on the germination of either *P. undulatum* or *E. ovata* were found. The seeds of both species germinated, although with different percentages (Figure 16 for *E. ovata* seeds; Figure 17 for *P. undulatum* seeds). In particular, for the seeds of *E. ovata* the germination rates and the Mean Germination Time (MGT) resulted similar under all substrates tested and in all watering treatments (Figure 16). For *P. undulatum* germination curves, a difference between watering treatments was found only for the filter-paper substrate. Indeed, seeds showed a higher germination rate in the control watering treatment compared to the other watering treatments. This phenomenon could derive from the lack of soil chelating action. Indeed, the soil is a very complex system influencing the qualitative and quantitative availability of allelopathic compounds (Cheng, 1995). In addition, when observing the selected indices (GP, MGT, t_{50} and VI), the values are not significantly different in all combinations of substrates and watering treatments, with the exception of paper substrate and watering treatments. This

result probably supports again the above-mentioned lack of soil chelating action of this substrate (more details in Appendix C).

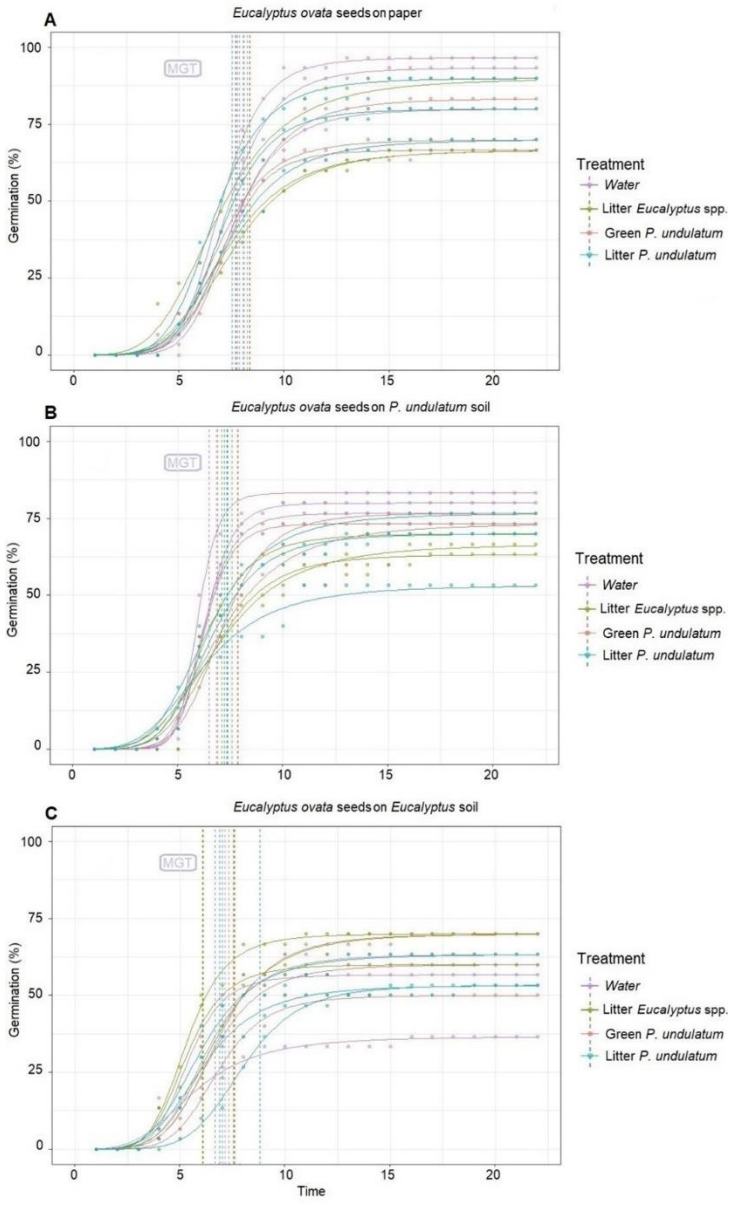

Figure 16. Germination curves for *E. ovata* seeds on different substrates (A = filter-paper substrates; B = *P. undulatum* soil; C = *Eucalyptus* soil). The colours show the different watering

treatments. The vertical dashed lines represent the Mean Germination Time for each treatment (MGT) for each treatment *x* replicates (ns for ANOVA test).

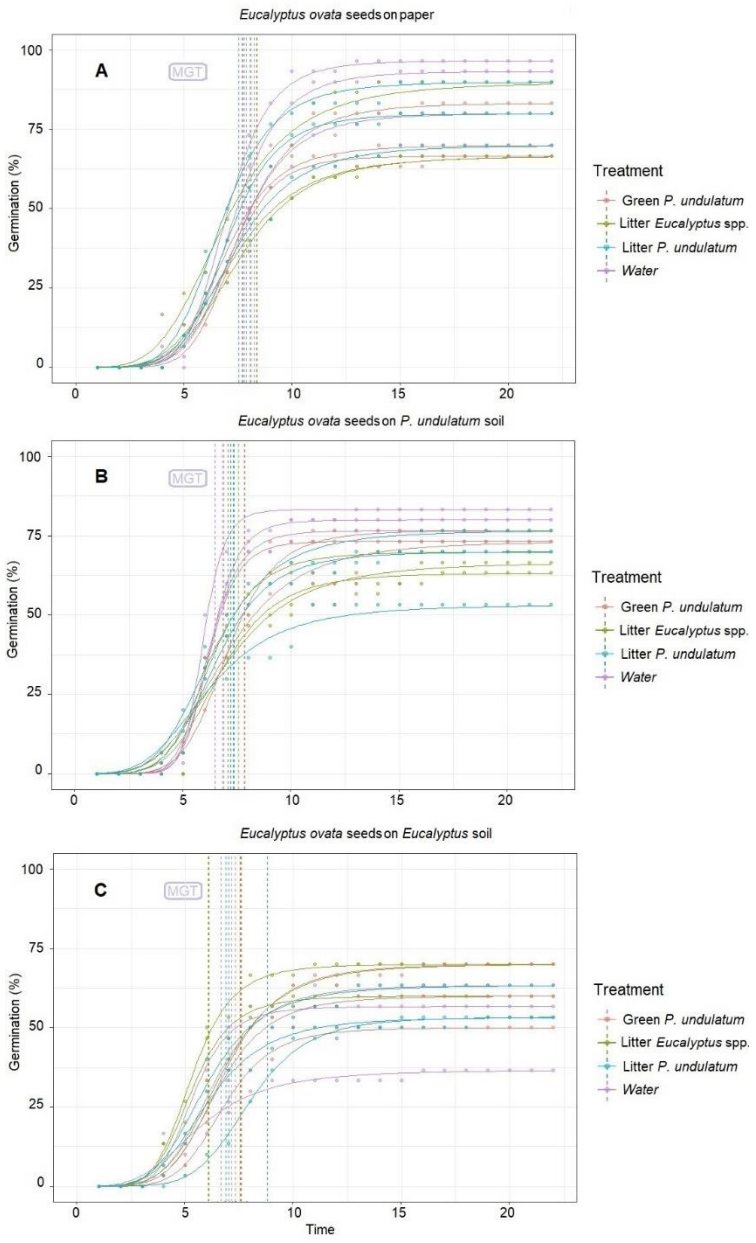

Figure 17. Germination curves for *P. undulatum* seeds on different substrates (A = filter-paper substrate; B = *P. undulatum* soil; C = *Eucalyptus* soil). The colours show the different watering

treatments. The vertical dashed lines represent the Mean Germination Time (MGT) for each treatment *x* replicates (ns for ANOVA test).

These results together with the absence of larger amounts of saponins (Total Saponins Content – TSC) and condensed tannins (Total Condensed Tannins content – TCT) in all extracts of *P. undulatum*, compared to the extracts of *Eucalyptus* spp. (Figure 18 – TSC; Figure 19 – TCT), are in agreement with the lack of detection of any possible allelopathic terpenes in the forest invaded by *P. undulatum* (I) (Table 7).

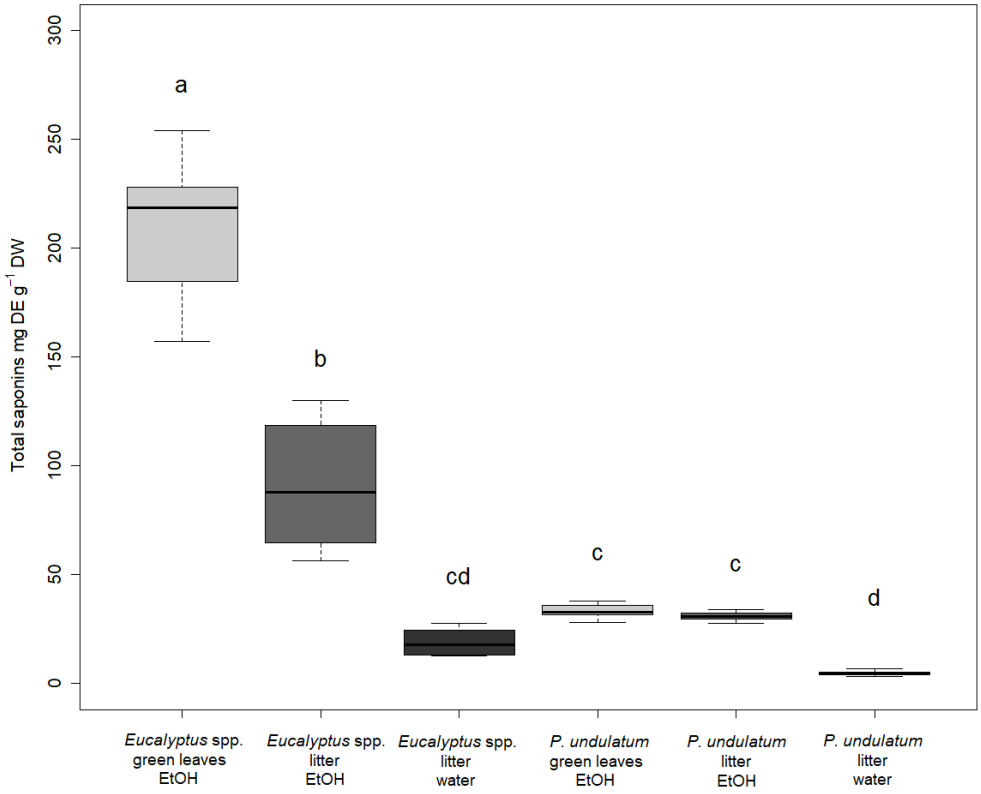

Figure 18. Total Saponins Content (mg of Diosgenin Equivalent (DE) g^{-1} DW) in *Eucalyptus* and *P. undulatum* extracts (ethanolic extracts of green leaves and litter, and water extracts of litter). Data was analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

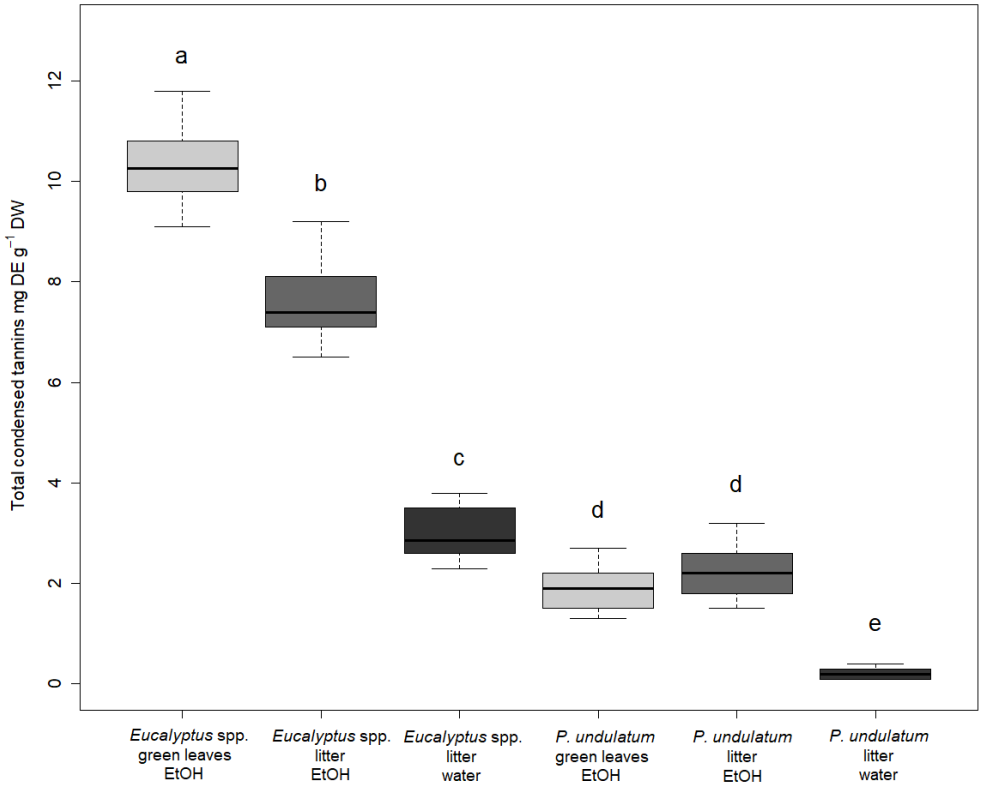

Figure 19. Content of-Total Condensed Tannins (TCT) (mg of Catechin Equivalent (CE) g^{-1} DW) in *Eucalyptus* and *P. undulatum* extracts (ethanol extracts of green leaves and litter and water extracts of litter). Data was analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

Indeed, in the I area (Invaded) monoterpenes, such as camphor and menthol, were not detected as well as in the area without *P. undulatum* (R). Additionally, the 1,8-cineole amount was lower in the I than in the R area (Remnant). These three monoterpenes were reported to have a high allelopathic and inhibiting actions during the mitosis, leading plant shoots and roots to grow in an abnormal manner (Duke et al., 2004; Fischer, 1986; Romagni et al., 2000; Schulz et al., 2007).

Table 7. List of detected MTs for each area (I and R) and the area where they are predominant.

MTs Identified	Presence	Area where are predominant
	I / R	
α-pinene	y/y	I
α-thujene	y/y	I
β-pinene	y/y	I
myrcene	y/n	I
α-phellandrene	y/y	I
α-terpinene	y/y	I
d-limonene	y/y	I
1,8-cineole	y/y	R
ocimene	y/y	I
p-cymene	y/y	I
α-terpineol	y/y	I
terpinyl-acetate	y/y	R
piperitone	y/y	I

Thus, all data showed that the invasiveness of *P. undulatum* seems not to be linked to the release of any allelopathic compounds. On the other hand, the data of polyphenols and the BVOCs identified in the two areas suggest that the biosynthesis of secondary metabolites in *P. undulatum* might increase its tolerance against abiotic stresses compared to *Eucalyptus* spp. Indeed, by the HPLC-DAD analyses it was possible to identify different classes of polyphenols and they amounts in each extract (both ethanolic

and aqueous) of *Eucalyptus* spp. and *P. undulatum* (Figure 20). In more details, hydrolysable tannins (identified as gallic and ellagic acid derivatives) were richest in all *Eucalyptus* extracts, especially in both *Eucalyptus* litter extracts, representing more than 90% of the total tannins content in the ethanolic extract, and more than 75% in the aqueous extract. Furthermore, these derivatives were not detected in any of the *P. undulatum* extracts. Instead, flavonoids were present in all extracts for both studied species (identified as myricetin derivatives), except in the aqueous extract of *Eucalyptus* spp. litter. In particular, the Total Flavonoids Content (TFC) was higher in the two ethanolic extracts of *Eucalyptus* compared to the two ethanolic extracts from *P. undulatum.* Finally, the hydroxycinnamic acid derivatives (identified as caffeic and *p*-coumaric acid derivatives) were found in all *P. undulatum* extracts but were missing from the *Eucalyptus* spp. extracts. These different polyphenol classes suggest different strategies adopted by the two species. Indeed, eucalypt extracts are richer in gallo- and ellagitannins and these compounds mainly provide defence against herbivores, by producing repellent and low-digestible compounds. On the other hand, *P. undulatum* extracts are characterised by high contents of hydroxycinnamic acid derivatives, that increase tolerance against abiotic stresses, *e.g.*, against salinity, drought stress and intense light (Khan et al., 2016; Klein et al., 2015; Król et al., 2014; Riaz et al., 2018).

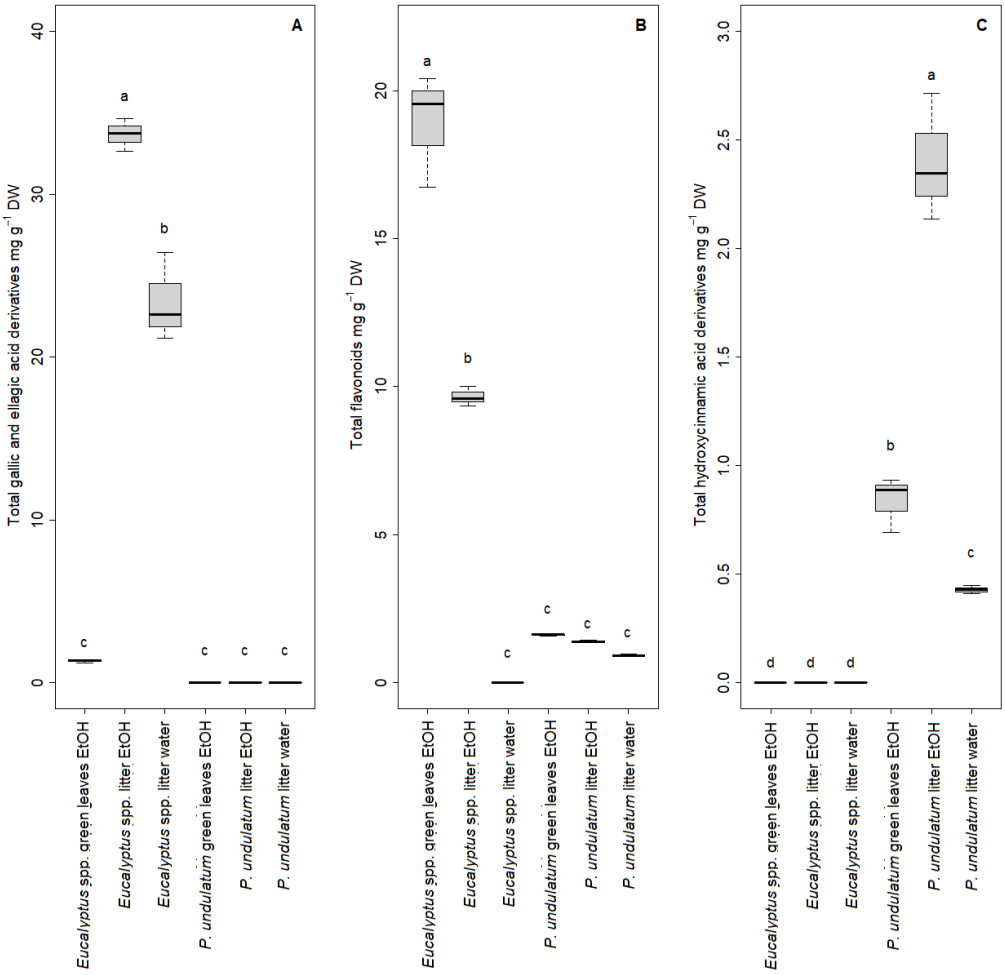

Figure 20. Total content of gallic and ellagic acids derivatives (A), Total Flavonoid Content (B) and Total hydroxycinnamic acid derivatives (C) of *Eucalyptus* and *P. undulatum* extracts (mg g^{-1} DW). Data was analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

Moreover, in the area invaded by *P. undulatum*, approximately 80% of BVOCs identified were MTs. On the other hand, in the area characterised by the presence of *Eucalyptus* spp. a lot of SQTs (ca. 57%) were detected. The percentages of each MTs and SQTs identified in both studied areas are reported in Figure 21 and Figure 22, respectively, where are also shown the results from a one-way ANOVA test.

Monoterpenes and monoterpenoids, emitted in higher concentrations in the area invaded by *P. undulatum*, play an important role in the defence against stresses in plants. Compounds such as α-pinene, α-thujene, β-pinene and d-limonene, which were higher in the I area, increase the defence against ROS and can improve thermo-tolerance during heat waves (Bertin and Staudt, 1996; Llusià et al., 2005; Tian et al., 2020). By contrast, compounds such as 1,8-cineole, were more abundant in the R area, being one of the main components of the *Eucalyptus* spp. terpenic profile (Keszei et al., 2010). This particular compound is of particular importance in the defence against biotic stresses, since it has a role in repelling herbivores (War et al., 2012), attracting pollinators (Boncan et al., 2020) and inducing allelopathic actions (Schulz et al., 2007). Lastly, sesquiterpene concentrations were higher in the R forest than in the I forest, with the exception of calamenene, a typical sesquiterpene present in *P. undulatum* essential oils (Medeiros et al., 2003), that has shown an important role in its prevention strategy against herbivores attacks.

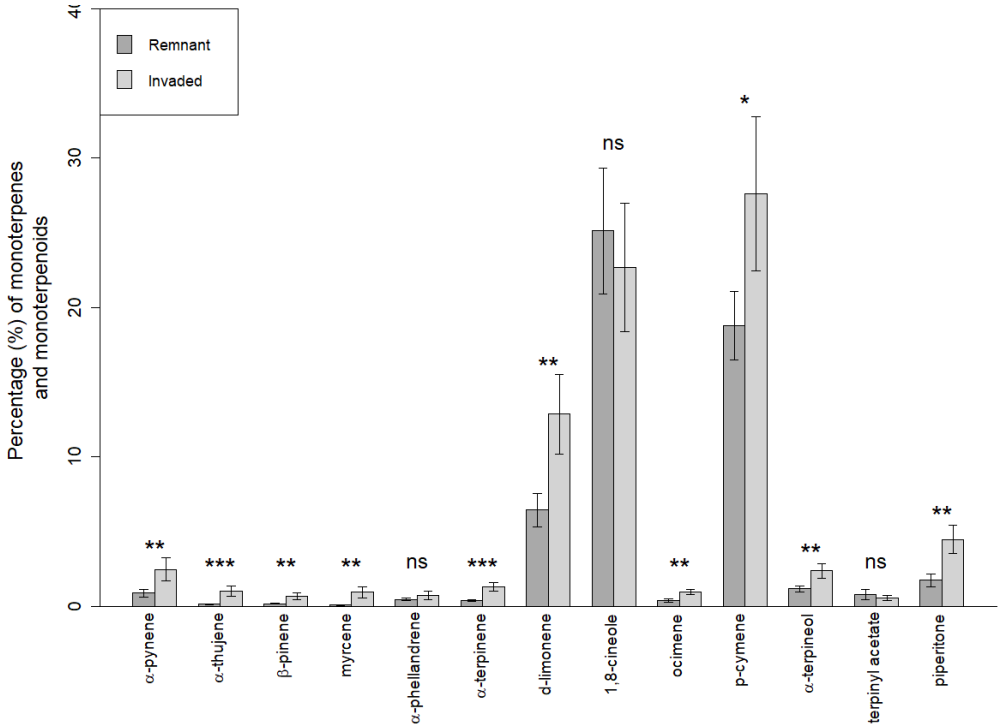

Figure 21. Histogram representing the % amount of monoterpenes and monoterpenoids identified in Remnant (R) and Invaded (I) area. Error bars indicate standard deviation, and the asterisks indicate significant differences between R and I: ns > 0.05, * 0.01 < p < 0.05, ** 0.001 < p < 0.1, *** p < 0.001.

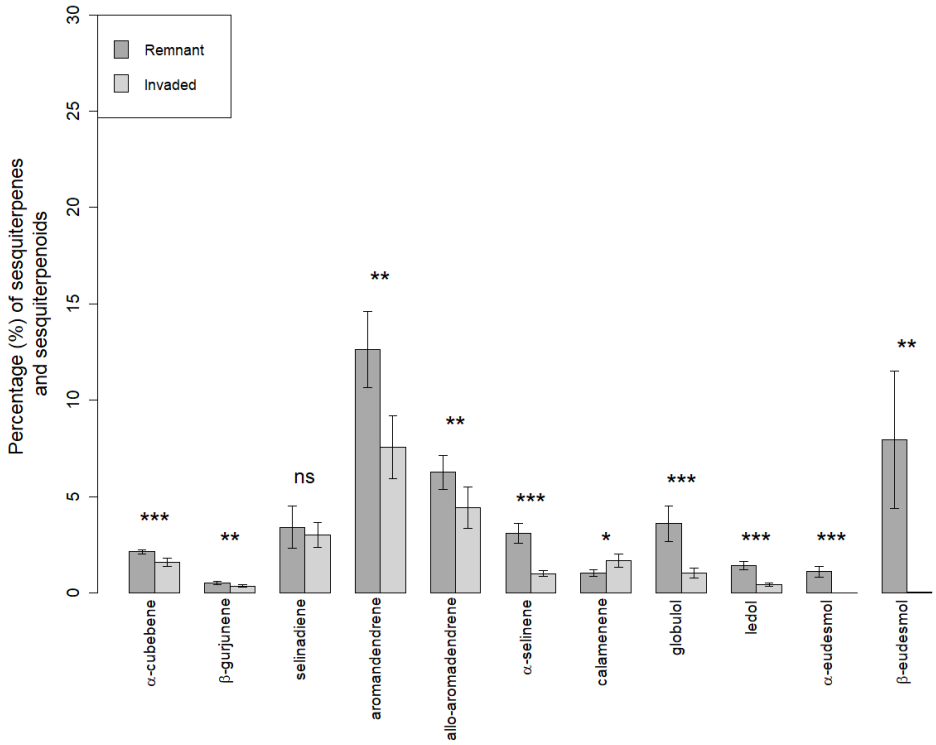

Figure 22. Histogram representing the % amount of sesquiterpenes and sesquiterpenoids identified in Remnant (R) and Invaded (I) area. Error bars indicate standard deviation, and the asterisks indicate significant differences between R and I: ns > 0.05, * 0.01 < p < 0.05, ** 0.001 < p < 0.1, *** p < 0.001.

4. Conclusions

In conclusion, we set the goal of studying multiple effects of climate change at ecosystem level in Mediterranean climate regions. It is known that plants interact with the environment by producing and emitting secondary metabolites (Pagare et al., 2015), which are synthetised to increase defence against biotic and abiotic stresses, playing essential functions in ensuring plants survival (Kessler and Kalske, 2018). In addition, some of these molecules are released by plants to communicate with other organisms: pollinators, natural antagonists of herbivores, or neighbouring plants (Holopainen et al., 2013). In this project, I have focused my research on secondary metabolites, particularly BVOCs (Biogenic Volatile Organic Compounds).

The main objective was to develop a better sampling strategy of BVOCs collection at canopy level, rather than at individual tree level (Appendix A). Afterwards, the terpenic profile and the trend of plant emissions was studied in two forest environments characterised by Mediterranean climate. The first forest was in the south of Tuscany (Italy) and the study looked at possible variations in the terpenic profile in a forest dominated by *Quercus ilex* following abiotic stress (mainly drought) (Appendix B). Then, in the second forest, located in Victoria (Australia), the study focused on the emission of volatile compounds and their possible role in explaining the invasiveness of alien species in habitats altered by climate change and human action (Appendix C). Finally, it was necessary to summarise the role of these compounds not only at ecological and plant level, but also at a wider level such as tropospheric chemistry and their potential benefits for human health (Appendix D).

The firs result (Appendix A) was the improvement of a pre-existing non-destructive BVOCs sampling method using SPME fibres coupled with a fan-sampler (Barreira et al., 2015), making it easier to be assembled and used in the field. The development of a new Dynamic BVOCs Sampling System (DBSS) allowed the acquisition of key information at canopy level and overcame the limitations of single plant measurements. In all sampling points this technique returned terpene profiles typical of Mediterranean

forests and the amounts of the single compounds collected by DBSS were higher when compared to the static technique. Finally, the fan-application allowed to reduce the sampling time necessary to obtain similar results with the static technique improving the efficiency and sensitivity of SPME fibres.

The second main goal (Appendix B) was to look at the ongoing health of a Mediterranean sclerophyll forest in a national reserve under stresses related to climate change (prolonged droughts). The monitoring of the forest health status was performed by the application of traditional methods such as species inventory and visual estimates of crown defoliation, as well as the use of the new and innovative DBSS technique. The visual evaluation of the health status measured through trees inventory, carried out over three years, and the seasonal and annual trends of terpenic profiles were in agreement with each other and showed rapid and significant damage to the tree canopy level, especially to the predominant oak species.

The third result interested a widespread phenomenon (Appendix C): alien species invasion in habitats altered by climate change. Specifically, the study regarded the invasiveness of the *Pittosporum undulatum,* an invasive native Australian species towards Eucalypt forests. Several approaches were tested to evaluate the problem on more levels: germination test, HPLC-DAD and spectrophotometer characterisation of secondary metabolites stored in the leaves and analysis of BVOCs at the environmental level using DBSS technique. In this study, the DBSS technique was also in agreement with the results obtained with other techniques demonstrating that the high invasiveness of *P. undulatum* it is not linked to allelopathic properties, but rather to greater tolerance against abiotic stresses thanks to biosynthesis of its secondary metabolites.

Thus, DBSS technique proved to be a particularly efficient tool for a fast and simple BVOCs field sampling, providing useful information in different studies (Appendix D). In addition, knowing and identifying organic compounds emitted by plants, and present in the air that we breathe, is fundamental even outside the context of ecological and environmental study. Indeed, due to BVOCs reactivity and oxidation processes in the atmosphere, they play an important role in atmospheric chemistry (Eerdekens et al.,

2009; Goldstein and Galbally, 2007; Slowik et al., 2010; Stroud, 2005). For example, isoprene lifetime is of 1.4 hours, when reacting with OH, 1.6 hours with NO_3 and 1.3 days with O_3. By contrast, α-pinene lifetime is 2.6 hours with OH, 4.6 hours with O_3 and 11 minutes with NO_3 (Atkinson and Arey, 2003). Therefore, since they represent the precursors of tropospheric O_3 and atmospheric aerosols (Goldstein et al., 2009; Griffin et al., 1999), these compounds are used as markers to estimate ambient air quality and pollution (Arneth et al., 2010). On the other hand, inhaling BVOCs in forest environments far away from urbanized, ozone-rich, areas and industrial factories can result in many health benefits for humans, as well as acting against mental fatigue and increasing cognitive performances (Cho et al., 2017; Peterfalvi et al., 2019; Tsunetsugu et al., 2010).

In conclusion, the DBSS technique shows potential applications in several conditions and studies, both ecological such as those presented in this thesis, as well as in urban environments, to monitor air quality and plant emissions. However, it is necessary to mention that the next step in this field of research would be the development of a reliable quantification procedure, required to further improve the DBSS sampling strategy, which will allow to provide quantitative data on BVOCs emitted in the environment.

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An Improvement of SPME-Based Sampling Technique to Collect Volatile Organic Compounds from *Quercus ilex* at the Environmental Level.

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An Improvement of SPME-Based Sampling Technique to Collect Volatile Organic Compounds from *Quercus ilex* at the Environmental Level

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Abstract: Biogenic Volatile Organic Compounds (BVOCs) include many chemical compounds emitted by plants into the atmosphere. These compounds have a great effect on biosphere–atmosphere interactions and may affect the concentration of atmospheric pollutants, with further consequences on human health and forest ecosystems. Novel methods to measure and determine BVOCs in the atmosphere are of compelling importance considering the ongoing climate changes. In this study, we developed a fast and easy-to-handle analytical methodology to sample these compounds in field experiments using solid-phase microextraction (SPME) fibers at the atmospheric level. An improvement of BVOCs adsorption from SPME fibers was obtained by coupling the fibers with fans to create a dynamic sampling system. This innovative technique was tested sampling *Q. ilex* BVOCs in field conditions in comparison with the conventional static SPME sampling technique. The results showed a great potential of this dynamic sampling system to collect BVOCs at the atmosphere level, improving the efficiency and sensitivity of SPME fibers. Indeed, our novel device was able to reduce the sampling time, increase the amount of BVOCs collected through the fibers and add information regarding the emissions of these compounds at the environmental level.

Keywords: BVOCs; GC-MS; monoterpenes; *Quercus ilex*; SPME

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1. Introduction

Trees evolved in arid and semi-arid environments, such as the Mediterranean basin, have developed several defense mechanisms to cope with drought, changing their physiology and metabolism. One of these mechanisms is the biosynthesis of Biogenic Volatile Organic Compounds (BVOCs), a large group of secondary metabolites, among which volatile isoprenoids (mono-, sesqui- and homo-terpenes) are the most important [1,2]. These compounds show noticeable functions in protection, defense and communication among plants, as well as between plants and other organisms. Their emissions are largely controlled by genetic and environmental conditions [3,4].

Most of the Mediterranean forest plants have been described as high BVOC emitters, in particular of monoterpenes, investing a great proportion of fresh assimilated carbon in their biosynthesis during the summer season [5–8]. Several studies have reported that BVOC emission is enhanced under abiotic stresses, such as drought and heat [9,10]. The estimated amount of isoprene and monoterpenes emitted by Mediterranean forests is about 4.5 kg km^{-2} day^{-1} [11], and it has a great impact on the atmospheric chemistry of this vulnerable habitat [11,12]. These compounds have a great effect on biosphere–atmosphere interactions by altering aerosol growth processes, cloud formation and, in

general, modifying atmospheric reactivity [13]. Indeed, BVOCs oxidation, especially of monoterpenes, plays an important role in the atmospheric chemistry as precursors of tropospheric ozone (O_3) and secondary organic aerosol (SOA) [14–18]. In particular, ozone is a greenhouse gas with detrimental effects on plant and human health [19,20]. In addition, monoterpenes have been shown to interact with human health in different ways, from stress relief to influencing immune function. In fact, recent studies have shown meaningful effects of BVOCs inhalation on the relief of stress-related mood disorders [21–23]. Terpenes, in addition to being largely utilized in the pharmaceutical industry, have recently been identified as the main chemical compounds responsible for the beneficial effects of the "forest bathing" therapy, an emerging popular practice consisting of immersing oneself in nature by mindfully using all five senses [24,25]. Increasing global concerns about the effects of atmospheric pollutants on human health and forest functioning are leading researchers to look for novel methods to collect and measure BVOCs in the atmosphere [26].

Up to date, BVOCs emission have been mainly investigated using plant and leaf enclosures connected to adsorption tubes or fibers followed by Gas Chromatography–Mass Spectrometry (GC–MS) analysis [27]. In particular, Solid-Phase MicroExtraction (SPME) fibers is a solvent free sampling technique and possesses useful characteristics to collect BVOCs in field conditions, since it is easy-to-handle, durable and provides repeatable results [28–30]. SPME can be used both in static (Static-HeadSpace—S-HS) and dynamic (Dynamic HeadSpace—D-HS) sampling enclosures of leaves or branches [31]. Between these two sampling systems, the D-HS has been recognized to provide more accurate measurements [32]. Indeed, measuring plants placed in an enclosed system, as in the case of S-HS, can result in unrealistically physiological conditions affecting gas exchanges and potentially altering BVOC emission rates of the plants [31,33]. SPME fibers can be also directly exposed to open air, a method largely applied to monitor and quantify volatile organic pollutants in urban environment through static sampling [34,35]. Moreover, SPME fibers have also been utilized for dynamic sampling at canopy level, by coupling with a fan-sampler [36] and, more recently, with a drone system [37]).

Several SPME sorbent coatings have been developed, such as divinylbenzene/polydimethylsiloxane (DVB/PDMS), which is used especially for the adsorption of semi-volatile analytes and larger volatile compounds, carboxen/polydimethylsiloxane (CAR/PDMS), suitable for small volatile molecules, and DVB/CAR/PDMS, which is indicated for the adsorption of an extended molecular weight range of analytes [30]. For this reason, in our study, we utilized the DVB/CAR/PDMS coating. In this study, DVB/CAR/PDMS fibers have been included in a dynamic sampling system characterized by a controlled and constant air flux, which allowed the adsorption of BVOCs on the fibers and the ability to reach the equilibrium retaining analytes with different molecular weights uniformly [38]. Our Dynamic BVOC Sampling System (DBSS), based on a previous study [36], was improved in the material used for the instrument to make it cheaper and easier to assemble. Our choice was to use aluminum instead of polyacetal plastic. Using this metal, all the components are easily available in any hardware store, and no glue was used for its assembly to avoid possible interferences in BVOCs analysis. The choice of aluminum was guided by the need to have a light and resistant instrument for field campaigns. Furthermore, the DBSS shape and its low weight allow it to be easily set up on a metal picket in the forest, while the metal net in front of the chamber provides a physical barrier to insects or debris, additionally creating a swirling flow. The DBSS was positioned at a height of 45 cm from the ground, which is optimal to limit wind action and manage the sampling operations [39]. Finally, the sampling duration of 4 h was chosen to obtain the best compromise between a long exposition time of SPME fibers [40] and an operationally feasible sampling time in the field.

In this work, we present a methodology for BVOC collection as specified above. Our technique for BVOC collection was tested under field conditions for qualitative and semi-quantitative analyses of BVOCs emitted from *Q. ilex* plants at the atmospheric level. The study was carried out on two sampling sites: the first one was located in the experimental

fields of CNR (National Research Council of Italy, Sesto Fiorentino, FI, Italy), where BVOCs were collected from three-year-old *Q. ilex* L. potted plants; the second experimental site consisted of a Mediterranean sclerophyll forest located in the Maremma Regional Reserve (Alberese, GR, Italy). The forest was dominated by *Q. ilex*, while other woody species were also present in a lower percentage, such as *Rubus* spp., *Phillirea latifolia, Cistus salviifolius, Quercus cerris, Pistacia lentiscus, Acer monspessulanum, A. campestre* and *Fraxinus ornus.*

Mediterranean forests are important for their ecological and socio-economic value providing several ecosystem services and goods to society [41–43]. To test our sampling method, we selected *Quercus ilex* as the most representative species of the Mediterranean forest ecosystem that, in recent years, has been subjected to repeated drought events [44]. Thus, the main aim of our study was to develop and test a new, rapid and simple sampling strategy to obtain reliable data on BVOC emission at the environmental level.

2. Results

2.1. BVOC Identification and Qualitative Analysis

Table 1 summarizes terpenes identified in the chromatograms (Figure S1) obtained from the analyses of samples collected at the three sampling points. In all three sampling points, the only terpenes identified were monoterpenes and monoterpenoids (MTs), while no sesquiterpenes or other BVOCs were detected. Indeed, the *Q. ilex* BVOC emission pattern is mainly characterized by monoterpenes [45–50]. In the first sampling point, there were no differences in MTs collected using the two sampling techniques (DBSS and static sampling). In both cases, the identified MTs were: monocyclic monoterpene hydrocarbons (d-limonene and *p*-cymene), one oxygenated derivative of monocyclic monoterpenes (1,8-cineole), and bicyclic monoterpene hydrocarbons (*α*-pinene, *α*-thujene, camphene, *β*-pinene, sabinene, car-3-ene). Similarly, qualitative differences between the two sampling techniques were not found in the second sampling point. In particular, the following acyclic monoterpene hydrocarbons were detected: myrcene and *β*-cis-ocimene. *β*-phellandrene, *α*-phellandrene, *α*-terpinene, d-limonene, *γ*-terpinene, *p*-cymene and terpinolene were detected among monocyclic monoterpene hydrocarbons. Finally, among the bicyclic monoterpene hydrocarbons, the following compounds were identified: *α*-pinene, *α*-thujene, camphene, *β*-pinene, sabinene and car-3-ene. Some qualitative differences were found during the third sampling point between DBSS and static sampling. Indeed, a higher number of MTs were identified using the DBSS compared to the static sampling. Among the acyclic monoterpene hydrocarbons, *β*-cis-ocimene were identified in both cases, while myrcene and *β*-trans-ocimene were found only using the DBSS. Among the monocyclic monoterpene hydrocarbons, d-limonene and *p*-cymene were detected using both sampling techniques, while only applying DBSS, *α*-terpinene, *γ*-terpinene and terpinolene were found. Finally, among the bicyclic monoterpene hydrocarbons, the following were found: *α*-pinene, *α*-thujene, camphene, *β*-pinene and sabinene in both cases, while car-3-ene only using DBSS.

2.2. Semi-Quantitative Analysis of Individual and Total MTs

In the first sampling point (Figure 1), a similar trend was common for both sampling techniques. In particular, *α*-pinene resulted as the most abundant compound sampled using both techniques, while *α*-thujene was the second most abundant compound only in samples collected by the DBSS technique. All peak areas were higher in samples collected by DBSS compared to the static technique.

A similar trend was also found in the second sampling point (Figure 2). The most abundant compounds were *α*-pinene and *β*-pinene, followed by *α*-thujene, car-3-ene, d-limonene and *p*-cymene. Similar to sampling point 1, the amounts of the single compounds collected by DBSS were higher compared to the static technique.

Table 1. A list of the identified compounds by comparison with the NIST 11 library. Their names and retention times (RT) are presented. The peak number (n. Peak) corresponds to the numbers reported in the chromatograms (Figure S1). It is also reported the presence (y: yes) or the absence (n: no) of each compound in each the different sampling points (SP 1, SP 2 or SP 3) and in the two sampling conditions (dynamic/static).

n. Peak	Compound Identified	RT	Presence		
		(min)	SP 1 (dynamic/static)	SP 2 (dynamic/static)	SP 3 (dynamic/static)
1	α-pinene	10.07 ± 0.02	y/y	y/y	y/y
2	α-thujene	10.15 ± 0.02	y/y	y/y	y/y
3	camphene	11.35 ± 0.05	y/y	y/y	y/y
4	β-pinene	12.61 ± 0.04	y/y	y/y	y/y
5	sabinene	12.76 ± 0.05	y/y	y/y	y/y
6	car-3-ene	12.97 ± 0.03	y/y	y/y	y/n
7	β-phellandrene	13.10 ± 0.03	n/n	y/y	n/n
8	myrcene	14.12 ± 0.02	n/n	y/y	y/n
9	α-phellandrene	14.35 ± 0.02	n/n	y/y	n/n
10	α-terpinene	14.83 ± 0.04	n/n	y/y	y/n
11	d-limonene	15.39 ± 0.02	y/y	y/y	y/y
12	1,8-cineole	15.68 ± 0.01	y/y	n/n	n/n
13	β-cis-ocimene	15.72 ± 0.02	n/n	y/y	y/y
14	β-trans-ocimene	13.99 ± 0.02	n/n	n/n	y/n
15	γ-terpinene	16.80 ± 0.01	n/n	y/y	y/n
16	*p*-cymene	17.62 ± 0.02	y/y	y/y	y/y
17	terpinolene	17.95 ± 0.01	n/n	y/y	y/n

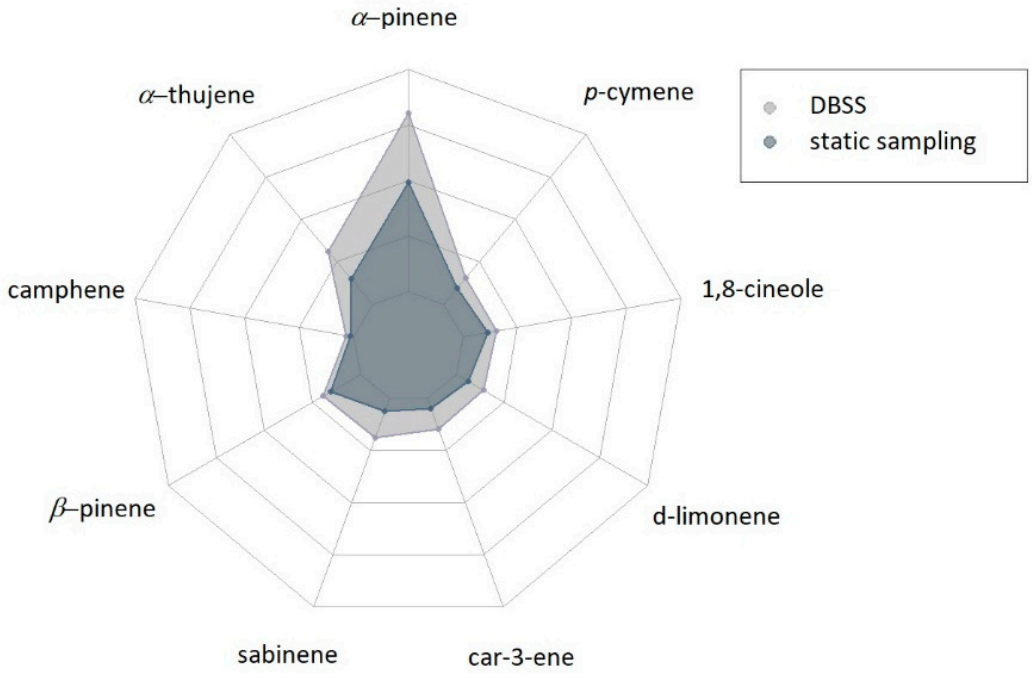

Figure 1. Radar-plot showing the results of the semi-quantitative analysis of BVOCs at sampling point 1 (n = 3) expressed as the peak area of each individual compound. The amounts of different compounds range between 0 and 350,000. Results of statistical analysis are reported in Table S1.

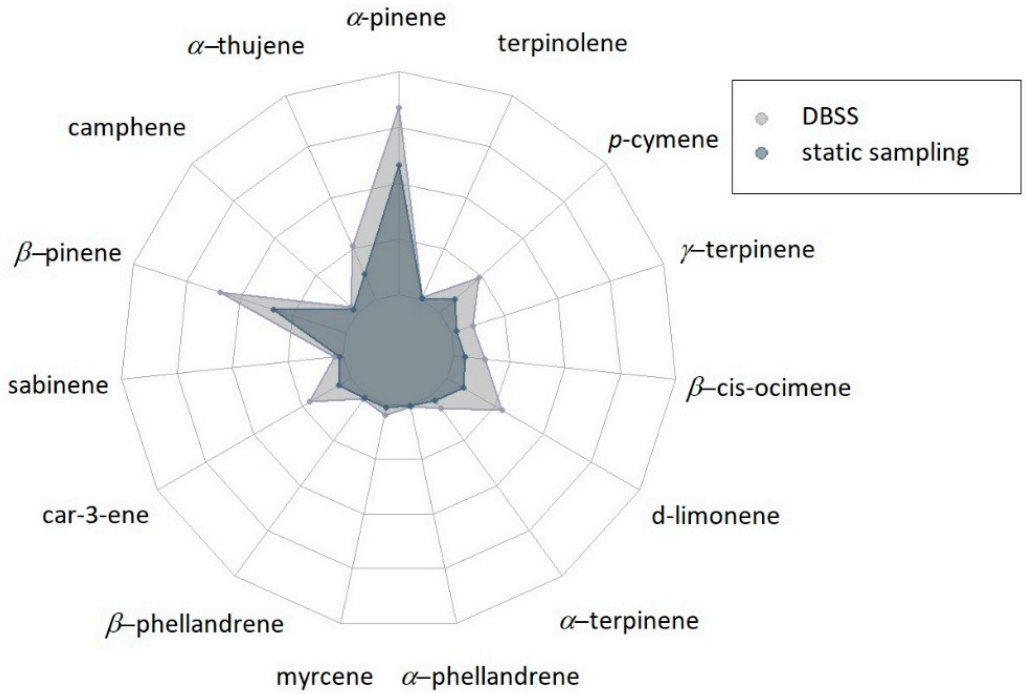

Figure 2. Radar-plot showing the results of the semi-quantitative analysis of BVOCs at sampling point 2 (n = 3) expressed as the peak area of each individual compound. The amounts of different compounds range between 0 and 2,000,000. Results of statistical analysis are reported in Table S2.

In the case of sampling point 3, the amounts of BVOCs collected using the DBSS were much higher than those found using the static sampling technique (Figure 3). In addition, at this sampling point, the DBSS allowed collecting a wider range of MTs compared to the samples collected using the static technique (Table 1). Indeed, compounds, such as car-3-ene, myrcene, α-terpinene, β-trans-ocimene, γ-terpinene and terpinolene, were detected only when using the DBSS. Finally, there is a slight difference in the trend of samples collected by DBSS and by static sampling. The samples collected by DBSS contained mainly α-pinene and *p*-cymene, followed by β-pinene and d-limonene, whereas in samples collected by the static technique, α-pinene and β-pinene were the most abundant compounds, followed by d-limonene and *p*-cymene (Figure 4).

Considering the total area of MTs in each sampling point, DBSS allowed collecting a higher total amount of MTs compared to the static technique (Figure 5). In particular, at sampling points 1 and 2, the sum of areas of MTs obtained from the DBSS was double compared to that of the static technique (56% for sampling point 1 and 50% for sampling point 2). In the third sampling point, the difference between the two techniques was considerably higher; indeed, the amounts of MTs collected by the static technique was only ~3% compared with the amounts obtained using the DBSS.

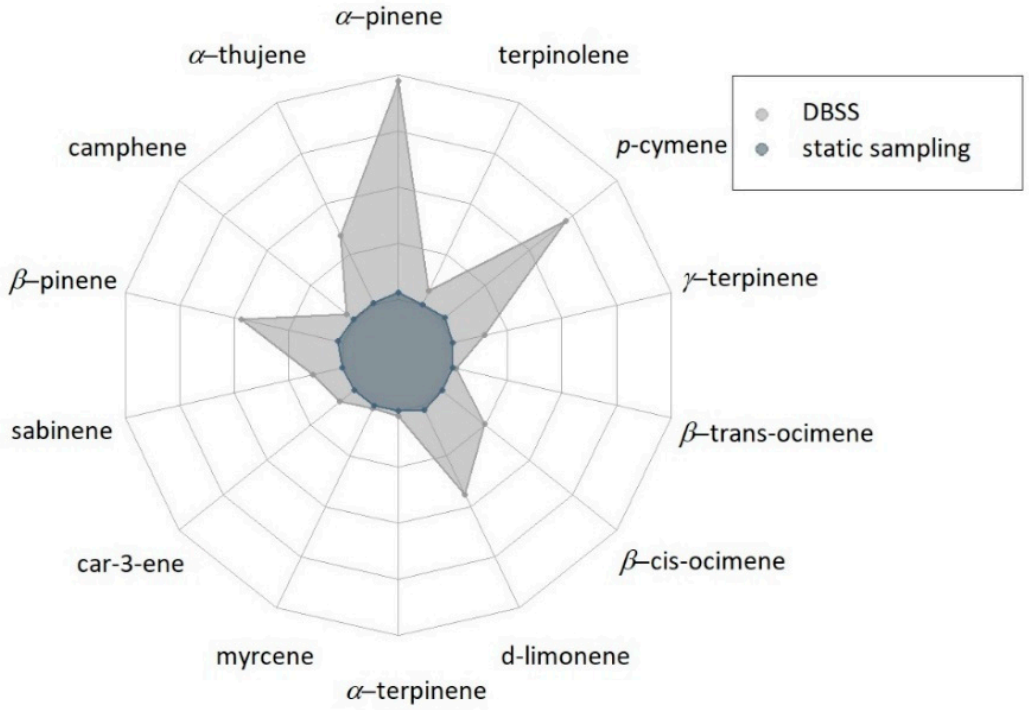

Figure 3. Radar-plot showing the results of the semi-quantitative analysis of BVOCs at sampling point 3 (n = 3) expressed as the peak area of each individual compound. The amounts of different compounds range between 0 and 1,350,000. Results of statistical analysis are reported in Table S3.

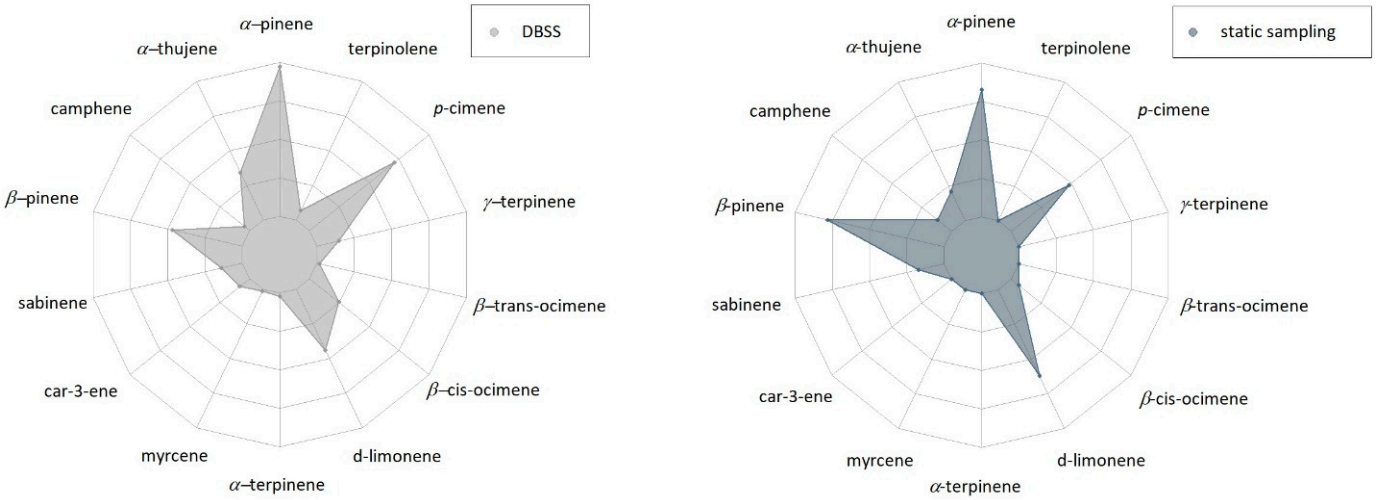

Figure 4. Radar-plots showing the results of the semi-quantitative analysis of BVOCs at sampling point 3 (n = 3) expressed as the peak area of each individual compound. The results are split in two graphs to highlight the quantitative differences between the two sampling techniques (different scales: DBSS from 0 to 1,350,000; static sampling from 0 to 45,000). Results of statistical analysis are reported in Table S3.

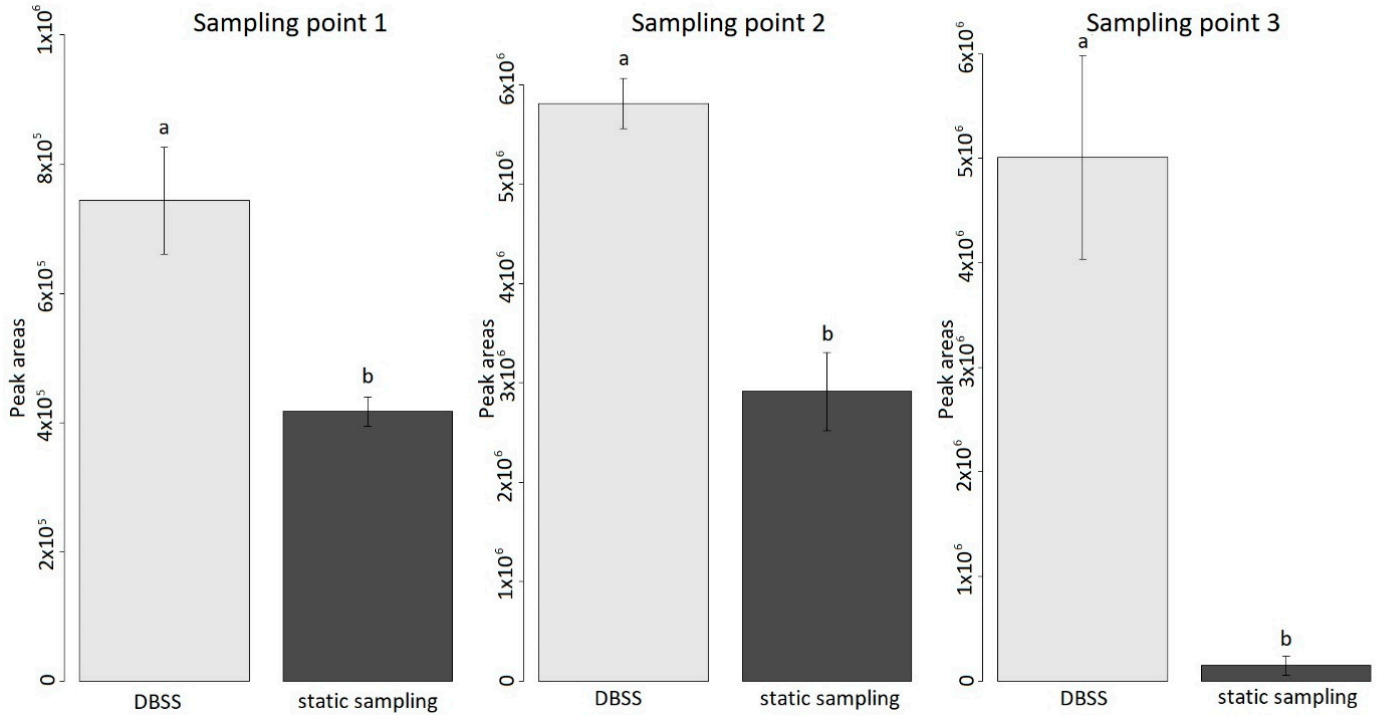

Figure 5. The amount of total MTs collected using DBSS (light grey) and static technique (dark grey) at sampling point 1, sampling point 2 and sampling point 3. Data are means ± standard deviation (n = 3). Data were analyzed by one-way ANOVA test, and the letters indicate statistical differences between the two sampling techniques in each sampling point obtained from a Tukey post hoc test ($p \leq 0.05$). Results of statistical analysis are reported in Table S4.

3. Discussion

In our study, air samples collected by static and DBSS techniques were compared to evaluate the most efficient and sensitive methodology for BVOC collection under field conditions. It is important to notice that the sampling site at the Maremma National Reserve allowed us to test the DBSS technique in a complex environment, in which many plants that emit MTs were present. Indeed, in this sampling site, in addition to *Q. ilex*, other species such as *Rubus* spp., *Phillirea latifolia*, *Cistus salvifolius*, *Q. cerris*, *Pistacia lentiscus*, *Acer monspessulanum*, *A. campestre* and *Fraxinus ornus* were observed in a lower percentage. Finally, two additional sources of BVOCs in the forest could be soil microbes and litter. However, microbial BVOC emission rates are very low in Mediterranean shrublands [51]. In addition, as recently observed by Viros et al. [52], BVOCs emitted from *Q. ilex* litter do not include monoterpenes, thus representing a negligible source of these compounds. On this basis, we can suggest that the compounds found in our study derived from green leaves and not from litter or soil microbes.

We used DVB/CAR/PDMS coating type fiber, and the choice was dependent on its technical characteristics [30]. Additionally, our choice was dependent on the fact that, in previous studies, the extraction efficiency of DVB/CAR/PDMS coating for terpenoid was better than that of other fibers on the market [53,54]. Yassaa et al. [45] showed that DVB/CAR/PDMS fibers might have competitive adsorption of isoprenoids and saturation condition of the coating. Nonetheless, in our study, DVB/CAR/PDMS fibers were the most appropriate choice for sampling BVOCs in field conditions because the exposure of the fibers directly to the atmosphere did not allow them to reach the saturation of the fiber coating. Additionally, the use of an open, dynamic headspace system removed the problems related to static headspace, such as increases in temperature and humidity [31].

Sampling parameters, such as time window (11 am–3 pm), fan height (40–45 cm) and sampling duration (4 h), were chosen following previous studies (see below). Several authors [55–57] have shown that the higher emissions of BVOCs occur between 9 am and

5 pm, with a maximum peak around 1–2 pm [56]. Indeed, their emission is linked with the time when both temperatures and solar radiation were higher. The height of 45 cm from the ground was chosen since terpene concentrations have been demonstrated to be high at heights from the forest floor to 4 m. This height is optimal to limit wind action, as it allows for a more sheltered sampling condition [39]. Moreover, the choice of the height at which the fan sampler has been positioned was made to allow ease of operation during sampling (i.e., to expose and to retract the fiber; to turn on and off the fan). Lastly, the sampling duration was set to 4 h to have a long exposition time of SPME (to collect as many BVOCs as possible while in the pre-equilibrium case—[40]) and to allow a feasible sampling time in the field during every season. Indeed, longer acquisition times could be difficult to implement in winter and fall because unsuitable weather conditions are more frequent, such as strong winds and rain and shorter daylight availability.

Our results showed that the DBSS was able to sample higher amounts of BVOCs in the two experimental sites (Sesto Fiorentino and Maremma National Reserve) at all sampling points (Figures 1–5). Indeed, in the first and second sampling point, the total amount of MTs obtained using DBSS was double that of the static technique (Figure 5). The differences in the amount of BVOCs collected between the two techniques were particularly noticeable at sampling point 3 (Figure 5), in which qualitative differences were also observed (Figure 4). These qualitative differences observed at sampling point 3 may be explained by considering the lower air temperature recorded in October compared to June. Indeed, since BVOC emission is temperature-dependent, with an optimum around 25 °C, the collection of BVOCs using DBSS resulted particularly efficient when environmental conditions limited their emission [3,58]. Furthermore, a potential sink effect played by humidity, reducing BVOC adsorption in SPME fibers, could exacerbate this outcome since these compounds are water soluble at low concentrations [59]. Therefore, the collection of BVOCs without the fan-sampler system may reduce the BVOC adsorption on SPME fibers when present in traces or under windy weather conditions [60,61]. The observed variations in the scale of the total monoterpenes obtained between sampling point 1 and sampling points 2 and 3 (Figure 5) could be explained by the differences in plant characteristics and site conditions. Indeed, in sampling point 1, the studied plants were represented by 15 three-year-old *Q. ilex* potted plants, while in sampling points 2 and 3, BVOCs were collected in a natural forest with mature trees. Therefore, the dimensions of the canopy were very different, as well as the environmental conditions of the sampling. In addition, the potted *Q. ilex* plants, with a mean height of 1.2 m, were positioned at the center of an open field subjected to wind gusts that could have reduced the deposition of BVOCs into the fibers compared to the under-canopy conditions of the forest. Finally, the site of sampling point 1 was located in Sesto Fiorentino, a semi-urban area in which BVOC degradation by atmospheric oxidants could have been occurred [62]. In particular, since the sampling was conducted during the central hours of the day, eventual monoterpene oxidation would have been caused by the presence of hydroxy radicals (OH) [63]. However, these types of degradation products were not detected in our experiment.

It is important to mention that the range of total MTs obtained in sampling points 2–3, which were carried out in the same sampling area but in different seasons, provided similar results when employing the DBSS strategy. This finding shows the high potential and good repeatability of the DBSS strategy.

Another advantage of the DBSS could derive from the homogenization of the sample through the fan. The creation of turbulent airflow, obtained by combining the fan and the net in front of the SPME fiber. BVOCs under forest canopy can have heterogeneous concentrations [39], and the emissions are influenced by several factors: different seasons, meteorological conditions, sunlight exposure, altitude, tree species and damages from herbivores [3,8,64]. Altogether, our results demonstrated the potential and versatility of the DBSS for the rapid in situ measurement of BVOCs under different environmental conditions. In particular, the DBSS allowed identifying the typical compounds emitted by *Q. ilex*. Indeed, qualitative MTs identification carried out in our study is consistent

with results reported in previous experiments conducted on the same species both in pots and in field conditions [49,50,65,66]. In all sampling points, α-pinene, α-thujene, β-pinene, sabinene and d-limonene were the most abundant compounds emitted by *Q. ilex* [5,46,48], which represented about 65–80% of the total detected monoterpenes. While other BVOCs such as car-3-ene, *p*-cymene and 1,8-cineole were also detected in lower amounts [46,48,67]. In addition, in agreement with Sabillo [47], our system was able to collect β-phellandrene, α-terpinene, γ-terpinene and terpinolene. Finally, consistently with Peñuelas et al. [58], we detected α-phellandrene (only at sampling point 2), emitted when *Q. ilex* is exposed to high air temperatures.

The main compounds detected in our study were α-pinene and α-phellandrene, which have shown anti-inflammatory and anti-cancer properties, respectively [68–70]; moreover, it has been demonstrated that d-limonene and *p*-cymene can act against allergic lung inflammation [71,72], while β-pinene and 3-carene, have shown to possess anti-depressive and anxiolytic functions when inhaled [73]. Finally, monoterpenes, in general, and myrcene and 1,8-cineole, in particular, display neuroprotective roles thanks to their antioxidant effects [74,75]. For these reasons, it could be interesting to monitor the air quality and the emission of Mediterranean forest plants, to further investigate the healing effects of BVOCs on human health.

4. Materials and Methods

4.1. Theoretical Background for SPME Sampling in Field Conditions

The SPME principle is explained by the Equation (1) [76]:

$$n = c_f^{\infty} V_f = c_0 \frac{K_{fs} V_s V_f}{K_{fs} V_f + V_s} \quad (1)$$

where n is the amount of analyte present in the sample matrix; c_0 is the initial concentration of the analyte in the sample matrix; C_f^{∞} represents the concentrations in sample and fiber coating at equilibrium; V_s and V_f represent volumes of the sample and fiber coating, respectively, while K_{fs} is the distribution coefficient of analyte between fiber coating and sample matrix. In this condition, whether the volume of sample (V_s) is very large (e.g., in-field sampling), the equation becomes [76]:

$$n = K_{fs} V_f c_0 \quad (2)$$

Thus, Equation (2) indicates that, in the case of a large volume of sample, the extracted analyte amount will directly coincide with its concentration in the sample matrix, and it is not linked with the sample volume. Thus, for field sampling, the SPME fiber can be exposed directly to the specific environment, and under stable agitation conditions and constant temperature and extraction time, a quantitative analysis is possible at pre-equilibrium conditions [77,78].

4.2. Instrumental Setup

The SPME fibers selected for the collection of BVOCs were divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS-50/30 µm layer, Supelco, Sigma-Aldrich Co., Darmstadt, Germany). Each SPME fiber was held in the middle of a 17 cm long aluminum cylinder, perpendicularly connected to a smaller vertical cylinder 7 cm long acting as a support. In Figure 6, a blueprint (I) and pictures (II, III, IV) of the dynamic sampling system for BVOC collection are represented. Observing panel I on the top, the DBSS is constituted by a vertical tube (A), a metal net (B) connected to the main cylinder (C) both in aluminum, and a small fan (D). The diameter of the cylinder was chosen to allow a proper fiber exposition. One face of the cylinder (external diameter 5 cm and internal diameter 4 cm) is closed by a metal net (B) to protect the fiber from debris and to create a swirling flow, while the other face is closed by a small fan (Jamicon®, Kaimei Electronic Corp., New Taipei City, Taiwan, 12 V, 6200 rpm, 13 m^3/h, 40 × 40 × 20 mm, panel IV) powered by

a lead–acid battery (Join®, Alpha Elettronica S.r.l., Collecchio, Italy, 12 V, 4.5 AH). The instrument aluminum body is designed to allow an easier sampling and movement in the forest and is, therefore, light and resistant; in addition, to connect the single parts of the instrument, 4 long screws were used instead of glue to allow the utilization under high-temperature conditions and avoid any interferences during the sampling.

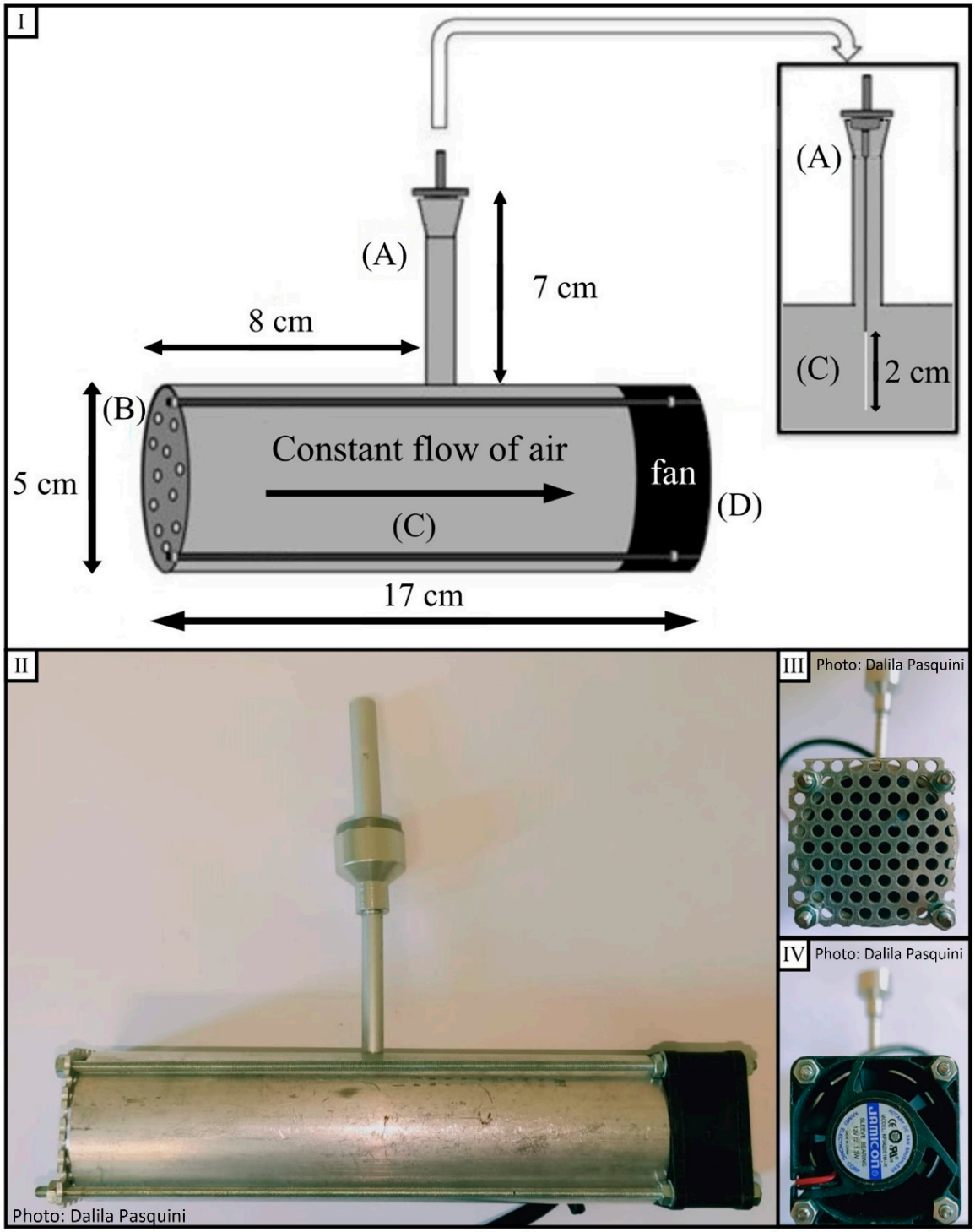

Figure 6. In **panel I**, the schematic drawing of the dynamic sampling system, adapted and modified by the BVOC system developed by Barreira et al. [36], is reported. The SPME fiber was firmly positioned in the appropriate vertical tube (**A**) by four small magnets. A metal net (**B**) was installed at one end of the cylinder (**C**) to protect the fiber, while at the opposite end, a fan (**D**) was positioned to create a swirling flow. All parts are linked together by screws. In **panel II**, a picture of the whole device is reported, while in **panel III** and **IV**, the details of the net located in the front of the device and the fan set on the back are shown.

After sampling, the fibers were placed in a special tray, within a hermetic case, with dedicated Teflon pressure supports to seal the needle until the fiber was transported to the laboratory, and all the fibers were subsequently desorbed within the same day, avoiding any degradation of compounds adsorbed into the SPMEs.

4.3. GC-MS Analysis

The desorption of SPME fibers was carried out with a 7820 A gas chromatograph coupled with a 5977E mass spectrometer (both from Agilent Technology, Santa Clara, CA, USA) operating in EI ionization mode at 70 eV energy. A DB-Wax (60 m × 250 µm × 0.5 µm, Agilent J&W) column was used for analytes separation. The other instrumental parameters were set as follows: injector temperature 260 °C, splitless mode and carrier flow (He) of 1.2 mL min^{-1}. The oven temperature program was initially set at 40 °C for one minute, then increased by 5 °C/min until 210 °C, and then of 10 °C/min until 260 °C, at which temperature, it was held for 10 min, with a total run time of 48 min. The mass spectra were acquired in the 29–205 m/z range at three scans sec^{-1}. Data were analyzed using the Agilent Mass Hunter software (Qualitative Analysis-Version B.06.00; Quantitative Analysis-Version B.07.01/Build 7.1.524.0), and the analytes were identified by matching their mass spectra and retention indices with those reported in NIST 11 spectral database library. Information from fragmentation patterns, retention times and data available from scientific literature was used for final identification [79,80]. The amounts of monoterpenes and monoterpenoids (MTs), expressed as peak areas, were reported both as single compounds and total MTs, and they have been related to Total Ion Current (TIC).

4.4. Test of DBSS in Field Conditions

BVOC samplings were carried out in two different experimental sites, utilizing SPME fibers with both the static and dynamic sampling system (DBSS). The first sampling site was located in the experimental fields of CNR (National Research Council of Italy, 43°49′05″ N, 11°12′12″ E, Sesto Fiorentino, FI, Italy) on the 5th of June 2019. BVOCs emissions were collected from 15 3-year-old *Quercus ilex* L. potted plants (kindly supplied by Vivaio Matteini, 51100, Pistoia) maintained under optimal irrigation (sampling point 1). The heights of individual plants were about 120 cm with a stem diameter of 1 cm (Table 2).

Table 2. The table shows the main atmospheric and sampling parameters: days of sampling, atmospheric temperature and humidity, speed and direction of wind, time window of sampling and site characteristics.

Sampling Point	Sampling Day	Temperature	Humidity	Wind	Sampling Time	Plants	Site
N	dd/mm/yyyy	°C	%	speed (km/h), direction			
1	6/6/2019	24 °C	45%	9–14 km/h, W/SW	12 pm 4 pm	*Q. ilex* in pot	Sesto Fiorentino (FI)
2	20/06/2019	26.5 °C	45%	10 km/h, W/SW	12 pm 4 pm	*Q. ilex* forest	Maremma Regional Park (GR)
3	1/10/2019	20 °C	60%	8km/h, S	12 pm 4 pm	*Q. ilex* forest	Maremma Regional Park (GR)

The second experimental site was located in the Maremma Regional Reserve at 320 m altitude (Alberese, 42°38′10″ N, 11°05′39″ E, Grosseto, GR, Italy), and BVOCs emissions were collected on the 20th of June 2019 (sampling point 2) and on the 1st of October 2019 (sampling point 3) (Table 2). This site was characterized by a Mediterranean sclerophyll forest with the predominance of *Q. ilex*, representing around 70% of the total number of individuals. At this site, SPME fibers were placed under the tree canopy, and the BVOC collection was carried out with the same settings mentioned above.

At both sampling sites, BVOC collection was conducted during rainless days and with light wind. The air temperature and humidity, as well as wind speed and direction, were recorded by weather stations installed in the proximity of the sampling sites by the Institute of Bioeconomy of the National Research Council of Italy (IBE, CNR). BVOCs were collected on DVB/CAR/PDMS coating fibers for 4 h, from 12 am to 4 pm, to cover the time interval with the highest presumable concentration of terpenes in the air as indicated in literature [60,61]. Indeed, several authors have shown a diurnal cycle in BVOCs emissions [55–57], with higher emissions between 9 am and 5 pm and a maximum peak around 1–2 pm [56]. The fibers were positioned at a height of 40–45 cm from the ground using a plastic band to connect them to a metal picket and at a distance of 30 cm from *Q. ilex* plants. All measurements were conducted in triplicate.

4.5. Statistical Analyses

All statistical analyses were carried out using R software (version 4.0.3). After carrying out the Shapiro and Levene tests, to check respectively the assumption of normality [81] and homoscedasticity [82,83], the data were analyzed using a one-way analysis of variance (ANOVA) and followed by a Tuckey post hoc test.

5. Conclusions

In recent years, there has been an increasing interest in alternative, fast and easy-to-handle methods to sample BVOCs emitted in the field. This would be of particular importance for monitoring changes in terpene emissions by forests under both abiotic and biotic stresses, as well as for evaluating changes in air quality for human well-being. In order to develop an innovative sampling method to measure and analyze BVOCs under environmental conditions, the use of a DBSS technique could be a particularly efficient tool for a fast and simple BVOC collection in future fieldworks. This innovative sampling method is able to collect efficiently different classes of MTs at the environmental level, overcoming low-temperatures and high-humidity limitations typical of the static techniques and providing key information at the ecological level without the limitations of single plant measurements. Thus, a potential future application of this sampling device could be to monitor BVOCs' environmental emissions from *Q. ilex* forests that experience high levels of tree mortality in comparison to healthy forests. Additional research, including comparison with other techniques (such as those employing cartridge sampling systems), acquisition of a larger dataset and development of a quantification procedure, are required in order to further improve the DBSS sampling strategy, which will provide quantitative data on BVOCs emitted at the environmental level.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11060388/s1, Figure S1: Chromatograms, Table S1: ANOVA summary sampling point 1, Table S2: ANOVA summary sampling point 2; Table S3: ANOVA summary sampling point 3; Table S4: ANOVA summary all sampling points (1, 2 and 3).

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Supplementary material

Appendix A

Supplementary Material

Supplementary Figures and Tables

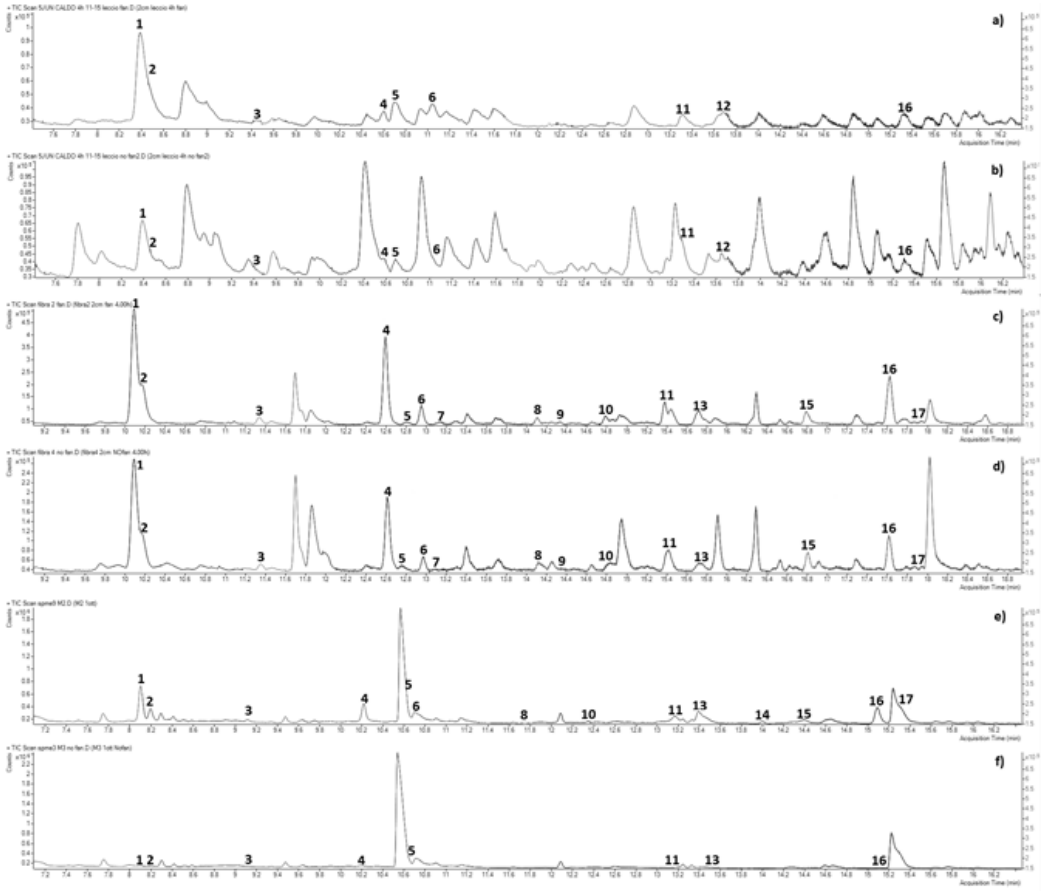


Figure S1. Representative Total Ion Chromatograms (TIC) for each sampling point using the two different methods (DBSS and static sampling): a) DBSS at sampling point 1; b) static method at sampling point 1; c) DBSS at sampling point 2; d) static method at sampling point 2; e) DBSS at sampling point 3; f) static method at sampling point 3. The numbered peaks correspond to: (1) α-pinene, (2) α-thujene, (3) camphene, (4) β-pinene, (5) sabinene, (6) car-3-ene, (7) β-phellandrene, (8) myrcene, (9) α-phellandrene, (10) α-terpinene, (11) d-limonene, (12) 1,8-cineole, (13) β-cis-ocimene, (14) β-trans-ocimene, (15) γ-terpinene, (16) *p*-cymene, (17) terpinolene. The shift in the retention time observed in the peaks in the chromatograms obtained in sampling point 3 (e-f) is due to a difference in the length of the column.

Table S2. Statistical results obtained from one-way Analysis of Variance (ANOVA) of the amounts of compounds found in sampling point 1, using DBSS and static sampling techniques. (ns $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Compounds	**F value**	***p***	
α-pinene	24.14	0.0080	**
α-thujene	10.65	0.0310	*
camphene	23.04	0.0086	**
β-pinene	0.12	0.7430	ns
sabinene	3.15	0.151	ns
car-3-ene	67.59	0.0012	**
d-limonene	7.66	0.0505	ns
1,8-cineole	4.81	0.0934	ns
p-cymene	1.84	0.2460	ns

Table S3. Statistical results obtained from one-way Analysis of Variance (ANOVA) of the amounts of compounds found in sampling point 2, using DBSS and static sampling techniques. (ns $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Compounds	F value	*p*	
α-pinene	5.27	0.0833	ns
α-thujene	38.78	0.0034	**
camphene	33.54	0.0044	**
β-pinene	25.16	0.0074	**
sabinene	33.3	0.0045	**
car-3-ene	3.46	0.1370	ns
β-phellandrene	3.97	0.1170	ns
myrcene	12.30	0.0247	*
α-phellandrene	47.88	0.0023	**
α-terpinene	54.69	0.0018	**
d-limonene	69.81	0.0011	**
β-cis-ocimene	119.40	0.0004	***
γ-terpinene	46.55	0.0024	**
p-cymene	9.81	0.0351	*
terpinolene	11.20	0.0286	*

Table S4. Statistical results obtained from one-way Analysis of Variance (ANOVA) of the amounts of compounds found in sampling point 3, using DBSS and static sampling techniques. (ns $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Compounds	**F value**	***p***	
α-pinene	30.56	0.0052	**
α-thujene	48.34	0.0022	**
camphene	86.29	0.0007	***
β-pinene	28.77	0.0058	**
sabinene	27.92	0.0061	**
car-3-ene	17.74	0.0136	*
myrcene	33.69	0.0044	**
α-terpinene	17.50	0.0139	*
d-limonene	101	0.0005	***
β-cis-ocimene	19.39	0.0117	*
β-trans-ocimene	43.63	0.0027	**
γ-terpinene	36.16	0.0038	**
p-cymene	55.03	0.0018	**
terpinolene	34.28	0.0042	**

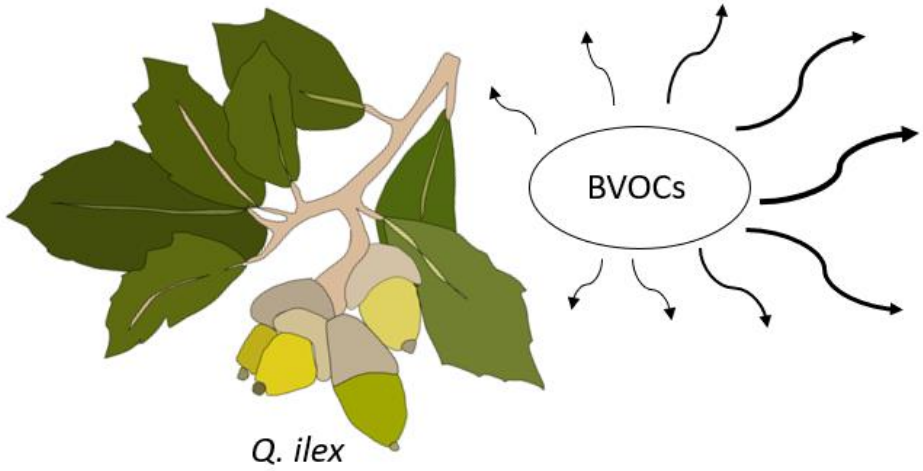
Table S5. Statistical results obtained from one-way Analysis of Variance (ANOVA) of the total amounts of compounds found in each sampling points (1, 2, 3), using DBSS and static sampling techniques. (ns $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Sampling point	**F value**	***p***	
1	43.77	0.0027	**
2	114.1	0.0004	***
3	73.63	0.0010	**

Appendix B – Ready for submission:

Effects of Drought-Induced Holm oak die-back on BVOC emissions in a Mediterranean forest.

Authors: **Pasquini, D.**, Gori A., Pollastrini M., Alderotti F., Ferrini F., Centritto M., Brunetti C.



Effects of Drought-Induced Holm oak die-back on BVOC emissions in a Mediterranean forest.

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Abstract - Trees of Mediterranean forests are capable to emit large amount of Biogenic Volatile Organic Compounds (BVOCs) and this emission is affected by climatic factors. Extreme weather conditions, such as heat waves and drought spells, may drastically impair plant physiology and growth. Furthermore, these conditions cause an increase in tree dieback, which unavoidably leads changes in plant community composition and functioning and land–atmosphere interactions. How trees dieback can alter BVOC emissions at the forest stand level is unknown. In our study, BVOC emissions *of Quercus ilex* L. (holm oak) forest in the Southern Tuscany (Central Italy) were examined. Site and climate effects on seasonal BVOC emissions were analysed. BVOCs were collected using Solid-Phase MicroExtraction (SPME) fibres in two forest stands characterised by two levels of tree dieback, estimated by crown defoliation. A floristic survey was carried out three times during the growing season for three years to identify herbaceous and woody plant species in the understorey of both holm oak stands. The abundance of each species was also assessed. The number of understorey species and their cover significantly differed between the two stands, with a greater number of shrubs in the stand characterised by a lower crown defoliation during the growing seasons. BVOC levels and composition significantly changed during the year and were affected by the degree of holm oak decline. Our results suggest that terpene emissions from Mediterranean forests would be modified by an increase of *Q. ilex* L. dieback, with

important consequences for functioning of this forest ecosystem and the atmospheric chemistry.

Key Words - BVOC, drought stress, climate change, Mediterranean basin, forests

1. Introduction

Climate-induced tree dieback represents one of the main current concerns throughout the world because of its severe consequences in terms of loss of biodiversity and ecosystem services (Allen et al., 2010; Anderegg et al., 2015; Shiels et al., 2015). In the Mediterranean basin, several studies have reported a widespread dieback of several woody species caused by anomalous drought and heat waves (Vila-Cabrera et al., 2011; Bussotti and Pollastrini, 2017; Colangelo et al., 2017). However, the impacts of these extreme climatic events are more pronounced in Mediterranean coastal areas, where the coverage of evergreen sclerophyll trees is projected to decrease significantly and to be replaced by shrubs which are better able to resist to harsh environmental conditions (Pollastrini et al., 2019). Some studies have detected plant species compositional changes in the understorey layer after dieback of the dominant tree layer, since the open canopy allows an increase of solar radiation reaching the ground, with several effects, including on soil moisture, evaporation, water distribution for understory plants as well as seed germination and soil microbiological activities (Barbier et al., 2008; Royer et al., 2011; Štursová et al., 2014).

In Mediterranean forests, a widespread dieback of holm oak (*Quercus ilex* L.) has been observed over the last decades and it has primary been attributed to drought and heat stress (Barbeta and Peñuelas, 2016; Pollastrini et al., 2019; Ogaya and Peñuelas, 2020). The typical symptoms of holm oak dieback are gradual defoliation, loss of tree vigour and ultimately, death. This dieback has triggered considerable attention since this evergreen sclerophyll oak dominates the landscape of several forest stands. Therefore, changes in canopy cover may influence light penetration and affect understorey microclimate and soil physical surface properties, with consequences in the composition of understorey vegetation (Terradas, 1999; Orwig et al., 2013; Kendrick et al., 2015; Nagel et al., 2018); Bartemucci et al., 2006; Valladares and Guzmán, 2006; Peñuelas et al., 2017a). *Q. ilex* trees are usually mixed with *Phillyrea latifolia* L., *Arbutus unedo* L., *Pistacia lentiscus* L., *Cistus* spp., *Myrtus communis* L. and *Erica* spp. (Ogaya and Peñuelas, 2004; Ogaya and Penuelas, 2006; Pollastrini et al., 2019). These species, which coexist and grow under the canopy of holm oaks, may take advantage of

microclimatic changes due to the greater penetration of light as well as changes in water and nutrient availability following the *Q. ilex* dieback. Therefore, it is important to understand if changes in understory species composition and richness may be related to different holm oak crown defoliation levels (Lloret et al., 1999; Liu et al., 2015; Peñuelas et al., 2017b).

Biogenic Volatile Organic Compounds (BVOCs), are secondary metabolites, highly volatile in nature, that are synthesised by plants to increase their tolerance to abiotic stresses, such as UV and high irradiance, ozone and heat waves, drought stress and reactive oxygen species (ROS) (Loreto and Schnitzler, 2010; Gil et al., 2013; Bonn et al., 2019; Babaei et al., 2021). To assess possible consequences of climate change on forest vegetation composition, in addition to traditional observational methods on the status of plant individuals, it is important to monitor BVOC emission patterns. Forest stands undergoing intense tree dieback phenomena, especially when changes are dominated by replacement of a dominant tree species with shrubs and herbaceous plants, might be particularly important in changing these patterns. Indeed, in Mediterranean maquis species the capacity to release or store BVOCs is strongly interspecific, with some plants that mainly store BVOCs in specialized compartments prior to emission, such as resin canals and ducts or surface trichomes (*C. monspeliensis* and *J. communis*), while others, such as *Q. ilex*, *P. latifolia* and *P. lentiscus*, directly release BVOCs in response to abiotic stresses (Ciccioli et al., 2014). In particular, *Q. ilex* is one of the highest emitters of monoterpenes in Mediterranean environment and its emissions are particularly sensitive to light , water availability and temperatures (Peñuelas and Llusià, 1999; Staudt et al., 2003; Lavoir et al., 2009; Pasquini et al., 2021). Thus, the monitoring of these terpene profiles over time can give and idea of the possible ecological change of the forest studied.

We investigated how changes in canopy vegetation cover may alter BVOC emission in two Mediterranean forest stands with different *Q. ilex* tree dieback rates. The specific objectives of this work are: 1) to test a possible relationship between changes in vegetation cover and changes in BVOCs composition; 2) to investigate whether the defoliation levels of *Q. ilex* affect woody and herbaceous understory species.

2 Materials and methods

2.1 Site and study areas

This study was carried out in the Maremma Regional Park in southern Tuscany (42°38'10" N, 11°05'39" E, Alberese, Grosseto, Italy), where holm oak forest is showing recent dieback (Figure 1). The Maremma Regional Park covers an area of about 18,000 ha, and comprises the *Monti dell'Uccellina*, modest reliefs reaching 413 m above sea-level (a.s.l.) with *Poggio Lecci*. The study area was a holm oak coppice forest harvested last time in 1975. The annual mean precipitation for the area is 600 mm, with most rain falling in November, and with the driest month being July. Mean warmest temperatures registered in summer and winter are 28.7 °C and 12.8 °C, respectively, while the mean coldest are 14.7 °C and 2.4 °C for summer and winter, respectively (Italian Meteorology Station of Alberese, 42°39'34.3" N and 11°02'29.57" E).

Two forest stands with a multistem structure (30 m × 30 m), one with a low rate (Low Dieback - LD) and one with a high rate (High Dieback - HD) of *Q. ilex* dieback, were selected. The first area (LD, 42°38'05.73'' N, 11°05'43.90'' E, 320 m a.s.l., facing S-SW) was characterised by a 7% of dead standing stems, and maximum tree height of 8.70 m; the second area (HD, 42°38'29.10'' N, 11°05'17.25'' E, 311 m a.s.l., facing W-SW) was characterised by 34% of all standing trees dead (78% *Q. ilex*) and maximum tree height of 12.60 m. Both areas had similar sloping ground (high slope of soil from 35 to 60%)). Three rectangular plots, each of ~ 200 m^2, were delimited inside both study areas (*i.e.*, LD1, LD2 and LD3; HD1, HD2 and HD3).

In both forest stands, the second most abundant species is the shrub *Phillyrea latifolia* L., followed by *Rubus ulmifolius* Schott., *Pistacia lentiscus* L. and *Cistus monspeliensis* L. In addition, other less abundant Mediterranean species found in this area are: *Quercus pubescens* Willd., *Quercus suber* L., *Fraxinus ornus* L., *Acer campestre* L., *Juniperus communis* L. (Table S1). The study area represents the natural habitat for many animal species, including wild boars and other ungulates. Soil texture and pH analyses, conducted in May 2019, revealed that LD and HD stands had similar

soil characteristics, with a loamy soil, rock outcrops and a neutral pH value (Figure S2 and Table S3).

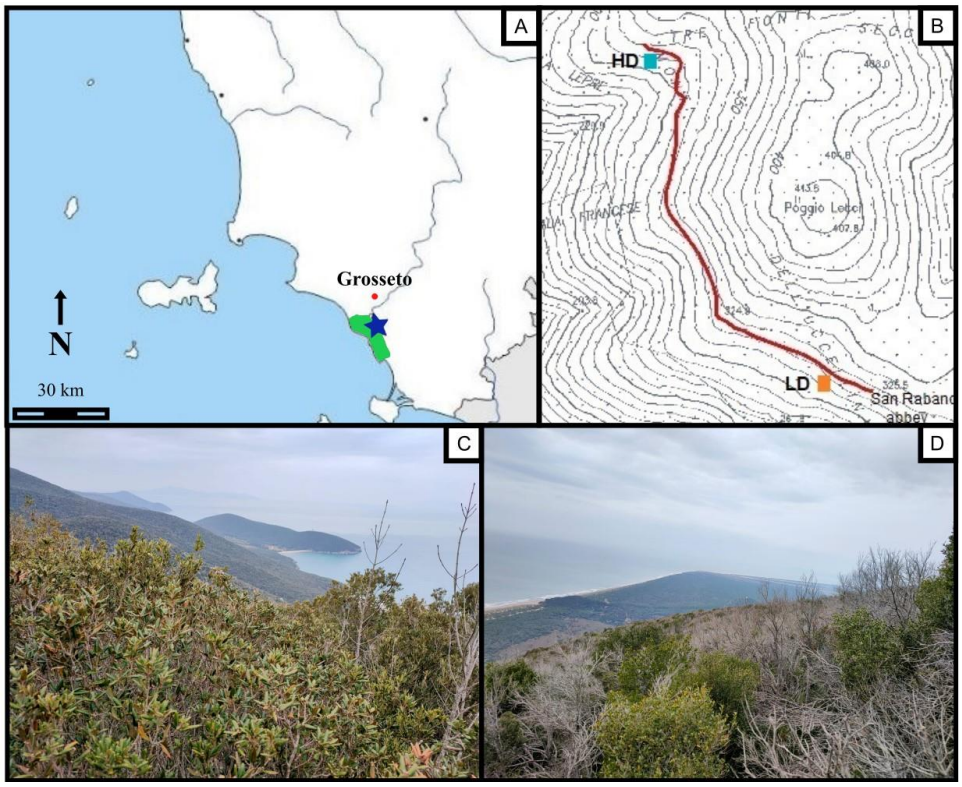

Figure 1. Maps of the study site: the map in panel A shows the location of the study areas (blue star) with respect to the Tuscany region (Italy), and to the city of Grosseto. In panel B, the orange rectangle (30 m × 30 m) represents the study area Low Dieback (LD), characterised by a low tree dieback rate. The light-blue rectangle (30 m × 30 m) represents the study area High Dieback (HD), characterised by a high tree dieback rate. The red line shows the path that links the two areas (ca. 1,200 m). Finally, the panels C and D represent the views of the two study areas LD and HD, respectively.

Visual assessments of *Q. ilex* crown conditions were carried out twice a year for three consecutive years (2019-2021) in the two areas (LD and HD). The level of crown defoliation has been recorded on in four categories: 1, low defoliation level (defoliation <25%); 2 low to medium defoliation level (defoliation 25 - 59%); 3, medium to high defoliation level (60 - 99%); 4 high defoliation level (defoliation 100%), in accordance with the ICP Forests protocol (Eichhhorn et al., 2020). LD and HD had different crown defoliation levels at the beginning of the study, showing a lower defoliation in the LD

area compared to the HD area (Table S4). Furthermore, over time, in both areas, the health status of the holm oak crowns gets worse. A seasonal decrement of the relative frequency of level 1 and 2 of defoliation (0-25% and 25-59%) was found in both areas along the three years; this trend was accompanied by an increment in relative frequency of level 3 and 4 of defoliation (60-99% and 100%) (Table S4). In addition, dead standing holm oaks were also counted: *Q. ilex* trees were considered dead if all stems and/or stumps were completely defoliation and with any signs of resprouting.

2.2 Ecological field work

The field work, with measurements and vegetation assessment, was carried out seasonally for three consecutive years (2019-2021). Measurements for spring 2020 are missing due country-wide COVID-19 restriction in Italy. For each plot, an inventory of plant species was carried out, registration plant species into three groups: (i) trees, including both overstory tree species (tree height over 2 meters) and understorey layer (tree height under 2 meters), (ii) shrubs species, (iii) herbaceous species. In each plot, it was estimated the surface (in cm^2) of soil covered by each species. Tree regeneration (seedling) were not considered in the present study due to their negligible number. All plants taller than 2 meters were tagged with yellow tag and their height and diameter at breast height (DBH) were recorded to calculate Basal Area (BA).

2.3 Terpenes collection and desorption

Biogenic Volatile Organic Compounds (BVOCs) were collected at the same time of the seasonal forestry inventories, choosing days with no rain and not very windy. The BVOCs were collected using Solid-phase microextraction (SPME) fibres with 2 cm of DVB/CAR/PDMS (50/30um layer) coating (Supelco, Sigma-Aldrich C., Darmstadt, Germany). Five SPME fibres in the two study areas (LD and HD) were installed under plants canopy (around at a height of 45 cm from the ground), as described in Pasquini et al (2021). BVOCs sampling was repeated for two consecutive days, with similar meteorological conditions, resulting in a total of 10 fibres per area per season. The terpenes were collected during the warmest hours of the day (at 11:00-15:00 CET).

Afterwards, the fibres were placed in a special air-tight sealed case and transported to the laboratory for analysis. In the laboratory, the fibres were desorbed with a 7890 B gas chromatograph coupled with a 7000 D mass spectrometer triple quadrupole (both from Agilent Technology, Santa Clara, CA, USA) operating in EI ionization mode at 70 eV energy. The other instrumental parameters and analytical conditions were reported in Pasquini et al., 2021. Data were analysed using the Agilent Mass Hunter software (Qualitative Analysis-Version B.06.00; Quantitative Analysis Version B.07.01/Build 7.1.524.0), and the analytes were identified by comparing their mass spectra and retention times with those reported in the NIST 11 spectral database library. The amounts of monoterpenes and monoterpenoids (MTs), expressed as peak areas, expressed as percentage on the total amount of the monoterpenes, identified in the two areas (LD and HD), for each season (summer and autumn), were reported as single compounds, and related to the Total Ion Current (TIC).

The collection was carried out two times a year: in summer, as the season with the highest monoterpenes emission, and in autumn, as the season with the lowest emissions (Peñuelas and Llusià, 1999; Peñuelas et al., 2005; Holopainen et al., 2013; Llusià et al., 2013). Winter was excluded for several reasons, as (i) it is a season characterised by very low terpene emissions (0.5% of the daily carbon fixed (Staudt and Bertin, 1998; Staudt et al., 2001), (ii) to avoid potential damages to the SPME fibres caused by high atmospheric humidity and (iii) because the amount of daylight and the temperatures are too short and low, respectively, for the type of measurements that we have adopted. During the study, air temperatures (mean max. and mean min.) and the precipitation (mm) were recorded daily by weather stations installed in the proximity of the sampling sites (Figure 2). Additionally, air temperature, air humidity and wind speed were punctually recorded *in-situ* using portable devices (Brift Hygrometer Bluetooth, SensorBlue, FC RoHS, for air temperatures and humidity – Infurider YF-876 anemometer, for wind speed and direction) (Table S5).

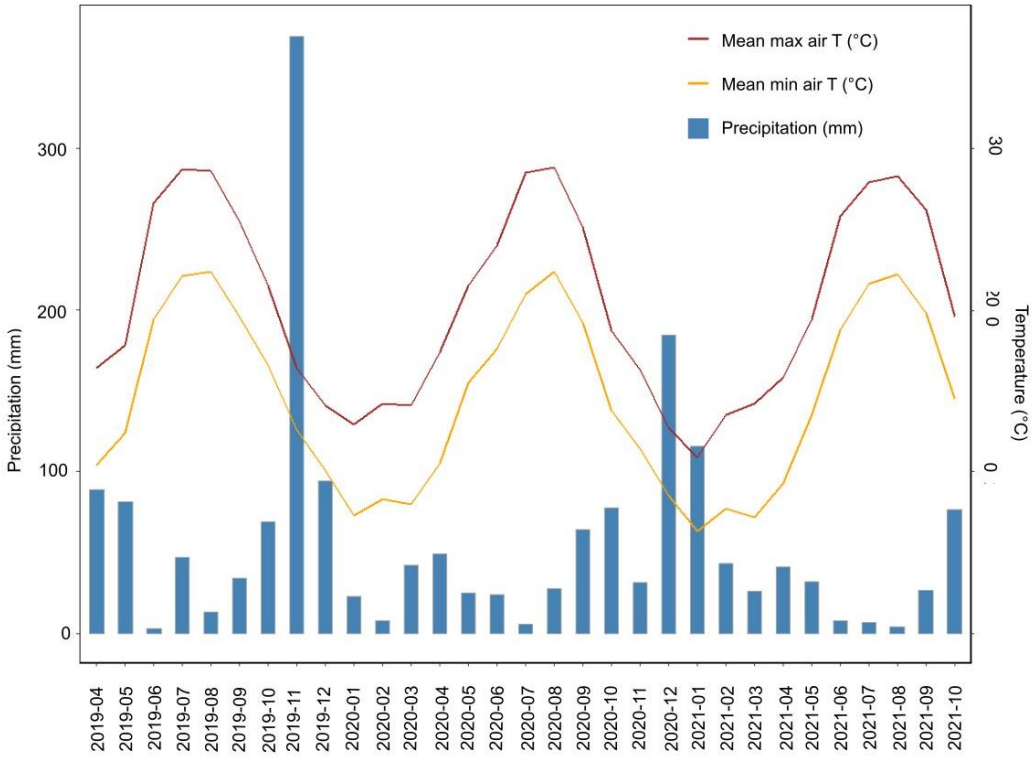

Figure 2. Monthly total precipitation (mm) and monthly average of maximum and minimum air temperature during the study (from April 2019 to October 2021).

2.4 Statistical analysis

Statistical analyses were carried out using R software (version 4.1.0) (R Core Team, 2021).

Due to a reasonably linear relationship between time and vegetation cover during the three years of the study (nine sampling points), a linear mixed effect model, that was structured as follows for the fixed part:

$$Surface\_Covered \sim Zone + DAYS + Zone{:}DAYS$$

where *Surface_Covered* is the percentage (%) of surface covered either by tree canopies or by shrubs (separate models) and it was the response variable, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days from the start of the sampling (16 July 2019).

Furthermore, both models were structured as follows in their random part:

$$\sim DAYS \mid Plot$$

where *DAYS* is as above-mentioned and *Plot* identifies the mean values of the tree plots in both study area (LD1-3; HD1-3). These analyses were carried out with the *nlme* package (Pinheiro et al., 2016).

For monoterpene compounds, the total amount of monoterpenes and monoterpenoids (MTs) was calculated seasonally to observe the trend of total emission during the whole study period The relative amount (percentages) of each MTs recorded seasonally was expressed as a percent value of total MTs (monoterpene profile). Then, Shapiro and Levene's test were carried out to check the assumptions of normality (Shapiro and Wilk, 1965) and homoscedasticity of the data (Gastwirth et al., 2009; Nordstokke and Zumbo, 2010), respectively. Once these assumptions were met, terpene amounts were analysed using a one-way analysis of variance (ANOVA), followed by a pairwise Tukey *post hoc* test. A 0.05 *p*-value threshold was set as cut-off value for significant differences.

To understand whether and how the terpenic profile (vectors) changed with changes in canopy cover of holm oak, with respect to other more abundant woody species in the two area (*Phillyrea latifolia* L., *Pistacia lentiscus* L., *Rubus ulmifolius* Schott.), a principal component analysis (PCA) was performed. The analysis was carried out using the *FactoMineR* package (Husson, 2011).

3. Results

3.1 Vegetation inventory and cover

In Summer 2019, the two stands showed different tree densities: the mean Basal Area (BA) for all alive tree species was 48.97 m^2/ha in area LD and 31.75 m^2/ha in area HD. The mean tree height and mean tree diameter of area LD were 6.5 m and 29 cm, respectively, while for area HD they were 10.5 m and 19 cm, respectively. The more abundant tree species found in the two stands at the beginning of the study (July 2019) were *Quercus ilex* L. in LD area and *Phillyrea latifolia* L. in HD area. Regarding the

shrubs layer, in the LD area the dominant species was *Pistacia lentiscus* L., while in the HD area was *Rubus ulmifolius* Schott (Table S6).

Along the three years, a decline in crown conditions of holm oak was observed with an increment of defoliation over the seasons (Table S4). Holm oak canopy showed a clear decline from July 2019 to October 2021 in both studied stands, as pointed out in the linear model (Figure 3 C-D). Canopy cover declined significantly in both stands ($p < 0.001$), passing from a starting condition, in which LD and HD were significantly different ($p = 0.0146$) (Table S7), to a new one in which the stands were similar ($p = 0.3245$) (Table S8). The decrease in tree canopy cover was accompanied by an increment in the cover of the shrub and herbaceous layer (Figure 3). This layer was dominated by *P. lentiscus* (representing ca. 95% of shrub cover in area LD in July 2019 and ca. 76% in October 2021) and *R. ulmifolius* (representing ca. 91% of shrub cover in HD area in July 2019 and ca. 95% in October 2021). These temporal changes in the shrub layer were reported in both stands, although significant only in HD area (area LD: $p = 0.1447$; area HD: $p < 0.001$). Furthermore, regarding the shrub layer, the two stands were significantly different ($p = 0.0073$) at the beginning of the study period (Table S9), then becoming similar and without statistical differences at end of the experimental campaign ($p = 0.9528$) (Table S10).

The surveys revealed that the cover of herbaceous species did not allow the implementation of a linear mixed model due their high variability (Figure S11), with no statistically significant differences in time and between the two study areas (Table S12 and S13).

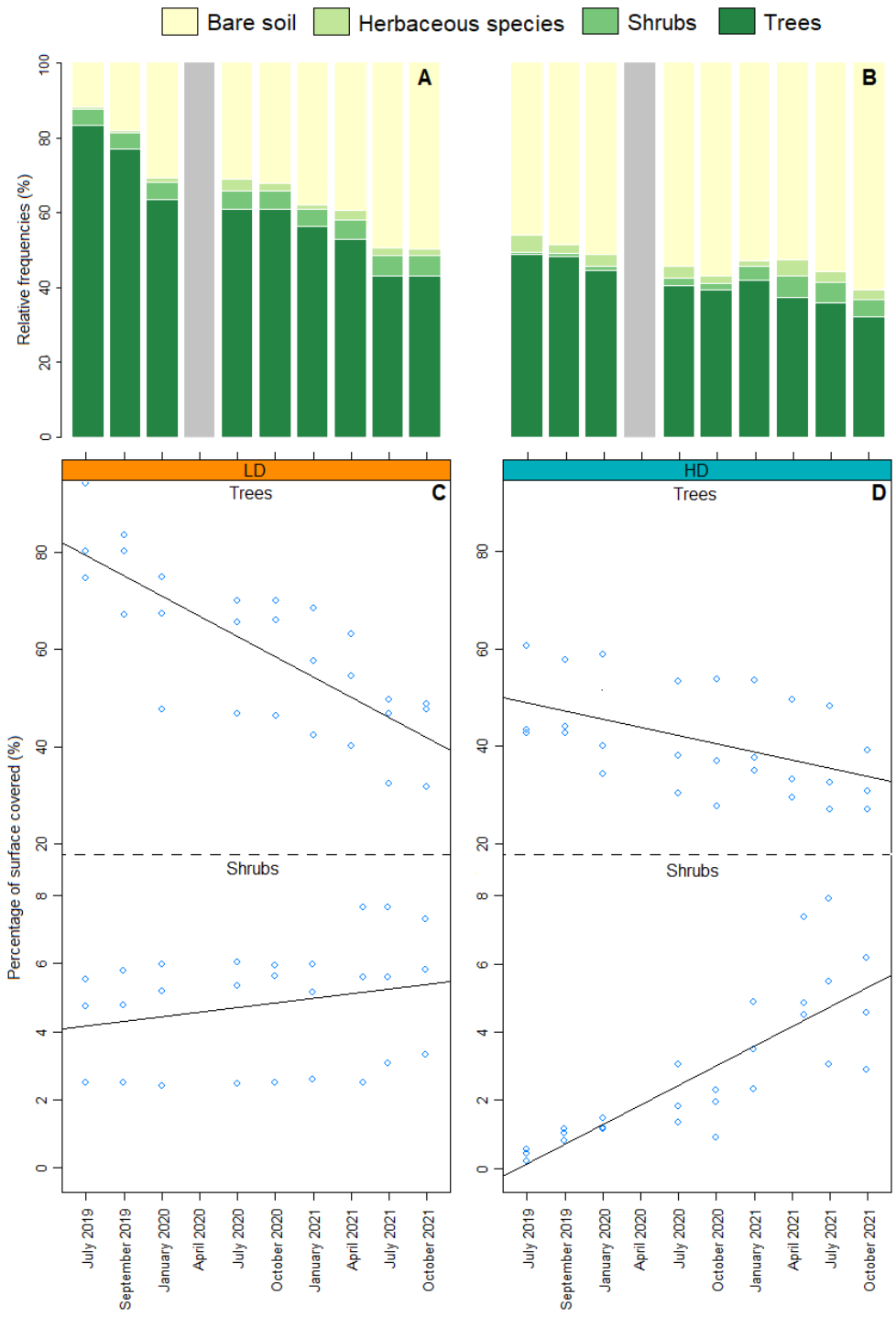

Figure 3. A-B) Relative frequencies of different classes of vegetation and bare soil. Different colours represent the different classes. C-D) Temporal progression of relative surface covered by

tree and shrub species (top row and bottom row, respectively) in the two studied areas LD (panel C) and HD (panel D). On the x axis are reported the sampling seasons. In each panel the blue dots represent the percentage of vegetation cover of the three plots (1, 2, 3). The black line represents the mean regression for each area.

3.2 Semi-Quantitative Analysis of Individual and Total monoterpenes (MTs)

A clear seasonality was found in the terpene emission rates of each year, with emission rates higher in summer than in autumn (Figure 4). Additionally, a decreasing trend in emission rates over the years was also observed (Figure 4). In addition, while MTs emission was significantly different at the beginning of study in the two forest stands, this difference was not observed in July and October 2021(summer 2019: $p < 0.001$; autumn 2019: $p = 0.011$; summer 2020: $p = 0.029$; autumn 2020: $p = 0.012$; summer 2021: $p = 0.999$; autumn 2021: $p = 0.554$).

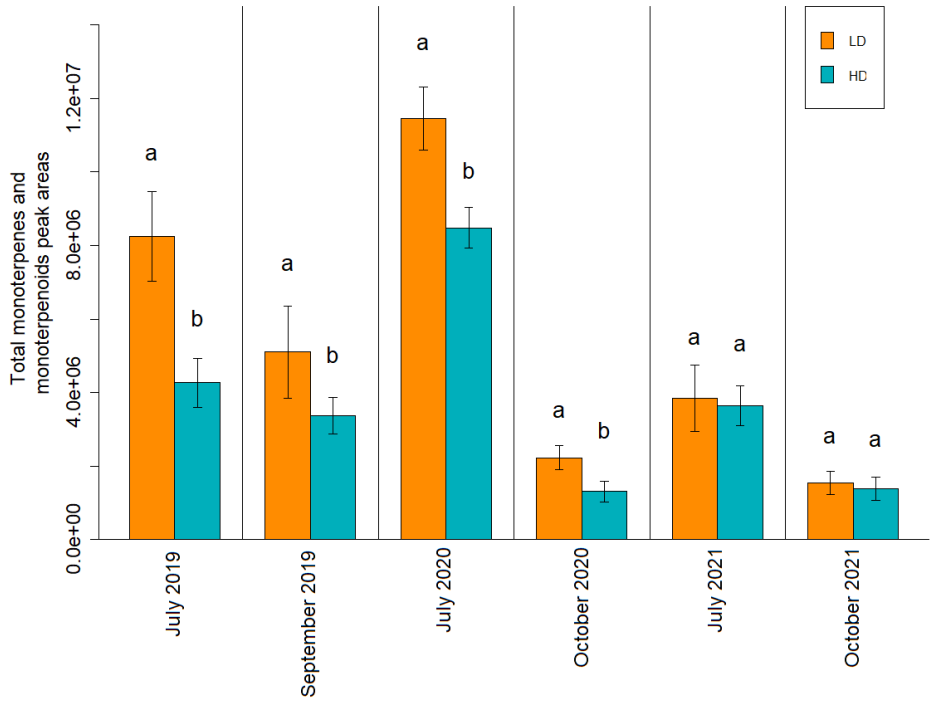

Figure 4. The amount of total MTs (Monoterpenes and Monoterpenoids) collected during the whole duration of the study period (2019-2021) (means ± standard deviations, $n = 10$). Data were

analysed by one-way ANOVA for each month separately (ns - $p > 0.05$, * - $0.01 < p < 0.05$, ** - $0.001 < p < 0.01$, *** - $p < 0.001$).

At all sampling times, the only terpenes identified belonged to the classes of monoterpenes and monoterpenoids (MTs). The identified MTs were: acyclic monoterpene hydrocarbons (myrcene, β-cisOcimene, β-transOcimene), monocyclic monoterpene hydrocarbons (α-phellandrene, α-terpinene, d-limonene, γ-terpinene, *p*-cymene and terpinolene) and bicyclic monoterpene hydrocarbons (α-pinene, α-thujene, α-fenchene, camphene, β-pinene, sabinene, car-3-ene) (Table 4, Figure S14). At all sampling times, α-pinene, α-thujene, β-pinene, sabinene, myrcene and d-limonene represented the most abundant compounds found in the understory air, resulting in more than 80% of the total monoterpenes detected, with the exception of the first season, July 2019, where they represented ca. 65% (Table 4). In addition, a reduction in the number of the detected compounds was observed from July 2019 (16 compounds) to July 2021 (11 compounds). Furthermore, over time, the two stands tended to be similar in the BVOC emission profile with less significant differences detected among the single compounds (Table 4).

Table 4. List of detected MTs (Monoterpenes and Monoterpenoids) for every season sampled. Values of detected MTs are given as mean percent of the total MTs peak area for each season (n = 20). Sd represents standard deviation. p-values presented were obtained using a one-way ANOVA for each season (ns - $p > 0.05$, * - $0.01 < p < 0.05$, ** - $0.001 < p < 0.01$, *** - $p < 0.001$). – represents non detected compounds. A representative chromatogram is shown in Figure S14.

	Summer 2019			Autumn 2019			Summer 2020		
	LD	HD		LD	HD		LD	HD	
Terpenes	(% mean ± sd)	(% mean ± sd)	*P*	(% mean ± sd)	(% mean ± sd)	*p*	(% mean ± sd)	(% mean ± sd)	*p*
α-pinene	16.17 ± 5.95	4.51 ± 1.59	***	19.01 ± 13.00	6.23 ± 5.09	n.s.	14.22 ± 3.50	5.10 ± 1.84	**
α-thujene	15.40 ± 5.61	5.24 ± 2.79	**	9.30 ± 3.91	8.25 ± 2.03	n.s.	5.85 ± 3.85	3.59 ± 1.99	n.s.
α-fenchene	0.59 ± 0.27	0.35 ± 0.22	n.s.	-	-		-	-	
camphene	5.65 ± 2.52	4.25 ± 1.03	n.s.	0.75 ± 0.53	0.43 ± 0.24	n.s.	1.47 ± 0.43	0.70 ± 0.11	**
β-pinene	3.67 ± 1.00	1.60 ± 0.98	***	12.28 ± 6.87	10.04 ± 4.71	n.s.	8.17 ± 4.02	1.74 ± 1.17	**
sabinene	3.37 ± 2.13	2.06 ± 0.93	n.s.	5.92 ± 4.65	2.47 ± 1.98	*	0.72 ± 0.40	0.38 ± 0.27	n.s.
car-3-ene	0.88 ± 0.26	1.31 ± 0.28	n.s.	-	-		1.61 ± 0.61	0.87 ± 0.11	*
myrcene	4.02 ± 1.08	2.06 ± 1.27	*	0.89 ± 0.34	0.48 ± 0.35	*	3.01 ± 1.22	3.24 ± 0.68	n.s.
α-phellandrene	1.16 ± 0.34	0.50 ± 0.14	n.s.	2.28 ± 0.30	1.64 ± 0.26	***	0.38 ± 0.19	0.25 ± 0.06	*
α-terpinene	1.01 ± 0.52	0.28 ± 0.16	*	0.79 ± 0.35	0.50 ± 0.11	n.s.	-	-	
d-limonene	11.57 ± 3.02	7.91 ± 3.30	n.s.	8.14 ± 3.71	4.29 ± 1.25	n.s.	19.19 ± 5.36	22.77 ± 3.76	n.s.
β-cisOcimene	0.94 ± 0.21	0.33 ± 0.21	***	0.37 ± 0.07	0.27 ± 0.09	n.s.	0.47 ± 0.15	0.47 ± 0.10	n.s.
β-transOcimene	1.73 ± 1.07	0.78 ± 0.79	n.s.	-	-		-	-	
γ-terpinene	0.91 ± 0.37	0.39 ± 0.21	**	3.05 ± 1.13	1.39 ± 0.65	**	0.32 ± 0.14	0.50 ± 0.16	n.s.
p-cymene	0.45 ± 0.14	0.29 ± 0.15	n.s.	0.41 ± 0.19	0.22 ± 0.12	n.s.	0.29 ± 0.09	0.37 ± 0.10	n.s.
terpinolene	0.34 ± 0.23	0.20 ± 0.18	n.s.	0.46 ± 0.15	0.13 ± 0.06	***	2.04 ± 0.69	2.29 ± 0.32	n.s.

Table 4. (continued).

	Autumn 2020			Summer 2021			Autumn 2021		
	LD	HD		LD	HD		LD	HD	
Terpenes	(% mean ± sd)	(% mean ± sd)	*P*	(% mean ± sd)	(% mean ± sd)	*P*	(% mean ± sd)	(% mean ± sd)	*p*
α-pinene	21.61 ± 10.52	7.04 ± 2.28	**	11.27 ± 10.60	11.52 ± 9.04	n.s.	16.38 ± 9.11	14.67 ± 9.26	n.s.
α-thujene	8.92 ± 2.65	2.98 ± 2.21	**	8.88 ± 5.66	10.80 ± 3.54	n.s.	11.75 ± 4.44	11.12 ± 5.71	n.s.
α-fenchene	-	-		-	-		-	-	
camphene	1.22 ± 0.44	0.58 ± 0.11	*	0.71 ± 0.54	0.77 ± 0.45	n.s.	0.91 ± 0.51	0.98 ± 0.58	n.s.
β-pinene	10.81 ± 3.90	4.08 ± 1.75	**	9.07 ± 7.01	9.38 ± 5.64	n.s.	11.76 ± 5.39	9.59 ± 5.04	n.s.
sabinene	2.20 ± 1.20	1.32 ± 0.75	n.s.	1.69 ± 1.01	1.41 ± 0.39	n.s.	2.31 ± 1.23	1.28 ± 0.46	n.s.
car-3-ene	0.71 ± 0.12	0.69 ± 0.13	n.s	-	-		-	-	
myrcene	2.20 ± 0.79	2.87 ± 0.70	n.s.	4.31 ± 1.72	4.70 ± 1.44	n.s.	-	-	
α-phellandrene	-	-		0.43 ± 0.18	0.36 ± 0.12	n.s.	-	-	
α-terpinene	0.64 ± 0.34	0.45 ± 0.14	n.s.	0.41 ± 0.15	0.47 ± 0.10	n.s.	0.52 ± 0.21	0.52 ± 0.23	n.s.
d-limonene	15.89 ± 3.31	13.89 ± 1.18	n.s.	10.44 ± 4.79	9.94 ± 4.17	n.s.	7.41 ± 2.37	7.87 ± 2.08	n.s.
β-cisOcimene	-	-		-	-		-	-	
β-transOcimene	-	-		-	-		-	-	
γ-terpinene	0.97 ± 0.41	0.37 ± 0.09	*	0.86 ± 0.42	1.00 ± 0.21	n.s.	0.84 ± 0.52	0.78 ± 0.28	n.s.
p-cymene	0.63 ± 0.21	0.40 ± 0.13	n.s.	0.68 ± 0.25	0.59 ± 0.21	n.s.	0.48 ± 0.17	0.56 ± 0.19	n.s.
terpinolene	-	-		-	-		-	-	

3.3 *Terpene profile changes with vegetational cover and sampling times*

The first two dimensions represented, cumulatively, over 70% of the variance, with the first principal component of the PCA explaining 48.5% and the second principal component explaining the 24.4% of the total variance (Figure S15). The last three sampling seasons (O_2020, J_2021 and O_2021) have a significantly lower content of detected monoterpenes (both LD and HD stands) and were associated with a lower *Q. ilex* cover and with a larger shrub cover (Figure 5 – panel A and B). Hence, the first principal component dimension clearly separates the sampling seasons with a higher percentage of *Q. ilex* canopy cover from the sampling times with a lower percentage of *Q. ilex* canopy cover, showing that this species is the greater contributor to this terpenic profile.

Between the two summers J_2019 and J_2020 (the two sampling seasons with the highest content of monoterpenes recorded), it is possible to observe a variation of the compositional blend (see Dim 2, Figure 5 panel A). This second dimension could be related to *Q. ilex* health, with higher emissions of camphene, α-phellandrene and α-thujene, when *Q. ilex* is under less prolonged water stress. On the other hand, higher emissions of d-limonene seem to be linked with increased water stress over time, likely linked to the reduction in rainfall (Figure 2). Another interesting observation from Figure 5, panels A and B is that, as also previously shown (see Figure 4 for the total MTs content, and Table S7, S8, S9, S10), the two areas are initially significantly different and distant from each other, while over time they tend to get closer and become more similar. Finally, in accordance with the ANOVA results for the total MTs content (Figure 4), all the samplings carried out in summer (J_2019, J_2020 and J_2021) are always placed to the right of their corresponding autumn sampling for the same year. This shows a more abundant amount of emissions and detection of terpenes during the warm seasons compared to the colder.

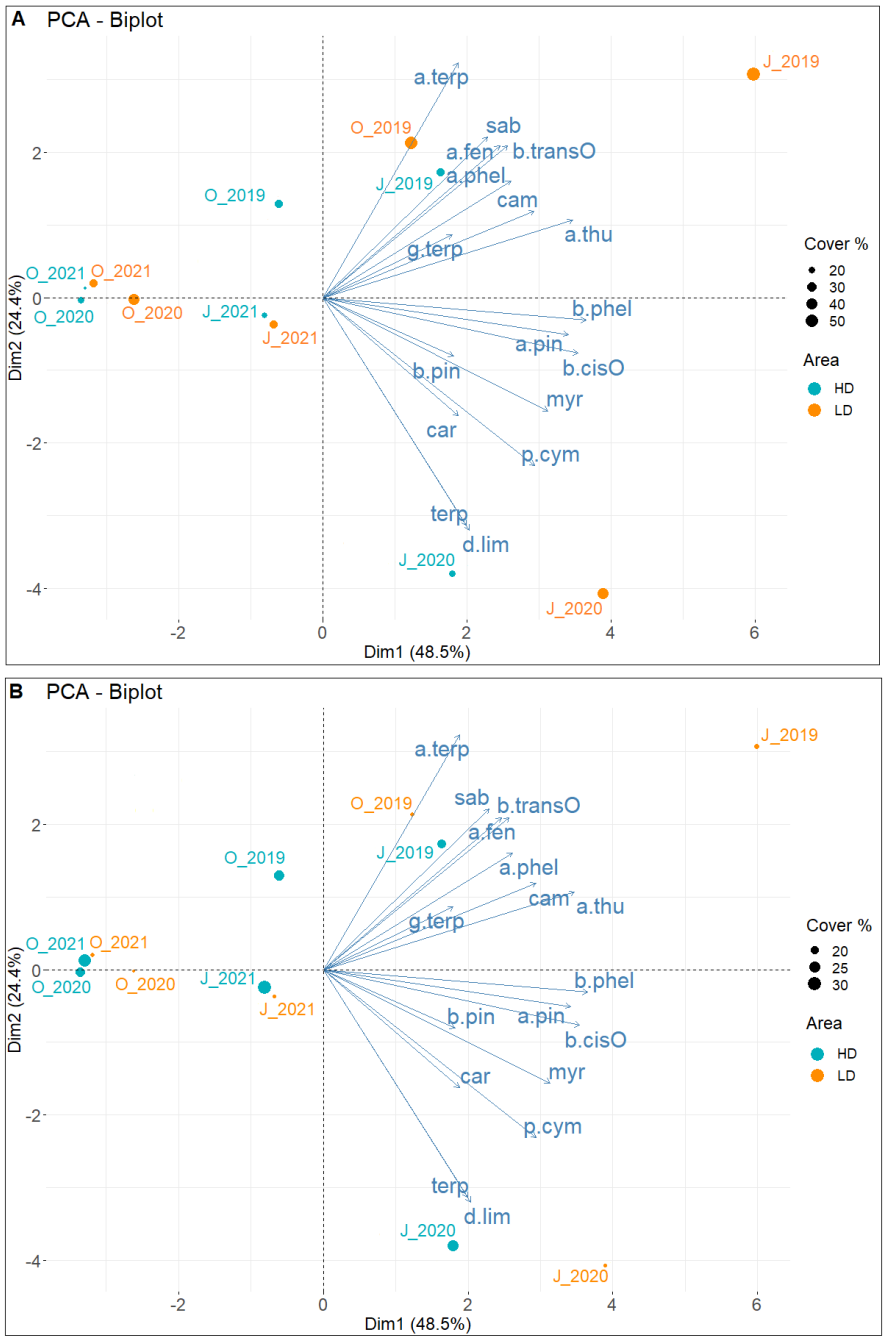

Figure 5. Principal component analysis biplots of monoterpene and monoterpenoid compounds. In all plots, loadings for each BVOCs are presented with arrows (a.pin: α-pinene, a.thu: α-

thujene, a.fen: α-fenchene, cam: camphene, b.pin: β-pinene, sab: sabinene, car: car-3-ene, myr: myrcene, a.phel: α-phellandrene, a.terp: α-terpinene, d.lim: d-limonene, b.cisO: β-cisOcimene, b.transO: β-transOcimene, g.terp: γ-terpinene, p.cym: *p*-cymene, terp: terpinolene). The variance explained by each principal component (Dim1 and Dim2) is shown in parentheses. (A) The points with different sizes represented the values of percentage canopy cover of *Quercus ilex* L. in the two stands, LD (orange) and HD (light-blue) stands, at the six sampling times: July 2019 (J_2019), October 2019 (O_2019), July 2020 (J_2020), October 2020 (O_2020), July 2021 (J_2021), October 2021 (O_2021). (B) The points with different sizes represented the values of percentage canopy cover of the shrubs (*Phillyrea latifolia* L., *Pistacia lentiscus* L., *Rubus ulmifolius* Schott.) in the two stands, LD (orange) and HD (light-blue) stands, at the six sampling times: July 2019 (J_2019), October 2019 (O_2019), July 2020 (J_2020), October 2020 (O_2020), July 2021 (J_2021), October 2021 (O_2021).

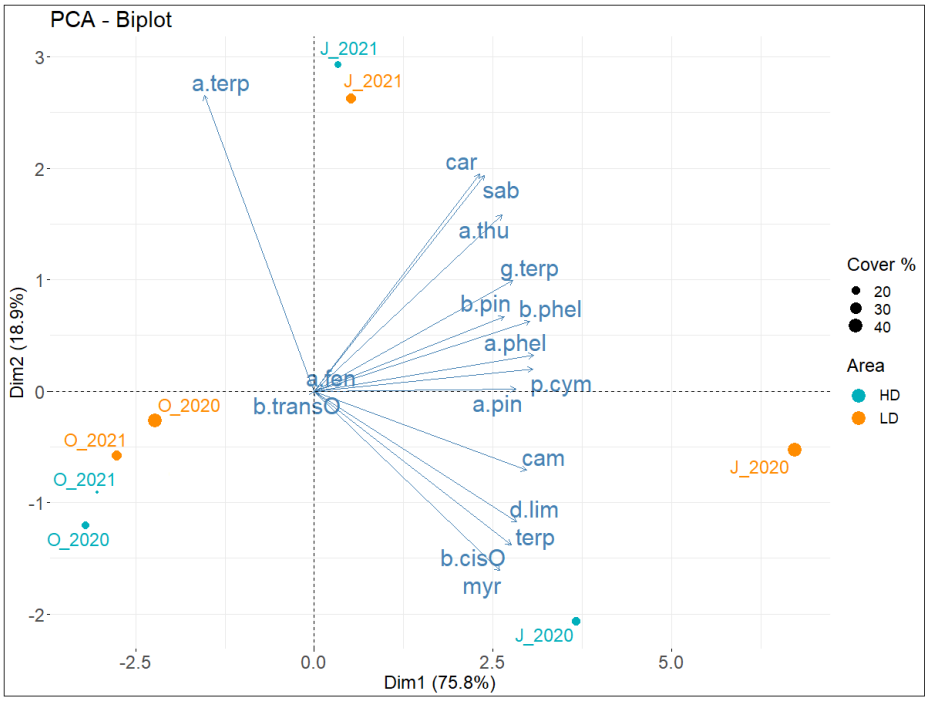

Figure 6. Principal component analysis biplots on monoterpene and monoterpenoid compounds. In all plots, loadings for each BVOCs are presented with arrows (a.pin: a-pinene, a.thu: a-thujene, a.fen: a-fenchene, cam: camphene, b.pin: b-pinene, sab: sabinene, car: car-3-ene, myr: myrcene, a.phel: a-phellandrene, a.terp: a-terpinene, d.lim: d-limonene, b.cisO: b-cisOcimene, b.transO: b-transOcimene, g.terp: g-terpinene, p.cym: *p*-cymene, terp: terpinolene). The variance explained by each principal component (Dim) is shown in parentheses. The individuals represented the values of percentage canopy cover (pointsize) of *Q. ilex* in the two areas, LD and HD (colours), during only four sampling times: July 2020 (J_2020), October 2020 (O_2020), July 2021 (J_2021), October 2021 (O_2021).

In Figure 6, the sampling seasons of 2019 (J_2019 and O_2019) were removed, in order to observe in details the sampling times of 2020 and 2021. Indeed, the very high MTs content found in the 2019 sampling seasons obscured the much smaller contents found during the subsequent sampling seasons (Figure 5 - Panel A and B). Thus, to provide a better interpretation of the results from 2020 and 2021, the values from 2019 were therefore omitted (Figure 6). In this case, we observe that a-terpinene is the only BVOC in the top left quadrant, separated from all other MTs and therefore it could be attributable to other species.

4. Discussion

The defoliation could be explained by some aspects of forest structure, such as tree density and tree height, as well as topography (Barbeta and Peñuelas, 2016). Indeed, trees grown in dense forests present a greater risk of defoliation and die-back because of great inter-tree competition for critical resources (Van Gunst et al., 2016), as well as trees with smaller basal areas, likely linked to their more superficial rooting system and to their smaller resource storage (Lloret et al., 2004; Galiano et al., 2012; Barbeta et al., 2013). Both of these conditions are present in our study areas (Ciancio et al., 2002). Moreover, the risk of tree dieback is linked to the soil characteristics and topography: if the soil is rocky, it does not allow for a deep rooting system, decreasing plants capacity to store water. Additionally, the presence of high slopes will result in more water run-off and less accumulation of water in the ground (Lloret et al., 2004; Liu et al., 2015), as in our case.

A significant decrement ($p < 0.001$) of tree canopy cover in both areas was observed (Figure 3). However, the slope of the linear mixed effects models for area LD is more pronounced than that of area HD, suggesting a more pronounced reduction of tree canopy cover in the area characterised by lower holm oak dieback. This result could be partially explained by the high rate of *Quercus ilex* L. mortality found at the beginning of the study (51% *Q. ilex* dead vs. 49% still alive). Because of this mortality, the dominant tree species in the area HD is *Phillyrea latifolia* L.. This species was visually

showing a good health status, with full canopy, where the *Q. ilex* presence was reducing, explaining the lower slope of the linear mixed effects models of tree cover in area HD (Saura-Mas et al., 2015; Ogaya and Peñuelas, 2021). Thus, the slope of the linear mixed effects models was more pronounced in area LD, since this area is currently undergoing the tree decline that had already affected area HD. Area LD, having more trees still alive, has a higher tree density (area LD: BA = 48.97 m^2/ha), and therefore the trees in LD were subjected to greater competition, compared to those in HD, where tree density was lower, (area HD: BA = 31.75 m^2/ha). Indeed, trees in dense areas are likely more susceptible to defoliation and dieback due to higher nutrient and water competition (Galiano et al., 2012; Van Gunst et al., 2016).

The significant decrease of tree canopy cover, in both areas, was likely caused by a change in microclimate conditions (*e.g.*, nutrients, light penetration, water availability) contributing to the understorey species changes (Saura-Mas et al., 2015). Indeed, an increment of understorey shrubs abundant was observed in both areas (Figure 3), even if the slope of the models is statistically significant only for the area HD ($p < 0.001$) and not for area LD ($p < 0.1447$). Indeed, in area LD several large shrubs were present at the beginning of the study (summer 2019), and, even if their number and size increased, this did not result in a significant variation of the linear model slope. The significant increment of shrubs cover in area HD could be related to the already high decline of canopy cover and the increased sunlight penetration. This has likely provided an advantage to *Rubus ulmifolius* Schott., which was only sporadically present at the start of the study. Indeed, the fruits of this species, being very palatable for mammals and birds, can take advantage of a large seed dispersal by animals (La Mantia et al., 2019).

The severe decline of *Q. Ilex* during the study period had a clear influence on the regeneration of the understorey herbaceous layer (Saura-Mas et al., 2015). In 2019 the herbaceous layer, although usually low, according to the typically herbaceous understorey of Mediterranean holm oak forest (Bacchetta et al., 2004; Biondi et al., 2004), was more abundant in area HD (under 1 m^2/ha) than in area LD (more than 4 m^2/ha) because of the higher canopy openness of area HD. Then, after the three years of

our study, in area LD several herbaceous seedlings germinated and grew-up, reaching coverage values of ca. 2 m^2/ha, while in area HD the herbaceous species layer had decreased, reaching coverage values of ca. 2.5 m^2/ha. This is likely due to the large expansion of the shrub layer (particularly *R. ulmifolius*) (Anderegg et al., 2012). The herbaceous species layer is highly sensitive to seasonal variations, and the period of our study was not sufficient to allow the application of statistical analysis (Figure S11). However, it is worth noting that the multiplicity of herbaceous plants found in both areas is probably due to the past human activity in this natural park.

Thus, over the study our results showed, in both areas (HD and LD), a continuing decrease in tree cover (especially to the detriment of *Q. ilex*) to the advantage of the understorey species, mainly shrubs (this increase was significant only in the HD area). In terms of tree and understorey cover, the two areas, as can be seen from the linear mixed models analysis, were significantly different at the start of the study, while this difference was no longer present at the end of the study. This similarity is confirmed at the biochemical level with the analysis of BVOCs. In fact, at the level of single identified compounds (Table 4), it is possible to observe that at the start of the study the two areas were significantly distinct in seven compounds (which ones), but this significance is not found at the end of the study. Furthermore, observing all monoterpenes identified (Figure 4), it is possible to observe how, during the first seasons, the total number of monoterpenes identified were significantly different between the two areas, while in the last two seasons this is no longer the case. The higher monoterpene emissions recorded in summer coincide with the higher mean temperatures, indeed it is widely reported that abiotic factors have a direct effect on monoterpene emission (Llusià and Peñuelas, 1999; Loreto and Schnitzler, 2010). A gradual reduction in the total amount of monoterpenes emitted by the forest trees, from 2019 to 2021, has been observed in both areas. This could be explained by the intensification of drought events and by the reduction of available soil water, due to reduced precipitations over the three years studied (Figure 2). Indeed, drought events have a great impact on monoterpene emissions: when drought events are short and of low intensity, they cause an increase in the emissions of

monoterpenes, while when they are severe and prolonged, the emissions could be reduced by ten times (Loreto et al., 2001). Other factors, such as leaf age (Staudt et al., 2003), attacks of insects (Staudt and Lhoutellier, 2007) and pathogen have a lower influence on monoterpene emissions. Differently, factors influencing this decreasing emission in monoterpenes could be the high defoliation of canopies and dieback of the dominant species, *Q. ilex*. Indeed, the largest portion of monoterpenes emissions are likely deriving from *Q. ilex* leaves, both because it is the predominant species in the forest and because it is considered one of the major emitters in the Mediterranean basin, even greater than *P. latifolia* (Llusià and Peñuelas, 1998; Ait Said et al., 2011).

Observing the single compounds detected at each sampling time, using our sampling technique based on SPME fibres, the typical terpenic profile of the Mediterranean maquis dominated by *Q. ilex* was obtained (Table 4). In all samplings α-pinene, α-thujene, β-pinene, sabinene, myrcene and d-limonene were the main compounds detected, representing approximately 65-80% of the total compounds emitted (Bertin and Staudt, 1996; Peñuelas and Llusià, 1999; Owen et al., 2001; Sabillo, 2001; Staudt et al., 2001). The variability of these compounds is quite large, partially depending on the species present in our study areas (mixed Mediterranean sclerophyll forest), as well as on the technique used to acquire BVOCs at plot level. For example, *P. latifolia* terpene emissions are richer in d-limonene than those of *Q. ilex* (Llusià and Peñuelas, 1998). This could explain, to some extent, the smaller concentration difference found between pinenes and d-limonene in area HD when compared to area LD (in area HD the 50-60% of live trees are represented by *P. latifolia*). Furthermore, even intraspecific variation in the composition of emitted compounds can be rather large. For *Q. ilex,* Staudt et al. (2001) defined three main chemotypes: the most widely distributed chemotype mainly emitted α-pinene, the second chemotype emitted high quantities of d-limonene, while the least abundant chemotype emitted myrcene. This is likely linked to the high variability of the enzymes (including isoforms) involved in the biosynthesis of terpenes (Fischbach et al., 2002). In addition to the mainly emitted monoterpenes, there were found camphene, car-3-ene, α-phellandrene, α-terpinene, β-cisOcimene, β-

transOcimene, γ-terpinene, p-cymene and terpinolene at lower emission rates, in agreement with (Peñuelas and Llusià, 1999; Staudt et al., 2001; Peñuelas et al., 2005; Blanch et al., 2009; Lavoir et al., 2009), while we detected α-fenchene only in the first year and with a low amount attributable only to *Q. ilex* (Owen et al., 2001). Finally, we did not detect 1,8cineole in any sampling point as reported by Sabillo (2001), even though this is not in agreement with Owen et al. (1997). Isoprene was also not detected, since *Q. ilex*, as other Mediterranean evergreen schlerophylls, is known to be a low emitter of this specific terpene (Peñuelas and Llusià, 1999; Owen et al., 2001). The compounds found in trace are those emitted more rarely during severe drought conditions, and whose presence is almost negligible in the terpene profile (Staudt et al., 2001).

What has just been said, is also found and obtained with the PCA analysis (Figure 5). Indeed, the sampling seasons characterised by a high cover of *Q. ilex* were located in the first and fourth quadrants where the total detected MTs are higher (Dim 1). While a totally opposite trend is seen when selecting the other species (*P. latifolia*, *P. lentiscus*, *R. ulmifolius*). Furthermore, a greater content of terpenes is visible during summers and a greater and significant diversity between the two areas in the first seasons (J_2019, O_2019 and J_2020), a difference that wades off in the last sampling (O_2020, J_2021, O_2021).

In conclusion, all results, showed a markedly significant difference between the two areas at the start of our study. The areas, even if similar in soil and topographic conditions (Figure S2 and Table S3), were markedly different in the vegetation coverage levels (Figure 3). Significant differences were also found regarding the total monoterpenes detected (Figure 4). Our results showed that this divergence decreased with the succession of the seasons, to the point that the two study areas were not significantly different by the end of our study, showing an overall high mortality trend of the *Q. ilex* forest.

Our study successfully estimated the variations of forest terpenic profiles over several seasons. Our results, in agreement with the scientific literature, showed that Q.

ilex contributes significantly to BVOCs emissions to the atmosphere, particularly in terms of monoterpenes. In addition, through these terpene measurements, it was possible to observe both a variation in the content of monoterpenes (by season, by area, and by year) and a modification linked to the health status of the plants. On the other hand, no great variations in the quality of the terpene profile were found since *Q. ilex* is one of the major emitters. Finally, the BVOCs in the atmosphere could react releasing CO2 or CH4 or O3 (Peñuelas and Staudt, 2010). The high ongoing dieback events of Q. ilex individuals found thus far, may lead to significant changes in the terpenic profiles of Mediterranean holm oak forests. Under future climatic scenarios, changes of BVOCs emissions may strongly impact a plethora of trophic interactions across their native ecosystem as well as affecting atmospheric chemistry.

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Author Contributions

Dalila Pasquini, Cecilia Brunetti and Antonella Gori contributed to the study conception and design. Material preparation and data collection were performed by Dalila Pasquini and Martina Pollastrini. Analysis and the first draft of the manuscript was written by Dalila Pasquini and all authors contributed to the final version of the manuscript. All authors have read and approved the final manuscript.

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Supplementary material

Appendix B

Supplementary Material

Supplementary Figures and Tables

Table S1. List of the species identified in both areas (LD and HD) in all the seasons measured during the three years of the study (2019-2021). The species name, family and the vegetation habitus are reported.

Species	Family	Vegetation habittus	2019						2020						2021							
			Summer		Autumn		Winter		Summer		Autumn		Winter		Spring		Summer		Autumn	
			LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Acer campestre L.	*Sapindaceae*	tree		√		√		√		√		√		√		√		√		√
Alliaria petiolata M. Bieb.	*Brassicaceae*	herbaceous											√	√	√		√		√	
Arum italicum Mill.	*Araceae*	herbaceous											√		√	√				
Asparagus acutifolius L.	*Asparagaceae*	herbaceous															√		√	
Atropa belladonna L.	*Solanaceae*	herbaceous	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Brachypodium rupestre Host.	*Poaceae*	herbaceous			√						√		√			√				
Cistus monspeliensis L.	*Cistaceae*	shrub	√	√	√	√	√	√		√	√	√	√		√	√	√	√	√	√
Clinopodium nepeta L.	*Lamiaceae*	herbaceous																	√	√
Cytinus hypocistis L.	*Cytinaceae*	herbaceous														√				
Datura stramonium L.	*Solanaceae*	herbaceous			√	√			√	√	√		√		√		√		√	√
Dioscorea communis L.	*Dioscoreaceae*	herbaceous													√					
Dittrichia viscosa L.	*Asteraceae*	herbaceous	√	√	√	√			√	√	√	√	√	√	√	√	√	√	√	√
Erigeron canadensis L.	*Asteraceae*	herbaceous															√	√	√	√
Euphorbia tinctoria L.	*Euphorbiaceae*	herbaceous													√	√				
Ferula communis L.	*Apiaceae*	herbaceous					√				√				√					
Fraxinus ornus L.	*Oleaceae*	tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Hedera helix L.	*Araliaceae*	shrub	√		√		√		√		√		√	√	√		√		√	
Hyoscyamus albus L.	*Solanaceae*	herbaceous							√		√		√	√	√		√			
Hypericum perforatum L.	*Hypericaceae*	herbaceous			√	√					√	√	√	√	√	√	√	√	√	√
Juniperus communis L.	*Cupressaceae*	shrub	√		√		√		√		√		√	√	√		√	√	√	√

Supplementary Material

Table S1. (continued).

Species	Family	Vegetation types	2019						2020						2021					
			Summer		Autumn		Winter		Summer		Autumn		Winter		Spring		Summer		Autumn	
			LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD
Lamium bifidum Cirillo	*Lamiaceae*	herbaceous											√							
Lathyrus tuberosus L.	*Fabaceae*	herbaceous											√		√	√				
Lotus ornithopodioides L.	*Fabaceae*	herbaceous													√	√				
Melissa officinalis L.	*Lamiaceae*	herbaceous														√		√		
Parietaria judaica L.	*Urticaceae*	herbaceous							√				√	√	√	√	√		√	
Phillyrea latifolia L.	*Oleaceae*	tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Piptatherum miliaceum L.	*Poaceae*	herbaceous							√		√		√	√		√	√	√	√	√
Pistacia lentiscus L.	*Anacardiaceae*	shrub	√		√		√		√	√	√	√	√	√	√	√	√	√	√	√
Pyrola rotundifolia L.	*Ericaceae*	herbaceous									√		√		√	√				
Quercus ilex L.	*Fagaceae*	tree	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Quercus pubescens Willd.	*Fagaceae*	tree	√		√		√		√		√		√		√		√		√	
Rubus ulmifolius Schott.	*Rosaceae*	shrub	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Ruscus aculeatus L.	*Asparagaceae*	shrub																	√	
Senecio sylvaticus L.	*Asteraceae*	herbaceous												√	√	√				
Sherardia arvensis L.	*Rubiaceae*	herbaceous													√					
Smilax aspera L.	*Smilacaceae*	herbaceous															√		√	
Sonchus oleraceus L.	*Asteraceae*	herbaceous									√								√	
Trachynia distachya L.	*Poaceae*	herbaceous							√				√		√		√		√	
Trifolium repens L.	*Fabaceae*	herbaceous											√			√	√		√	
Urtica dioica L.	*Urticaceae*	herbaceous					√						√		√	√				
Verbascum thapsus L.	*Scrophulariaceae*	herbaceous			√	√			√	√	√	√			√	√		√	√	

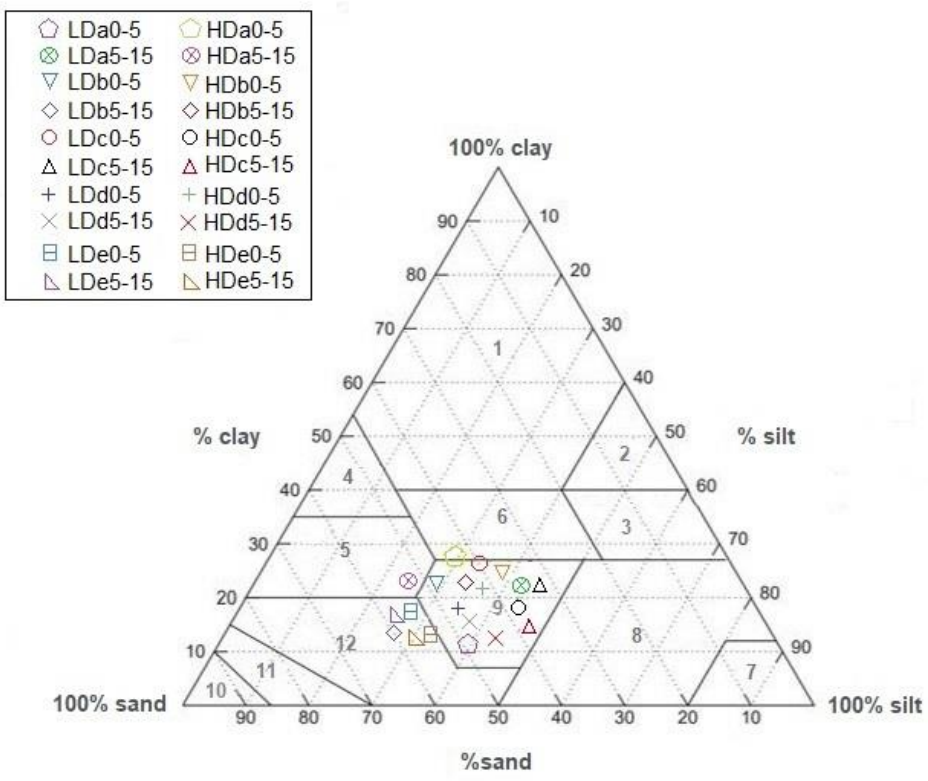

Figure S2. USDA Soil Textural Triangle with the 12 principal textural classes: 1) Clay, 2) Silt clay, 3) Silt clay loam, 4) Sandy clay, 5) Sandy clay loam, 6) Clay loam, 7) Silt, 8) Silt loam, 9) Loam, 10) Sand, 11) Loamy sand, and 12) Sandy loam. The colored symbols represent the five samples (low letters a-e) of soil collected on May 2019 in the two studied areas (capital letters LD and HD) at two different depths: from 0 to 5 cm below-ground (0-5), and from 5 to 15 cm below-ground (5-15).

Table S3. pH values of 5 soil samples (low letters a-e) collected in the two studied areas (capital letters LD and HD) on two different depths: from 0 to 5 cm below-ground (0-5), from 5 to 15 cm below-ground (5-15). The pH value was calculated in H_2O and 1 mol L^{-1} KCl.

Sample	0-5 cm	5-15cm	Sample	0-5 cm	5-15cm
LDa	6.87	6.97	**HDa**	6.54	6.69
LDb	6.75	6.93	**HDb**	6.94	7.09
LDc	7.02	7.10	**HDc**	6.77	7.04
LDd	7.01	7.12	**HDd**	6.80	7.12
LDe	6.74	6.94	**HDe**	7.04	7.17
mean	6.88	7.01	*mean*	6.82	7.02
sd	0.13	0.09	*sd*	0.19	0.19

Table S4. Relative frequencies of crown defoliation of *Q. ilex* (mean ± standard deviation, in %) in LD (Low Dieback) and HD (High Dieback) stands in the three years (2019, 2020, 2021) of the study. The defoliation level is reported as category correspond to: 1 - low defoliation level (<25%); 2 – low to medium defoliation level (25 - 59%); 3 – medium to high defoliation level (60 - 99%); 4 – high defoliation level (100%).

Defoliation category	**2019**		**2020**		**2021**	
	Area LD	**Area HD**	**Area LD**	**Area HD**	**Area LD**	**Area HD**
1	12.82 ± 0.01	7.96 ± 1.15	12.96 ± 1.20	9.09 ± 0.23	8.38 ± 0.82	4.12 ± 1.02
2	25.64 ± 0.01	14.17 ± 2.67	27.88 ± 4.12	19.14 ± 4.35	22.79 ± 1.82	17.44 ± 4.18
3	46.15 ± 0.01	29.19 ± 0.88	42.02 ± 4.43	20.86 ± 3.32	48.24 ± 9.03	32.47 ± 8.20
4	15.38 ± 0.01	48.66 ± 0.64	16.76 ± 0.91	50.89 ± 1.25	20.58 ± 0.53	45.97 ± 4.72

Table S5. Meteorological parameters (air temperature, (°C), humidity (%) and wind speed (km/h) and direction) values collected during the study.

Season	**Sampling Data** (dd/mm/yyyy)	**Air temperature** (°C)	**Air humidity** (%)	**Wind speed** (km/h)	**Wind direction**
Summer	16/07/2019	31	50	12	NE
2019	17/07/2019	30	55	10	NW
Autumn	30/09/2019	24	70	10	SW
2019	01/10/2019	21	65	8	S
Summer	14/07/2020	30	37	14	NE
2020	15/07/2020	29	35	13	W
Autumn	06/10/2020	20	80	8	SW
2020	07/10/2020	22	83	10	W
Summer	12/07/2021	30	53	12	SE
2021	14/07/2021	29	50	12	W
Autumn	25/10/2021	21	75	10	NE
2021	26/10/2021	19	77	6	NW

Table S6. List of species with number of alive and dead individuals identified in both studied areas (LD and HD) at the beginning (September 2019) and the end (October 2021) of the study period.

Specie	Sept 2019 alive inviduals		Sept 2019 dead individuals		Oct 2021 alive individuals		Oct 2021 dead individuals	
	LD	HD	LD	HD	LD	HD	LD	HD
Acer campestre L.	0	1	0	1	0	1	0	1
Asparagus acutifolius L.	0	0	0	0	1	0	0	0
Atropa belladonna L.	19	131	0	0	25	53	0	0
Cistus monspeliensis L.	11	6	0	0	43	44	0	0
Clinopodium nepeta L.	0	0	0	0	3	0	0	0
Datura stramonium L.	0	0	0	0	13	31	0	0
Dittrichia viscosa L.	80	35	0	0	94	45	0	0
Erigeron canadensis L.	0	0	0	0	3	43	0	0
Fraxinus ornus L.	2	4	0	1	2	3	0	2
Hedera helix L.	2	0	0	0	5	0	0	0
Juniperus communis L.	2	0	0	0	5	1	0	0
Parietaria judaica L.	0	0	0	0	17	0	0	0
Phillyrea latifolia L.	9	40	0	6	9	34	0	12
Piptatherum miliaceum L.	0	0	0	0	44	10	0	0
Pistacia lentiscus L.	10	0	0	0	12	1	0	0
Quercus ilex L.	42	27	4	28	24	17	22	38
Quercus pubescens Willd.	6	0	2	0	6	0	2	0
Rubus ulmifolius Schott.	8	34	0	0	23	279	0	0
Ruscus aculeatus L.	0	0	0	0	1	0	0	0
Smilax aspera L.	0	0	0	0	4	0	0	0
Sonchus oleraceus L.	0	0	0	0	1	0	0	0
Trachynia distachya L.	0	0	0	0	10	0	0	0
Trifolium repens L.	0	0	0	0	1	0	0	0
Verbascum thapsus L.	0	0	0	0	1	0	0	0

Table S7. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either

by tree canopies, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days passed from the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{LD}$)	79.80039	5.289899	46	15.085429	<.0001
Zone_HD	-30.84918	7.481046	4	-4.123645	0.0146
$DAYS_{LD}$	-0.04542	0.002974	46	-15.272397	<.0001
Zone_HD:DAYS	0.02729	0.004206	46	6.489605	<.0001

Table S8. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either by tree canopies, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days from the end of the sampling (26 October 2021) to the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{T\_f}$)	42.42170	5.283341	46	8.029333	<.0001
Zone_HD	-8.38710	7.471772	4	-1.122504	0.3245
$DAYS_{LD}$	-0.04542	0.002972	46	-15.282685	<.0001
Zone_HD:DAYS	0.02729	0.004203	46	6.493977	<.0001

Table S9. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either by woody shrubs, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days passed from the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{LD}$)	4.160983	0.5716497	46	7.278903	<.0001
Zone_HD	-4.073960	0.8084347	4	-5.039318	0.0073
$DAYS_{LD}$	0.001464	0.0009870	46	1.483687	0.1447
Zone_HD:DAYS	0.004844	0.0013958	46	3.470287	0.0011

Table S10. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either by woody shrubs, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days from the end of the sampling (26 October 2021) to the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{T\_f}$)	5.366177	0.9815953	46	5.466792	<.0001
Zone_HD	-0.087429	1.3881854	4	-0.062981	0.9528
$DAYS_{LD}$	0.001464	0.0009870	46	1.483743	0.1447
Zone_HD:DAYS	0.004844	0.0013958	46	3.470417	0.0011

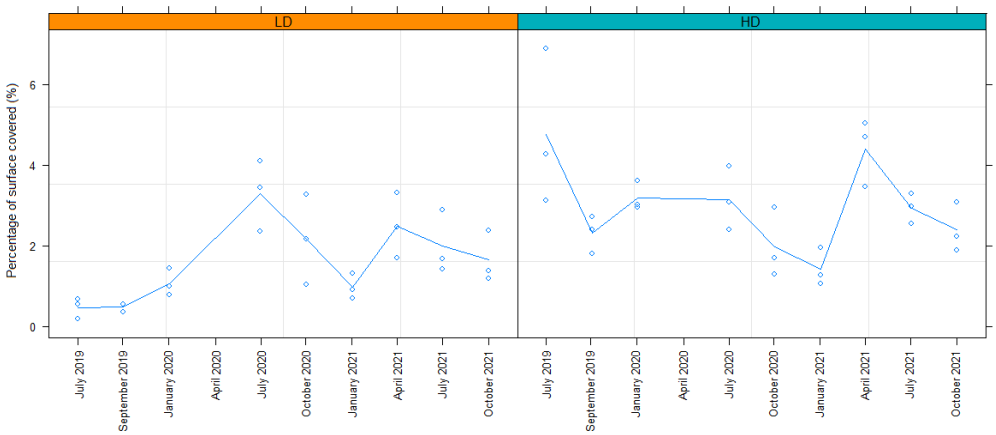

Figure S11. Temporal progression of relative surface covered by shrubs in the three plots (1, 2, 3) from area LD (top row) and HD (bottom row). On the x axis are reported the sampling seasons. In each panel the blue line represents the values of percentage of surface covered by herbaceous species. A linear model was not possible to obtain due the high variability of the data.

Table S12. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either by herbaceous species, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days passed from the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{LD}$)	1.9388035	0.4308671	46	4.499771	<.0001
Zone_HD	0.7785777	0.6093381	4	1.277743	0.2705
$DAYS_{LD}$	-0.0007364	0.0008350	46	-0.881968	0.3824
Zone_HD:DAYS	0.0012624	0.0011808	46	1.069070	0.2906

Table S13. Summary of linear mixed effects model with fixed part: *Surface_Covered ~ Zone + DAYS + Zone:DAYS;* where *Surface_Covered* is the percentage (%) of surface covered either by herbaceous species, *Zone* is a categorical variable with two levels (*i.e.*, LD and HD) and *DAYS* are the number of days from the end of the sampling (26 October 2021) to the start of the sampling (16 July 2019).

	Value	*Std.Error*	*DF*	*t-value*	*p-value*
($Intercept_{T\_f}$)	1.3327415	0.3949578	46	3.374389	0.0015
Zone_HD	1.8175057	0.5585547	4	3.253944	0.3130
$DAYS_{LD}$	-0.0007364	0.0008350	46	-0.881968	0.3824
Zone_HD:DAYS	0.0012624	0.0011808	46	1.069070	0.2906

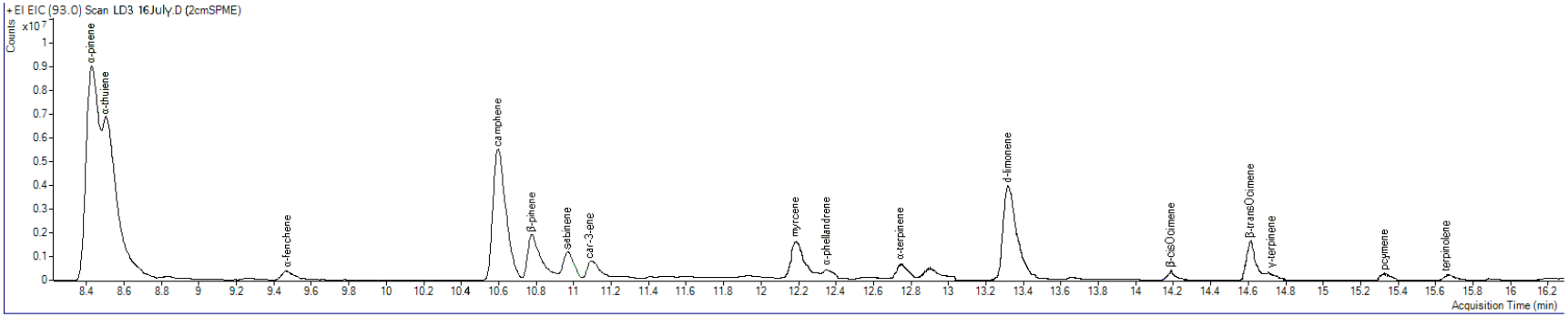

Figure S14. Chromatogram of all monoterpenes and monoterpenoids identified in all sampling season of 2019. The extracted Ion Chromatogram (EIC - ion 93m/z), typical of monoterpenes, was selected to highlight the terpenic profile: 1) α-pinene, 2) α-thujene, 3) α-fenchene, 4) camphene, 5) β-pinene, 6) sabinene, 7) car-3-ene, 8) myrcene, 9) α-phellandrene, 10) α-terpinene, 11) d-limonene, 12) β-cisOcimene, 13) β-transOcimene, 14) γ-terpinene, 15) p-cymene, 16) terpinolene.

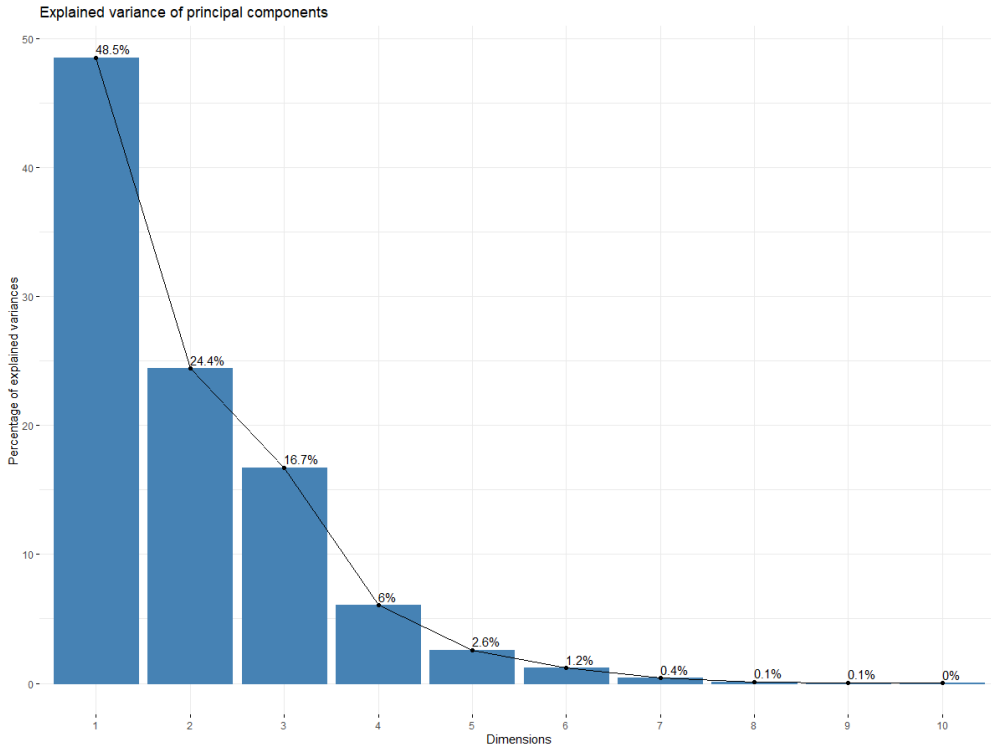

Figure S15. Compositional Screeplot of the amounts of monoterpenes and monoterpenoids (expressed as peak areas). Above each bar is reported the value of the variance of the corresponding component.

Appendix C – Submitted:

Chemical responses of *Pittosporum undulatum* in eucalypt forest show stronger defence mechanisms to abiotic stresses.

Authors: **Pasquini D.**, Dos Santos Nascimento L.B., Brunetti C., Ferrini F., Gleadow R.

Chemical responses of *Pittosporum undulatum* in eucalypt forest show stronger defence mechanisms to abiotic stresses.

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Abstract - The rate of current climate changes will likely modify the composition of native plant communities, often favouring invasive species. Studies conducted in south-eastern Australia have reported *Pittosporum undulatum* to be an aggressive invader of *Eucalyptus* forests. We tested if *P. undulatum* negative impact on floristic diversity is due to the release of allelopathic compounds inhibiting the germination of other species, or if its invasiveness is linked to the biosynthesis of secondary compounds, which may improve abiotic stress tolerance of this species. We compared the germination of *Pittosporum undulatum* and *Eucalyptus ovata* on different substrates, watering seeds with three different leachates and distilled water. Polyphenolic compounds of *Eucalyptus* spp. and *P. undulatum* leaf extracts were analysed by high-performance liquid chromatography, while saponins and tannins were spectrophotometrically quantified. Finally, using Solid Phase Microextraction fibres, Biogenic Volatile Organic Compounds were collected and analysed to compare the emissions in eucalypt forests with and without *P. undulatum.* There were no significant treatment effects on germination rates and no allelopathic compounds were identified in the leachate from *P. undulatum* leaves. *P. undulatum* leachates showed high levels of hydroxycinnamic acid derivatives while flavonoids and tannins characterised *Eucalyptus* leachates. A high monoterpene content was found in the invaded forest,

whereas the forest lacking *P. undulatum* were dominated by sesquiterpenes. Our results suggest that the invasiveness of *P. undulatum* may be linked to the biosynthesis of compounds that play a protective role against abiotic stresses. By contrast, the secondary metabolites of *Eucalyptus* spp. may confer a better protection against biotic attacks.

Key Words - Allelopathy, biodiversity, climate change, environmental stresses, germination test, secondary metabolites.

1. *Introduction*

Changing climatic conditions have played a major role in the evolution of plants, which may implement defence strategies changing their traits to survive (Agrawal et al. 2015; Kijowska-Oberc et al. 2020; Larson et al. 2020). Across Mediterranean climate regions around the world (*e.g.*, Mediterranean basin, Southwest and South Australia, the Cape Region in South Africa, California in the USA, and Central Chile - Myers, 1990), the general increase in the temperature and reduction in precipitation, associated with high human-driven impacts on land, is leading to a wide change in the richness and distribution of many species (Fahrig 2003; Sardans and Peñuelas 2014; Lagacherie et al. 2018). Indeed, these regions are experiencing a change at the environmental level influenced by abiotic and biotic stresses, resulting in consequences in biodiversity which may impair ecosystem function (Rocha et al. 2020; Newbold et al. 2020). Forests and land ecosystems have been subject to nutrient losses and increases in the frequency and severity of extreme climatic events (Bachelot et al. 2020; Prietzel et al. 2020). Furthermore, an increase in CO_2 concentrations, acid deposition on soil as well as diseases and pathogens have also occurred (Kijowska-Oberc et al. 2020; Oliva et al. 2020). Such changes may lead to an imbalance between native plants mortality and exotic species invasion, these last sometimes being more competitive in the new and degraded habitats (Simberloff 2011). Species invasion (either indigenous or exotic) is a serious threat to natural environments (Cronk and Fuller 1995; Adair and Groves 1998), posing difficult challenges to ecological management worldwide (Usher 1988; Humphries et al. 1991; Klepeis et al. 2009).

Invasive species can modify the composition, structure and functionality of native plant communities (O'Leary et al. 2018) and can also influence the ecosystem with the production and release of allelopathic compounds (Foy and Inderjit 2001; Gris et al. 2019). Despite several studies stating that disturbance of natural ecosystems is a precursor of exotic species invasion (Rejmanek 1989; D'Antonio et al. 1999; MacDougall et al. 2013), other studies conducted in south-eastern Australia have demonstrated that native trees and shrubs can also promote decline in species richness,

since they may possess invasive characteristics (Gleadow and Ashton 1981; Molnar et al. 1989; McMahon et al. 1996; Lunt 1998). One of these native species, which is often considered to be an aggressive invader in several areas of south-eastern Australia, is *Pittosporum undulatum* Vent. (Gleadow and Ashton 1981; Mullet and Simmons 1995; Rose and Fairweather 1997; Head and Muir 2004; Bellingham et al. 2018).

Pittosporum undulatum is an evergreen tree (~5-15 m tall) native to south-east Australia that has a high invading potential thanks to its strong capacity to colonize different habitats (Gleadow and Narayan 2007; Nunes et al. 2014). In fact, *P. undulatum* is a notorious invader of forests around the world, and it is also spreading outside its natural range in Australia (Gleadow and Ashton 1981; Gleadow and Rowan 1982; Gleadow and Walker 2014). A decline of many native species has been found in forests invaded by *P. undulatum*, causing a serious reduction in floristic and structural diversity (Gleadow 1982; Rose and Fairweather 1997) and birds (O'Leary et al. 2021). Wet and dry sclerophyll forests, dominated by *Eucalyptus* are invaded by *P. undulatum* right across the continent. Indeed, the species preferentially establishes around the bases of established, threatening the survival of the natural stands of mixed eucalypt woodlands (Gleadow and Ashton 1981). The invasive capacity of *P. undulatum* is linked to a wide range of favourable traits, such as high germination capacity, high competitiveness of its seedlings, and a dense crown (Gleadow and Ashton 1981; Gleadow 1982; Gleadow and Rowan 1982; Gleadow et al. 1983; Kentish et al. 1995; Bradstock et al. 1997; Goodland and Healey 1997). Another possibility could be the presence of allelopathic compounds (*i.e.* terpenoids, alkaloids, glycosides, flavonoids, saponins and tannins) in the leaves, emitted as volatiles or secreted into the soil that inhibit germination (Waller 1989; Li et al. 2010; Chaudhuri and Ray 2016) and growth of other plants, as proposed by Gleadow and Ashton 1981. *P. undulatum* is known to contain high concentrations of saponins (Gleadow and Ashton 1981; Faizal and Geelen 2013). In addition, another factor, not yet investigated, could be related to the higher capacity of *P. undulatum* to better respond to abiotic stresses when compared to other species (*i.e.*, *Eucalyptus* spp.).

In this study we investigate whether the invasiveness of *P. undulatum* is related to the emission of allelopathic compounds, or based on its secondary metabolism, this last allowing *P. undulatum* to withstand changing environments imperilling native species. In detail, we carried out: (i) germination analyses to test the allelopathic action of different leachates obtained from litter and green leaves of *P. undulatum*, and from litter of *Eucalyptus* spp.; (ii) analyses of secondary metabolites (saponins, tannins and polyphenols) in leaf and litter extracts of *P. undulatum* and *Eucalyptus* spp. to quantify the content of compounds with allelopathic properties of these species; and finally, (iii) analyses of Biogenic Volatile Organic Compounds (BVOCs) at the environmental level, using Solid Phase Microextraction (SPME) fibres to observe possible differences of volatile profiles between the two areas in pure *Eucalyptus* spp. woodlands and in woodlands invaded by *P. undulatum.*

2. Methods and materials

2.1 Study Area

The study site is the Bunjil Reserve (north-east of Melbourne, Victoria, Australia, Figure 1), one of the seven Phantom Hill Bushland Reserves. The seven reserves cover an area of 140 hectares, extending from Smiths Gully, in the north, to Watsons Creek, in the south. The annual average precipitation for the area is 660 mm, with the most rain falling in November (73.3 mm), and the driest month being March (42.7 mm). Mean warmest temperatures are approximately 27.1 °C and 14.5 °C for summer and winter, respectively (according to meteorological data acquired by the Australian Bureau of Meteorology Station 086068, situated in Viewbank, approximately 15 km far from the Bunjil Reserve). The reserve contains two different conditions: an area invaded by *P. undulatum* (I), nearby Bishops Rd (37°38'31.94"S, 145°14'27.14"E), around 700 meters from the Bunjil Reserve gate; and an area with high-quality remnant native vegetation, (R) (37°38'49"S, 145°14'57.75"E). The invaded area at the Phantom Hill was characterised by the presence of *P. undulatum* (ca. 40% of woody species), *Eucalyptus* spp. (*E. goniocalyx, E. polyanthemos, E. ovata*) (ca. 55% of woody species) and *Acacia* spp. (ca. 5% of woody species). The natural area (R) was

characterised by extensive stands of mixed eucalypt woodland (95-98% of *Eucalyptus* spp.: *E. rubida, E. goniocalyx, E. polyanthemos, E. ovata*) and 2-5% of *Acacia* spp.

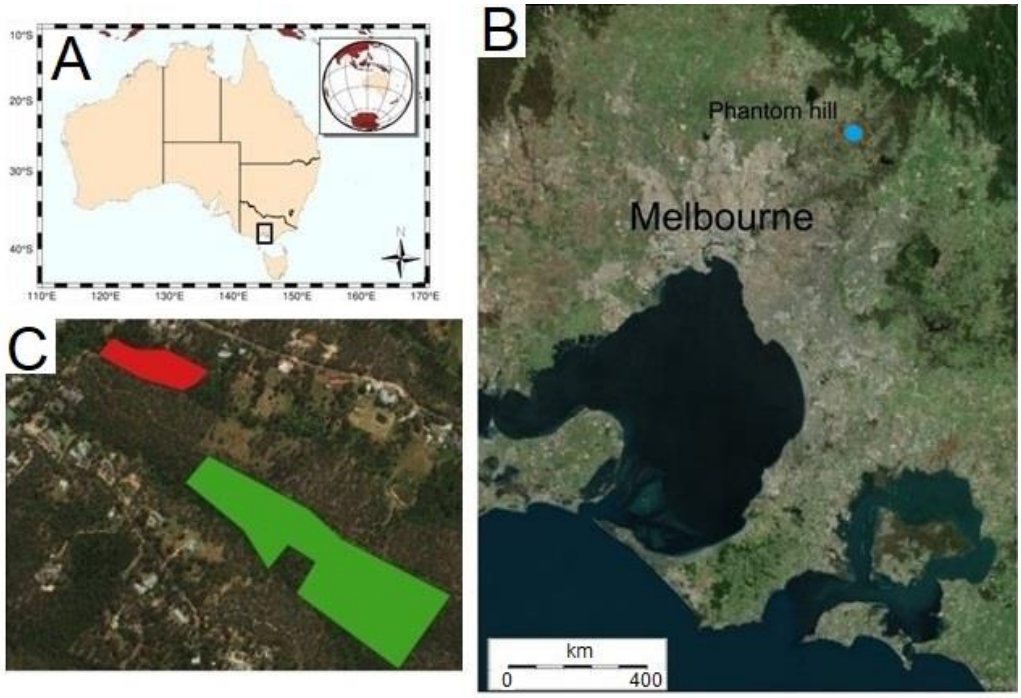

Figure 1. Location of the study sites. Panel A: location of Greater Melbourne Area within Australia. Panel B: location of the study area of Phantom Hill (light-blue dot) in respect to the city of Melbourne. Panel C: the red area represents the study site with the presence of *P. undulatum* (denominated I, invaded), and the green area represents the site characterised by the presence of *Eucalyptus* spp. and *Acacia* spp. (denominated R, remnant vegetation).

2.2 Study Species

Pittosporum undulatum (Sweet Pittosporum) is an Australian native tree species with a natural distribution spanning from south-east Queensland to eastern Victoria (Goodland and Healey 1997). Recently, authors have reported the spread of *P. undulatum* through US, Mexico, Guatemala, the Caribbean (Jamaica and Puerto Rico), South America (Colombia, Ecuador, Bolivia and Brazil), South Africa, Spain and Portugal (Lourenço et al. 2011; Negrelle et al. 2018), in addition to the already known invasions in Hawaii, Bermuda, Canary Islands, New Zealand, Lord Howe Island, Norfolk Islands and other parts of Australia (Gleadow and Ashton 1981). *P. undulatum*

is an aggressive invader species, growing up to 15 m, with a dense crown and dark evergreen leaves blocking up to 90% and 75% of sunlight during winter and summer, respectively (Gleadow and Narayan 2007). In autumn it produces orange capsules, usually carrying around 20 sticky seeds (Cooper 1959), which facilitates its distribution by birds and animals (Gleadow and Ashton 1981). The main characteristics of *P. undulatum* that could explain its spread are the high germination rates (Mullett 1999) and its capacity to grow in environments altered by human activity and without the need of forest fires (Negrelle et al. 2018). Additionally, it has a great adaptability to a wide range of climatic and a root system capable to grow in different edaphic conditions (Gleadow and Ashton 1981; Mullett 1999).

Eucalyptus polyanthemos (Red Box), *Eucalyptus goniocalyx* (Long-leaf Box), *Eucalyptus ovata* (Swamp Gum) and *Eucalyptus rubida* (Candlebark) are evergreen small/medium-size tree species with heights of 8-30 m and 30-40 m, respectively). They are all native to south-eastern Australia (southern New South Wales to Victoria) (Goodger et al. 2004). Unlike *P. undulatum*, their canopies are high and relatively open, and vertically oriented leaves resulting in low sunlight interception. Their flowering season is very long, and it can spread from austral winter to summer (June-March) (Field et al. 2011). The small seeds are contained in conical shaped woody capsules 3-8 mm long (Costermans 2000; van Dijk et al. 2020). The regeneration cycle of many eucalypt species is linked to fire, a natural factor in the Australian environment. Fire releases the seed from the canopy (there is little seed stored in the soil) and increases the amount of light reaching the forest floor. The absence of fires in peri-urban areas, due to fire-suppression policies carried out since 1939 (Gleadow and Ashton 1981; McMahon et al. 1996; Mullett 1999; Gleadow and Narayan 2007), might therefore have encouraged *P. undulatum*, which is very drought tolerant at the seedling stage (Gleadow et al. 1984), at the expense of *Eucalyptus* species. Another factor is that *Eucalyptus* in in this study are all known to be able to resprout from epicormic buds after even very hot fires, whereas *P. undulatum* (Narayan and Gleadow 2007).

2.3 Germination Experiment

A germination study was conducted in order to test whether biotic contaminations or allelopathic chemicals in the soil and leaves influence germination success or germination trajectories, as proposed by Gleadow (1982). To do this, natural soil was collected *in situ* collected from the invaded and reference (*Eucalyptus*-dominated) areas in the study site at Phantom Hill (Figure 1) and used as the substrate base in Petri dishes. Seeds were placed on filter papers laid over the soil and watered with leachates of green leaves and litter from *P. undulatum* and *Eucalyptus* spp. (details below). The field sampling was conducted on the 20th November 2019, during the Austral spring season. Seeds were sown on 25th November 2019.

In detail, in the invaded area we collected: 500 g of soil (layer 15-30 cm deep) under *P. undulatum* plants, approximately 250 g of *P. undulatum* green leaves and 250 g of *P. undulatum* litter. In the reference area we collected: 500 g of soil (layer 15-30 cm deep) under the mixed *Eucalyptus* canopy and approximately 250 g of *Eucalyptus* litter. For the latter, only leaf litter was collected, as it has been demonstrated that the litter of eucalypt has high concentration of allelopathic compounds compared with other plant parts (Abdelmigid and Morsi 2017). All leaves (green and litter) were placed in plastic bags and stored at -20°C overnight. The following day, soil samples were sieved using a 2 mm sieve and the leachates were prepared as follows: 50 g of green *P. undulatum* leaves, 50 g of *P. undulatum* litter and 50 g of *Eucalyptus* spp. litter were washed twice with distilled water, put separately in 2500 ml volumetric flasks without tissue disruption and filled up to 1000 ml with distilled water. After 72 h of soaking at room temperature (21–22 °C), the 5% aqueous extracts were filtered and stored at 4 °C until use (Dorning and Cipollini 2006; Parepa et al. 2012).

Seed of *P. undulatum* were collected from a naturally sown tree growing on the Clayton campus of Monash University (Melbourne, Victoria). Seeds of *E. ovata* were obtained from the Australian Tree Seed Centre (CSIRO, ACT, Australia, Seedlot 20808), *E. ovata* was selected because its distribution overlaps with both the native and *P. undulatum* range and seeds were readily available.

Germination tests were carried out using three substrates and four watering regimes in a factorial design. The four substrates were: filter-paper alone (control); 5 g of soil collected under *P. undulatum* plants and 5 g of soil collected under *Eucalyptus* trees. The four water regimes were: distilled water (control); *Eucalyptus. spp.* litter extract*; P. undulatum* green leaves extract and *P. undulatum* litter extract. Thus, the experimental design consisted of 12 combinations (3 substrates × 4 watering regimes) per species and three replicates for each combination. Each replicate consisted of a Petri dish (90 mm, Filter-paper Advantech type 2), with 30 seeds giving a total of 72 Petri dishes and 2160 seeds. Dishes were placed in an incubator with at a constant temperature of 20°C (Boland et al. 1980) and a photoperiod of 12 h light/ 12 h darkness (Bell 1994; Close and Wilson 2002). All seeds were sowed on the 25th of November. Daily visual inspections were carried out to count germinated seeds and to remove are ones that became infected with fungi. Seeds were considered to have germinated as soon as the embryo ruptured the seed coat (Battaglia 1993) and the radicle was visible. Additionally, the state of cotyledons and seedlings tissues were noted. At the end of the experiment (22 days for *E. ovata* and 38 days for *P. undulatum*) the length of hypocotyl, root and cotyledons for each seedling was also noted and a squash test was conducted to detect the vitality of the seeds that did not germinate (Battaglia 1993).

In addition to germination curves, for each species and combination of substrate and watering treatments, the following measures have also been calculated, all using the package "*germinationmetris*" (Aravind et al. 2021):

- Germination Percentage (GP) (ISTA 2015),

$$GP = \frac{N_g}{N_t} \times 100 \qquad \text{(Eq. 2.1)}$$

  where, N_g is the number of germinated seeds and N_t is the total number of seeds;
- Time for the first germination (t_0), which represents the first day of germination and the time for the last germination (t_g), which represent the last day of germination. In addition, the time spread of germination, was calculated as the difference between the last and first day of germination $t_g - t_0$ (Kader 2005);

- The Mean Germination Time (MGT) was calculated as the average length of time required for maximum germination of a seed lot

$$MGT = \frac{\sum_{i=1}^{t} N_i T_i}{\sum_{i=1}^{t} N_i} \quad \text{(Eq. 2.2)}$$

where N_i is the number of seeds germinated in the $i \ldots t$ time and T_i is the time from the beginning of the experiment (Benvenuti 2003; Ranal and Santana 2006);

- the germination time (t_{50} - method "farooq") represents the time required to reach 50% of final germination

$$t_{50} = t_i + \frac{\left(\frac{N}{2} - N_i\right)(t_j - t_i)}{N_j - N_i} \quad \text{(Eq. 2.3)}$$

where N is the final number of germinated seeds and n_i, n_j are the partial numbers of seeds germinated in adjacent counts at times t_i and t_j respectively, when $n_i < N/2 < n_j$ (Farooq et al. 2005);

- the Vigor Index (VI) of seedlings was measured as

$$VI = \text{GP} \times (L_r + L_s) \quad \text{(Eq. 2.4)}$$

where GP is the germination percentage and L_r, L_s are root and shoot lengths of seedling, respectively (Vashisth and Nagarajan 2010).

2.4 Analyses of Saponins and Total Condensed Tannins in leaf litter leachates

Saponins and total condensed tannins were measured on water leachate prepared for the germination experiment (water extracts of *P. undulatum* litter and of *Eucalyptus* spp. litter) and on ethanol extracts prepared as reported below in the following section. For the water leachate, before performing these spectrophotometric assays, 3 mL of each sample was evaporated under vacuum and re-dissolved in 3 mL of ethanol. All the analyses, both for saponins and tannins, were conducted in triplicate.

Total Saponins Content (TSC) was measured following the procedure described by Le et al. (2018). In detail, 0.15 mL of sample extract, 0.15 mL of vanillin in ethanol (8% w/v) and 1.5 mL of sulphuric acid in water (72% v/v) were mixed and placed in a warm bath at 60 °C for 15 minutes. The TSC was determined using UV/VIS

spectrophotometer (Lambda 25, Perkinelmer) at 535 nm. Diosgenin (Extrasynthese, Genay Cedex France) was used as external standard to create a five-points calibration curve (0.025-0.25 mg/ml) and the TSC obtained was expressed as milligram diosgenin equivalents (mg DE) per g of Dry Weight (DW) of plant material.

The Total Condensed Tannin content (TCT) was measured following the protocol described by St-Pierre et al. (2019), adding 1 mL of a solution composed of 0.1% of 4-dimethylaminocinamaldehyde (DMCA) in methanol-HCl 9:1 (v/v) to each extract (water-based and ethanol. The mixture was mixed vigorously for 1 minute and then incubated in the dark at room temperature, for 15 minutes. The TCT was determined using UV/VIS spectrophotometer at 640 nm. Epicatechin (Extrasynthese, Genay Cedex France) was used as external standard to create a seven-point calibration curve (0.0025-0.1 mg/ml) and the TCT was expressed in catechin equivalents (mg CE) per g of DW.

2.5 HPLC Analysis of Polyphenols in leaves and leaf litter

To carry out a characterisation of polyphenols profiles, in addition to the green leaves and litter samples described above, also approximately 50 g of green leaves of *Eucalyptus* spp. were collected during the same sampling day (20th November). Lyophilized powdered samples (green leaves and litter) of *P. undulatum* and *Eucalyptus* spp. were weighed (150 mg), placed into test tubes and added with 5 mL of a ethanol:water solution (80:20, v/v), acidified to pH 2.5 with 0.1% of HCOOH. Each sample was sonicated in an ultrasonic bath for 20 minutes and the entire procedure was replicated three times. After the extraction the supernatant was defatted by adding 3 mL of n-hexane and repeating this procedure for four times.

Of the 15 ml of ethanolic extract, 3 mL were used for the spectrophotometer analyses of saponins and tannins (mentioned above) and the remaining 12 mL were evaporated under vacuum and re-dissolved in 1 mL of methanol:water (50:50, v/v). An aliquot of 5 µl of the extracts was injected into the Perkin® Elmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and coupled with a LC 200 diode array detector (DAD) (Perkin Elmer®, Bradford®, CT, USA). The separation

was achieved on a Zorbax® SB-18 column (250 × 4.6 mm, 5 µm) (Agilent, Santa Clara, CA, USA), kept at 30 °C. The mobile phase consisted of acidified water (pH 2.5 adjusted with HCOOH; solvent A) and acidified acetonitrile (pH 2.5 adjusted with HCOOH; solvent B). The gradient used was similar for all extracts: 97% of solvent A and 3% of solvent B (0-10 minutes); minutes 10-11 of hold time; 60% of solvent A and 40% of solvent B (12-66 minutes); minutes 67-71 of hold time; 97% of solvent A and 3% of solvent B (71-72 minutes). The flow rate was kept constant at 0.6 mL/min. The extracts were analysed in triplicate, in a wavelength ranging from 180 to 900 nm, while the wavelengths used to quantify the different compounds were 280 nm and 350 nm for *Eucalyptus* spp. extracts, and 280 nm and 330 nm for *P. undulatum*.

Each compound was identified based on a combination of retention time and spectral matching with data comparison against authentic standards (gallic, caffeic and *p*-coumaric acids, all from Sigma-Aldrich Chemie Germany; and ellagic acid, rutin, luteolin-7-*O*-glucoside, all from Extrasynthese, Genay Cedex France) and literature. The quantitative results of polyphenols were reported as mg/g of dry weight (DW) and are expressed as Total Gallo Ellagic Tannins Content (TTC), Total Flavonoid Content (TFC) and the Total Hydrocinnamic Acid derivatives Content (THC), as a sum of the content of individual compounds belonging to each phenolic class.

2.6 Collection and Analysis of BVOCs.

During the same sampling day for the collection of materials for the germination experiment (20th November 2019), five Solid Phase Microextraction (SPME) fibres were used at each sampling site to collect Biogenic Volatile Organic Compounds (BVOCs) at environmental level. The local temperature was 37 °C, with 29% humidity, and 13 km/h wind speed with a NE direction. Ten fan-samplers mounted with SPME fibres (Sigma-Aldrich of 2cm and assembly Divinylbenzene/Carboxen /Polydimethylsiloxane) were installed at a height of 45 cm from the ground (Pasquini et al. 2021). This height was chosen to simplify the sampling, since terpene concentrations have been shown to be higher at heights from 0 to 4 m (Noe et al. 2012). Sampling time was set between 11 am and 3 pm. The fibres were then put in a

special tray, within a hermetic case and dedicated Teflon pressure supports to seal the needles for the transport to the laboratory.

The SPME fibres were desorbed in an Agilent 7890B gas chromatograph coupled with a 5977A mass spectrometer with EI ionization operating at 70eV. A chromatographic column Agilent DB-Wax 60 m x 250 μm x 0.5 μm was used. The injector temperature was set to 260 °C, splitless mode, with a flow of 1.2 mL/min. The oven temperature program consisted of an initial temperature of 40 °C for one minute, which was then increased by 5 °C/min until 210 °C, and then by 10 °C/min until 250 °C (max temperature for this column). Lastly, the temperature was decreased to 240 °C and was held for 10 minutes, resulting in a total run time of 51 minutes. The lowest mass acquired was 29 m/z and the higher was 350 m/z at three scans per second.

The data was analysed using the Agilent Mass Hunter software (Qualitative Analysis-Version B.06.00; Quantitative Analysis Version B.07.01/Build 7.1.524.0), and the terpenes were identified by matching their mass spectra and retention indices with those reported in the NIST 11 spectral database library. Information related to the fragmentation patterns and retention times available from scientific literature was used for the final compound identification (Goodner 2008; Vezzola et al. 2019). The amount of monoterpenes and sesquiterpenes, expressed as peak areas, were related to Total Ion Current (TIC).

2.7 Statistical Analyses.

All statistical analyses were carried out using R (version 4.1.0) and RStudio (version 1.4.1717). For every index calculated at the end of the germination experiment and for every species individually, a One-way Analysis of Variance (ANOVA) was conducted between watering treatments for each substrate, separately. Before carrying out the ANOVA, the assumption of normality and homoscedasticity were checked using Shapiro and Levene's tests, respectively (Shapiro and Wilk 1965; Gastwirth et al. 2009; Nordstokke and Zumbo 2010).

For TSC and TCT of the different extracts (*Eucalyptus* green leaves in EtOH, *Eucalyptus* litter in EtOH, *Eucalyptus* litter in water, *P. undulatum* green leaves in

EtOH, *P. undulatum* litter in EtOH and *P. undulatum* litter in water) and for the total content of polyphenols of each class of identified metabolites (TTC, TFC and THC) a one-way non-parametric analysis of variance (Kruskal-Wallis Test) was conducted. This test was carried out, since the ANOVA's assumptions of normality tested with Shapiro's was not met, while the heteroscedasticity tested with Levene's test was met. After that, Dunnett post hoc test was carried out.

For BVOCs compounds, in order to test differences between the two studied areas (I and R), we calculated the relative amount of each monoterpene (MT) and sesquiterpene (SQT) identified, expressed as a percentage of total terpenes profiles (TMTs + TSQTs) for both areas. The mean percentages of each terpene were analysed by a one-way analysis of variance. A 0.05 *p*-value threshold was set as cut-off value.

3. Results

3.1 Leachates did not affect the germination of P. undulatum or E. ovata but substrate type did

Leachates of fresh *P. undulatum* leaves and leaf litter collected from areas invaded by *P. undulatum* or in a area of remnant bushland dominate by *Eucalyptus* were applied to Petri dishes containing *P. undulatum* and *E. ovata seeds* and compared to seeds watered only with water. Germination was assessed as per cent of total viable seeds, rate of germination and time to 50% germination. Under all combination of treatments, the seeds were able to germinate, but with different percentages for each species, as shown with the germination curves of *E. ovata* (Figure 2) and *P. undulatum* (Figure 3). The Mean Germination Times (MGT) for *E. ovata* seeds were similar for all watering. The germination curves showed a similar trend in all watering treatments (Figure 2). For *P. undulatum* seeds, likewise, differences were not detected either in the germination curves and in MGT for all watering treatments (Figure 3 B-C). However, for *P. undulatum* seeds tested on the filter-paper substrate (Figure 3) there was a higher germination rate in the control watering treatment, compared to seeds that were watered with leachates. Applying the various extracts as watering treatments did

not affect the germination of either *P. undulatum* or *E. ovata*. studied. Observing all the calculated indices, regarding *E. ovata* seedlings, the Vigor Index (VI) was affected by the substrate, changing significantly in paper and *P. undulatum* soil under the different water treatments (Table 1). In particular, the highest VI was obtained using water as watering treatment for filter-paper substrate, while the *P. undulatum* green leaf leachate was the best watering treatment for *P. undulatum* soil substrate. For *P. undulatum* seedlings the two types of soils (*i.e.,* soils collected under *P. undulatum* and under *Eucalyptus* plants) did not present any significantly differences, while, for the paper substrate, all indices, except the spread germination time, were significantly different (Table 2). In the case of filter-paper substrate, using water as watering treatment allowed to obtain 85.6% of Germination Percentage (GP) while the other watering methods resulted in GP lower than 50%. Furthermore, the highest VI was recorded when water was used as watering method.

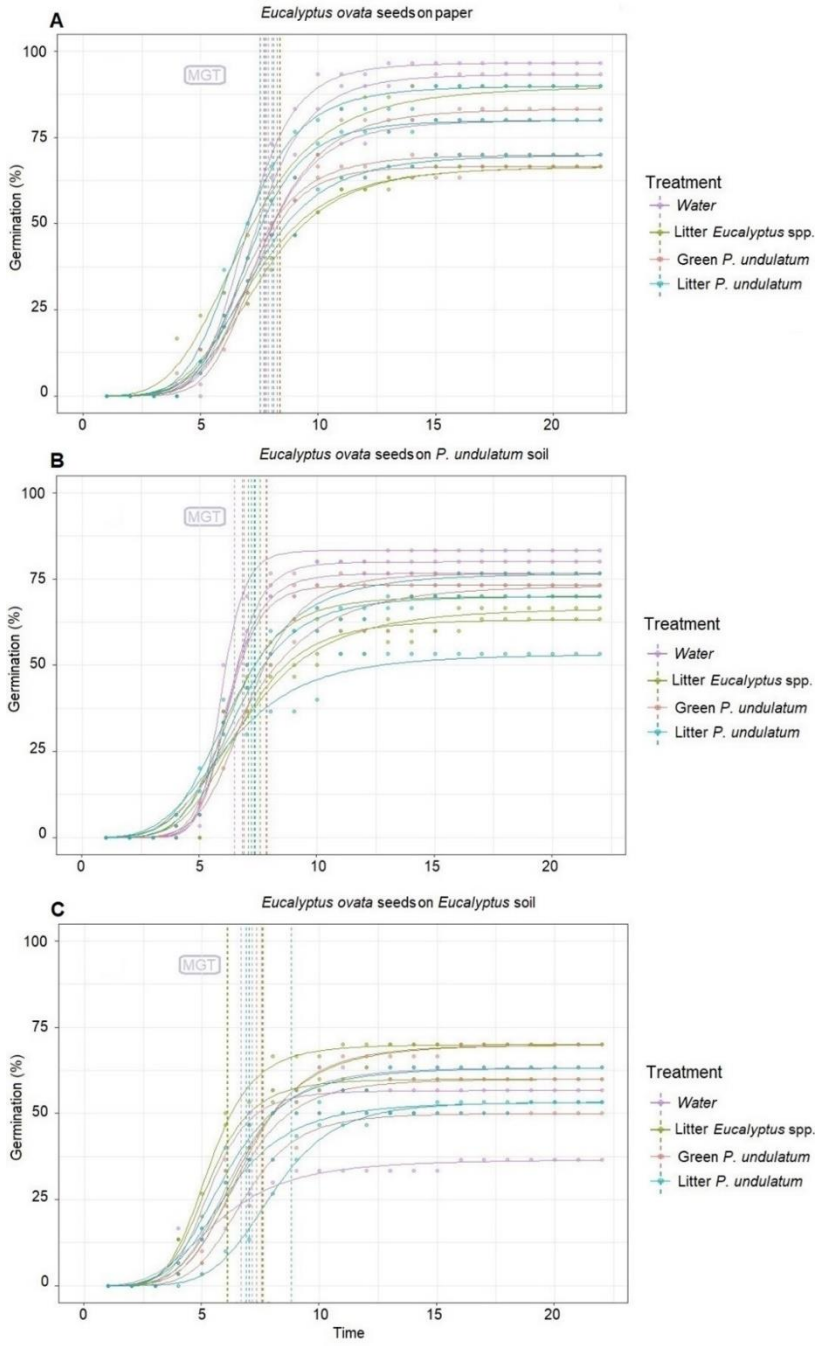

Figure 2. Germination curves for *E. ovata* seeds on different substrates (A= filter-paper substrate; B = *P. undulatum* soil; C = *Eucalyptus* soil). The colours show the different watering treatments. The vertical dashed lines represent the Mean Germination Time (MGT) for each treatment *x* replicates (ns for ANOVA test).

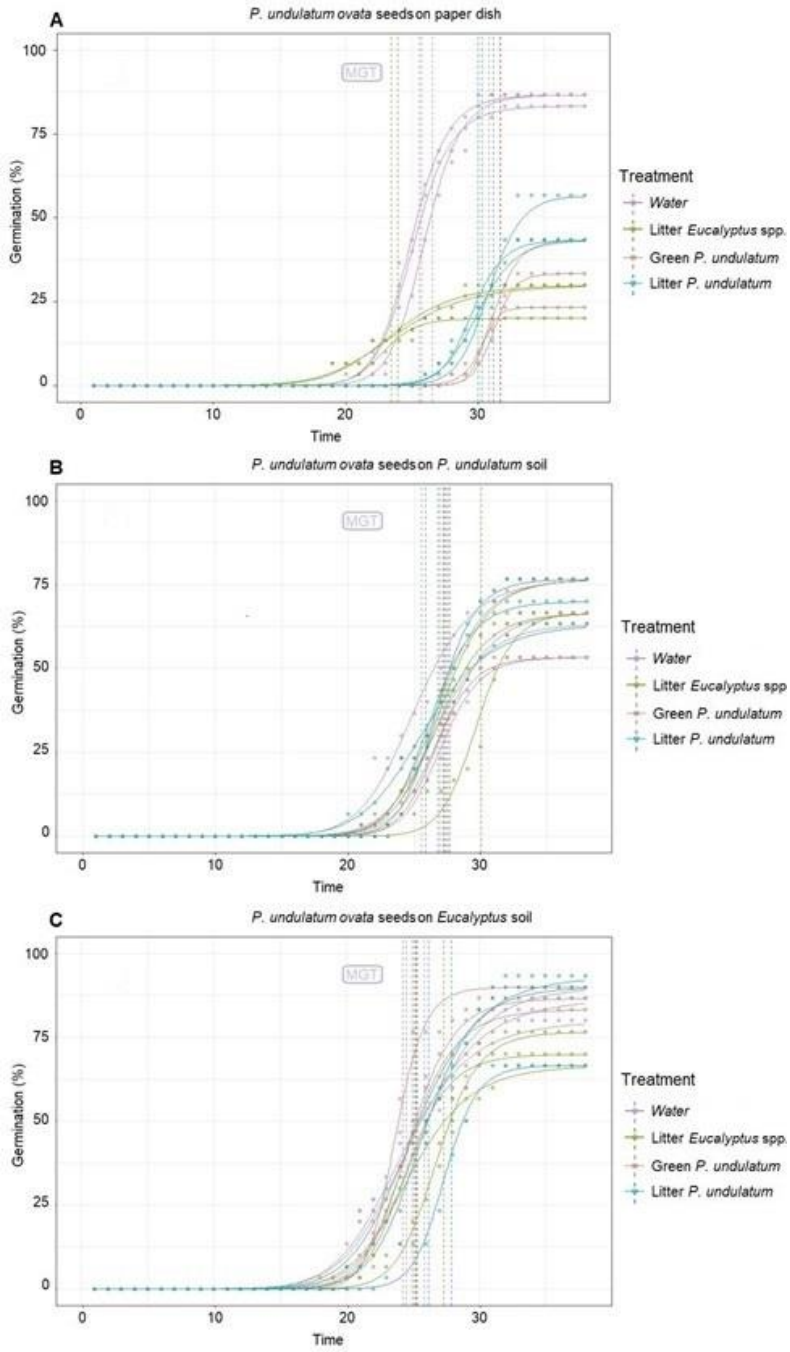

Figure 3. Germination curves for *P. undulatum* seeds on different substrates (A= filter-paper substrate; B = *P. undulatum* soil; C = *Eucalyptus* soil). The colours show the different watering treatments. The vertical dashed lines represent the Mean Germination Time for each treatment (MGT) *x* replicates (ns for ANOVA test).

Table 1 Mean and standard deviation of germination indices between replicates for *E. ovata* seedlings: Germination Percentage (%, GP), First Germination time (day, t_0), Last Germination Time (day, t_g), Time spread of germination (day, $t_g - t_0$), Mean Germination Time (day, MGT), Median germination time (day, t_{50} - Farooq) and Vigor Index (% *x* cm, VI). The symbols next to each value represents one-way ANOVA results between treatments (*ns* > 0.05, * 0.01< *p* < 0.05, ** 0.001< *p* < 0.01, *** *p* < 0.001).

Substrate	Watering treatment	GP (%)	t_0 (day)	t_g (day)	$t_g - t_0$ (day)	MGT (day)	t_{50} (Farooq) (day)	VI (% *x* mm)
Filter-paper	Water (control)	90.0±8.8^{ns}	5.0±1.0^{ns}	14.0±1.0^{ns}	9.0±1.0^{ns}	8.0±0.4^{ns}	7.3±0.1^{ns}	1390.5±164.4***
(no soil)	Litter *Eucalyptus*	74.4±13.5^{ns}	4.7±0.6^{ns}	14.3±0.6^{ns}	9.7±0.6^{ns}	8.1±0.3^{ns}	7.3±0.4^{ns}	550.9±140.6***
	Green *P. undulatum*	73.3±8.8^{ns}	4.7±0.6^{ns}	15.6±1.5^{ns}	11.0±1.7^{ns}	8.0±0.3^{ns}	7.3±0.3^{ns}	446.0±54.9***
	Litter *P. undulatum*	80.0±10.0^{ns}	5.0±0.1^{ns}	15.0±0.1^{ns}	10.0±0.1^{ns}	7.9±0.2^{ns}	6.9±0.3^{ns}	1179.1±189.4***
P. undulatum soil	Water (control)	79.9±3.3^{ns}	4.7±1.2^{ns}	11.7±1.5^{ns}	7.0±2.6^{ns}	6.9±0.1^{ns}	5.9±0.3^{ns}	1310.0±217.3**
	Litter *Eucalyptus*	66.7±3.3^{ns}	5.3±1.1^{ns}	15.7±1.5^{ns}	10.3±0.6^{ns}	7.9±0.6^{ns}	5.9±0.1^{ns}	1044.2±93.8**
	Green *P. undulatum*	74.4±1.9^{ns}	4.7±0.6^{ns}	11.3±3.5^{ns}	6.7±4.0^{ns}	7.5±0.7^{ns}	6.6±0.5^{ns}	1414.6±18.1**
	Litter *P. undulatum*	66.7±12.0^{ns}	4.3±0.6^{ns}	14.0±2.6^{ns}	9.7±2.3^{ns}	7.5±0.2^{ns}	5.9±0.2^{ns}	927.2±108.8**
Eucalyptus soil	Water (control)	52.2±13.9^{ns}	4.0±0.1^{ns}	11.3±4.2^{ns}	7.3±4.2^{ns}	6.6±0.6^{ns}	5.9±0.7^{ns}	876.3±278.9^{ns}
	Litter *Eucalyptus*	66.7±5.8^{ns}	4.0±0.2^{ns}	12.3±1.1^{ns}	8.3±1.1^{ns}	6.6±0.9^{ns}	5.7±0.5^{ns}	1134.1±99.8^{ns}
	Green *P. undulatum*	60.0±10.0^{ns}	4.0±4.0^{ns}	13.0±3.0^{ns}	9.0±3.0^{ns}	7.5±0.2^{ns}	6.7±0.4^{ns}	723.6±78.1^{ns}
	Litter *P. undulatum*	56.7±5.8^{ns}	4.3±0.6^{ns}	15.7±3.1^{ns}	11.3±2.5^{ns}	7.8±1.1^{ns}	6.5±1.3^{ns}	1036.9±160.0^{ns}

Table 2 Mean and standard deviation of germination indices between replicates for *P. undulatum* seedlings: Germination Percentage (%, GP), First Germination time (day, t_0), Last Germination Time (day, t_g), Time spread of germination (day, $t_g - t_0$), Mean Germination Time (day, MGT), Median germination time (day, t_{50} - Farooq) and Vigor Index (% *x* cm, VI). The symbols next to each value represents one-way ANOVA results between treatments (*ns* > 0.05, * 0.01< *p* < 0.05, ** 0.001< *p* < 0.01, *** *p* < 0.001).

Substrate	Watering treatment	GP (%)	t_0 (day)	t_g (day)	$t_g - t_0$ (day)	MGT (day)	t_{50} (Farooq) (day)	VI (% *x* mm)
Filter-paper (no soil)	Water (control)	$85.6\pm1.9^{***}$	$21.3\pm1.5^{***}$	$31.0\pm1.0^{*}$	9.7 ± 2.1^{ns}	$26.0\pm0.5^{***}$	$25.36\pm0.6^{***}$	$4494.3\pm126.7^{***}$
	Litter *Eucalyptus*	$26.7\pm5.8^{***}$	$19.7\pm1.1^{***}$	$28.7\pm2.5^{*}$	9.0 ± 3.6^{ns}	$24.1\pm0.5^{***}$	$22.8\pm0.3^{***}$	$1260.3\pm285.1^{***}$
	Green *P. undulatum*	$33.2\pm9.8^{***}$	$27.3\pm1.1^{***}$	$33.0\pm0.1^{*}$	5.7 ± 1.1^{ns}	$31.1\pm0.4^{***}$	$31.1\pm0.6^{***}$	$493.3\pm238.5^{***}$
	Litter *P. undulatum*	$47.8\pm7.7^{***}$	$26.7\pm0.6^{***}$	$32.7\pm0.6^{*}$	6.0 ± 1.0^{ns}	$30.5\pm0.5^{***}$	$30.3\pm0.9^{***}$	$1282.5\pm289.1^{***}$
P. undulatum soil	Water (control)	64.4 ± 11.7^{ns}	22.7 ± 1.5^{ns}	32.0 ± 0.1^{ns}	9.3 ± 1.5^{ns}	26.9 ± 0.9^{ns}	26.6 ± 0.9^{ns}	3272.6 ± 488.1^{ns}
	Litter *Eucalyptus*	70.0 ± 5.8^{ns}	22.3 ± 1.5^{ns}	32.3 ± 0.6^{ns}	10.0 ± 1.0^{ns}	28.2 ± 1.3^{ns}	28.0 ± 2.0^{ns}	3376.4 ± 312.1^{ns}
	Green *P. undulatum*	65.5 ± 11.7^{ns}	22.7 ± 1.3^{ns}	32.7 ± 0.6^{ns}	10.2 ± 1.1^{ns}	27.5 ± 0.5^{ns}	26.9 ± 0.1^{ns}	3829.2 ± 985.2^{ns}
	Litter *P. undulatum*	70.0 ± 6.7^{ns}	22.0 ± 1.7^{ns}	32.0 ± 1.0^{ns}	10.0 ± 2.6^{ns}	26.9 ± 0.5^{ns}	26.4 ± 0.5^{ns}	3511.6 ± 728.7^{ns}
Eucalyptus soil	Water (control)	85.5 ± 5.1^{ns}	20.0 ± 1.0^{ns}	32.0 ± 1.2^{ns}	12.0 ± 0.2^{ns}	26.7 ± 0.6^{ns}	24.1 ± 0.7^{ns}	5424.3 ± 581.2^{ns}
	Litter *Eucalyptus*	71.1 ± 5.1^{ns}	19.3 ± 1.1^{ns}	31.0 ± 0.9^{ns}	11.7 ± 0.6^{ns}	26.0 ± 1.0^{ns}	25.1 ± 1.8^{ns}	3794.9 ± 203.7^{ns}
	Green *P. undulatum*	86.7 ± 3.3^{ns}	20.1 ± 0.1^{ns}	31.3 ± 0.6^{ns}	11.3 ± 0.5^{ns}	25.0 ± 0.5^{ns}	24.4 ± 0.7^{ns}	4525.3 ± 428.9^{ns}
	Litter *P. undulatum*	83.3 ± 14.5^{ns}	21.0 ± 1.7^{ns}	30.9 ± 1.0^{ns}	10.1 ± 2.6^{ns}	26.5 ± 1.0^{ns}	26.1 ± 1.5^{ns}	3508.9 ± 1622.4^{ns}

3.2 Total Saponins and Total Condensed Tannins Content differed between species and between fresh leaves and leaf litter

In all the extracts of *Eucalyptus* green leaves and litter the amount of saponins were higher than those correspondent of *P. undulatum* extracts (Figure 4). TSC were almost 6-fold lower in the ethanolic extract of *P. undulatum* green leaves (33.42 ± 3.32 mg DE/g DW) than in the corresponding eucalypt extract (208.78 ± 28.56 mg DE/g DW) (Figure 4).

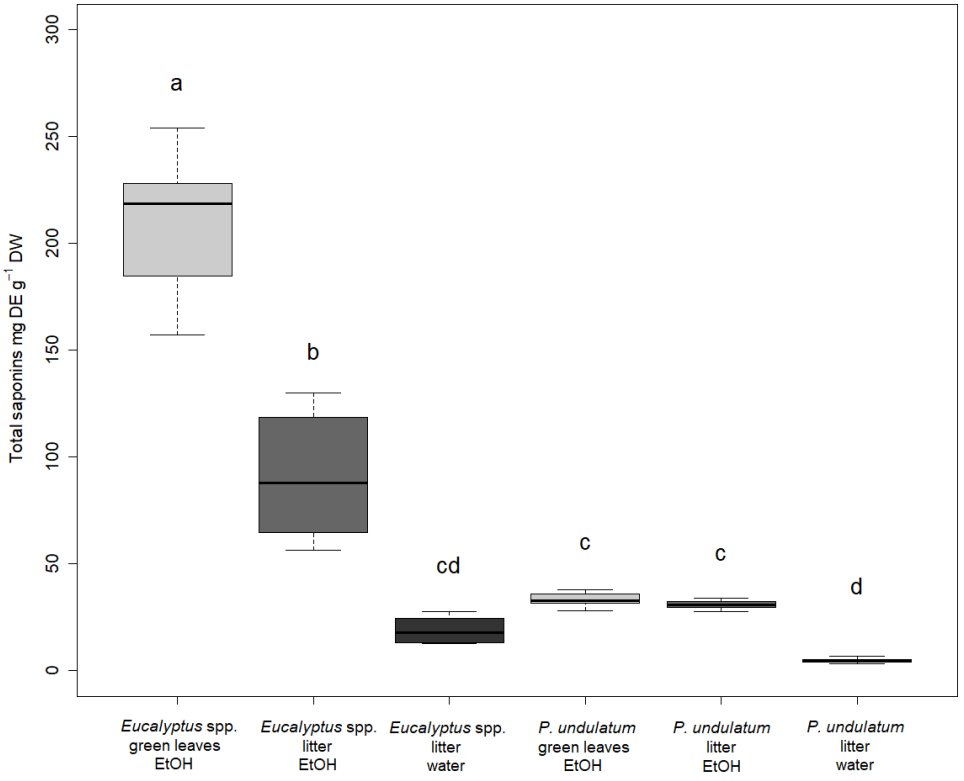

Figure 4. Total saponin content (mg of Diosgenin Equivalent (DE) g^{-1} DW) in *Eucalyptus* and *P. undulatum* extracts (ethanolic extracts of green leaves and litter, and water extracts of litter). Each value is the mean of X measurement (±1SD). Bars with the same letter are not significantly different when analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

In all extracts of *Eucalyptus* (green leaves and litter) the content of tannins was higher than that of *P. undulatum* extracts (Figure 5). In the litter water extract of

Eucalyptus, TCT were about 10-fold higher (2.99 ± 0.55 mg CE/g DW) than in the litter water extract of *P. undulatum* (0.27 ± 0.09 mg CE/g DW).

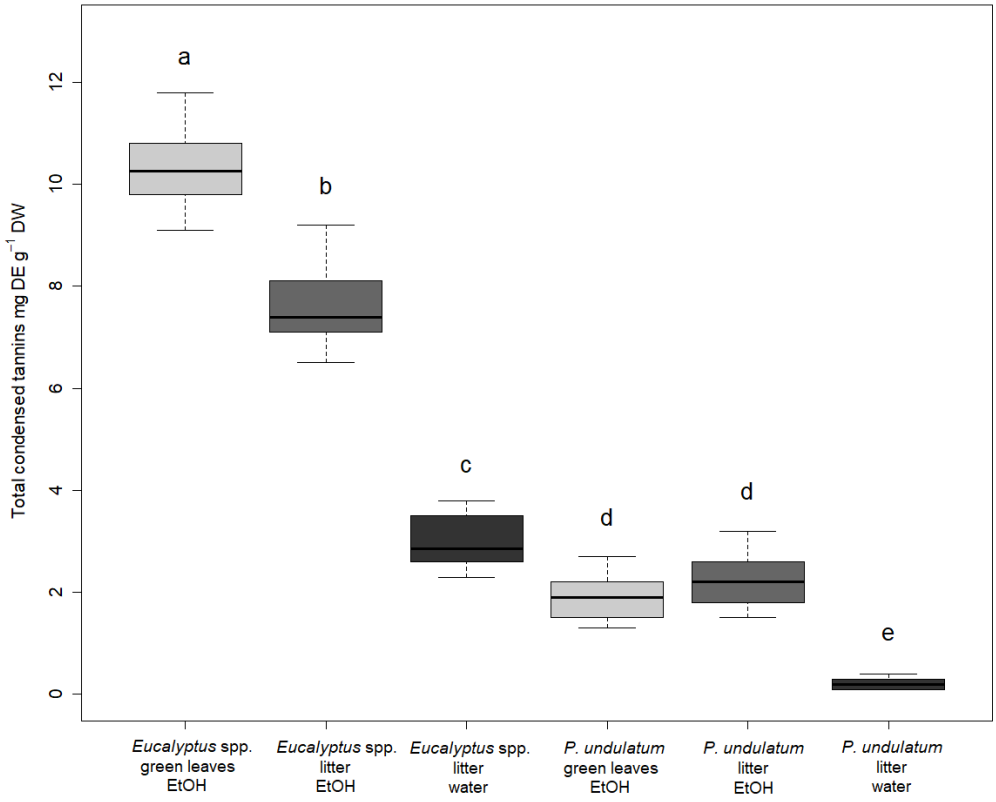

Figure 5. Content of total condensed tannins (TCT) (mg of Catechin Equivalent (CE)) g^{-1} DW) in *Eucalyptus* and *P. undulatum* extracts (ethanol extracts of green leaves and litter and water extracts of litter). Each value is the mean of X measurement (±1SD). Bars with the same letter are not significantly different when analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

3.3 HPLC-DAD Analyses of Polyphenol Content.

Different polyphenols were identified and quantified in each extract (ethanolic and aqueous) of *Eucalyptus* spp. and *P. undulatum* (Figure S1-S6, Table S1-S6). In detail, hydrolysable tannins derived from both ellagic and gallic acids were detected and here identified as gallic and ellagic acids derivatives. The ellagic acid derivatives were the richest in both *Eucalyptus* litter extracts, representing more than 90% of the total tannin content in ethanolic extracts and more than 75% in aqueous extracts). On

the other hand, in eucalyptus green leaves extracts the most abundant tannins were derived from gallic acid (around 65% of total tannins content). Gallic and ellagic acids derivatives were significantly higher in litter extracts of *Eucalyptus* compared to the extracts obtained from green leaves of the same species (Figure 6A). Instead, Total Flavonoid Content (TFC) was higher in the two ethanolic extracts of *Eucalyptus* compared to the two ethanolic extracts from *P. undulatum* (Figure 6B), and it was not detected in the water *Eucalyptus* litter extract. The myricetin derivatives were the only flavonoids identified in the extracts of *Eucalyptus* and *P. undulatum*. Caffeic and *p*-coumaric acids derivatives (reported as here as hydroxycinnamic acid derivatives, Figure 6) were identified only in *P. undulatum* extracts. The hydroxycinnamic acid derivatives were detected only in the *P. undulatum* ethanolic extracts of green leaves (0.84± 0.13 mg/g of DW), litter (2.40 ± 0.29 mg/g of DW) and aqueous extract (0.43 ± 0.02 mg/g of DW).

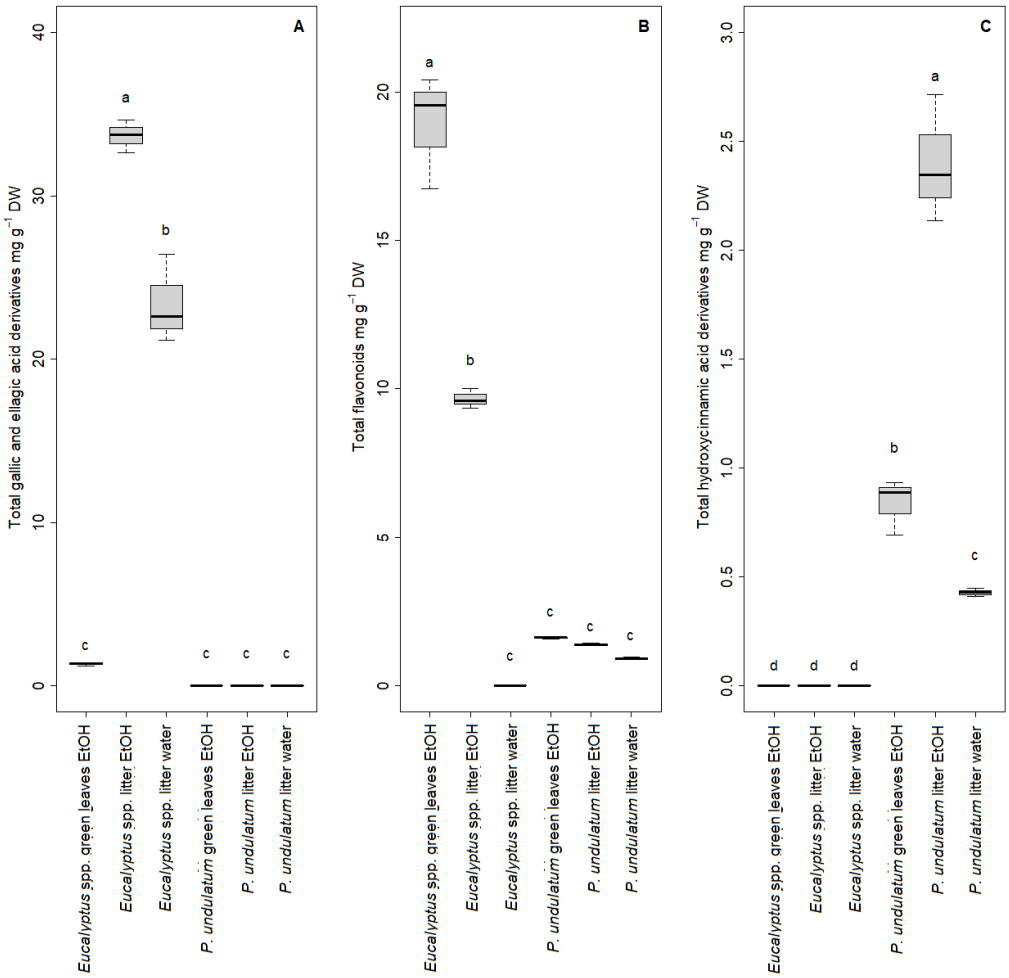

Figure 6. Total content of gallic and ellagic acids derivatives (A), Total Flavonoid Content (B) and Total hydroxycinnamic acid derivatives (C) of *Eucalyptus* and *P. undulatum* extracts (mg g^{-1} DW). Bars are value is the mean of X measurement (±1SD). Bars with the same letter are not significantly different when analysed by a one-way non-parametric analysis of variance (Kruskal-Wallis Test) followed by a Dunnet *post-hoc* test.

3.4 BVOC Analysis reveals difference in monoterpenes and sesquiterpenes in invaded sites

Approximately the 80% of BVOCs identified in the area invaded by *P. undulatum* (I) are represented by MTs and only 20% by SQTs. In the area of natural vegetation characterised by the presence of *Eucalyptus* spp. and *Acacia* spp. (R), the percentages

of MTs and SQTs are more similar to each other: 57% of the BVOCs identified are represented by MTs and 43% by SQTs. The relative concentrations of monoterpenes and monoterpenoids found in the two studied areas are shown in Figure 7. Results from one-way ANOVA test showed that all the identified MTs are significantly different between the two sites, excepted for α-phellandrene, 1,8-cineole e terpinyl acetate. Additionally, it is possible to note that all MTs, excepted the 1,8-cineole and terpinyl acetate, are higher in the area invaded by *P. undulatum* (I) compared to the air collected in the remnant area (R). The percentages of sesquiterpenes and sesquiterpenoids identified in the two studied areas are reported in Figure 8. All SQTs (excepted the selinadiene) are significantly higher in the remnant vegetation compared to the area invaded by *P. undulatum*.

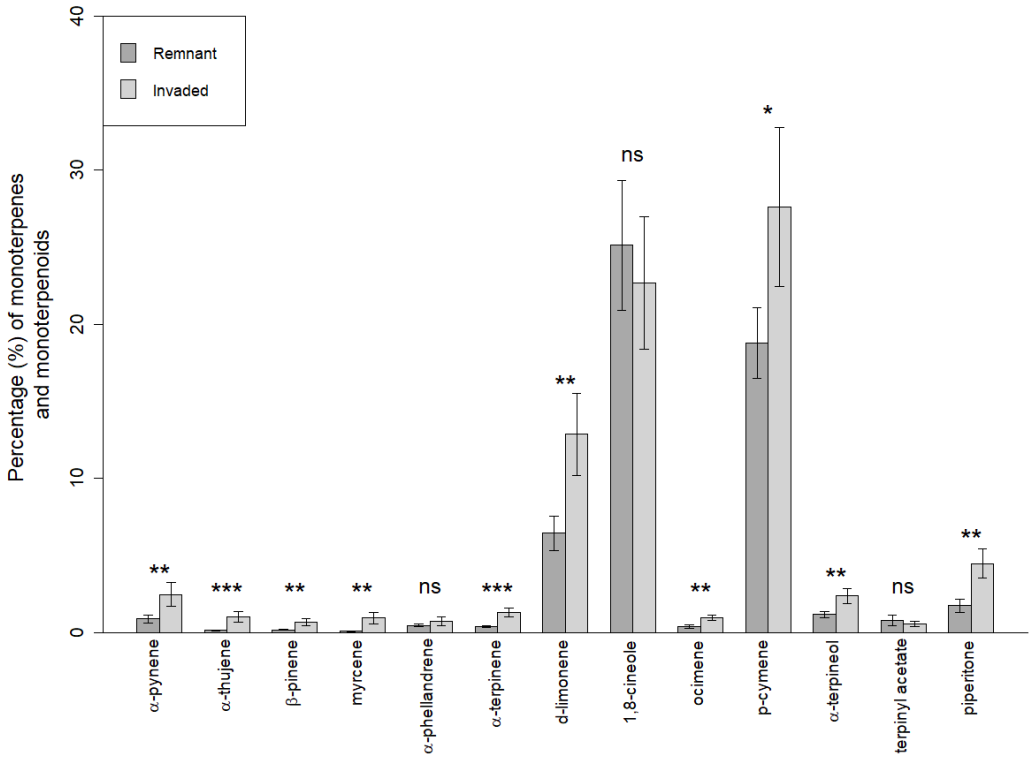

Figure 7. Histogram representing the amount (%) of monoterpenes and monoterpenoids identified in volatiles collected in remnant area of natural vegetation (R) and areas invaded by *P. undulatum* (I). Error bars indicate standard deviation, and the asterisks indicate significant differences between R and I: *ns* > 0.05, * 0.01< p < 0.05, ** 0.001< p < 0.01, *** p < 0.001.

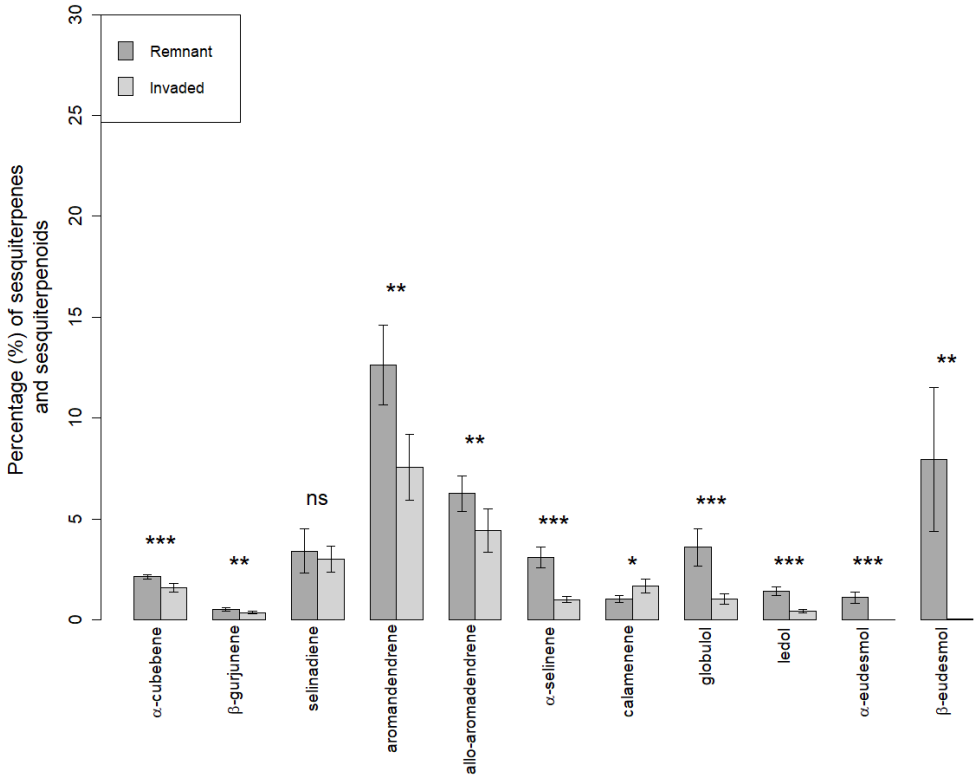

Figure 8. Histogram representing the amount (%) of monoterpenes and monoterpenoids identified in volatiles collected in remnant area of natural vegetation (R) and areas invaded by P. undulatum (I). Error bars indicate standard deviation, and the asterisks indicate significant differences between R and I: *ns* > 0.05, * 0.01< p < 0.05, ** 0.001< p < 0.01, *** p < 0.001.

4. Discussion

This study investigated the possible biochemical bases of *P. undulatum* invasiveness in a *Eucalyptus* sclerophyll forest located in south-eastern Australia. To do this we first tested the hypothesis that the success of *P. undulatum* is due, in part, due to suppression of germination of native plants by comparing the germinability of *P. undulatum* and a co-occurring tree, *E. ovata*, on leaf and soil extracts. We then tested the hypothesis that the high invasiveness of *P. undulatum* may be due differences in the storing and emission of secondary metabolites.

Does P. undulatum inhibit germination of eucalypts?

The germination curves indicate the absence of inhibition or suppression of seed germination due to *P. undulatum* aqueous extracts. Additionally, for the *E. ovata* and *P. undulatum* seeds, observing the selected indices (GP, t_0, t_g, MGT, t50 and VI), the values are significantly different between watering treatments only for the paper substrate. This could be explained by the lack of soil chelating action. Indeed the soil is a very complex system and it influences the qualitative and quantitative availability of phenolics and other allelopathic compounds (Cheng 1995). This influence happens because once phenolic allelochemicals enter the soil system, they undergo transformation and retention processes (Cheng 1995). Phenolics, such as caffeic acid, or tannins are adsorbed by clay minerals forming chelate complexes with metals (Shindo and Kuwatsuka 1975). When evaluating the possible allelopathic action of some plants it is necessary to consider the physiochemical properties of the soil and thus, when carrying out allelopathic tests of germinability, it is important to use the same soils that plants find in nature (Inderjit 1996). The only soil substrate showing a significant difference of Vigour Index (VI) between treatments was *P. undulatum* soil for the *E. ovata* seedlings. In particular, we can see the highest value (1414.6 ± 18.1) for green *P. undulatum* leachate, which is even greater than with water. On the other hand, litter extracts of both eucalypt and *P. undulatum* had the lowest VI values. These results do not allow to clearly link *P. undulatum* invasiveness to allelopathic actions under natural conditions.

In the literature, phytotoxic effects of *P. undulatum* leaf extracts on the germination of native Australian species varied considerably. Gleadow and Ashton (1981) carried out a germination test, where they reported an allelopathic action of this invasive species that could explain its rapid spread: *P. undulatum* leaves leachate suppressed germination in *E. obliqua* (47%), in *E. melliodora* (8%) and in *E. goniocalyx* (48%). Our results disagree with these authors, but the absence of allelopathic action from *P. undulatum* leaf extract agrees with Tunbridge et al. (2000), that reported a significant increment of germination rates of *E. viminalis* when seeds were treated with *P. undulatum* leaf leachate (+70%) compared to untreated seeds

(watering with distilled water). These results could be explained by the fact that allelopathic actions of *P. undulatum* could differ depending on different species and subspecies exposed to them. Moreover, regarding our result of no inhibition of *P. undulatum* leaf leachate on *P. undulatum* seeds germination, other authors reported an inhibitory action on *P. undulatum* seedlings under mature *P. undulatum* canopy (Richardson et al. 1985). This result was not obtained through a germination experiment using leaf extracts but by observing the lack of young seedlings of *Pittosporum* beneath established mature *Pittosporum* trees. Thus, the reasons could be others, such as different microclimate or light availability. Finally, our data do not indicate an allelopathic action of *Eucalyptus* spp. leaf extracts on *P. undulatum* and *E. ovata* seeds germination and this result is contrasting with the literature. Indeed, previous studies have shown that many eucalypt species present allelopathic actions which may inhibit and suppress seed germination and seedling establishment of other species (Ahmed et al. 2008; Khan et al. 2009; Rassaeifar et al. 2013; Brumati et al. 2018; Hoogar et al. 2019; Bayle 2019). However, *Eucalyptus* spp. allelopathic action against *P. undulatum* and on the germination of *E. ovata* itself was not previously investigated.

As shown by the germination test results, the lack of allelopathic action of *P. undulatum* might be correlated with the low values of Total Saponins Content (TSC) and Total Condensed Tannin Content (TCT) found in the water extract of *P. undulatum*. On the other hand, the higher content of saponins in eucalypt extracts, concomitantly with a higher tannin content respect to the corresponding extracts obtained from *P. undulatum*, suggests a major investment of carbon for the biosynthesis of these secondary metabolites in *Eucalyptus* (Fox and Macauley 1977; Bhuyan et al. 2015). As previously reported for other species (Oleszek 1993; Scognamiglio et al. 2012; Faizal and Geelen 2013), saponins and tannins may help protecting eucalypt leaves against insects and herbivores by decreasing the digestibility of their tissues (Barbehenn and Constabel 2011; War et al. 2012). Since phytotoxicity of saponins and tannins has been linked to a reduction in the growth of seedlings and to the inhibition of germination (Waller 1989; Li et al. 2010; Chaudhuri and Ray 2016),

our results cannot support a possible negative role of *P. undulatum* on floristic diversity caused by the release of allelopathic compounds inhibiting the germination and growth of eucalypt seedlings.

Is the invasiveness of P. undulatum due to the storing and emission of secondary metabolites?

Our data support the hypothesis that the high invasiveness of *P. undulatum* may be due to the storing and emission of secondary metabolites. Firstly, different polyphenol classes were found in the *Eucalyptus* spp. and *P. undulatum* extracts. Both species present myricetin derivatives, however eucalypt extracts are richer in gallo- and ellagitannins, with the ellagic acid derivatives being the most abundant compounds. Tannin biosynthesis is induced by the interactions between plants and herbivores (Hoste et al. 2006; Chen et al. 2018), as well as in response to environmental stresses, since tannins may also play antioxidant actions (Williams et al. 2014). By contrast, *P. undulatum* extracts are characterised by higher contents of hydroxycinnamic acid derivatives, with caffeic acid derivatives as the most abundant compounds, in agreement with findings of Nunes et al. (2014). The role of caffeic acid in cell plants may be linked to their functional against abiotic stresses (Riaz et al. 2018). Indeed, caffeic acid is utilized by plants to synthesise lignin to increase the thickness of cell walls as a response to salinity (Klein et al. 2015), drought stress (Riaz et al. 2018) and intense light (Król et al. 2014). Furthermore, caffeic acid derivatives have a stable structure to trap free radicals, thus playing an antioxidant role against reactive oxygen species (ROS), creating (dos Santos et al. 2011; Khan et al. 2016).

Here, the forest invaded by *P. undulatum* had a higher percentage of monoterpenes, such as α- and β- pinene, α-thujene and d-limonene than the uninvaded forests. These compounds play an important role in plants to counteract the production of ROS in response to drought (Bertin and Staudt 1996) or to improve thermo-tolerance under heat waves (Loreto et al. 1998; Llusià et al. 2005; Tian et al. 2020). By contrast, 1,8-cineole and terpinyl acetate concentrations tended to higher in the areas of natural, remnant vegetation than in the areas invaded by *P. undulatum*, although this difference was not statistically significant. It is important to note that 1,8-cineole is one

of the main components of *Eucalyptus* spp. terpenic profile (Keszei et al. 2010): around 95% for *E. polybractea* (King et al. 2004); 80% for *E. rubida* (Lucia et al. 2012); 70% for *E. polyanthemos* (Lee and Shibamoto 2001); 40% for *E. ovata* (Elaissi et al. 2011); 20% for *E. goniocalyx* (Menut et al. 1995). This monoterpene has been linked to a lower efficiency in thermal protection (Copolovici et al. 2005), while its principal role is regulating plant-insect interactions such as attracting pollinators (Boncan et al. 2020), repelling herbivores (War et al. 2012), and providing antimicrobial properties (Su et al. 2008; Elaissi et al. 2011). Monoterpenes play an important role in the defence against abiotic and biotic stresses: thermo tolerance (Atkinson and Arey 2003; Jardine et al. 2020), high light tolerance (Holopainen et al. 2013), attracting natural enemies of herbivores (Sánchez-Osorio et al. 2019) and attracting pollinators (McFrederick et al. 2008). Regarding the SQTs content, it is possible to observe a different trend: all the identified sesquiterpenes are more abundant in the remnant *Eucalyptus* forest (R) than in the forest invaded by *P. undulatum* (I). The only sesquiterpene more abundant in the I is calamenene, a bicyclic sesquiterpene typical of *P. undulatum*. Indeed in the terpenic profile of its leaves the content of this compound is around 40% (Medeiros et al., 2003). Several studies reported that the sesquiterpene roles are related to plant biotic interactions (Köllner et al. 2008; Ashour et al. 2010; Holopainen et al. 2013; Raguso 2016; Chiu et al. 2017).

The differences in the terpene concentrations, together with the higher content of saponins and tannins in *Eucalyptus* spp. leaves, could explain the hypothesis that eucalypts invest more carbon in defence mechanisms against herbivore attack, while *P. undulatum* invests in secondary metabolites that may increase its tolerance against abiotic stresses, such as drought and heat. Indeed, Gleadow and Rowan (1982) have shown that *P. undulatum* is drought tolerant rather than drought avoiding, since severely wilted leaves of seedlings of *P. undulatum* can regain their turgidity after rewatering, while this was not observed in that study for *Eucalyptus viminalis* seedlings. Many species of *Eucalyptus*, including *E. ovata* used here, are able to recover quickly after fire from epicormic buds. *P. undulatum*, can also do this, but only when the fires are relatively low in temperature. It is possible with the increase in fire

frequency and intensity associated with climate change in south-eastern Australia, the balance may change to favour of *Eucalyptus* again.

In conclusion, our study shows that the invasiveness of *P. undulatum* may be due to the biosynthesis of secondary metabolites that increase its tolerance against abiotic stresses rather than the release of allelopathic compounds. On the other hand, the secondary metabolites identified in *Eucalyptus* spp. suggest a different strategy of this species, mainly focused on the prevention of herbivores attacks by producing repellent and low-digestible compounds. Additionally, the characterisation of the BVOCs present under canopy at environmental level allowed to obtain a broader understanding of the terpenes emitted in the air instead of single measurements of each plant.

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Authors' contributions

Dalila Pasquini, Cecilia Brunetti and Roslyn Gleadow contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dalila Pasquini and Luana Beatriz Dos Santos Nascimento. The first draft of the manuscript was written by Dalila Pasquini and all authors contributed to the final version of the manuscript. All authors have read and approved the final manuscript.

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Supplementary material

Appendix C

Supplementary Material

Supplementary Figures and Tables

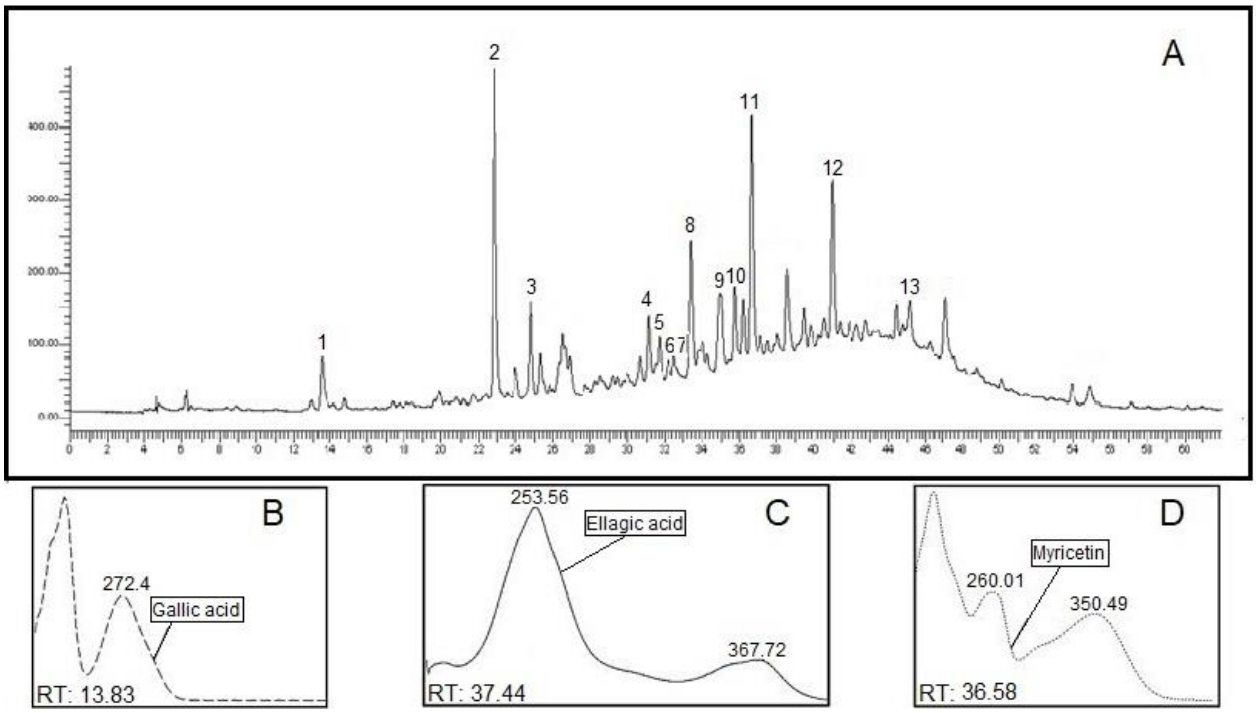

Figure S1. A: representative HPLC-DAD chromatogram (at 280 nm) of ethanolic extracts of *Eucalyptus* green leaves. The numbers indicate the major peaks: 1, 2, 3 and 4) gallic acid derivatives, 5, 7, 8, 10, 11 and 12) myricetin derivatives, 6, 9 and 13) ellagic acid derivatives. B, C and D: UV spectra of authentic standards (gallic acid, ellagic acid and myricetin) reported with their Retention Time (RT).

Table S2. List of the compounds detected in ethanolic extracts of green leaves of Eucalyptus spp. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S1A). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	13.87 ± 0.03	272	Gallic acid derivative
2	22.90 ± 0.12	273	Gallic acid derivative
3	24.86 ± 0.12	273	Gallic acid derivative
4	22.69 ± 0.02	272	Gallic acid derivative
5	26.71 ± 0.03	263-354	Myricetin derivative
6	31.13 ± 0.07	254-364	Ellagic acid derivative
7	31.72 ± 0.09	264-352	Myricetin derivative
8	32.27 ± 0.13	261-354	Myricetin derivative
9	32.55 ± 0.07	254-366	Ellagic acid derivative
10	33.45 ± 0.10	259-353	Myricetin derivative
11	35.02 ± 0.11	259-350	Myricetin derivative
12	36.67 ± 0.09	258-349	Myricetin derivative
13	41.00 ± 0.08	253-364	Ellagic acid derivative

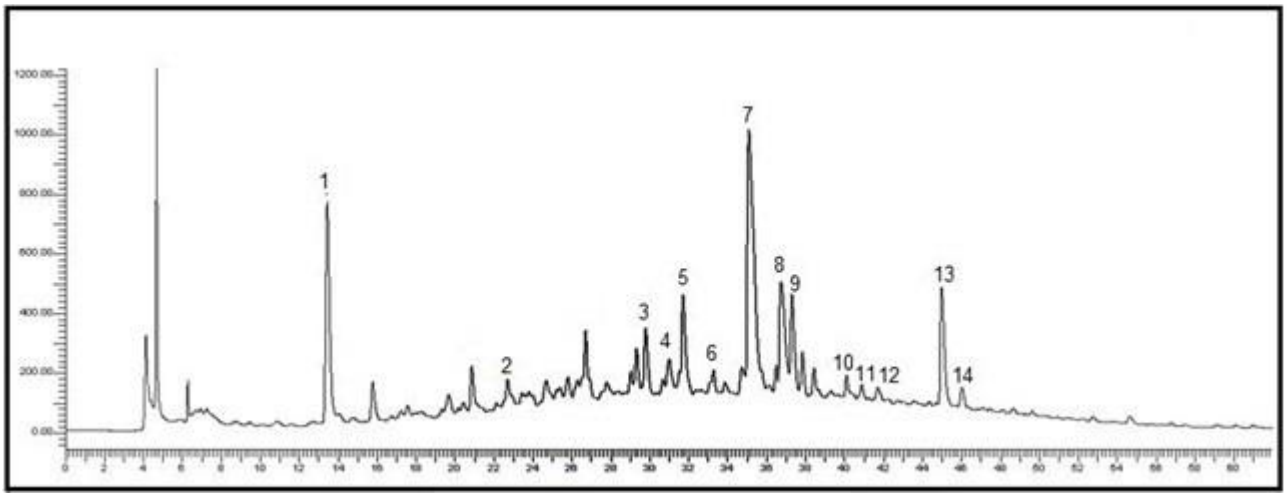

Figure S3. Representative HPLC-DAD chromatogram (at 280 nm) of ethanolic extract of *Eucalyptus* litter. The numbers indicate the major peaks: 1 and 2) gallic acid derivatives, 3, 4, 5, 7, 8, 10, 12, 13 and 14) ellagic acid derivatives, 6, 9 and 11) myricetin derivative.

Table S4. List of the compounds detected in ethanolic extracts of *Eucalyptus* spp. litter. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S3). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	13.43 ± 0.03	272	Gallic acid derivative
2	22.69 ± 0.02	273	Gallic acid derivative
3	29.79 ± 0.03	253-363	Ellagic acid derivative
4	31.00 ± 0.03	255-368	Ellagic acid derivative
5	31.70 ± 0.02	251-367	Ellagic acid derivative
6	33.43 ± 0.3	259-358	Myricetin derivative
7	35.07 ± 0.02	251-366	Ellagic acid derivative
8	36.73 ± 0.04	252-368	Ellagic acid derivative
9	37.30 ± 0.02	260-355	Myricetin derivative
11	40.09 ± 0.02	250-366	Ellagic acid derivative
11	40.87 ± 0.02	260-356	Myricetin derivative
12	41.69 ± 0.02	251-367	Ellagic acid derivative
13	44.97 ± 0.04	252-369	Ellagic acid derivative
14	46.01 ± 0.02	251-369	Ellagic acid derivative

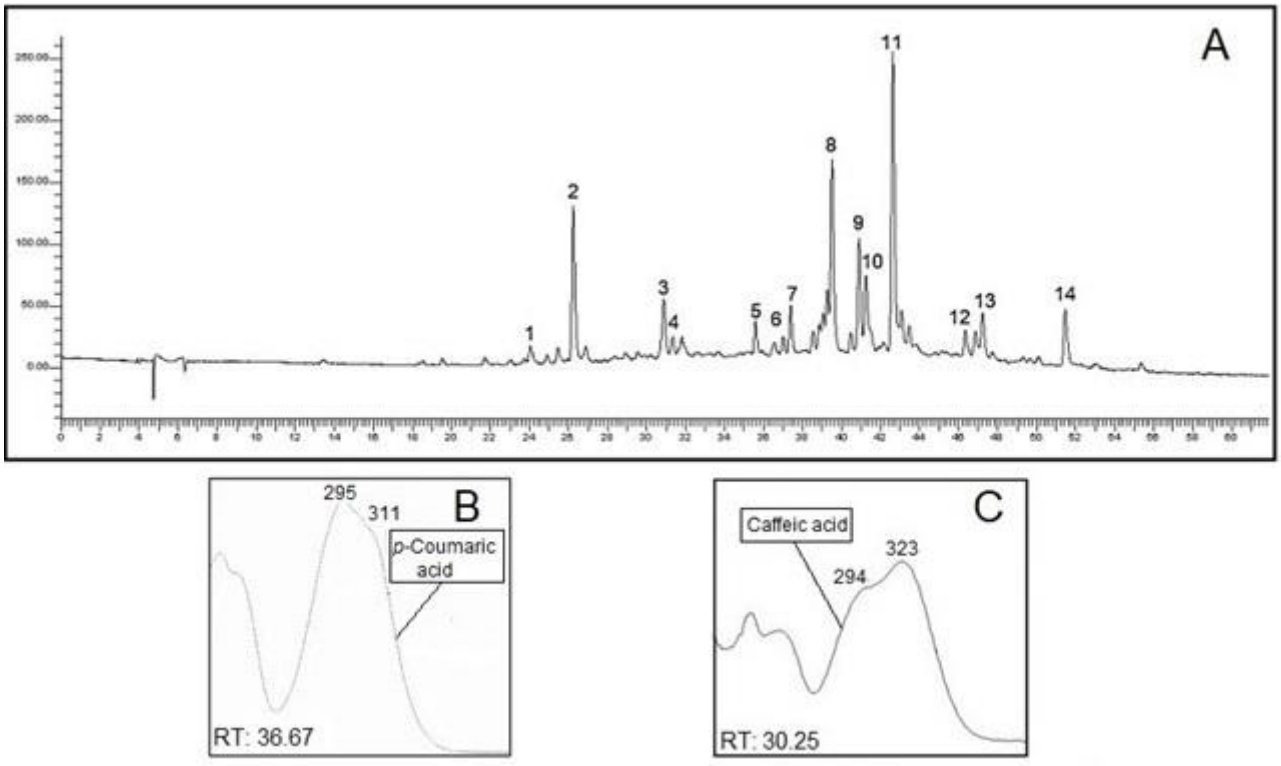

Figure S5. A: representative HPLC-DAD chromatogram (at 330 nm) of ethanolic extracts od *Pittosporum undulatum* green leaves. The numbers indicate the major peaks: 1, 2, 4, 8, 9, 10, 11, 12, 13 and 14) caffeic acid derivatives, 3) *p*-coumaric acid derivative, 5, 6 and 7) myricetin derivatives. B, C: UV spectra of authentic standards (*p*-coumaric and caffeic acid) reported with relative Retention Time (RT).).

Table S6. List of the compounds detected in ethanolic extracts of green leaves of *Pittosporum undulatum*. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S5A). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	23.60 ± 0.88	296-324	Caffeic acid derivative
2	25.72 ± 1.02	297-323	Caffeic acid derivative
3	30.16 ± 1.28	295-312	*p*-Coumaric acid derivative
4	30.70 ± 1.16	295-323	Caffeic acid derivative
5	35.76 ± 0.06	259-352	Myricetin derivative
6	37.53 ± 0.01	258-353	Myricetin derivative
7	39.42 ± 0.03	258-352	Myricetin derivative
8	38.71 ± 1.41	297-325	Caffeic acid derivative
9	40.09 ± 1.42	297-324	Caffeic acid derivative
10	40.44 ± 1.42	296-324	Caffeic acid derivative
11	41.84 ± 1.38	296-325	Caffeic acid derivative
12	45.82 ± 0.90	297-324	Caffeic acid derivative
13	46.37 ± 0.84	298-323	Caffeic acid derivative
14	51.31 ± 0.32	297-321	Caffeic acid derivative

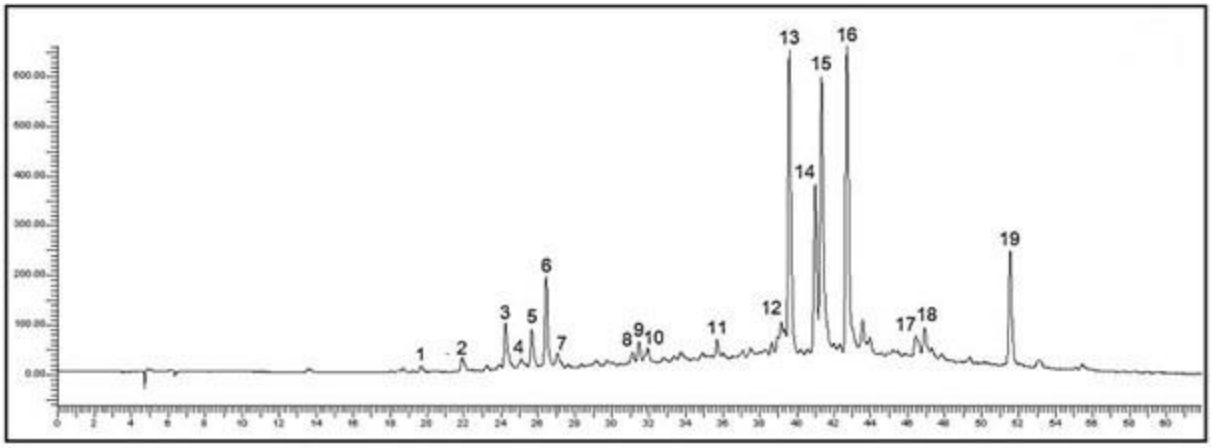

Figure S7. Representative HPLC-DAD chromatogram (at 330 nm) of ethanolic extracts of *Pittosporum undulatum* litter. The numbers indicate the major peaks: 1, 2, 3, 4, 5, 6, 7, 9, 10, 13, 14, 15, 16, 17, 18 and 19) caffeic acid derivatives, 8) *p*-coumaric acid derivative,11 and 12) myricetin derivatives.

Table S8. List of the compounds detected in ethanolic extracts of litter of *Pittosporum undulatum*. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S7). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	19.77 ± 0.03	296-325	Caffeic acid derivative
2	21.86 ± 0.04	294-324	Caffeic acid derivative
3	24.21 ± 0.03	297-324	Caffeic acid derivative
4	25.07 ± 0.03	294-326	Caffeic acid derivative
5	25.63 ± 0.04	295-325	Caffeic acid derivative
6	26.39 ± 0.04	297-323	Caffeic acid derivative
7	27.02 ± 0.04	297-325	Caffeic acid derivative
8	31.06 ± 0.05	292-313	*p*-Coumaric acid derivative
9	31.40 ± 0.04	296-324	Caffeic acid derivative
10	31.89 ± 0.04	295-325	Caffeic acid derivative
11	35.77 ± 0.03	259-350	Myricetin derivative
12	39.24 ± 0.02	259-350	Myricetin derivative
13	39.54 ± 0.05	297-325	Caffeic acid derivative
14	40.93 ± 0.05	296-327	Caffeic acid derivative
15	41.28 ± 0.05	298-326	Caffeic acid derivative
16	42.66 ± 0.05	298-326	Caffeic acid derivative
17	46.42 ± 0.08	297-325	Caffeic acid derivative
18	46.85 ± 0.05	296-323	Caffeic acid derivative
19	51.48 ± 0.05	298-326	Caffeic acid derivative

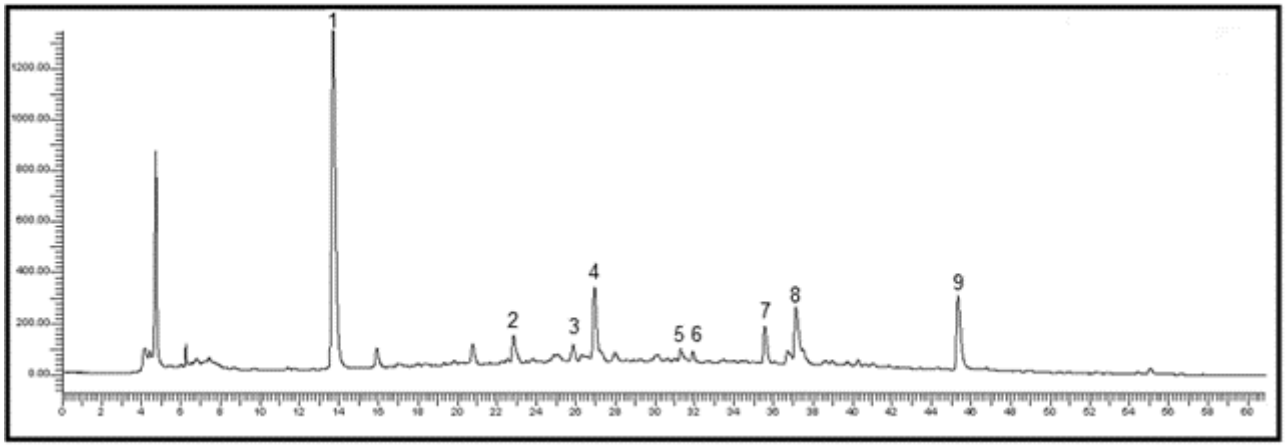

Figure S9. Representative HPLC-DAD chromatogram (at 280 nm) of aqueous extracts of *Eucalyptus* litter. The numbers indicate the major peaks: 1, 2 and 4) gallic acid derivatives, 3, 5, 6, 7, 8 and 9) ellagic acid derivatives.

Table S10. List of the compounds detected in aqueous extracts of litter of *Eucalyptus* spp. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S9). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	13.78 ± 0.08	272	Gallic acid derivative
2	22.99 ± 0.02	273	Gallic acid derivative
3	25.98 ± 0.03	258-369	Ellagic acid derivative
4	27.08 ± 0.02	273	Gallic acid derivative
5	31.43 ± 0.07	258-369	Ellagic acid derivative
6	31.88 ± 0.04	258-369	Ellagic acid derivative
7	35.66 ± 0.04	258-368	Ellagic acid derivative
8	37.23 ± 0.05	258-369	Ellagic acid derivative
9	45.42 ± 0.04	258-368	Ellagic acid derivative

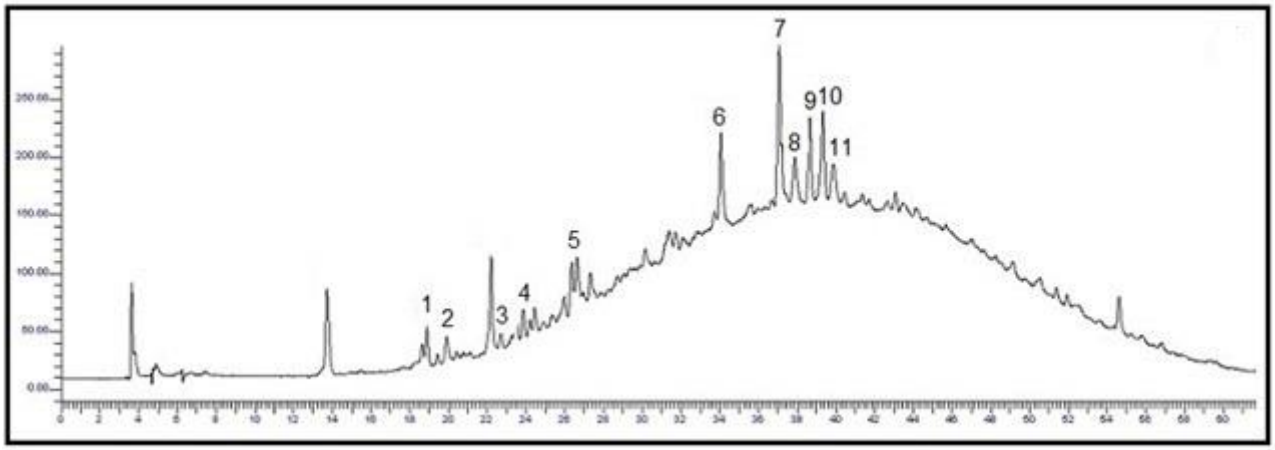

Figure S11. A**:** representative HPLC-DAD chromatogram (at 330 nm) of aqueous extracts of *P. undulatum*. The numbers indicate the major peaks: 1,2, 3, 4, 5, 8, 9, 10 and 11) caffeic acid derivatives, 6) myricetin derivatives, 7) *p*-coumaric acid derivative.

Table S12. List of the compounds detected in aqueous extracts of litter of *P. undulatum*. The putative identification of the compounds was based on the comparison of UV spectra and RT with authentic standards. The peak numbers (n. peak) correspond to the numbers reported in the chromatogram (Figure S11). Their RT and maximum wavelength (λ_{max}) are reported.

n. peak	RT (min)	λ_{max} (nm)	Putative identification
1	18.61 ± 0.02	291-319	Caffeic acid derivative
2	19.93 ± 0.02	294-323	Caffeic acid derivative
3	22.67 ± 0.05	290-319	Caffeic acid derivative
4	23.89 ± 0.04	290-321	Caffeic acid derivative
5	26.42 ± 0.02	290-318	Caffeic acid derivative
6	34.04 ± 0.05	262-352	Myricetin derivative
7	37.10 ± 0.04	293-312	*p*-Coumaric acid derivative
8	37.89 ± 0.02	292-325	Caffeic acid derivative
9	38.68 ± 0.03	294-321	Caffeic acid derivative
10	39.33 ± 0.02	290-321	Caffeic acid derivative
11	39.85 ± 0.02	293-323	Caffeic acid derivative

Appendix D – Published:

Polyphenols and terpenes in Mediterranean plants: an overview of their roles and possible applications.

Authors: **Pasquini D.**, Detti C., Ferrini F., Brunetti C., Gori A.

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Polyphenols and terpenes in Mediterranean plants: an overview of their roles and possible applications

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Abstract: The Mediterranean basin represents one of the key hotspots in terms of biodiversity and endemic floristic richness in the world (*i.e.*, a reservoir of plant biodiversity). With ongoing climate change, the Mediterranean vegetation is increasingly exposed to different sources of environmental stresses, such as drought, heat, and solar irradiance. To cope with these severe abiotic stresses, beside morpho-anatomical traits, Mediterranean endemic species enhance the production of secondary metabolites, especially terpenes and polyphenols. These compounds have different roles in plants. Terpene and polyphenol compounds play a key antioxidant function (quenching Reactive Oxygen Species) thus improving ozone and drought tolerance, while also acting as pollinator attractors and repellents for dangerous herbivorous insects (contributing to the taste and odour of different plant tissues). In addition to their roles in plants, these bioactive compounds provide multiple health-promoting benefits for humans. Indeed, they can be used in different types of industries, such as pharmaceutical, nutraceutical, green (as supplements to fossil fuel and insecticides) and cosmetic industries. In conclusion, these compounds may be considered as key innovative components in different technological domains.

Keywords: antioxidant activity; environmental stresses; human health; Mediterranean basin; pharmaceutical industry; secondary metabolites.

1. Introduction

Mediterranean plants are rich in secondary metabolites, particularly phenolics (Sardans and Peñuelas, 2013) and terpenes (Ormeño et al., 2007). Many of them have an important physiological and ecological role: improving ozone tolerance, protecting proteins and cell membranes from reactive oxygen species (ROS), quenching ROS (Andre et al., 2010; Di Ferdinando et al., 2014; Griesser et al., 2015), photo-protecting against UV-radiation (Edreva et al., 2008), providing protection against herbivores as well as attracting pollinators (Holopainen et al., 2013).

Secondary metabolite biosynthesis is stimulated by abiotic stress intensification, such as the increase in temperatures and drought waves imposed by global climate change to Mediterranean regions (Akula and Ravishankar, 2011; Tattini and Loreto, 2014). Mediterranean plant species, especially woody evergreens, make a high investment of fresh assimilated carbon in their biosynthesis during the dry season (Di Ferdinando et al., 2013). Recently, Gori et al. (2020) have reported a significant seasonal

and diurnal variation of polyphenol amounts in leaves of several Mediterranean endemic species. These results show that the content of polyphenols presents a monthly variation, with higher amounts in summer rather than spring and autumn, and a clear daily trend, with higher concentrations at midday. This could explain their protective role against UV and drought stress (Agati et al., 2002; Barnes et al., 2016). A similar seasonal trend was reported by Llusià and Peñuelas (2000) for terpenes. Terpene emissions in Mediterranean woody species are prevalently linked with changes in temperature, the maximum emission rate in spring for the least volatile monoterpenes and in summer for the most volatile monoterpenes (Llusià and Peñuelas, 2000). In addition, Tattini et al., (2015) showed an interesting daily orchestration of the different components of the antioxidant machinery of *Platanus* × *acerifolia* in drought-stressed leaves during summer. It was suggested that a higher isoprene emission early in the afternoon, when the decline in sunlight irradiance is coupled with an increasing air temperature, may complement the antioxidant functions of carotenoids and improve membrane stability.

These organic compounds, in addition to providing the abovementioned benefits to plants, have gained scientific attention for their promising effects on human health. In the last decades, several studies have highlighted the potential applications of secondary metabolites, in particular polyphenols, in pharmacology due to their anti-inflammatory, antioxidant and neuroprotective activities (Finley, 2004; Paduch et al., 2007; Gosslau et al., 2011; Gori et al., 2016). In addition, the link between a polyphenol-based diet and decreased risks of cancer (Soto-Vaca et al., 2012; Hardman, 2014) has been demonstrated as well as their healing effects in chronic pain therapy (Guimarães et al., 2014). Terpenes, instead, are mainly used as natural bio-pesticides and herbicides (Smith et al., 2018).

The main aim of this review is to describe the roles of polyphenols and terpenes in plants and their possible applications for health and human life (Figure 1). Many studies report multiple applications of

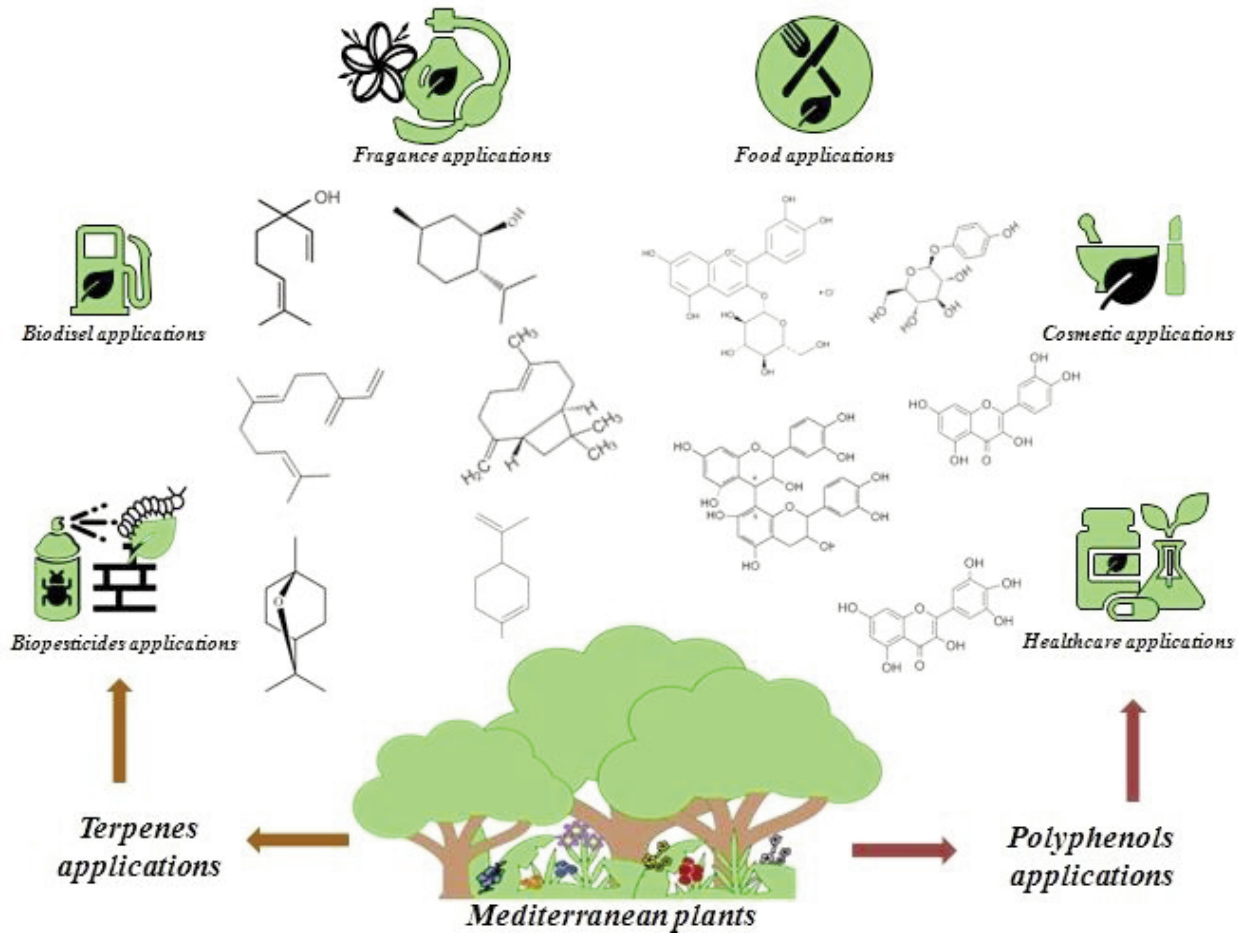

Figure 1. Roles of polyphenols and terpenes in plants and their possible applications for health and human life.

polyphenol enriched extracts obtained from Mediterranean plants; however, the studies are focused on potential applications of terpenes referred to pure isolated molecules. Indeed, most of the reports are mainly focused on aromatic plants utilised for the food and fragrance sectors (Li et al., 2017; Kutyna and Borneman, 2018). Therefore, although several studies report the richness of terpene emissions from Mediterranean woody plants (Loreto et al., 1998; Llusià, and Peñuelasa, 2000; Staudt et al., 2001; Bonn et al., 2019; Bach et al., 2020), only a few of them are related to the possible industrial applications of terpene leaf extracts of Mediterranean woody species.

Thus, given the high importance of these secondary metabolites, it could be crucial to focus on polyphenols and terpenes extracted from Mediterranean plants and their potential health benefits and industrial applications.

2. Polyphenols and their roles in plants

The polyphenols group is the biggest and most variable group of plant secondary metabolites (Scalbert and Williamson, 2000). These compounds can be classified into flavonoids and non-flavonoids (phenolic acids, stilbenes, lignans) (Belščak-Cvitanović et al., 2018). All flavonoids share the basic structural skeleton (C6-C3-C6), consisting of two aromatic rings (ring A and B) and a heterocyclic ring (ring C) that usually contains one oxygen atom (pyran). Due to the hydroxylation pattern and variations of the aromatic rings, flavonoids can be further divided into different subclasses: flavones, flavonols, flavanones, isoflavones, flavan-3-ols and anthocyanidins (Saxena et al., 2012) (Figure 2). In addition, within the flavonoids group, tannins are classified into two main groups: hydrolysable tannins (gallotannins and ellagitannins) and condensed tannins (also called proanthocyanins) (Porter, 1989; Romani et al., 2006; Romani et al., 2012).

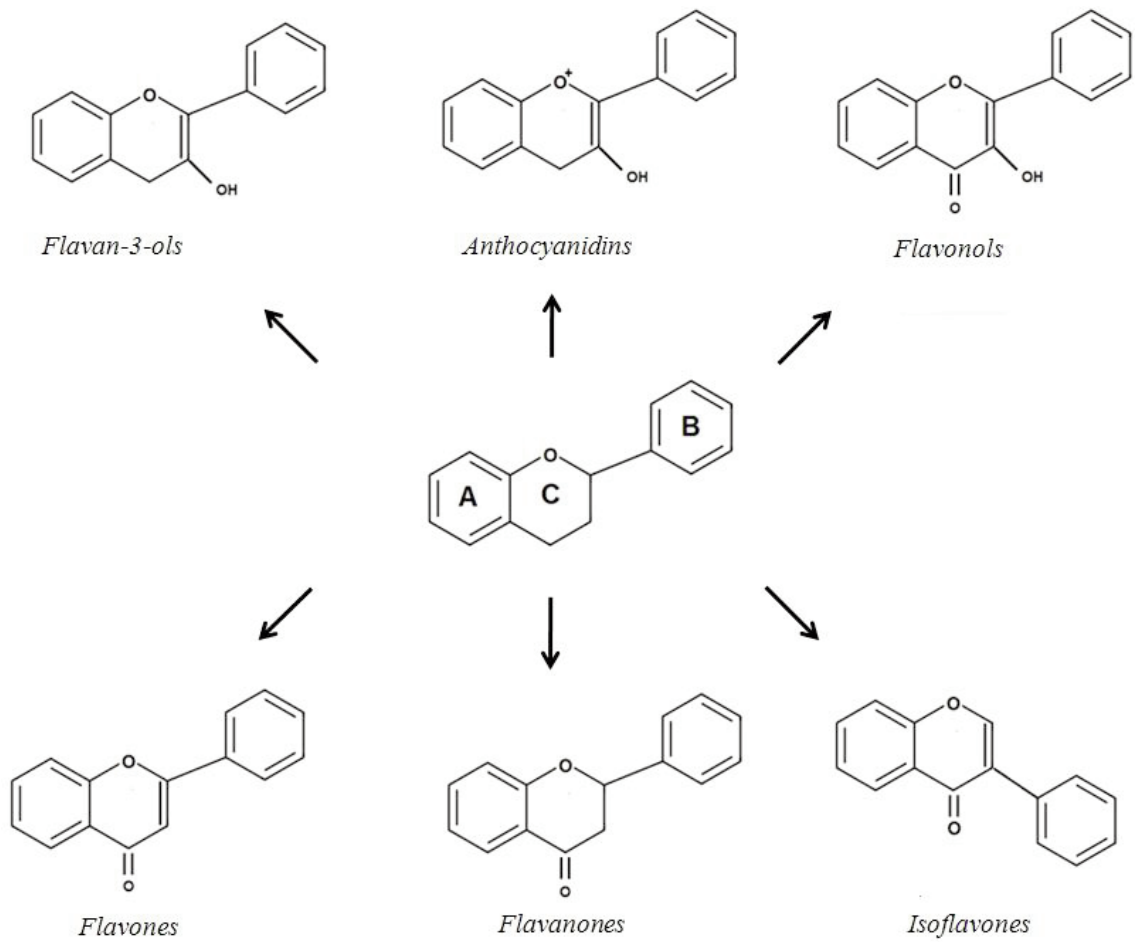

Figure 2: The basic structure of flavonoid subclasses.

Among non-flavonoid compounds, the main group is represented by phenolic acids that can be subdivided into two main types: benzoic acid, constituted by a benzene ring linked to a carboxyl group (C6-C1) and cinnamic acids, derived from a benzene ring linked to a three-carbon side acid chain (C6–C3) (Tsao et al., 2010; Singla et al., 2018).

Polyphenols exhibit a large variety of roles in plants, especially related to growth and development including seed germination and cell division (Tanase et al., 2019). In addition, these compounds act as the major yellow, red, blue, and purple pigments and may act as attractors for pollinators (Crozier et al., 2008; Vinha et al., 2018). Anthocyanins, for example, are water-soluble, are stored in vacuoles and are responsible for the orange to blue colours found in many flowers, leaves, fruits, seeds and other tissues (Tanaka et al., 2008), whereas tannins provide protection against pathogen infection and predator attack (Sieniawska et al., 2017) by conferring bitterness and astringency (Acamovic et al., 2005; Soares et al., 2020). Polyphenols are utilised for many functions within the plant cell acting mainly as defence and protecting molecules against abiotic stresses (Sharma et al., 2019). These compounds play a key role in maintaining the redox balance due to photo-oxidative stress (Caverzan et al., 2016), avoiding the generation of ROS (Reactive Oxygen Species) and quenching ROS, including singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), once they are formed (Rice-Evans et al., 1997; Agati and Tattini, 2010; Brunetti et al., 2013). For example, vacuolar flavonoids (in conjunction with guaiacol peroxidases) may help in maintaining whole-cell H_2O_2 within a sub-lethal concentration range (Ferreres et al., 2011).

In addition, polyphenols with their high absorptivity at 250-270 and 335-360 nm act as good UV screeners not only of UV-radiation but also of short-wave visible radiation (e.g., ellagitannins and anthocyanins, Manetas, 2006; Tattini et al., 2007; Hatier and Gould, 2008). The ability to absorb the solar shortest wavelengths is displayed by most polyphenols and it is not a peculiar capacity of hydroxycinnamates and flavonoids (which are well known UV-B and UV-A absorbers, Harborne and Williams, 2000). UV-absorbing flavonoids located in the epidermal cells strongly reduce highly energetic solar wavelengths from reaching ROS-generating cells, and the consequential photo-oxidative stress and damage (Brunetti et al., 2015; Guidi et al., 2016). Furthermore, another important role that they have is the ability to provide heavy metal stress protection by chelation of transition metals (i.e., Fe, Cu, Ni, Zn), which generate the hydroxyl radical via Fenton's reaction (Mira et al., 2002). Kidd et al., (2001) revealed that the chelation of these metals in the soil may be an effective form of defence against the effects of heavy metal concentration toxicity.

2.1. Plant polyphenols and their potential applications

Studies on plant secondary metabolites, especially on polyphenols, have increased over the last decades and have shown that these molecules show an antioxidant function in plants and have been suggested to perform the same reducing functions in humans (Brunetti et al., 2013). As a consequence, in the last few years, there is a renewed interest in medicinal plant-based products and, in this context, many Mediterranean phenolic-rich species may represent unrivalled sources for nutraceutical, cosmetic and pharmacological industries (Gurib-Fakim, 2014).

2.1.1. Polyphenol applications for human healthcare

In recent years, polyphenols have been reported to play key roles as signalling molecules in mammals through their ability to interact with a wide range of receptors and enzymes, which, in turn, are responsible for mediating ROS-induced signalling cascades vital to cell growth and differentiation (Scalbert et al., 2005; Brunetti et al., 2013). Furthermore, some flavonoids (such as quercetin and myricetin derivatives) have been proven to form hydrogen bonds with the Ser212 residue of MAPKs (mitogen-activated protein kinase) through the 3′-OH group, thus showing the capacity to inhibit their activity (Lee et al., 2008). MAPKs can control the expression of antioxidant enzymes, inhibiting cell cycle progression and cell proliferation and the expression and functional activation of oncogenes (Hu and Kong, 2004). Indeed, polyphenols have been shown to modulate cellular signalling processes dur-

ing inflammation, blocking carcinogenesis, and inhibiting tumour growth in both in vitro and in vivo tests (Benvenuto et al., 2016; Clementino et al., 2017). These capabilities, together with an apoptosis-inducing effect, appear to be mostly due to the polyphenolic non-scavenging and antioxidant roles, suggesting their preeminent function in chemoprevention and chemoprotection (Piccolella et al., 2015).

Mediterranean wild species have a long history in ethnic and popular medicine for their health-promoting properties. For example, the medicinal benefit of *Cistus x incanus* L. has been known since the 4th century BC. The leaf extracts of this species were used for treating skin diseases and gastric problems (Riehle et al., 2014). Furthermore, polyphenol-containing aqueous extracts of its leaves have been shown to have anti-bacterial, anti-inflammatory, antioxidant and anti-mycotic effects (Wittpahl et al., 2015; Gori *et al*., 2016; D'Ambrosio et al*.,* 2020). In addition, the CYSTUS052 aqueous extract derived from *C.* × *incanus* aerial parts has shown to have a potent anti-influenza virus activity in mice (Droebner et al., 2007) and to inhibit human immune deficiency virus (HIV) in vitro (Rebensburb et al., 2016). Another example concerns *Myrtus communis* L. whose leaf extracts are indicated against diseases affecting the respiratory system such as emphysema or bronchitis (Romani et al., 2012; Jabri et al., 2018). Furthermore, some authors reported the importance of the different compounds found in myrtle extracts, including berry and seed polyphenol extracts, that are used to treat various disorders, such as gastric ulcers and esophageal reflux (Sumbul et al*.,* 2010; Jabri et al*.,* 2016). In a recent study conducted by Ebrahimi (2020), the authors tested the anti-hemorrhagic activity of myrtle leaf extracts that could be attributed also to tannins present in the polyphenolic aqueous leaf extract. Finally, the anti-inflammatory properties of pulp and seed extracts of myrtle were proven in human skin fibroblasts exposed to oxidative stress (Cruciani et al., 2019).

There is a growing interest in plant extracts that could be used as an alternative to current antimicrobial agents with increasing antimicrobial resistance (Ferreira et al., 2012). In this sense, the importance of phenolic compounds and their antimicrobial activity has been well-documented (Cushnie et al., 2003). Some antimicrobial constituents such as tannins, flavonoids and other phenolic acids have been detected in leaf extracts of *Arbutus unedo* L., including arbutin (Tenuta et al., 2020). Indeed, in one recent study, the antimicrobial activity of arbutin and its metabolite hydroquinone against several uropathogenic strains was demonstrated (Jurica et al., 2017). Results of this study showed that the aqueous extract of *A. unedo* leaves could be used as a phyto-therapeutic agent in many clinical applications. Another Mediterranean species that was shown to have antimicrobial properties against a broad range of bacterial and fungal pathogens is the mastic tree, *Pistacia lentiscus* L. (Salhi et al., 2019). Its leaves, rich in polyphenols (representing 7.5% leaf dry weight), including phenolics acids, flavonoids, mostly myricetin derivatives, and hydrolysable tannins (Rodríguez-Pérez et al. 2013; Detti et al., 2020), were shown to possess also anticancer and anti-inflammatory activities. In fact, Remila et al. (2015) demonstrated that the extracts of this species have an anticancer potential against melanoma (B16F10). In addition, Mehenni et al. (2016) demonstrated the hepatoprotective activity against paracetamol-induced hepatic necrosis and the antidiabetic activity against streptozotocin-induced in rats.

2.1.2. Nutraceutical and food industry applications

In the last decades, studies regarding the physiological activities of food-derived bioproducts and food components with functional properties have greatly increased (Erdmann et al., 2008). In fact, the food sector is currently focused in developing functional food additives and food products with improved health promoting benefits (Hernández-Ledesma et al., 2017). Indeed, foods containing bioactive compounds are important for diminishing of risks of important chronic diseases that are becoming the leading causes of global morbidity and mortality (Annuzzi et al., 2014). In this context, polyphenol plant extracts with their properties can be one of the most interesting candidates (Vattem and Maitin, 2015). In fact, after a microencapsulation process, they can be utilised as functional additives in foods (Nazzaro et al., 2012). For example, the enrichment of dairy food products has been recently investigated (Cutrim et al., 2018). The polyphenolic rich aqueous extract of *Rosmarinus officinalis* L., for exam-

ple, was added as a functional ingredient in cottage cheese. The incorporation of the extract increased the antioxidant properties of the cheese. In addition, the nutritional value of cottage cheese was not affected by the introduction of the extract in free and microencapsulated forms (Ribeiro et al., 2016). Moreover, Petrotos et al*.,* (2012) investigated the influence of the application of polyphenolic compounds from olive mill wastewater to yoghurt. In this study, it was observed that the added polyphenols provided a protective effect that avoided an unwanted drop in the pH during storage.

There is an increasing interest in polyphenol plant extracts as potential functional ingredients for improving health in bakery products (Ou et al., 2019; Xu et al., 2019). Cacak-Pietrzak (2019) tested *Cistus x incanus* herbal extracts as functional supplement of food products in bread. The bread sample incorporated with the extract was characterised by significantly higher total polyphenolic concentration, and much higher antioxidant activity, when compared to a control bread sample. Bread with increased antioxidant activity should be consumed because of its role in the prevention and treatment of various chronic and degenerative human diseases (Dziki et al., 2014).

The addition of antioxidants is known to delay or inhibit deterioration of food products and benefit consumers by prolonging their shelf life (Lindley, 1998). Besides, antioxidants also have antimicrobial activities (Babuskin et al., 2014). Primary synthetic antioxidants have been used in the food industry over the last 50 years. Nevertheless, despite their superior efficacy and high stability, there is an increasing concern about their safety because they can have toxigenic, mutagenic and carcinogenic effects (Nanditha and Prabhasankar, 2008). According to Ramarathnam *et al*. (1995) and Kiokias *et al*. (2008), the current trend for consumers requires the utilization of natural antioxidants in the food industry since these compounds are healthier than chemical food preservatives. Galanakis et al. (2018) showed that polyphenols from olive, in a concentration of 200 mg/Kg flour, were able to induce antimicrobial properties in bakery products and subsequently prolong their shelf-life.

Finally, polyphenol plant extracts can also be utilised as colorant agents. In the case of colorants, anthocyanins represent an attractive and natural alternative to the artificial ones (Espín et al., 2000). For example, *Arbutus unedo* fruits, rich in anthocyanins (and in particular of cyaniding 3-*O*-glucoside), were studied to be used as natural colorants with bioactive properties (such as antioxidant, antimicrobial and cytotoxic effects) in wafers (Lopez et al., 2019). Results of this study showed that the polyphenolic extract provided colorant properties without altering the main organoleptic characteristics of the food sample, demonstrating the potential uses of *A. unedo* fruit extracts for industrial applications (Lopez et al., 2019).

2.1.3. Applications in cosmetic industry

The interest in cosmetics prepared with natural products rich in polyphenols is mainly based on the antioxidant action of these compounds against oxidative stress (de Lima Cherubim et al., 2020). In particular, polyphenols have been shown to have a protective function against photoaging (Zillich et al., 2015) and polyphenolic rich extracts are proposed as one of the most effective functional raw material for the production of antiaging cosmetic products (Ratz-Lyko et al., 2012). Indeed, photoaging is caused by overexposure to UV radiations, which increases the production of reactive oxygen species (ROS), causing lipid peroxidation, DNA damage, and protein alterations (Rittié and Fisher, 2002). Moreover, ROS can also contribute to skin ageing by direct activation of enzymes that are responsible for the cleavage of extracellular matrix (ECM) components (Mukherjee et al., 2011). Phenolic compounds can absorb ultraviolet radiation, avoiding penetration of the solar radiation into the skin, and allowing a proven decrease in free radical formation and consequently preventing DNA damage (Petruk et al., 2018). Moreover, it has been shown that the post-treatment of human epidermal keratinocytes after UV exposition with plant polyphenols (such as resveratrol, quercetin and verbascoside) was effective against the overproduction of peroxides and inflammatory mediators (Potapovich et al., 2013). These studies suggested that polyphenolic extracts can be useful ingredients for both sunscreens and after-sun cosmetic products (Zillich et al., 2015; de Lima Cherubim et al., 2020). In this context, some

Mediterranean shrub species such as *Cistus incanus* L. and *Cistus ladanifer* L., naturally rich in bioactive polyphenols, represent a potential source of ingredients for skin protecting cosmetics (Kubica et al., 2016). For example, Gaweł-Bęben (2020) showed that *C. incanus* polyphenol leaf extracts are effective tyrosinase inhibitors (30–70% inhibition at 100 μg/mL). Tyrosinase is a copper containing enzyme catalysing the rate limiting conversion of L-tyrosine to dihydroxyphenylalanine (L-DOPA) and subsequently to dopaquinone. Tyrosinase inhibitory activity is particularly interesting for cosmetic applications due to the increasing problem of hyperpigmentation disorders and thanks to the growing consumer demand for safe and effective skin lightening cosmetics (Pillaiyar et al., 2017). In addition, another Mediterranean wild species, *Pistacia lentiscus* L. has been shown to be a potent elastase inhibitor (74% of elastase inhibitory activity tested at 50 μg/mL) (Chiocchio et al., 2018). Elastase belongs to the chymotrypsin family of proteases and it is responsible for the breakdown of elastin and other proteins, such as collagen and fibronectin, which are fundamental for the elastic properties of ECM (Imokawa and Ishida, 2015). Mis-regulations of this enzyme are involved in skin ageing processes (Korkmaz et al., 2010). In fact, the excessive hydrolysis of the dermal elastin fibre network can lead to the loss of skin elasticity and consequent skin sagging (Thring et al., 2009). For this reason, elastase inhibitors are important for their anti-wrinkle activities, promoting the preservation of skin elasticity (Jadoon et al., 2015).

Finally, polyphenol extracts are important in the cosmetic industry for their antimicrobial activity (Panzella, 2020). Indeed, these molecules can be used to minimise the deterioration caused by microorganisms in order to keep the microbiological purity of cosmetic products during the manufacturing process and also to extend their shelf-life to ensure consumer safety (Herman, 2019; Mellou et al., 2019).

Table 1 summarises all the information about polyphenol compounds present in the plants described in this study. Furthermore, the principal functions carried out by their principal compounds are also included, and the possible applications in different types of industry.

Table 1. List of the main polyphenols extracted from Mediterranean plants and their potential industrial and healthcare applications.

Compounds	Species	Functions	Applications	References
Gallic acid Myricetin derivatives Quercetin derivatives	*Pistacia lentiscus*	Anticancer Elastase inhibitors	Human Healthcare Cosmetic industry	Remila *et al.,* 2015 Chiocchio *et al.,* 2018
Hydrolyzable tannins	*Myrtus communis*	Antiradical activity Anti-hemorrhagic	Human Healthcare	Romani *et al*., 2012 Ebrahimi *et al*., 2020
Arbutin Cyanidin-3-*O*-glucoside	*Arbutus unedo*	Anti-microbial Coloring agent	Cosmetic industry Food industry	Tenuta *et al.,* 2020, Jurica *et al.,*2017
Myricetin derivatives Quercetin derivatives Epigallocatechin gallate Epicatechin	*Cistus incanus*	Anti-inflammatory Antioxydants Tyrosinase inhibitors	Human Healthcare Food Industry Cosmetic industry	D'Ambrosio *et al.,* 2020 Cacak-Pietrzak *et al.,* 2019

3. Terpenes and their roles in plants

Terpenes are a large class of organic compounds and include more than 50.000 molecules (Maimone and Baran, 2007; Mewalal et al., 2017; Keasling and Eiben, 2019). All terpenes are hydrocarbons derived from isoprene, a molecule with five atoms of carbon; for this reason, the terpenes, are classified according to isoprene units: hemiterpenes (C_5H_8), monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) (Ashour et al., 2010; Mewalal et al., 2017; Tetali, 2019) (Figure 3).

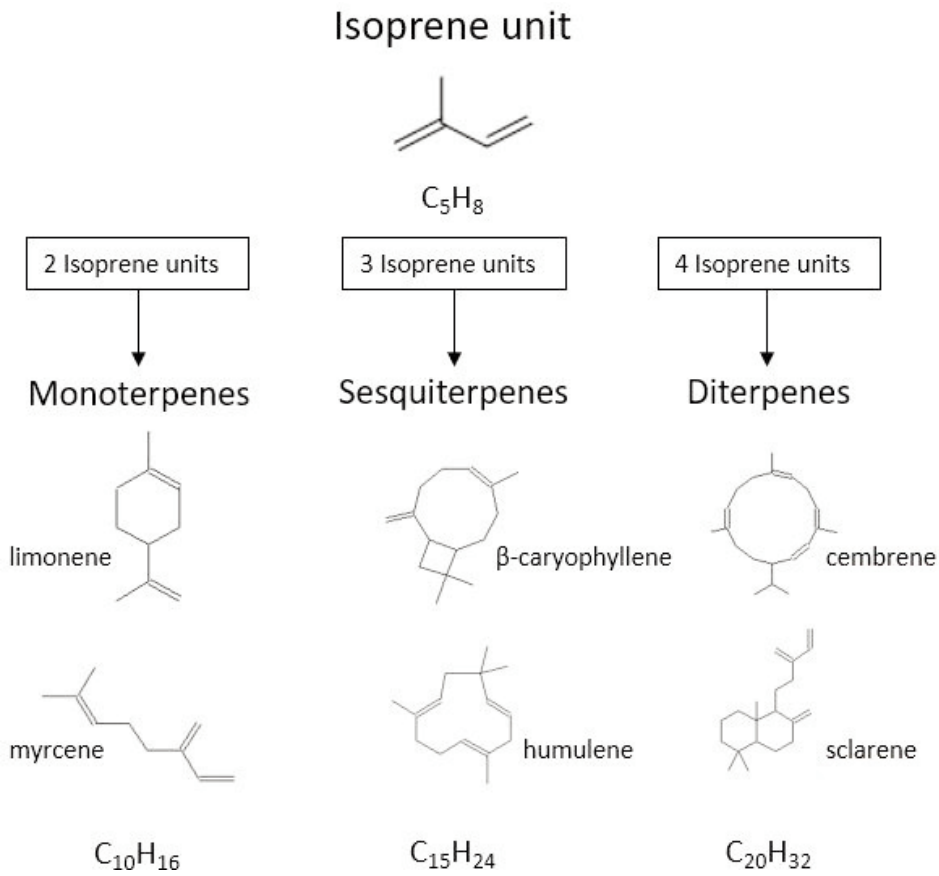

Figure 3. The molecular structure of isoprene and the three classes of terpenes (monoterpenes, sesquiterpenes and diterpenes) deriving from isoprene units with two examples per class.

The primary function of terpenes is their action as signal molecules: plant-plant for allelopathic purposes or to warn neighbouring plants of dangers (Baldwin et al., 2006; Polechońska et al., 2019) and plant-insects to attract pollinators or natural enemies of herbivorous insects (Dicke and Baldwin, 2010). Another important role is related to their antimicrobial and insecticidal activity, for this reason some Biogenic Volatile Organic Compounds (BVOCs) have been defined as "phytoncides" (*phyton* in ancient Greek is "plant", plus *cide*, which indicates "killing") (Antonelli et al., 2020). Their emission is linked with abiotic and biotic factors and stresses, such as: high temperature, UV, drought, fire, air pollution and competition, or herbivore attacks (Holopainen et al., 2013; Bonn et al., 2019). The emission mode of these volatile molecules depends on the species: some plants release terpenes directly after their biosynthesis such as *Quercus* spp. (Staudt et al., 2001), while other plants can accumulate them in the foliar tissues, using specific structures such as resin canals and ducts or surface trichomes (Ormeño et al., 2011).

Isoprene emission increases under abiotic stress conditions, such as drought and heat stress. Experimental research shows that isoprene emission can improve photosynthetic performance at high temperatures, stabilizing cells and particularly chloroplast thylakoid membranes (Sharkey et al., 2008; Velikova et al., 2012). Indeed, utilizing an inhibitor of isoprene biosynthesis (fosmidomycin), restored thermotolerance was observed when isoprene was supplied in the airstream flowing over the leaf (Sharkey et al., 2001). Further similar experiments led to the conclusion that thermotolerance of photosynthesis is a substantial benefit to plants that produce isoprene and that this benefit may explain why plants produce isoprene (Sharkey et al., 2008; Tattini et al., 2014; Tattini et al., 2015). In addition, isoprene improves plant tolerance to ozone by quenching reactive oxygen species (ROS) (Loreto et al., 2001; Holopainen et al., 2013). During the Mediterranean dry season,

stomatal closure is the main, early response of plants against drought to avoid tissue dehydration (Gallé et al., 2007), and consequently, a decrease of plant carbon balance (McDowell, 2011). Several authors showed that during drought stress, around 20-50% of the newest photosynthetically assimilated carbon is used to emit isoprene (Sharkey and Loreto, 1993; Brilli et al., 2007). This could be explained by considering the benefits that isoprene, and other secondary metabolites confer to plants (Sharkey et al., 2008; Velikova et al., 2012). In addition, to sustain isoprene production, plants can also use alternative carbon pools, such as xylem-transported glucose from root and stem storage, conferring more resistance against water stress (Genard-Zielinski et al., 2014).

Monoterpene emission allows the plant to reduce ROS induced damages and to improve ozone and thermo-tolerance (Atkinson and Arey, 2003; Jardine et al., 2020). Monoterpenes also play an important role against biotic stresses: to attract natural enemies of herbivores, to defend plants against pathogens and to deter herbivore animals (Sánchez-Osorio et al., 2019). In addition, they have a pivotal function as pollinator attractants and in limiting neighbouring plant's growth due to their allelopathic effects (Holopainen et al., 2013). Some examples of monoterpenes emitted by plants to lure the pollinators are linalool, β-ocimene and β-myrcene (McFrederick et al., 2008), whereas β-ocimene is a signal emitted to warn neighbouring plants of herbivore attacks (Blande et al., 2010).

Finally, sesquiterpenes act prevalently in plant biotic interactions, playing a repellent action against herbivores and neighbouring plants (Ashour et al., 2010; Holopainen et al., 2013). Furthermore, they can also attract pollinators (Raguso, 2016) or natural predators against herbivorous insects (Ashour et al., 2010; Chiu et al., 2017).

3.1. Plant terpenes and their potential applications

Mediterranean plant species produce a wide range of volatile and semi-volatile terpenes (Bonn et al., 2019; Bach et al., 2020). Some species, such as *Rosmarinus officinalis* L., *Cistus albidus* L. and *Pinus halepensis* Mill. (Llusià and Peñuelasa, 2000), store volatile terpenes in specific structures before releasing them to the atmosphere; others, such as *Quercus ilex* L. and *Quercus coccifera* L., release them directly after being synthesised (Loreto et al., 1998; Staudt et al., 2001). Several authors studied the variation of these emissions, for abiotic and biotic influences (Loreto and Schnitzler, 2010; Akula and Ravishankar, 2011) or seasonal trends (Llusià and Peñuelas, 2000; Steinbrecher et al., 2009), focusing mainly on the plant-plant (Baldwin et al., 2006) or plant-insect (Holopainen et al., 2013) interaction and their influences on air quality (Koistinen et al., 2007). Regarding potential applications of terpenes for human health and life, Mediterranean plants have been mainly exploited for the production of essential oils, which constitute an important part of traditional medicinal and food applications (Ali et al., 2015). For example, aromatic and medicinal plants like rosemary, lavender, thyme, and oregano have long been studied for the biological activity of their essential oils (Mancini et al., 2014; Ali et al., 2015). However, considering the growing interest in monoterpenes and sesquiterpenes applications for several industrial and medicinal uses (Tetali, 2019), here we report a summary of the main applications of these compounds even if they are not exclusively extracted from Mediterranean plants.

3.1.1. Terpenes application for human healthcare

Several natural products have been used by mankind as a source of medicinal products since ancient times for their disinfectant and preservative properties. In modern pharmaceutical industry, terpenes are used as active principles for drugs. In 2002, the worldwide sales of terpene-based pharmaceuticals were estimated to US$12 billion (Guimarães et al., 2014). The recent increasing interest in the clinical application of these compounds is due to the wide range of their biological properties, including cancer chemo-preventive effects, antimicrobial, antiviral, analgesic, anti-inflammatory, antifungal and antiparasitic activities (Paduch et al., 2007; Quintans et al., 2013; Rufino et al., 2014; Ma et al., 2015; Li et al., 2016). For example, α-pinene presents anti-inflammatory activity reducing the production of tumour necrosis factor-α (TNF-α) and interleukin-6, that have pro-inflammatory and anti-inflammatory

functions (Bae et al., 2012; Kim et al., 2015). Moreover, β-caryophyllene and α-phellandrene are used as tumour pre-treatments, causing a reduction of levels of interleukins and TNF-α (Cho et al., 2007; Siquerira et al., 2016). Another bicyclic monoterpene that shows anti-inflammatory effects is borneol (Zhong et al., 2014). Among monocyclic monoterpenes, D-limonene and p-cymene have been shown to reduce allergic lung inflammation in mice (Amorim et al., 2016; Games et al., 2016; Hansen et al., 2016). Regarding the acyclic monoterpenes, linalool inhibits acute lung inflammation by producing IL-6, IL-1β, IL-8, TNF-α and monocyte chemoattractant protein-1 (MCP-1), which are involved in neuroinflammatory processes and several diseases (Peana et al., 2002; Peana et al., 2003; Sabogal-Guáqueta et al., 2016; Kim et al., 2019). Additionally, after *in vitro* studies, 1,8 cineole (Kahn et al., 2014), D-limonene (Shin et al., 2020) and β-caryophyllene (Hu et al., 2017) show inhibitory actions against neuro-inflammation. A natural sesquiterpene with neuro-protective activity against Parkinson's disease is β-caryophyllene (Ojha et al., 2016) and this sesquiterpene shows reduction of alcohol-induced liver injury (Varga et al., 2018).

Several terpenes show antiparasitic functions, due to their interaction with Fe (II) groups, resulting in the release of free radicals that can kill parasites, as in the case of *Plasmodium falciparum* (Rodrigues Goulart et al., 2004). They present also antimicrobial functions linked to their lipophilic structure. For example, terpinen-4-ol, α-terpineol, 1,8-cyneol and linalool have antibacterial proprieties against Gram-positive and Gram-negative bacteria. Menthol is toxic for *Escherichia coli* (Trombetta et al., 2005), while (4R)-(+)-carvone shows important effects against *Listeria monocytogenes* and *Escherichia coli*, while also showing antifungal properties against *Saccharomycetes* (Carvalho and Fonseca, 2006).

The terpenes are also widely used in dermatology and cosmetology as vehicles, because they increase the therapeutic value of drugs, allowing an easier skin penetration through intercellular lipid disruption; thus, improving drug diffusion and action (Moghimi, 1996).

Recently, α-pinene, β-pinene, car-3-ene, borneol, verbenol, pinocarveol and linalool, when inhaled, have shown anti-depressive and anxiolytic functions (Linck et al., 2010; Souto-Maior et al., 2011; Kessler et al., 2014; Guzmán-Gutiérrez et al., 2015; Woo and Lee, 2020).

Some studies have shown the role of terpenes against neuronal diseases and tumours (Crowell, 1999; Legault and Pechette, 2007; Cheng et al., 2014; Sobral et al., 2014; Porres-Martinez et al., 2016). For example, borneol has a free radical scavenging activity, thus performing an important neuroprotective function against Alzheimer's disease (Hong et al., 2011; Liu et al.; 2015). Also, β-caryophyllene, myrcene, linalool, 1,8 cineole, α- and γ-terpinene, show neuroprotective functions thanks to their antioxidant effects (Calleja et al., 2013; Cheng et al., 2014), by decreasing the production of ROS, Matrix Metallo-Proteinase (MMP) and Nitric Oxide (NO) (Cutillas et al., 2018; de Christo Scherer et al., 2019). Terpenes have been used as chemotherapeutic agents for treating tumours. They present multiple mechanisms: during the initial phase of carcinogenesis, they prevent interaction of carcinogens with DNA; during the promotion phase, they inhibit cancer cells developing and migrating; in later stages, they allow cancer cell apoptosis (Crowell, 1997) and consequently, tumour regression (Vigushin et al., 1998). Some studies have shown that β-pinene and p-cymene have an acceptable chemotherapeutic potency (Li et al., 2009; Ferraz et al., 2013; Bakarnga-Via et al., 2014). Myrcene shows significant antiproliferative actions in various tumour cell lines such as breast carcinoma (MCF-7), human lung carcinoma (A549) and leukemia (P388) (Silva et al., 2007). One of the most important monoterpenes is D-limonene, well tolerated by humans (Vigushin et al., 1998; Kris-Etherton et al., 2002), it presents protective actions against several tumours: breast, intestine, pancreas, liver and colon by inhibiting the proliferation of cancer cells, thus allowing apoptosis (Lu, 2004).

The important benefits of volatile terpenes in the forest atmosphere for human health are evident in the recent study conducted by Cho et al. (2017), where the authors state that a short trip to the forest is beneficial for humans through showering of biogenic aerosols. This kind of healthy program has been practiced in the United States since 1960 as "forest recreation" (Douglass, 1982). In Germany there is a similar practice called "Kneipp therapy" created by the priest Sebastian Kneipp (Joos et al., 2006;

Spievogel and Spalek, 2012), while in Japan the Japanese Forest Agency introduced the "*Shinrin-yoku*" (*i.e.,* Forest bathing) in 1982, and established the "Therapeutic effects of forest plan" in 2005 (Tsunetsugu et al., 2010). A recent review suggested that even a 2 hour walk in the forest could increase Natural Killer (NK) cells acting against cancer and promoting health (Peterfalvi et al., 2019). It is worth mentioning that inhaling BVOCs in a forest setting does not provide constant effects, as there is a high degree of variability, associated to environmental and individual characteristics. A Japanese study, involving a small group of healthy subjects, measured monoterpene blood concentrations before and after a 60-min visit to the forest (Sumitomo et al., 2015). The authors noticed that blood concentrations of some BVOCs exhibited a marked increase after visiting the forest, and in particular, average plasma levels of α-pinene changed from 2.6 nM (baseline) to 19.4 nM (post- visit). In another toxicokinetic study, the participants were exposed for 2 hours in a chamber with different concentrations of limonene (10, 225, and 450 mg/m^3) and the pulmonary uptake was estimated to be up to 70% of the amount supplied, with an increase of plasma concentrations of limonene (Kohlert et al., 2000). Thus, for now, we can only affirm that plasma concentrations of BVOCs tend to rise whenever a subject is exposed to a forest, but it is not possible to quantify the specific beneficial effects for human health.

Additionally, in the last decades, other authors have studied the relationship between human well-being and indoor plants, showing that these plants can play an important role in reducing air pollutants and promoting health and comfort in our houses (Bringslimark et al., 2009; Deng and Deng, 2018). In particular, Yang et al. (2009) suggested that indoor plants can improve the quality of air both removing pollutants and releasing terpenes which have anxiolytic and anti-inflammatory effects.

Lastly, another new use of terpenes is as analgesic drugs. Pain can cause a loss of physical and mental functioning, thus a decrease in life quality and a significant economic damage for society (Guimaraes et al., 2014). Several clinical studies, conducted on human patients, showed an improvement in general health and increased quality of life using drugs with 10-40% of terpenoids (Guimaraes et al., 2014). For example, patients with several types of pain (*i.e.,* headaches, arthritis, muscular aches and back pain) found the use of a combination of natural oils rich in carvacrol, thymol, 1,8-cineol, limonene, α- and β-pinene and cineole very useful (Woolf, 2010; De Sousa, 2011; Guimaraes et al., 2013).

3.1.2. Further industrial application of plant terpenes

Plant-derived terpenes are identified as an alternative, low cost and sustainable source of energy, potentially capable of replacing or supplementing fossil fuel (Mewalal et al., 2017; Tetali, 2019), contributing to decreased CO_2 concentration in the atmosphere and thus climate change. Many qualities of terpenes are appreciated; among them their low hygroscopy, high viscosity, their freezing point, and their high energy density (Melis, 2017). Monoterpenes could be used to replace gasoline (Melis, 2017), while sesquiterpenes and diterpenes could be use as biodiesels (Tippmann et al., 2013). The cyclic forms are preferred because of their higher energy density (*i.e.,* by increasing combustion heat): for example, limonene could be added to diesel fuels (Tracy et al., 2009) and α-pinene could be used as a replacement for fossil fuels (Harvey et al., 2012). Nonetheless, from hydrogenated linalool, myrcene and farnesene (*i.e.,* acyclic terpenes) high-density fuels have also been synthesised (Mewalal et al., 2017). Furthermore, some terpenes are used to obtain adhesive, coating and emulsifier materials in the manufacturing industry (Ashour et al., 2010).

Terpenes and the essential oils containing them are widely used and studied for their applications as flavouring in food and beverages, and in the perfume industry (Ashour et al., 2010). In 2017, the worldwide flavour and fragrance industry was estimated to be of US$30 billion (Kutyna and Borneman, 2018). The food industry is interested in compounds with antioxidant characteristics, and with a good taste and aroma (Putnik et al., 2019). The most used terpenes are monoterpenes such as limonene, linalool and 1,8-cineole for lemon's aroma (Kutyna and Borneman, 2018); additionally, 3-carene, α-pinene, caryophyllene and β-myrcene are used for the aroma of mango (Li et al., 2017) while woody

Table 2: List of the main terpenes synthesised by Mediterranean plants and their potential industrial and healthcare applications.

Compounds	Species	Functions	Applications	References
1,8-cineole	*Hissopus* spp., *Laurus* spp., *Ocimum* spp., *Origanum* spp., *Salvia* spp., *Thymus* spp., *Verbena* spp.	Neuro-protective	Human healthcare	Khan et al., 2014
		Analgesic	Human healthcare	De Sousa, 2011
		Aromatic agent	Food industry	Kutyna and Borneman, 2018
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016
α-pinene	*Anethum* spp., *Mentha* spp., *Ocimum* spp., *Origanum* spp., *Pinus* spp., *Pistacia* spp., *Querus* spp., *Salvia* spp., *Thymus* spp., *Verbena* spp.	Anti-inflammatory	Human healthcare	Bae et al., 2012; Kim et al., 2015; Cho et al., 2017
		Anxiolytic	Human healthcare	Woo and Lee, 2020
		Analgesic	Human healthcare	De Sousa, 2011; Guimarães et al., 2013
		Chemotherapeutic	Human healthcare	Silva et al., 2007; Cho et al., 2017
		Replacing fossil fuel	Green energy	Harvey et al., 2012
		Aromatic agent	Food industry	Li et al., 2017
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016
β-pinene	*Anethum* spp., *Foeniculum* spp., *Laurus* spp., *Ocimum* spp., *Origanum* spp., *Petroselinum* spp., *Salvia* spp.	Anti-inflammatory	Human healthcare	Woo and Lee, 2020
		Anti-depressive	Human healthcare	Kessler et al., 2014; Guzmán-Gutiérrez et al., 2015
		Chemotherapeutic	Human healthcare	Li et al., 2009; Bakarnga-Via et al., 2014
		Analgesic	Human healthcare	Woo and Lee, 2020
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016
β-caryophyllene	*Hissopus* spp., *Laurus* spp., *Lavander* spp., *Mentha* spp., *Ocimum* spp., *Origanum* spp., *Salvia* spp., *Thymus* spp., *Verbena* spp.	Anti-inflammatory	Human healthcare	Cheng et al., 2014; Amorim et al., 2016; Cho et al., 2017
		Anti-depressive	Human healthcare	Kessler et al., 2014
		Neuro-protective	Human healthcare	Cheng et al., 2014; Ojha et al., 2016
		Chemotherapeutic	Human healthcare	Silva et al., 2007
γ-terpinene	*Foeniculum* spp., *Lavander* spp., *Mentha* spp., *Origanum* spp., *Petroselinum* spp., *Salvia* spp., *Thymus* spp.	Chemotherapeutic	Human healthcare	Ferraz et al., 2013
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016

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limonene	*Citrus* spp., *Hyssopus* spp., *Lavander* spp., *Mentha* spp., *Ocimum* spp., *Origanum* spp., *Quercus* spp., *Salvia* spp. *Thymus* spp., *Verbena* spp.	Anti-inflammatory	Human healthcare	Amorim et al., 2016; Hansen et al., 2016
		Neuro-protective	Human healthcare	Shin et al., 2020
		Chemotherapeutic	Human healthcare	Vigushin et al., 1998; Kris-Etherton et al., 2002
		Analgesic	Human healthcare	De Sousa, 2011
			Food industry	Kutyna and Borneman, 2018
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016
linalool	*Citrus* spp., *Hyssopus* spp., *Lavander* spp., *Mentha* spp., *Ocimum spp., Origanum spp., Quercus* spp., *Salvia* spp., *Thymus* spp., *Verbena* spp.	Anti-inflammatory	Human healthcare	Peane et al., 2003; Sabogal-Guáqueta et al., 2016; Kim et al., 2019
		Anti-bacterial	Human healthcare	Rodrigues Goulart et al., 2004
		Anti-depressive	Human healthcare	Linck et al., 2010; Kessler et al., 2014; Guzmán-Gutiérrez et al., 2015
		Neuro-protective	Human healthcare	Sabogal-Guáqueta et al., 2016;
		Chemotherapeutic	Human healthcare	Silva et al., 2007
		Replacing fossil fuel	Green energy	Mewalal et al., 2017
		Aromatic agent	Food industry	Kutyna and Borneman, 2018
		Antiparasitic and toxic for insects	Insecticide industry	Gallardo et al., 2012; Dambolena et al., 2016
myrcene	*Anethum* spp., *Foeniculum* spp., *Hyssopus* spp., *Lavander* spp., *Mentha* spp., *Ocimum* spp., *Origanum* spp., *Petroselinum* spp., *Salvia* spp.	Chemotherapeutic	Human healthcare	Silva et al., 2007
		Replacing fossil fuel	Green energy	Mewalal et al., 2017
		Aromatic agent	Food industry	Li et al., 2017
		Antiparasitic and	Insecticide industry	Dambolena et al., 2016
p-cymene	*Anethum* spp., *Foeniculum* spp., *Lavander* spp., *Mentha* spp., *Origanum* spp., *Petroselinum* spp., *Salvia* spp., *Thymus* spp.	Anti-inflammatory	Human healthcare	Games et al., 2016; Cho et al., 2017
		Chemotherapeutic	Human healthcare	Ferraz et al., 2013
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016
thymol	*Origanum* spp., *Thymus* spp., *Verbena* spp.	Anti-inflammatory	Human healthcare	Games et al., 2016; Cho et al., 2017
		Analgesic	Human healthcare	De Sousa, 2011
		Chemotherapeutic	Human healthcare	Ferraz et al., 2013
		Antiparasitic and toxic for insects	Insecticide industry	Dambolena et al., 2016

notes are linked to α-humulene, α-bulnesene, γ-cadinene (Campelo et al., 2020). Some terpenes derived from herbs and spices have been commercialised for products like toothpastes, shampoos and soaps (Tetali, 2019).

Another function of terpenes, which is also one of their roles in nature, is their use as insecticides, as they have a short persistence in the environment (Ashour et al., 2010). Terpenes could be considered as an important alternative to chemical insecticides as insects do not seem to develop resistance to them; in addition, terpenes do not contaminate food or the environment (Isman, 2006). Limonene and pyrethrins, that act at the nerve cell membranes level, are a natural insecticide and deterrent for several insect species, while also having low toxicity to mammals (Isman, 2006). Moreover, 1,8-cineole, anisole, β-pinene, linalool, menthone, α-pinene, pulegone, and myrcene have demonstrated fumigant properties against insects (Dambolena et al., 2016), while citronellol and geraniol showed the highest toxicity for lice (*Pediculus humanus capitis*; Gallardo et al., 2012).

Mediterranean plants are very rich in essential oils and for this reason they can be considered very important resources for the above-mentioned sectors. For example, the principal common Mediterranean aromatic species used in food and fragrance industries belong to the *Lamiaceae*, *Verbenaceae* and *Rutaceae* families (Elshafie and Camele, 2017). The *Lamiaceae* family contains several aromatic plants, such as *Lavandula* spp., *Oregano* spp., *Thymus* spp., *Mentha* spp., *Sage* spp. The *Marjoram* spp.; the *Verbenaceae* family hosts *Verbena officinalis*, traditionally used in herbalism and flower remedies (Vohra, 2004). The *Rutaceae* family contains *Citrus limon* with its terpene rich extracts, especially D-limonene, α-pinene, α- and β- phellandrene and sesquiterpene (Price, 1993). The common aromatic compounds synthetised by Mediterranean plants are especially, α-thujene, camphene, myrcene, p-cymene, β-phellandrene, which are all emitted by lavender, marjoram, oregano, and sage. Additionally, γ-terpinene, linalool, limonene, α-pinene are the main compounds all synthetised by mint, thyme, lemon, and vervain (Elshafie and Camele, 2017).

Table 2 summarises all the information about terpene compounds found in the plants described in this study.

4. Conclusions

In the Mediterranean basin, abiotic stresses can pose a serious environmental threat to plants due to increasing aridity and heat waves. Plants adopt various mechanisms capable of ensuring their survival in this harsh environment, such as the ability to synthesise an extraordinary arsenal of secondary metabolites (*e.g.*, terpenes and polyphenols) which act primarily as protective compounds against environmental pressures.

Apart from their role in plants, these bioactive compounds have a wide array of health-promoting benefits. For example, polyphenols, due to their antioxidant effects, can be exploited in different types of industries, such as pharmaceutical, nutraceutical and cosmetic, demonstrating that these compounds are innovation hotspots in the most diverse technological domains. Additionally, as highlighted by recent studies on forest bathing therapy, inhaling BVOCs in forest environments can bring many health benefits, such as the alleviation of mood-related disorders. In particular, the inhalation of some terpenes can reduce mental fatigue by inducing relaxation and increasing cognitive performance (Figure 1).

In conclusion, the Mediterranean region, with its species richness, represents a biological hotspot for the discovery of novel drugs and for developing innovative industrial applications. Furthermore, with its forests and natural parks, it represents a sanctuary where people can obtain physiological and psychological benefits.

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