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Climate change on olive physiology, on flower biology, on ripening of drupe and on olive oil quality

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Abstract

Olive (*Olea europaea* L.) is one of the most important fruit trees of the Mediterranean region with an enormous economic, ecosystemic and environmental value and its growth and development are mainly affected by atmospheric conditions. In recent years, with the scenario of climate change, this species is being strongly exposed to thermal and hydric stresses during the growing season, mostly during summer. The changes in temperature and precipitation can negatively influence: the flower biology, the production, and quality of drupes and oil. For this reason, it is essential to adopt agronomic practices that allow better adaptability for drought and high temperatures. Among the traditional agronomic techniques to mitigate the effects of climate changes on plants there are soil management techniques which aim at containing water losses like the controlled water deficit techniques which lead to saving water resources. Over the past decade the application of biostimulants and anti-transpirants products as a sustainable practice to mitigate the negative impact of environmental stresses and to ameliorate or maintain plant productivity has become increasingly widespread.

The first aim of this research was to investigate the effects of different biostimulants classes like: *Trichoderma,* Micro-Algae, Seaweed and Glycine betaine (**Chapter 2)**, and two anti-transpirants products such as Kaolin and Pinolene (**Chapter 3**) in order to mitigate the damages of high temperature and water stress conditions on young olive plants growing in greenhouse conditions. Subsequently, based on the results obtained in the two preliminary studies (mentioned above), we tested some of the pervious biostimulants in open field conditions to observe their effects on the quantitative-qualitative parameters of the drupes and oil of a native Campania cultivar "Racioppella" (**Chapter 4**).

The results obtained from these studies have shown the efficiency in the use of biostimulants and anti-transpirant products in improving biometric parameters of young olive trees, and qualitative and quantitative parameters of drupes and oil, in particular they improved

fundamental parameters that determine the consumer satisfaction, as well as their antioxidant capacity and nutraceutical potential.

The imminent climate changes in the Mediterranean areas, are rising concerns not only for gradual warming but also for extreme variations of seasonal temperatures. In fact, excessively hot springs influence the floral biology of plants, from the development of floral organs to pollination. Other solutions to mitigate climate changes consist in the selection of cultivars resistant or tolerant to high temperatures conditions during flowering and to clarify the physiological processes involved in heat-stress responses.

A second aim of this study was to highlight possible differences among different olive cultivars in the time-course response of pollen viability to different combination of temperature and humidity treatments. We used pollen from 12 olive cultivars belonging to germplasm of the Campania region in Southern Italy and growing in the same site (**Chapter 5**). Most of the olive cultivars showed a significant decrease of pollen viability already after 24 h incubation under 36 °C and 100% RH. In a current scenario of climate change, it is critical to evaluate the effect of temperature on reproductive traits to predict the future impact of global warming on crop yield. Based on our results the cultivar that showed greater tolerance to extreme temperatures and humidity was “Biancolilla”, which could be used to survive in extreme environmental conditions.

Altogether, results obtained in this thesis provide novel sustainable solutions to ameliorate and increase the yield of olive trees and the quality of their products,oil and drupes, under unfavorable environmental conditions.

Chapter 1. General Introduction

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Olive (*Olea europaea* L.) is a typical and widespread tree of the Mediterranean region, where its cultivation started in the third millennium B.C. [1]. The introduction of olive cultivation coincided with the expansion of the Mediterranean civilizations, and the olive has been used widely in traditional remedies in European Mediterranean islands and countries such as Spain, Italy, France, Greece, Israel, Morocco, Tunisia, and Turkey. Moreover, there are at least nine biblical references citing the medicinal use of the plant in ancient times. Apart from the Mediterranean region, the plant is also cultivated widely in the Arabian Peninsula, the Indian subcontinent, and Asia [2].

This perennial and evergreen tree has a strong socio-economic importance for many southern European countries, which encompass 80% of the worldwide olive tree area (and produce roughly 95% of the world olive oil supply [3]. Olive production is concentrated in the Mediterranean-type climatic regions of Southern Europe, particularly Spain (53%), Italy (24%), Greece (15%) and Portugal (7%) (**Fig. 1**).

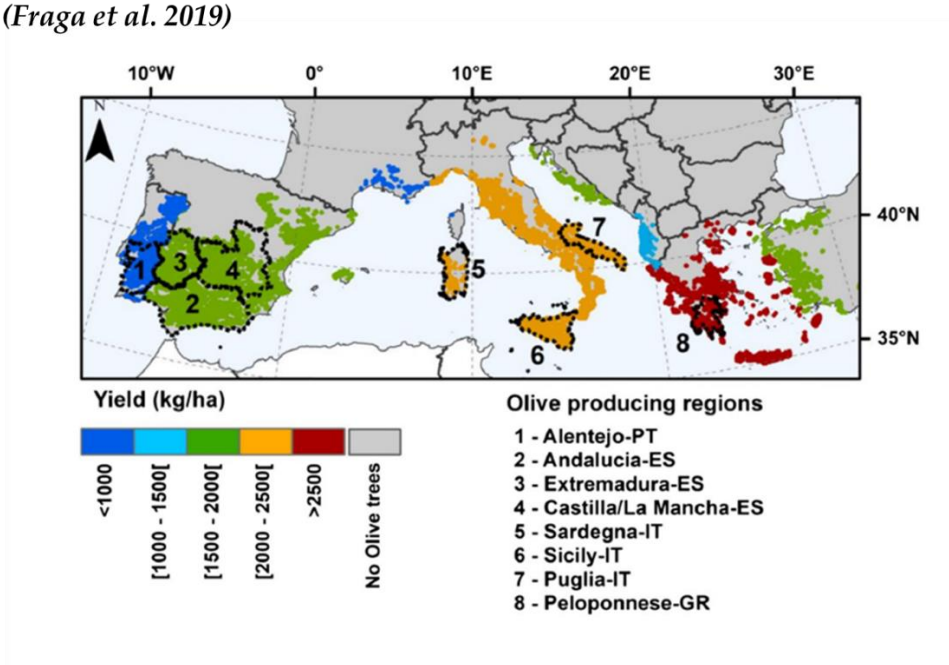

Figure 1. Olive orchard distribution in Europe. The different colour represents country yields according to FAO statistics.

The olive has significant phenotypic and and genetic variability [4,5]. Factors that have limited genetic erosion and favored the preservation of genetic diversity include its longevity, a limited selection pressure, and the fact that the replacement with new genotypes was not frequently practiced in the past [6]. At present more than 600 varieties are known, not to mention synonymies and homonymies, which would increase the number to almost 1300 varieties [7]. It is well known that, among countries that cultivate the olive tree, Italy has the richest germplasm in terms of number of varieties (several hundred), and this number is certainly destined to increase as molecular analysis will be extended, not only to the *ex situ* collection [8], but also to ancient varieties cultivated locally for centuries by farmers. Amongst the Italian regions, where cultivation of the olive tree is widely practiced, Campania has one of the largest germplasm collections. In a recent study, Muzzalupo et al. [8] fingerprinted more than 210 Italian varieties and the Campania region accounted for more than 20% (43 varieties) of the total number. Many of these varieties are characterized by an extensive morphological diversity and adaptation to local environmental conditions [9]. Italy produces 15% of the world EVOO market and it is the first EVOO world consumer. From the most recent population surveys, it emerges that Italian consumers are attracted to the increase EVOO quality as they are appreciating the health benefits of EVOO and can recognize the organoleptic properties, associated with agronomic and technological factors [10]. Olives and olive derived are an important valuable source of natural phenolic antioxidants [11]. In fact, an increasing number of epidemiologic and experimental studies report that the olive oil may have a role in the prevention of coronary heart disease [12] , cognitive impairment, e.g., Alzheimer’ s disease [13], protective effects against of the cancer of the colon, breast and ovary [14], diabetes accompanied by hypertriglyceridemia and inflammatory and autoimmune diseases, such as rheumatoid arthritis [13]. The high quality EVOO is considered as a true pharm-food. This property is due both to the fat composition, i.e., high oleic acid concentration, which ranges from 56% to 84%; the essential polyunsaturated fatty acids: linoleic acid ranging from 3.5% to 21% and linolenic acid <1.5%. Besides, EVOOs contain a relevant concentration of effcient chemo preventive molecules,

including tocopherols (vitamin E), β-carotene, and phenolic compounds (PCs) [10]. It is therefore known that the olive tree represents a real social, economic, health, ambiental and cultural wealth; in this thesis we have investigated some aspects concerning the relationship between olive trees cultivation and climate change, in particular, we have carried out studies varietal screening of Campanian cultivars more resistant to high temperature scenarios and to application of biostimulant and antiperspirant products to improve resistance, quality and yield of olives and oil in extreme environmental conditions.

1.1 Climate changes effects on olive trees growth and development

Climate change is an undeniable fact that is challenging society and every economic sector, including agriculture. In the Mediterranean region, recent reports show that significant warming has occurred in the last 40 years and annual temperatures are now about 1.5 ◦C higher with respect to the preindustrial period (1880–1899) and well above current global warming trends (+1.1 ◦C), and since 2014, we experienced the six warmest years on record, globally [15]. Increasing temperatures were accompanied by a series of extreme heat events that occurred at an unprecedented trend in terms of duration, intensity, and frequency [16,17] and there has been a substantial decrease in the frequency of cold extremes [18,19]. For the Mediterranean region, future climate projections tend to be particularly severe. In this region, precipitation projections point to an overall decrease, which will lead to a lowering of soil water availability. The Mediterranean region is already characterized by plant heat and water stresses, due to the harsh summertime weather conditions, including low precipitation, excessive heat, and high solar radiation. Moreover, nocturnal temperatures will also tend to increase, leading to an even higher thermal stress level, and there will be modification in the frequency of the occurrence of extreme weather events, such as heatwaves, hail, floods and wildfires [20–22].These events are projected to increase in frequency and magnitude under climate change scenarios, leading to a rise in the severity

of drought and heatwave spells over the Mediterranean Basin, also in conjunction with the flowering period. Future climatic changes have great importance for the agricultural sector, and the olive tree sector. Regarding perennial crops, such as olive trees, under future climatic conditions these projections are expected to cause severe adverse effects, particularly on water relations, on floral biology, oxidative pathways, and other physiological processes, phenological timings, final yield and quality attributes [23]. Proper olive cultivation areas have a mean annual temperature of 15–20 °C, with a minimum of 4 °C and a maximum of 40 °C [24]. Usually, the optimum temperature for olive vegetative growth ranges between 10 °C and 30 °C, while carbohydrate synthesis occurs at higher rates at temperatures ranging from 20 °C to 30 °C [25]. Olive trees require a period of low temperatures (0–7 °C) for flowering bud differentiation [24]. On the other hand, temperatures constantly above 16 °C prevent bud differentiation [25]. However, the minimum temperature should not drop below −7 °C, which can seriously damage trees, and if the temperature reaches −12 °C, can kill them.

Due to climate change, there is a risk that the needs of the plant are not met, and this can cause serious problems in particular the temperature is the most significant environmental factor that limits olive growing areas, while water availability is the most significant factor that limits olive yield.

1.2 Heat stress

In the scenario of climate changes, one of the main abiotic stress plants faces, is heat stress, which causes a series of biochemical, morphological, physiological, and molecular changes that adversely affect plant development (**Fig. 2**). Heat stress exerts negative impacts on various crops though the ranges of temperatures vary largely on crop species [26,27]. Reduced germination percentage, plant emergence, abnormal seedlings, poor seedling vigor, and reduced radicle and plumule growth of geminated seedlings are major impacts caused by heat stress documented in various cultivated plant species [26,28]. High-temperature causes loss of cell water content for which the cell size and the growth are

reduced [29,30]; the morphological symptoms of heat stress include scorching and sunburns of leaves and twigs, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration and damage [29].

Photosynthesis is one of the most heat-sensitive physiological processes in plants. Heat markedly affects the leaf water status, leaf stomatal conductance (gs), and intercellular CO_2 concentration [31]. Closure of stomata under high temperatures is another reason for impaired photosynthesis that affects the intercellular CO_2 [30]. High temperatures at flowering are known to decrease pollen viability [32] and elevated temperatures are raising apprehension regarding crop productivity and food security [33]. Further important consequence of high temperature is the alteration of the oxidative metabolism, which causes membrane instability. In particular, the accumulation of reactive oxygen species (ROS), such as H_2O_2, in both chloroplasts and mitochondria, can have negative impacts, such as severe DNA damage, the autocatalytic peroxidation of lipids and membrane pigments, the loss of semi-permeability membranes and the breakdown of photosynthetic pigments and decreased enzyme activity [34].

Various studies about the consequences high-temperature stress on the olive tree are reported in the bibliography; in particular, problems have been reported on pollen vitality [35], yield reduction [36], the high temperatures influence fruit development as well as oil accumulation, diminish oil quality by modifying fatty acid composition and causing a reduction of polyphenols and oleic acid, the most important components of olive oil [37–41]. However, high temperature effects are genotype dependent and each cultivar responds differently to this stress [42].

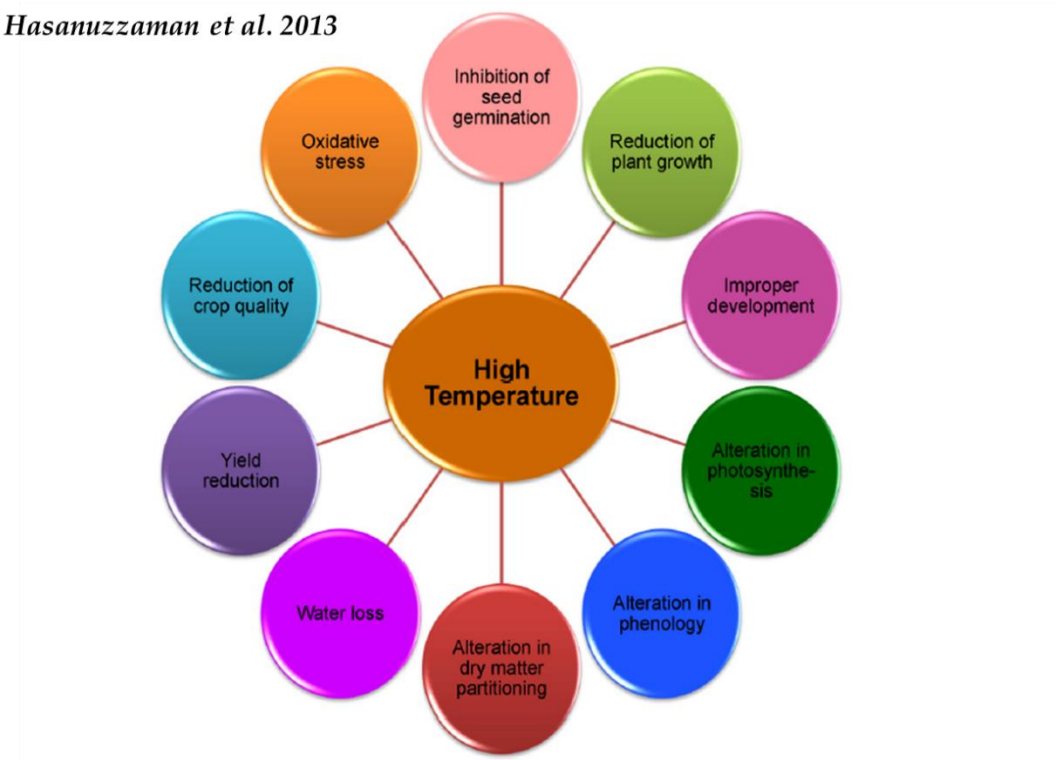

Figure 2. Major effects of high temperature on plants.

1.3 Drought stress

Global climate changes are leading to increases in temperature and atmospheric CO_2 levels as well as alterations in rainfall patterns. Periods of inadequate rainfall leading to drought are predicted to arise more frequently under such conditions. Terminal drought conditions bring about a progressive decrease in soil water availability to plants and cause premature plant death, while intermittent drought conditions affect the plant growth and development but are not usually lethal [43]. It has been established that drought stress is a very important limiting factor at the initial phase of plant growth and establishment [44–47], in fact a primary response of plants subjected to drought stress is growth arrest. The alarm indicators of water stress are different (**Fig. 3**). Plant water status is the key factor affecting the yield and quality of horticultural crops [48], it is crucial to maintain the optimum plant water status. If the plant water status declines beyond a certain limit (threshold level), there

is a severe decline in the yield and quality of vegetable crops [49]. The response to drought stress in crops may range from partial stomatal closure under moderate stress to drying and death of the plant at the wilting point [50]. Under water-stressed conditions, stomatal conductance decreases due to closure of stomata to maintain the leaf water status [51] and the maintaining optimum canopy temperature is crucial for the metabolic activities of a plant. Among the various methods used to determine the water status of plants, there is leaf water potential (LWP) where a more negative (lower) value of LWP of a plant indicates a more dehydrated leaf (water stressed); the RWC is a comparison of the water content of a leaf to the maximum possible water content of that leaf at its full turgor [52]. The RWC is highly associated with the cell turgor and cell turgor further controls cell expansion [48]. L'inibizione della crescita dei germogli in condizioni di siccità riduce le richieste metaboliche della pianta e mobilita i metaboliti per la sintesi di composti protettivi necessari per l'aggiustamento osmotico [53]. Under water stress, chlorophyll content decreases due to damage to chloroplast membrane and structure, photo-oxidation of chlorophyll, increased activity of chlorophyllase, and suppression of biosynthesis of chlorophyll [54]. The olive trees s has developed a series of physiological mechanisms to tolerate drought stress, the activation of the phenylpropanoid biosynthetic pathway leading to the accumulation of phenolic compounds is a well-known metabolic response to water deficit as well as to other environmental stresses. Such metabolic responses of the plants to unfavorable environmental conditions play a key role in preventing cellular damage caused by oxidative stress. However, the constitutive tolerance to water deficit not sufficient to protect olive trees from the combined effects of extreme heat waves, water stress, and high irradiance, which are all linked to climate change [55].

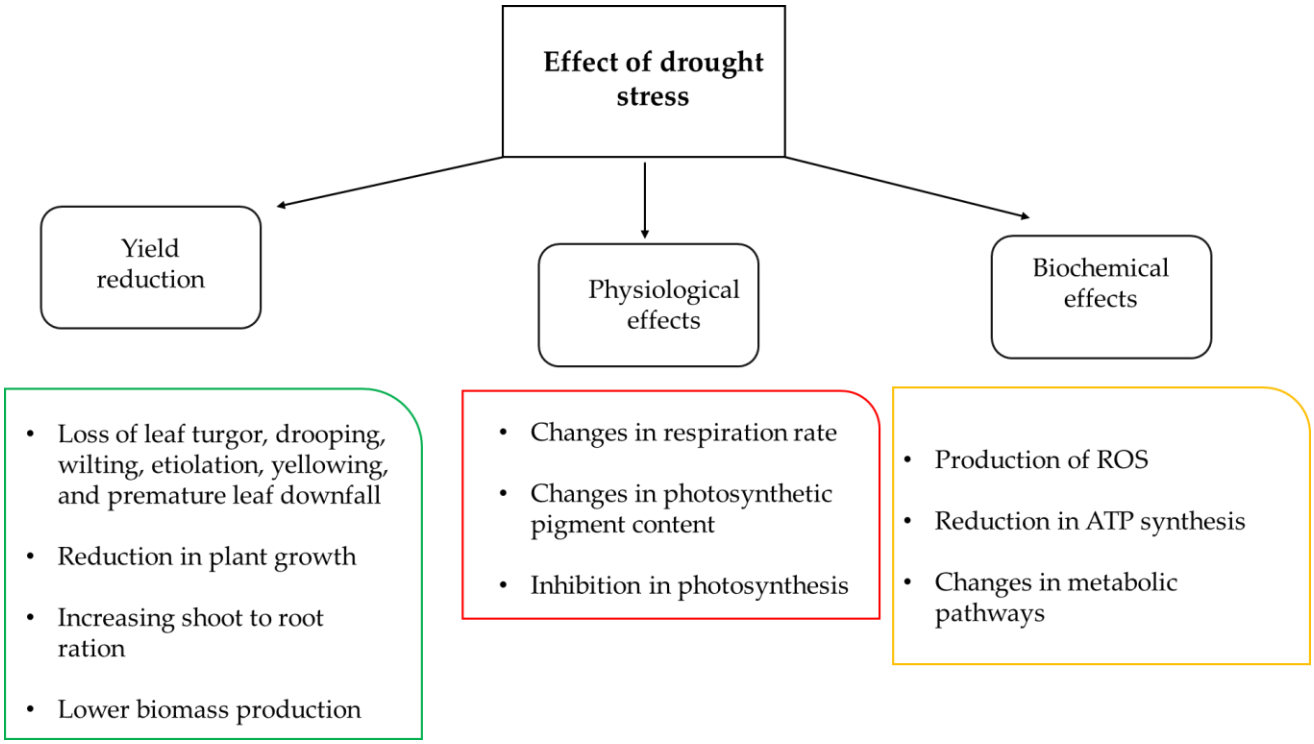

Figure 3. Main effects of drought stress on physiological and biochemical processes and yield related factors.

Understanding how olive trees respond to abiotic stresses is the first step to improving their profitability, allowing the selection of more resistant cultivars and identification of tolerant characteristics useful in breeding programs, as well as the development of accurate adaptation strategies or solutions according to necessities. In the scenario of climate change, various solutions have been adopted to try to reduce the effects of high temperatures on crops: defoliation on the canopy [56], use of shading nets [57], use of antitraspirant products [58], use of biostimulants [59] and the use of resistant species in extreme environmental conditions.

In the present study we wanted to test the efficiency of antiperspirant and biostimulant products in reducing the effects of high temperatures on olive trees and we screened the most resistant CVs to extreme temperature and humidity conditions.

1.4 Strategies to mitigate the climate changes effects: use of natural biostimulants

The regulation of plant growth and the development and alleviation of the negative effects of environmental stresses, are important factors determining the productivity of cultivated plants. While it is well recognized that biotic and abiotic stress prevents essentially all crop systems from achieving their yield potential, current understanding of the mechanisms involved, and the strategies to mitigate these effects are limited. Abiotic stresses may be prevented by optimizing plant growth conditions and through provision of water and nutrients and plant growth regulators (PGRs—auxins, cytokinins, gibberellins, strigolactones, brassinosteroids). Another strategy is to improve soil management with agronomic techniques to contain water losses or implement controlled water deficit techniques that lead to saving water resources. In addition to these traditional approaches, biostimulants are increasingly being integrated into production systems with the goal of modifying physiological processes in plants to optimize productivity [60–62]. Biostimulants can be treated as an additive to fertilizers and support the uptake of nutrients, promote plant growth, and increase tolerance to abiotic stress. The definition of biostimulants is wide and not sufficiently precise. However, there are two main features that distinguish biostimulants from other growth and plant-protection agents. A biostimulant may be any substance or mixture of substances of natural origin or microorganism which improves the condition of crops without causing adverse side effects [59], they cannot be defined as fertilizers because they do not provide nutrients directly to plants. Biostimulants may facilitate the acquisition of nutrients by supporting metabolic processes in the soil and plants and they may be used in the form of soil preparations (powders, granules, or solutions added to the soil) or as liquid foliar application products [63]. Biostimulants can be introduced into the irrigation system and taken up by plants along with water [64]. The use of biostimulants can increase yields, increase the concentration of foliar pigments, secondary metabolites, and vitamins, increase the resistance to numerous biotic and abiotic stress of cultivated plants, reduce fertilizer delivery and encourage agriculture close to organic farming, thereby reducing the

environmental contamination. Additional benefits include increased germination rate, improved root depth, and reduced post-transplant stress (**Fig. 4).** Various compounds with bioactive properties can be utilized as biostimulants to boost plant growth and development under normal and stressful conditions [65,66], while among the distinctive characteristics a biostimulatory product must improve nutrients use efficiency, tolerance to abiotic stressors, quality of the final product and nutrients availability in soil [67]. So far, six distinct categories of biostimulants are recognized, including microbial inoculants, humic substances, such as humic and fulvic acids, protein hydrolysates and amino acids, biopolymers, inorganic compounds. and seaweed extracts, all of which are commercially available with wide applications in agriculture [68].

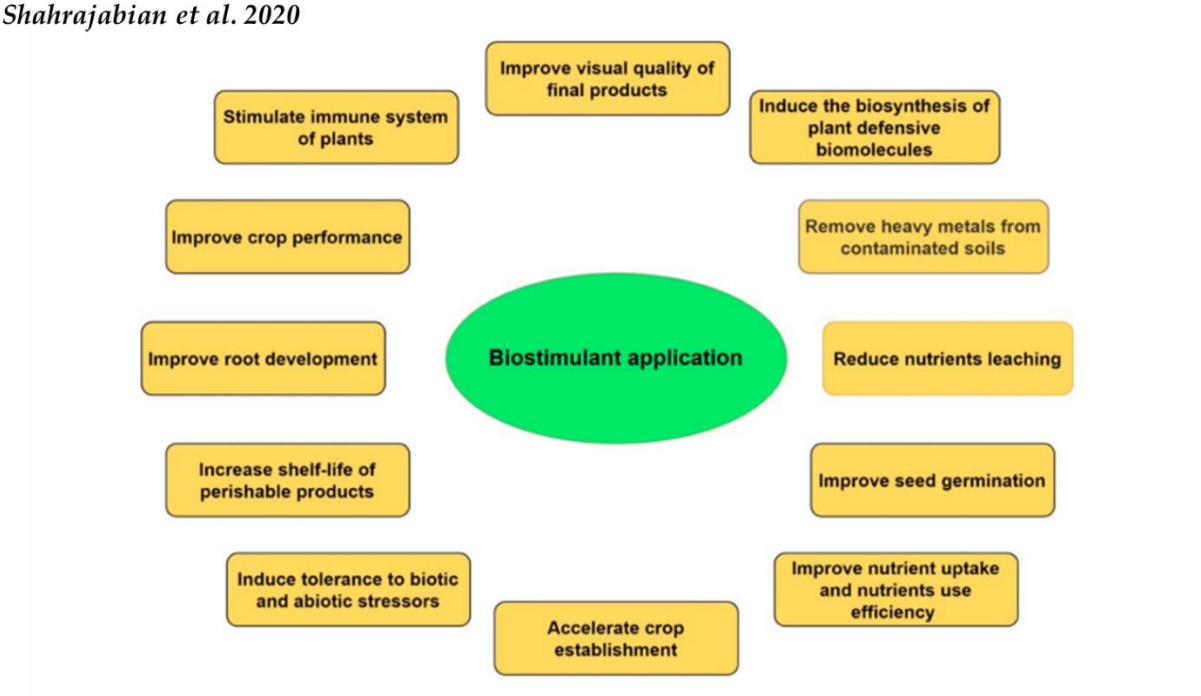

Figure 4. The most important biostimulants effects on crops.

In recent years the use of biostimulant products has also extended to the cultivation of the olive tree, in fact in the bibliography there are some studies showing the positive effects of these products on the yield and quality of the oil. In adult olive trees, biostimulants were reported as being able to improve the yield and fruit characteristics [69,70], to replace fertilizers of inorganic origin [71] and to have positive effects on oil quality. Almadi et al.

[73] demonstrated that biostimulants increased the growth of young olive trees stimulating physiological processes, that is, by improving assimilate production through photosynthesis. Very few studies have been carried out to evaluate the possibilities of using biostimulants on olive to increase vegetative and/or reproductive growth and/or olive oil quality, in fact one of the purpose of our study was to test the response in qualitative-quantitative terms of both the olives and the oil with different biostimulants application.

1.5 Another strategy to mitigate climate change effects: use of anti-transpirant products

It is important to adopt agronomic practices that allow a better adaptability for drought and high temperature, and therefore the capacity to integrate both tolerance and recovery capacity [34] of olive orchards. Olive withstands to a high degree the low water availability, by means of morphological, physiological, and biochemical adaptations. Nonetheless, water deficit has significant impacts on water relations, oxidative phenomena and use and biomass production in terms of growth and yield [74]. In addition, the simultaneous occurrence of multiple abiotic constraint factors in summer months, such as high heat loads and high irradiance levels, can aggravate drought effects. Since the region where the majority of olive culture is located, i.e. Mediterranean basin, is particularly vulnerable to climate change one may expect more severe heat and drought stress during the following years [75].

One neglected agronomic technique that has the potential to significantly contribute to abiotic stress amelioration in food crop production is the use of anti-transpirants. Anti-transpirants product are materials that reduce transpiration, can potentially result in greater food production by releasing more of a crop's potential yield during drought [76]. They are compounds applied to plant leaves to reduce transpiration since they reduce the stomatal opening and increase the leaf resistance to water vapor diffusion, and they have often been used to prevent water stress. Reducing rates of transpiration directly by anti-transpirant

sprays avoided the need for drastically altering environmental conditions experiments designed to evaluate the effects of transpiration [77]. Anti-transpirants have been used successfully in agriculture to control leaf transpiration and improve the quality of different cultures [58,78,79].

Anti-transpirant products can be divided into three categories that have been extensively described by Mphande et al. [76]: reflective anti-transpirants (kaolin), metabolic or stomata-closing antitranspirant (prominent in this class is exogenous abscisic acid – ABA) and film-forming anti-transpirants (di-1-p-menthene and poly-1-p-menthen). Originally, some of these products, as the kaolin was developed for the suppression of pests in many crops, but later, it has been demonstrated that the white kaolin film formed on the leaf surface increases the reflection of incoming solar radiation [80].

Studies on the application of anti-transpirant products on various crops are reported in the bibliography [58,77,78], while there are still few studies conducted on the efficiency of these products on the characteristics of both the olives and the oil, in fact for this reason in the present study we evaluated the effect of anti-transpirant products on the qualitative-quantitative parameters of olives and oil.

1.6 Cultivars resistant to abiotic stress

The projections of climate changes in the Mediterranean areas are rising concerns not only for gradual warming but also for abrupt variations of seasonal temperatures. Consequently, researchers commit to select genotypes tolerant to high temperatures or heat waves and to clarify the physiological processes involved in heat-stress responses [81,82]. Climate change is impacting on the biological dynamics of plants showing phenological aberrancies [83,84]. In pome and stone-fruit trees of temperate regions, extreme weather events are reported to affect production in key phases (as the erratic break of the bud dormancy) that ultimately results in plant yield reductions and financial losses [85–87]. Temperature exposure over

time is the main driver for spring development, including bloom timing and leaf-out [88,89]. Among reproductive traits, pollen interaction with the environment change represents a potential bottleneck in plant life cycle that can limit the reproductive fitness and productivity in plant species [90].

Indeed, pollen functionality has been widely reported to be extremely sensitive to environmental constraints [91–93]. Particularly, pollen viability and germination are mostly dependent on temperature and humidity exposure of pollen grains [94].

The olive has significant phenotypic and genetic variability [4,95]. Factors including plant longevity, limited selection pressure, and limited replacement with new genotypes have reduced genetic erosion and favored the preservation of genetic diversity of olive varieties [96]. Temperature also influences both drupe development and oil composition in olive. For example, in very hot sites, olives can show early pigmentation due to the rapid degradation of chlorophyll due to high temperatures [96], whereas in sites with lower temperatures, olive oil has a high content of unsaturated fatty acids [97]. Temperature can also influence the aromatic components of olive oil, reducing the content of volatile substances [98]. Hence, it is arguable that the growing environment is crucial in expressing the typical characteristics and quality of olive cultivars [99]. In most crop species, including *Olea europaea* L., the production of fruits and seeds relies on pollen functionality. Since pollen viability and germinability are both essential to ensure fertilization, the interaction of pollen with extreme weather events can significantly limit crops productivity in the current climate change scenario [100]. For this reason, it is important to select cultivars resistant to extreme climatic conditions to guarantee good production and high-quality oil.

1.7 Aim of the research

This PhD project aims to advance the understanding of plant responses to applications of biostimulants and anti-traspirants products in abiotic stress conditions and to select Campanian olive cultivars that are more resistant to high temperature and humidity conditions during the flowering. Therefore, the main aims of this research are:

1. evaluation of biostimulants effect on plant growth and bioactive compounds on young olive trees under abiotic stress conditions.
2. effects of anti-transpirant products to assess: biometric, eco-physiological and nutraceutical parameters of young olive trees under abiotic stress conditions;
3. effect of biostimulants application on *Olea europaea* L. in Mediterranean conditions on production and bioactive compounds of drupes and oil;
4. varietal screening of Campanian olive cultivars resistant to high temperature and different humidity conditions during flowering.

1.1 References

1. Loukas, M.; Krimbas, C.B. History of Olive Cultivars Based on Their Genetic Distances. Journal of Horticultural Science 1983, 58, 121–127, doi:10.1080/00221589.1983.11515099.
2. El, S.N.; Karakaya, S. Olive Tree (*Olea Europaea*) Leaves: Potential Beneficial Effects on Human Health. Nutrition Reviews 2009, 67, 632–638, doi:10.1111/j.1753-4887.2009.00248.x.
3. Besnard, G.; Khadari, B.; Navascués, M.; Fernández-Mazuecos, M.; El Bakkali, A.; Arrigo, N.; Baali-Cherif, D.; Brunini-Bronzini de Caraffa, V.; Santoni, S.; Vargas, P.; et al. The Complex History of the Olive Tree: From Late Quaternary Diversification of Mediterranean Lineages to Primary Domestication in the Northern Levant. Proc. R. Soc. B. 2013, 280, 20122833, doi:10.1098/rspb.2012.2833.

4. Ayed, R.B.; Grati-Kamoun, N.; Moreau, F.; Rebaï, A. Comparative Study of Microsatellite Profiles of DNA from Oil and Leaves of Two Tunisian Olive Cultivars. Eur Food Res Technol 2009, 229, 757–762, doi:10.1007/s00217-009-1111-3.
5. Poljuha, D.; Sladonja, B.; Šetić, E.; Milotić, A.; Bandelj, D.; Jakše, J.; Javornik, B. DNA Fingerprinting of Olive Varieties in Istria (Croatia) by Microsatellite Markers. Scientia Horticulturae 2008, 115, 223–230, doi:10.1016/j.scienta.2007.08.018.
6. Rallo, P.; Dorado, G.; Martín, A. Development of Simple Sequence Repeats (SSRs) in Olive Tree (*Olea Europaea* L.). Theor Appl Genet 2000, 101, 984–989, doi:10.1007/s001220051571.
7. Ganino, T.; Bartolini, G.; Fabbri, A. The Classification of Olive Germplasm. The Journal of Horticultural Science and Biotechnology 2006, 81, 319–334, doi:10.1080/14620316.2006.11512069.
8. Muzzalupo, I.; Stefanizzi, F.; Salimonti, A.; Falabella, R.; Perri, E. Microsatellite Markers for Identification of a Group of Italian Olive Accessions. Sci. agric. (Piracicaba, Braz.) 2009, 66, 685–690, doi:10.1590/S0103-90162009000500014.
9. Cicatelli, A.; Fortunati, T.; De Feis, I.; Castiglione, S. Oil Composition and Genetic Biodiversity of Ancient and New Olive *(Olea Europea* L.) Varieties and Accessions of Southern Italy. Plant Science 2013, 210, 82–92, doi:10.1016/j.plantsci.2013.05.011.
10. Lanza, B.; Ninfali, P. Antioxidants in Extra Virgin Olive Oil and Table Olives: Connections between Agriculture and Processing for Health Choices. Antioxidants 2020, 9, 41, doi:10.3390/antiox9010041.
11. Briante, R.; Febbraio, F.; Nucci, R. Antioxidant Properties of Low Molecular Weight Phenols Present in the Mediterranean Diet. J. Agric. Food Chem. 2003, 51, 6975–6981, doi:10.1021/jf034471r.
12. Escrich, E.; Ramírez-Tortosa, M.C.; Sánchez-Rovira, P.; Colomer, R.; Solanas, M.; Gaforio, J.J. Olive Oil in Cancer Prevention and Progression. Nutrition Reviews 2006, 64, S40–S52, doi:10.1111/j.1753-4887.2006.tb00262.x.

13. Scarmeas, N.; Stern, Y.; Tang, M.-X.; Mayeux, R.; Luchsinger, J.A. Mediterranean Diet and Risk for Alzheimer's Disease. Ann Neurol. 2006, 59, 912–921, doi:10.1002/ana.20854.
14. Braga, C.; Vecchia, C.L.; Franceschi, S.; Negri, E.; Parpinel, M.; Decarli, A.; Giacosa, A.; Trichopoulos, D. Olive Oil, Other Seasoning Fats, and the Risk of Colorectal Carcinoma. Cancer 1998, 82, 448–453, doi:10.1002/(SICI)1097-0142(19980201)82:3<448::AID-CNCR4>3.0.CO;2-L.
15. Kennedy, J.J.; Dunn, R.J.H.; Titchner, H.A.; Morice, C.P.; Killick, R.E.; McCarthy, M.P. Global and Regional Climate in 2017. Weather 2018, 73, 382–390, doi:10.1002/wea.3421.
16. King, A.D.; Karoly, D.J.; Henley, B.J. Australian Climate Extremes at 1.5 °C and 2 °C of Global Warming. Nature Clim Change 2017, 7, 412–416, doi:10.1038/nclimate3296.
17. Kuglitsch, F.G.; Toreti, A.; Xoplaki, E.; Della-Marta, P.M.; Zerefos, C.S.; Türkeş, M.; Luterbacher, J. Heat Wave Changes in the Eastern Mediterranean since 1960: HEAT WAVES IN THE EASTERN MEDITERRANEAN. Geophys. Res. Lett. 2010, 37, doi:10.1029/2009GL041841.
18. Alexander, L.V. Global Observed Long-Term Changes in Temperature and Precipitation Extremes: A Review of Progress and Limitations in IPCC Assessments and Beyond. Weather and Climate Extremes 2016, 11, 4–16, doi:10.1016/j.wace.2015.10.007.
19. Frich, P.; Alexander, L.; Della-Marta, P.; Gleason, B.; Haylock, M.; Klein Tank, A.; Peterson, T. Observed Coherent Changes in Climatic Extremes during the Second Half of the Twentieth Century. Clim. Res. 2002, 19, 193–212, doi:10.3354/cr019193.
20. Porter, J.R.; Semenov, M.A. Crop Responses to Climatic Variation. Phil. Trans. R. Soc. B 2005, 360, 2021–2035, doi:10.1098/rstb.2005.1752.
21. Costa, R.; Fraga, H.; Fernandes, P.M.; Santos, J.A. Implications of Future Bioclimatic Shifts on Portuguese Forests. Reg Environ Change 2017, 17, 117–127, doi:10.1007/s10113-016-0980-9.

22. Fraga, H.; García de Cortázar Atauri, I.; Malheiro, A.C.; Moutinho-Pereira, J.; Santos, J.A. Viticulture in Portugal: A Review of Recent Trends and Climate Change Projections. OENO One 2017, 51, 61, doi:10.20870/oeno-one.2016.0.0.1621.
23. Fraga, H.; Moriondo, M.; Leolini, L.; Santos, J.A. Mediterranean Olive Orchards under Climate Change: A Review of Future Impacts and Adaptation Strategies. Agronomy 2020, 11, 56, doi:10.3390/agronomy11010056.
24. Therios, I.N. Olives; Crop production science in horticulture; CABI: Wallingford ; Cambridge, MA, 2009; ISBN 978-1-84593-458-3.
25. Brito, C.; Dinis, L.-T.; Moutinho-Pereira, J.; Correia, C. Kaolin, an Emerging Tool to Alleviate the Effects of Abiotic Stresses on Crop Performance. Scientia Horticulturae 2019, 250, 310–316, doi:10.1016/j.scienta.2019.02.070.
26. Kumar, S.; Kaur, R.; Kaur, N.; Bhandhari, K.; Kaushal, N.; Gupta, K.; Bains, T.S.; Nayyar, H. Heat-Stress Induced Inhibition in Growth and Chlorosis in Mungbean (Phaseolus Aureus Roxb.) Is Partly Mitigated by Ascorbic Acid Application and Is Related to Reduction in Oxidative Stress. Acta Physiol Plant 2011, 33, 2091–2101, doi:10.1007/s11738-011-0748-2.
27. Hasanuzzaman, M.; Nahar, K.; Alam, Md.; Roychowdhury, R.; Fujita, M. Physiological, Biochemical, and Molecular Mechanisms of Heat Stress Tolerance in Plants. IJMS 2013, 14, 9643–9684, doi:10.3390/ijms14059643.
28. Toh, S.; Imamura, A.; Watanabe, A.; Nakabayashi, K.; Okamoto, M.; Jikumaru, Y.; Hanada, A.; Aso, Y.; Ishiyama, K.; Tamura, N.; et al. High Temperature-Induced Abscisic Acid Biosynthesis and Its Role in the Inhibition of Gibberellin Action in Arabidopsis Seeds. Plant Physiology 2008, 146, 1368–1385, doi:10.1104/pp.107.113738.
29. Rodríguez, M.; Canales, E.; Borrás-Hidalgo, O. Molecular Aspects of Abiotic Stress in Plants.
30. Ashraf, M.; Foolad, M.R. Roles of Glycine Betaine and Proline in Improving Plant Abiotic Stress Resistance. Environmental and Experimental Botany 2007, 59, 206–216, doi:10.1016/j.envexpbot.2005.12.006.

523 31. Greer, D.H.; Weedon, M.M. Modelling Photosynthetic Responses to Temperature of
524 Grapevine (Vitis Vinifera Cv. Semillon) Leaves on Vines Grown in a Hot Climate:
525 Photosynthetic Temperature Responses of Semillon. Plant, Cell & Environment 2012,
526 35, 1050–1064, doi:10.1111/j.1365-3040.2011.02471.x.
527 32. Tan, W.; Meng, Q. wei; Brestic, M.; Olsovska, K.; Yang, X. Photosynthesis Is Improved
528 by Exogenous Calcium in Heat-Stressed Tobacco Plants. Journal of Plant Physiology
529 2011, 168, 2063–2071, doi:10.1016/j.jplph.2011.06.009.
530 33. Passarella, V.S.; Savin, R.; Slafer, G.A. Breeding Effects on Sensitivity of Barley Grain
531 Weight and Quality to Events of High Temperature during Grain Filling. Euphytica
532 2005, 141, 41, doi:10.1007/s10681-005-5068-4.
533 34. Wang, S. Genotypic Variation in Growth and Physiological Response to Drought
534 Stress and Re-Watering Reveals the Critical Role of Recovery in Drought Adaptation
535 in Maize Seedlings. Frontiers in Plant Science 2016, 6, 15.
536 35. Ribeiro, H.; Cunha, M.; Abreu, I. Quantitative Forecasting of Olive Yield in Northern
537 Portugal Using a Bioclimatic Model. Aerobiologia 2008, 24, 141–150,
538 doi:10.1007/s10453-008-9094-2.
539 36. Orlandi, F.; Rojo, J.; Picornell, A.; Oteros, J.; Pérez-Badia, R.; Fornaciari, M. Impact of
540 Climate Change on Olive Crop Production in Italy. Atmosphere 2020, 11, 595,
541 doi:10.3390/atmos11060595.
542 37. Alowaiesh, B.; Singh, Z.; Fang, Z.; Kailis, S.G. Harvest Time Impacts the Fatty Acid
543 Compositions, Phenolic Compounds and Sensory Attributes of Frantoio and
544 Manzanilla Olive Oil. Scientia Horticulturae 2018, 234, 74–80,
545 doi:10.1016/j.scienta.2018.02.017.
546 38. García-Inza, G.P.; Castro, D.N.; Hall, A.J.; Rousseaux, M.C. Opposite Oleic Acid
547 Responses to Temperature in Oils from the Seed and Mesocarp of the Olive Fruit.
548 European Journal of Agronomy 2016, 76, 138–147, doi:10.1016/j.eja.2016.03.003.
549 39. García-Inza, G.P.; Castro, D.N.; Hall, A.J.; Rousseaux, M.C. Responses to Temperature
550 of Fruit Dry Weight, Oil Concentration, and Oil Fatty Acid Composition in Olive (*Olea*

Europaea L. Var. 'Arauco'). European Journal of Agronomy 2014, 54, 107–115, doi:10.1016/j.eja.2013.12.005.

40. Lombardo, N.; Marone, E.; Alessandrino, M.; Godino, G.; Madeo, A.; Fiorino, P. Influence of Growing Season Temperatures in the Fatty Acids (FAs) of Triacilglycerols (TAGs) Composition in Italian Cultivars Of.
41. Mailer, R.J.; Ayton, J.; Graham, K. The Influence of Growing Region, Cultivar and Harvest Timing on the Diversity of Australian Olive Oil. J Am Oil Chem Soc 2010, 87, 877–884, doi:10.1007/s11746-010-1608-8.
42. Nissim, Y.; Shloberg, M.; Biton, I.; Many, Y.; Doron-Faigenboim, A.; Zemach, H.; Hovav, R.; Kerem, Z.; Avidan, B.; Ben-Ari, G. High Temperature Environment Reduces Olive Oil Yield and Quality. PLoS ONE 2020, 15, e0231956, doi:10.1371/journal.pone.0231956.
43. Bhargava, S.; Sawant, K. Drought Stress Adaptation: Metabolic Adjustment and Regulation of Gene Expression. Plant Breed 2013, 132, 21–32, doi:10.1111/pbr.12004.
44. Anjum, F.; Yaseen, M.; Rasool, E.; Wahid, A.; Anjum, S. WATER STRESS IN BARLEY (HORDEUM VULGARE L.) I. EFFECT ON MORPOHOLOGICAL CHARACTERS.
45. Bhatt, R.M.; Rao, N.K.S. INFLUENCE OF POD LOAD ON RESPONSE OF OKRA TO WATER STRESS. 2005, 10.
46. Kusaka, M.; Ohta, M.; Fujimura, T. Contribution of Inorganic Components to Osmotic Adjustment and Leaf Folding for Drought Tolerance in Pearl Millet. Physiologia Plantarum 2005, 125, 474–489, doi:10.1111/j.1399-3054.2005.00578.x.
47. Shao, H.-B.; Chu, L.-Y.; Jaleel, C.A.; Zhao, C.-X. Water-Deficit Stress-Induced Anatomical Changes in Higher Plants. Comptes Rendus Biologies 2008, 331, 215–225, doi:10.1016/j.crvi.2008.01.002.
48. Jones, H.G. Physiological Aspects of the Control of Water Status in Horticultural Crops. HortSci 1990, 25, 19–25, doi:10.21273/HORTSCI.25.1.19.
49. Nezhadahmadi, A.; Prodhan, Z.H.; Faruq, G. Drought Tolerance in Wheat. The Scientific World Journal 2013, 2013, 1–12, doi:10.1155/2013/610721.

579 50. Parkash, V.; Singh, S. A Review on Potential Plant-Based Water Stress Indicators for
580 Vegetable Crops. Sustainability 2020, 12, 3945, doi:10.3390/su12103945.
581 51. Liu, F.; Jensen, C.R.; Andersen, M.N. Hydraulic and Chemical Signals in the Control
582 of Leaf Expansion and Stomatal Conductance in Soybean Exposed to Drought Stress.
583 Functional Plant Biol. 2003, 30, 65, doi:10.1071/FP02170.
584 52. Ihuoma, S.O.; Madramootoo, C.A. Recent Advances in Crop Water Stress Detection.
585 Computers and Electronics in Agriculture 2017, 141, 267–275,
586 doi:10.1016/j.compag.2017.07.026.
587 53. Hsiao, T.C.; Xu, L. Sensitivity of Growth of Roots versus Leaves to Water Stress:
588 Biophysical Analysis and Relation to Water Transport. Journal of Experimental
589 Botany 2000, 51, 1595–1616, doi:10.1093/jexbot/51.350.1595.
590 54. Kingston-Smith, A.H.; Foyer, C.H. Bundle Sheath Proteins Are More Sensitive to
591 Oxidative Damage than Those of the Mesophyll in Maize Leaves Exposed to Paraquat
592 or Low Temperatures.
593 55. Petridis, A. Effect of Water Deficit on Leaf Phenolic Composition, Gas Exchange,
594 Oxidative Damage and Antioxidant Activity of Four Greek Olive (*Olea Europaea* L.)
595 Cultivars. Plant Physiology and Biochemistry 2012, 11.
596 56. Hunter, J.J.; Visser, J.H. The Effect of Partial Defoliation, Leaf Position and
597 Developmental Stage of the Vine on the Photosynthetic Activity of Vitis Vinifera L.
598 Cv Cabernet Sauvignon. SAJEV 2017, 9, doi:10.21548/9-2-2297.
599 57. Retamales, J.B.; Montecino, J.M.; Lobos, G.A.; Rojas, L.A. COLORED SHADING NETS
600 INCREASE YIELDS AND PROFITABILITY OF HIGHBUSH BLUEBERRIES. Acta
601 Hortic. 2008, 193–197, doi:10.17660/ActaHortic.2008.770.22.
602 58. Di Vaio, C.; Marallo, N.; Di Lorenzo, R.; Pisciotta, A. Anti-Transpirant Effects on Vine
603 Physiology, Berry and Wine Composition of Cv. Aglianico (Vitis Vinifera L.) Grown
604 in South Italy. Agronomy 2019, 9, 244, doi:10.3390/agronomy9050244.
605 59. du Jardin, P. Plant Biostimulants: Definition, Concept, Main Categories and
606 Regulation. Scientia Horticulturae 2015, 196, 3–14, doi:10.1016/j.scienta.2015.09.021.

60. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. Front. Plant Sci. 2017, 7, doi:10.3389/fpls.2016.02049.
61. Graziani, G.; Ritieni, A.; Cirillo, A.; Cice, D.; Di Vaio, C. Effects of Biostimulants on Annurca Fruit Quality and Potential Nutraceutical Compounds at Harvest and during Storage. Plants 2020, 9, 775, doi:10.3390/plants9060775.
62. Di-Vaio, C.; Cirillo, A.; Cice, D.; El-Nakhel, C.; Rouphael, Y. Biostimulant Application Improves Yield Parameters and Accentuates Fruit Color of Annurca Apples. Agronomy 2021, 11, 715, doi:10.3390/agronomy11040715.
63. Kocira, A.; Świeca, M.; Kocira, S.; Złotek, U.; Jakubczyk, A. Enhancement of Yield, Nutritional and Nutraceutical Properties of Two Common Bean Cultivars Following the Application of Seaweed Extract (Ecklonia Maxima). Saudi Journal of Biological Sciences 2018, 25, 563–571, doi:10.1016/j.sjbs.2016.01.039.
64. Drobek, M.; Frąc, M.; Cybulska, J. Plant Biostimulants: Importance of the Quality and Yield of Horticultural Crops and the Improvement of Plant Tolerance to Abiotic Stress—A Review. Agronomy 2019, 9, 335, doi:10.3390/agronomy9060335.
65. Askari-Khorasgani, O.; Hatterman-Valenti, H.; Flores Pardo, F.B.; Pessarakli, M. Plant and Symbiont Metabolic Regulation and Biostimulants Application Improve Symbiotic Performance and Cold Acclimation. Journal of Plant Nutrition 2019, 42, 2151–2163, doi:10.1080/01904167.2019.1648681.
66. Massa, D.; Lenzi, A.; Montoneri, E.; Ginepro, M.; Prisa, D.; Burchi, G. Plant Response to Biowaste Soluble Hydrolysates in Hibiscus Grown under Limiting Nutrient Availability. Journal of Plant Nutrition 2018, 41, 396–409, doi:10.1080/01904167.2017.1404611.
67. Rouphael, Y.; Colla, G. Editorial: Biostimulants in Agriculture. Front. Plant Sci. 2020, 11, 40, doi:10.3389/fpls.2020.00040.
68. Shahrajabian, M.H.; Chaski, C.; Polyzos, N.; Petropoulos, S.A. Biostimulants Application: A Low Input Cropping Management Tool for Sustainable Farming of Vegetables. Biomolecules 2021, 11, 698, doi:10.3390/biom11050698.

635 69. Ali, A.H.; Aboohanah, M.A.; Abdulhussein, M.A. Impact of Foliar Application with
636 Dry Yeast Suspension and Amino Acid on Vegetative Growth, Yield and Quality
637 Characteristics of Olive (*Olea Europaea* L.) Trees.
638 70. Chouliaras, V.; Tasioula, M.; Chatzissavvidis, C.; Therios, I.; Tsabolatidou, E. The
639 Effects of a Seaweed Extract in Addition to Nitrogen and Boron Fertilization on
640 Productivity, Fruit Maturation, Leaf Nutritional Status and Oil Quality of the Olive (
641 *Olea Europaea* L.) Cultivar Koroneiki: Effect of Seaweed Extract on Olive Tree
642 Nutritional Status and Productivity. J. Sci. Food Agric. 2009, 89, 984–988,
643 doi:10.1002/jsfa.3543.
644 71. Hernández-Hernandez, G.; Salazar, D.M.; Martínez-Tomé, J.; López-Cortés, I. The Use
645 of Biostimulants in High-Density Olive Growing: Quality and Production. AJAAR
646 2019, 1–14, doi:10.9734/ajaar/2019/v10i430034.
647 72. Zouari, I.; Mechri, B.; Tekaya, M.; Dabbaghi, O.; Cheraief, I.; Mguidiche, A.; Annabi,
648 K.; Laabidi, F.; Attia, F.; Hammami, M.; et al. Olive Oil Quality Influenced by
649 Biostimulant Foliar Fertilizers. Braz. J. Biol. Sci. 2020, 7, 3–18,
650 doi:10.21472/bjbs(2020)071501.
651 73. Almadi, L.; Paoletti, A.; Cinosi, N.; Daher, E.; Rosati, A.; Di Vaio, C.; Famiani, F. A
652 Biostimulant Based on Protein Hydrolysates Promotes the Growth of Young Olive
653 Trees. Agriculture 2020, 10, 618, doi:10.3390/agriculture10120618.
654 74. Bacelar, E.A.; Santos, D.L.; Moutinho-Pereira, J.M.; Goncalves, B.C.; Ferreira, H.F.;
655 Correia, C.M. Immediate Responses and Adaptative Strategies of Three Olive
656 Cultivars under Contrasting Water Availability Regimes: Changes on Structure and
657 Chemical Composition of Foliage and Oxidative Damage. Plant Science 2006, 10.
658 75. Denaxa, N.-K.; Damvakaris, T.; Roussos, P.A. Antioxidant Defense System in Young
659 Olive Plants against Drought Stress and Mitigation of Adverse Effects through
660 External Application of Alleviating Products. Scientia Horticulturae 2020, 259, 108812,
661 doi:10.1016/j.scienta.2019.108812.

662 76. Mphande, W.; Kettlewell, P.S.; Grove, I.G.; Farrell, A.D. The Potential of
663 Antitranspirants in Drought Management of Arable Crops: A Review. Agricultural
664 Water Management 2020, 236, 106143, doi:10.1016/j.agwat.2020.106143.
665 77. del Amor, F.M.; Cuadra-Crespo, P.; Walker, D.J.; Cámara, J.M.; Madrid, R. Effect of
666 Foliar Application of Antitranspirant on Photosynthesis and Water Relations of
667 Pepper Plants under Different Levels of CO2 and Water Stress. Journal of Plant
668 Physiology 2010, 167, 1232–1238, doi:10.1016/j.jplph.2010.04.010.
669 78. Di Vaio, C.; Villano, C.; Lisanti, M.T.; Marallo, N.; Cirillo, A.; Di Lorenzo, R.; Pisciotta,
670 A. Application of Anti-Transpirant to Control Sugar Accumulation in Grape Berries
671 and Alcohol Degree in Wines Obtained from Thinned and Unthinned Vines of Cv.
672 Falanghina (Vitis Vinifera L.). Agronomy 2020, 10, 345,
673 doi:10.3390/agronomy10030345.
674 79. del Amor, F.M.; Rubio, J.S. Effects of Antitranspirant Spray and Potassium: Calcium:
675 Magnesium Ratio on Photosynthesis, Nutrient and Water Uptake, Growth, and Yield
676 of Sweet Pepper. Journal of Plant Nutrition 2009, 32, 97–111,
677 doi:10.1080/01904160802531043.
678 80. Lombardini, L.; Harris, M.K.; Glenn, D.M. Effects of Particle Film Application on Leaf
679 Gas Exchange, Water Relations, Nut Yield, and Insect Populations in Mature Pecan
680 Trees. 2005, 40, 5.
681 81. Hedhly, A.; Hormaza, J.I.; Herrero, M. Global Warming and Sexual Plant
682 Reproduction. Trends in Plant Science 2009, 14, 30–36,
683 doi:10.1016/j.tplants.2008.11.001.
684 82. Mesihovic, A.; Iannacone, R.; Firon, N.; Fragkostefanakis, S. Heat Stress Regimes for
685 the Investigation of Pollen Thermotolerance in Crop Plants. Plant Reprod 2016, 29, 93–
686 105, doi:10.1007/s00497-016-0281-y.
687 83. Walther, G.-R.; Post, E.; Convey, P.; Menzel, A.; Parmesan, C.; Beebee, T.J.C.;
688 Fromentin, J.-M.; Hoegh-Guldberg, O.; Bairlein, F. Ecological Responses to Recent
689 Climate Change. Nature 2002, 416, 389–395, doi:10.1038/416389a.

84. White, M.A.; de BEURS, K.M.; Didan, K.; Inouye, D.W.; Richardson, A.D.; Jensen, O.P.; O'Keefe, J.; Zhang, G.; Nemani, R.R.; van LEEUWEN, W.J.D.; et al. Intercomparison, Interpretation, and Assessment of Spring Phenology in North America Estimated from Remote Sensing for 1982-2006. Global Change Biology 2009, 15, 2335–2359, doi:10.1111/j.1365-2486.2009.01910.x.
85. Byrne, D.H.; Sherman, W.B.; Bacon, T.A. Stone Fruit Genetic Pool and Its Exploitation for Growing under Warm Winter Conditions. In Temperate Fruit Crops in Warm Climates; Erez, A., Ed.; Springer Netherlands: Dordrecht, 2000; pp. 157–230 ISBN 978-90-481-4017-6.
86. Legave, J.M.; Blanke, M.; Christen, D.; Giovannini, D.; Mathieu, V.; Oger, R. A Comprehensive Overview of the Spatial and Temporal Variability of Apple Bud Dormancy Release and Blooming Phenology in Western Europe. Int J Biometeorol 2013, 57, 317–331, doi:10.1007/s00484-012-0551-9.
87. Bartolini, S.; Massai, R.; Iacona, C.; Guerriero, R.; Viti, R. Forty-Year Investigations on Apricot Blooming: Evidences of Climate Change Effects. Scientia Horticulturae 2019, 244, 399–405, doi:10.1016/j.scienta.2018.09.070.
88. Rathcke, B. Phenological Patterns of Terrestrial Plants. 2023.
89. Bertin, R.I. Plant Phenology And Distribution In Relation To Recent Climate Change. The Journal of the Torrey Botanical Society 2008, 135, 126–146, doi:10.3159/07-RP-035R.1.
90. Aronne, G. Identification of Bottlenecks in the Plant Life Cycle for Sustainable Conservation of Rare and Endangered Species. Front. Ecol. Evol. 2017, 5, 76, doi:10.3389/fevo.2017.00076.
91. Aronne, G.; Buonanno, M.; De Micco, V. Reproducing under a Warming Climate: Long Winter Flowering and Extended Flower Longevity in the Only Mediterranean and Maritime Primula. Plant Biol J 2015, 17, 535–544, doi:10.1111/plb.12239.
92. Asma, B.M. Determination of Pollen Viability, Germination Ratios and Morphology of Eight Apricot Genotypes.

718 93. Pirlak, L. The Effects of Temperature on Pollen Germination and Pollen Tube Growth
719 of Apricot and Sweet Cherry.
720 94. Iovane, M.; Izzo, L.G.; Cirillo, A.; Romano, L.E.; Di Vaio, C.; Aronne, G. Flowering and
721 Pollen Resilience to High Temperature of Apricot Cultivars. Scientia Horticulturae
722 2022, 304, 111261, doi:10.1016/j.scienta.2022.111261.
723 95. Poljuha, D.; Sladonja, B.; Šetić, E.; Milotić, A.; Bandelj, D.; Jakše, J.; Javornik, B. DNA
724 Fingerprinting of Olive Varieties in Istria (Croatia) by Microsatellite Markers. Scientia
725 Horticulturae 2008, 115, 223–230, doi:10.1016/j.scienta.2007.08.018.
726 96. Rallo, P.; Dorado, G.; Martín, A. Development of Simple Sequence Repeats (SSRs) in
727 Olive Tree (*Olea Europaea* L.). Theor Appl Genet 2000, 101, 984–989,
728 doi:10.1007/s001220051571.
729 97. Mohamed Mousa, Y.; Gerasopoulos, D.; Metzidakis, I.; Kiritsakis, A. Effect of Altitude
730 on Fruit and Oil Quality Characteristics of 'Mastoides' Olives. J. Sci. Food Agric. 1996,
731 71, 345–350, doi:10.1002/(SICI)1097-0010(199607)71:3<345::AID-JSFA590>3.0.CO;2-T.
732 98. Ridolfi, M.; Terenziani, S.; Patumi, M.; Fontanazza, G. Characterization of the
733 Lipoxygenases in Some Olive Cultivars and Determination of Their Role in Volatile
734 Compounds Formation. J. Agric. Food Chem. 2002, 50, 835–839, doi:10.1021/jf0109118.
735 99. Di Vaio, C.; Nocerino, S.; Paduano, A.; Sacchi, R. Characterization and Evaluation of
736 Olive Germplasm in Southern Italy: Olive Germplasm in Southern Italy. J. Sci. Food
737 Agric. 2013, 93, 2458–2462, doi:10.1002/jsfa.6057.
738 100. Ledesma, N.A.; Kawabata, S. Responses of Two Strawberry Cultivars to Severe High
739 Temperature Stress at Different Flower Development Stages. Scientia Horticulturae
740 2016, 211, 319–327, doi:10.1016/j.scienta.2016.09.007.
741

Chapter 2. Biostimulants Improve Plant Growth and Bioactive Compounds of Young Olive Trees under Abiotic Stress Conditions

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Abstract: The negative impacts of extreme heat and drought on olive plants have driven the quest for mitigation approaches based on the use of biostimulants, which have proved to be effective in contrasting environmental stresses. The aim of our study was to evaluate the effectiveness of six biostimulants in mitigating high temperature and water stress in young olive trees in terms of vegetative and eco-physiological parameters as well as bioactive compound content. Biostimulants based on glycine betaine and macro- and micro-algae effectively protected the plants from abiotic stress by improving their eco-physiological and vegetative parameters. At the end of the growing season, olive plants were experiencing water deficit which had built up through the summer months. At this time, the glycine betaine-treated plants had a three-fold higher stomatal conductance compared with the control, while plants sprayed with the seaweed mix had a relative water content 33% higher than the control. The kaolin treatment resulted in higher total phenolics and antioxidant activities (DPPH, FRAP and ABTS) in water stress conditions and caused an increase of 238.53 and 443.49% in leaves total polyphenols content in 100% and 50% water regime, respectively. This study showed the effectiveness of biostimulants in mitigating the damage from abiotic stress on young olive trees, by improving some vegetative, eco-physiological and leaf nutraceutical parameters. Further studies are needed to test the efficiency of these biostimulants in open field conditions on olive trees in full production.

2.1. Introduction

Olive (*Olea europaea* L.) is one of the most important fruit trees of the Mediterranean region with an enormous economic and ecological value. In its climatic region, olive is often subject to drought periods during the warm season; nevertheless, it is characterized by high morphological and physiological adaptation capacities [1]. Water stress was reported to induce in olive plants the activation of antioxidant enzyme systems such as ascorbate

peroxidase, catalase and superoxide dismutase [2]. Moreover, the activation of the phenylpropanoid biosynthetic pathway leading to the accumulation of phenolic compounds is a well-known metabolic response to water deficit as well as to other environmental stresses [2]. Such metabolic responses of the plants to unfavorable environmental conditions play a key role in preventing cellular damage caused by oxidative stress. However, the constitutive tolerance to water deficit alone is not sufficient to protect olive trees from the combined effects of extreme heat waves, water stress and high irradiance, which are all linked to climate change. For this reason, the application of biostimulants as a sustainable practice to mitigate the negative impact of environmental stresses and to meliorate or maintain plant productivity has become increasingly popular over the past decade [3]. In this respect, different categories of biostimulants have been proved effective to minimize the effects of abiotic stresses on plants. Among these, plant-derived biostimulants are particularly interesting due to their biocompatibility and low environmental impact [4]. The osmolyte glycine betaine is widely studied and its effectiveness towards numerous sources of stress such as drought, cold or salinity, were reported [5]. Kaolin clay reflective particles are also well known as an effective tool to reduce the effects of abiotic stress on crop performance by affecting the plant at the morphological, physiological and biochemical levels [6]. In this case, however, contrasting results were reported, depending on the species or even the genotype [6,7]. Pinolene, is a therpenic polymers (Di-1-p-menthene), which is inactive from a biochemical point of view, and it is mainly used in crops to limit leaf water loss since it acts as film-forming antitranspirant [8,9]. Pinolene has been proposed as an alternative to defoliation in hot climates [10–13]. Among the algal extracts, foliar applications of a biostimulant based on Ascophyllum nodosum and Laminaria digitata were shown to be involved in the regulation of secondary metabolism, resulting in improved fruit quality and nutritional value of apples cv. “Annurca” [14]. In addition, Kusvuran [15] reported that under drought stress, the foliar application of micro-algae such as Chlorella vulgaris, significantly improved secondary metabolites, such as polyphenolic compounds and an- tioxidant enzyme activities. Furthermore, Graziani et al. [14] reported that treatments with micro-algae on apple trees cv. “Annurca” also improved

post-harvest fruit conservation by preserving the nutritional quality, in terms of polyphenols content after 120 days of cold storage. Fungi of the Trichoderma genus are widely studied and commonly used as biostimulants in agriculture. Rudresh et al. [16] showed that a mixture of *T. harzianum, T. viride* and *T. virens* increased plant biomass and nutrient uptake. Some reports showed beneficial effects of *Thricoderma* on the alleviation of salt stress effects [17,18]. Dini and co-workers recently reported that Trichoderma strains increase phenolics concentration both in olive leaves and in oil, as a result of improved plant nutrient uptake and enhanced nitrogen use efficiency [19]. On the basis of this knowledge, we tested the application of six different biostimulant treatments as an approach to overcome/balance the negative impact of high temperatures and water deficit on young olive trees. In this study we monitored agronomic and eco- physiological parameters as well as the antioxidant activities and the phenolic composition of leaf extracts. Such results would be of interest to olive producers, presenting solutions for stress mitigation and crop conservation.

2.2. Materials and Methods

2.2.1. Plant Material, Biostimulants Treatments and Experimental Design

The trial was conducted in a greenhouse at the Department of Agriculture of the University of Naples Federico II, Portici - Italy, between the end of May and the end of September 2020. Two-year-old potted olive trees of the cultivar "Salella", were grown in 5 L pots containing a substrate made up of sand:peat:clayey soil (1:1:1, v/v/v). At the beginning of the experiment, all olive plants had homogeneous vegetative characteristics and 50 g/pot of "Nitrophoska Gold" (a slow-release fertilizer based on N (15%), P_2O_5 (9%), K_2O (15%) supplemented with micronutrients) by COMPO EXPERT Italia Srl (Cesano Maderno, Monza and Brianza, Italy) was mixed with the substrate.

The experimental design was based on seven biostimulant treatments:

(1) Control (C) plants only treated with water, no biostimulant applied.

(2) *Trichoderma* based product (TR), "Trianum-P" by Koppert Biological Systems (Koppert Italy, Bussolengo, VR - Italy), with active ingredient *Trichoderma harzianum* Rifai

858 strain T-22 (also known as KRL-AG2*). The product was applied to the root system by
859 irrigation at the dose of 6.67 g/L of water.
860 (3) Micro-Algae-based product (MA), "AgriAlgae® Biologico Originale" by AgriAlgae®
861 (Madrid, Spain), a biological biostimulant. The product was applied to the root system by
862 irrigation at the dose of 6.67 g/L of water.
863 (4) Seaweed based product (P), "Seaweed Mix®" by L. Gobbi Srl (Campo Ligure, Genoa,
864 Italy), made of Ascophyllum nodosum and Laminaria digitate extract. The product was
865 applied to the root system by irrigation at the dose of 4 mL/L of water.
866 (5) Glycine betaine-based product (B), "BIO-HELP" by Biolchim SPA (Bologna, Italy), a
867 bio-promoter of resistance to environmental stress. The product was applied to the root
868 system by irrigation at the dose of 10 g/L of water.
869 (6) Kaolin (K), "Manisol" by Manica S.p.A (Rovereto, Italy). The product was applied as
870 foliar spray at the dose of 40 g/L of water.
871 (7) A water emulsifiable organic concentrate of di-1-p-menthene (C20H34) (V), "Vapor
872 Gard®" by BIOGARD® (Bergamo, Italy), a terpenic polymer also known as pinolene. The
873 product was applied as foliar sppray at the dose of 10 mL/L of water.
874 All biostimulants were applied five times during the growing season at 20-day intervals.
875 Olive trees were divided into two groups, corresponding to two watering regimes: 100%
876 and 50% of the evapotranspiration (ET). Evapotranspiration was calculated on the well-
877 watered plants of the 100% watering regime group using a gravimetric method as follows:
878 every two days the pots were weighed up and water loss by evapotranspiration was
879 calculated. Subsequently, 100% or 50% of water loss, corresponding to X mL or X/2 mL
880 water per pot, respectively, was restored by drip irrigation. The drip irrigation system was
881 controlled by a programmable timer, and it was powered by an electric pump, feeding water
882 to drippers at a flow rate of 2 L/h. One or two drippers were installed into each pot for
883 irrigation at 50% or 100%, respectively. A total of 14 treatments were compared based on a
884 factorial combination of seven biostimulant treatments (including control) and two
885 irrigation regimes (100% and 50%). The treatments were arranged in a randomized split-

plot design with irrigation levels as main factor and biostimulants as sub-factor. Each treatment consisted of 10 plants.

2.2.2. *Determination of Vegetative and Eco-Physiological Parameters of Leaves*

On fully developed leaves, the stomatal conductance was measured using a Porometer (Li-1600 Steady State Porometer, TR. Turoni Srl, Forli, Italy) at 12:00 a.m. The leaf SPAD index was measure with a chlorophyll meter SPAD-502 (Konica-Minolta, Osaka, Japan). The leaf relative water content (RWC) was calculated following the previously described procedure [7] according to the formula:

$$RWC\ (\%) = ((fw - dw)/(rw - dw)) * 100$$

The number of leaves per plant was recorded at the beginning and at the end of the experiment in order to calculate the increase in leaf number. Leaf area per plant was measured using imageJ software version 1.50 (Wayne Rasband, National Institute of Health, Bethesda, MD, USA) at the end of the experiment. Leaf dry weight was determined by drying sub-samples in a forced air oven until constant weight was reached. Polyphenolic content and antioxidant activity assays were determined on lyophilized leaves. The eco-physiological measurements were carried out in June (one month after the first biostimulant application) and in September (at the end of the growing season), taking six measurements per treatment.

2.2.3. *Chemicals Analyses and Ultrasound-Assisted Extraction of Polyphenolic Compounds*

All standards for the analysis were supplied by Sigma Aldrich St. Louis, MO, USA, while hydroxytyrosol was purchased from Indofine Hillsborough, NJ, USA), secologanoside from ChemFaces Biochemical Co., Ltd. (Wuhan, China) and oleuropein form Extrasynthese (Genay, France). Acetonitrile and water (LC-MS grade) were acquired from Carlo Erba reagents (Milan, Italy), whereas acetic acid (98–100%) was purchased from Fluka (Milan, Italy). Lyophilized samples were extracted using the method reported in the literature [20] with few modifications. In particular, 0.3 gr of dried sample were extracted with 15 mL of methanol/water (80:20 *v*/*v*, 0.1% formic acid) by sonication at room temperature for 15 min. Samples were centrifuged to 4000 rpm at 4 ◦C for 10 min, and the pellet was extracted in the

same way. The supernatants were collected, filtered through 0.45 mm nylon syringe membranes and then used for high-resolution mass spectrometry analysis and antioxidant activity assays.

2.2.4. UHPLC-HRMS Analysis of Polyphenolic Compounds

An Ultra-High-Pressure Liquid Chromatograph (UHPLC, Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Q-Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, Waltham, MA, USA) was used to investigate the quali-quantitative profile of polyphenolic compounds applying conditions reported in our previous work [19]. An Accucore aQ 2.6 μm (100 2.1 mm) column (Thermo Sci- entific, Waltham, MA, USA) was applied for chromatographic separation of polyphenols with a column temperature set at 30 ◦C. The mobile phase consisted of water containing 0.1% of acetic acid (eluent A) and acetonitrile (eluent B). Polyphenolic compounds were eluted using the following gradient program: 0–5 min 5% B, 5–25 min 5–40% B, 25–25.1 min 40–100% B, 25.1–27 min 100% B, 27–27.1 min 100–5% B, 27.1–35 min 5% B. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 μL. The mass spectrometer was operated in negative ion mode (ESI–) setting two scan events (Full ion MS and All ion fragmentation, AIF) for all compounds of interest. Full scan data were acquired setting a resolving power of 35,000 FWHM (full width at half maximum) at m/z 200. The key parameters were as follows: spray voltage 2.8 kV, sheath gas flow rate 35 arbitrary units, auxiliary-gas flow rate, 10 arbitrary units, capillary temperature 275 ◦C, auxiliary gas heater temperature 350 ◦C, S-lens RF level 50. For the scan event of AIF, the resolving power was set at 17,500 FWHM, the collision energies were 10, 20, and 45 eV, and the scan range was m/z 80–1200. Data acquisition and processing were performed with Quan/Qual Browser Xcalibur software, v. 3.1.66.10 (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA).

2.2.5. Antioxidant Activity: ABTS Assay

Determination of the ABTS free radical scavenging activity was carried out following the method described by Re et al. [21]. Briefly, 44 μL of aqueous potassium persulfate (2.45 mM) were added to 2.5 mL of aqueous ABTS (7 mM) and incubated in the dark at room temperature for 12–16 h. The ABTS solution was diluted with ethanol (1:88) to obtain an

944 ABTS radical working solution with an absorbance value of 0.75 0.050 at 734 nm. The assay
945 was performed by adding 100 μL of properly diluted sample to 1 mL of ABTS radical
946 working solution and the absorbance was monitored after 2.5 min at 734 nm. Results were
947 expressed as Trolox equivalent antioxidant capacity (TEAC, mmol Trolox equivalents kg^{-1}
948 dry weight of leaves). All determinations were performed in triplicate.
949 *2.2.6. Antioxidant Activity: DPPH Assay*
950 The DPPH assay was carried out according to the procedure reported by Brand- Williams
951 et al. [22] with minor modifications. Briefly, methanolic DPPH radical working solution was
952 prepared diluting methanolic DPPH (4 mg in 10 mL) with methanol, until an absorbance
953 value of 0.900 0.020 at 517 nm. For the assay, 200 μL of sample were added to 1 mL of
954 radical working solution and the absorbance value was monitored after 10 min. The results
955 were expressed as Trolox equivalents antioxidant capacity (TEAC, mmol Trolox equivalent
956 kg–1 of dry weight of leaves). All determinations were performed in triplicate.
957 *2.2.7. Antioxidant Activity: FRAP Assay*
958 The FRAP assay was conducted according to the method reported by Benzie and Strain [23]
959 with slight adjustments as mentioned in Formisano et al. [24]. Briefly, the FRAP reagent was
960 made up of 10 μM TPTZ in 40 μM HCl, 20 μM of aqueous $FeCl_3$ and acetate buffer (300 μM,
961 pH 3.6) at 1:1:10 (v/v/v). Sample solutions, properly diluted, (10 μL) and FRAP reagent (300
962 μL) were mixed and the absorbance was monitored at 593 nm after 10 min. The results were
963 expressed in mmol Trolox® Kg^{-1} dry weight (dw). The results were corrected for dilution
964 and expressed as Trolox® equivalent antioxidant capacity (TEAC, mmol Trolox equivalents
965 Kg^{-1} dry weight of leaves). All determinations were performed in triplicate.
966 *2.2.8. Total Polyphenol Content: FOLIN Test*
967 Total phenolics were determined according to a Folin-Ciocalteu procedure with slight
968 changes [25]. Briefly, 125 μL of diluted extract or blank was mixed with 500 μL of deionized
969 water and 125 μL of the Folin-Ciocalteu reagent for 6 min at room temperature. Subse-
970 quently, 1.25 mL of 7.5% of sodium carbonate solution and 1 mL of deionized water were
971 added in the mixture. The absorbance at 760 nm after 90 min. of incubation in the dark was
972 measured. Concentrations of total phenolic were expressed in terms of mg of gallic acid

973 equivalents (GAE) per gram dry weight (DW), based on a calibration curve ($R2 > 0.993$) that
974 was computed over a dynamic range 0.05–2.5 g/L gallic acid. Each extract was analyzed in
975 triplicate.
976 *2.2.9. Statistical Analysis*
977 All data were subjected to analysis of variance (ANOVA). Duncan's multiple range test
978 (DMRT) was performed for means separation of each of the measured variables at $p = 0.05$.
979 A principal component analysis (PCA) was executed on vegetative, physiological and
980 bioactive parameters at the end of growing season to detect the interrelationship. The PCA
981 results are shown as a biplot to highlight the interaction between samples and variables.
982 Samples are displayed as points while variables are displayed as vectors. A correlation
983 analysis between the total phenolic content (Folin) and each of the antioxidant capacity
984 assays (ABTS, DPPH and FRAP) was performed. The statistical package XLStat Version
985 2013 (New York, NY, USA) was implemented for all the analyses.
986
987 **2.3. Results and Discussion**
988 *2.3.1. Vegetative and Eco-Physiological Parameters*
989 Figure 1 shows the temperature values recorded in the greenhouse during the growing
990 season, from May to September. The young olive trees were exposed to prolonged thermal
991 stress: the maximum daily temperature ranged between 23.42 ◦C (4 June) and 51.74 ◦C (2
992 June), while the minimum daily temperature ranged between 10.20 ◦C (31-May) and 25.01
993 ◦C (2 June). The average daily temperatures were between 19.44 ◦C (5 June) and 36.97 (2
994 June).
995 In Table 1, the vegetative parameters determined on the leaves are reported: leaf dry weight
996 (g), leaf number and leaf area (cm^2). A significant interaction emerged between the two
997 tested factors (watering regime biostimulant treatment). Leaf dry weight and leaf area were
998 higher in plants at the 100% water regime and were not influenced by the different
999 biostimulant treatments. Leaf dry matter did not show significant differences between the
1000 various treatments compared with the control (C) in both watering regimes. The two
1001 watering regimes also affected the production of new leaves: plants in the group at the 100%

watering regime produced 89% new leaves, compared with 48% new leaves at the 50% watering regime. Therefore, the plant group exposed to water deficit produced 45% less new leaves compared to the well-watered group. These results are consistent with previous literature on the effects of water deficit: vegetative growth is closely related to plant water status since a loss of turgor impairs cell expansion and results in reduced plant growth in terms of plant height, leaf area, dry weight and other vegetative parameters [26,27]. Compared with the untreated control under the 50% watering regime, plants treated with glycine betaine (B) had a significantly higher leaf number (+44%) and 56% larger leaf area, while the micro-algae (M) and seaweed (P) treated plants had a 26% and 44% larger leaf area, respectively. Our results are in agreement with previous studies reporting that glycine betaine improved plant resistance to high temperature stress [28]. Moreover, Alia et al. [29] reported that tolerance to abiotic stress during the imbibition and germination of seeds, as well as during the growth of young seedlings was enhanced in transformed *Arabidopsis thaliana* accumulating glycine betaine. The exogenous application of glycine betaine was also reported to positively affect plant growth and final crop yield under drought stress [30,31]. Seaweed extracts are employed in agriculture for their beneficial effects on plant growth, root development, mineral nutrition and fruit setting as well as improved resistance to abiotic stresses (drought, salinity and temperature), pests and diseases as recently reviewed by Mukherjee and Patel [32].

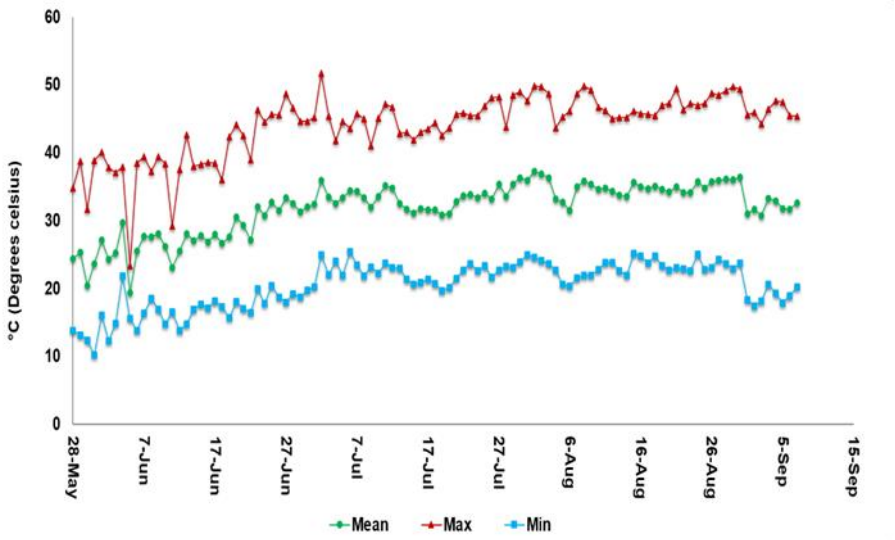

Figure 1. Temperature trend recorded during the growing season from May to September under greenhouse conditions: maximum, minimum, and mean temperature (◦C).

Table 1. Leaf dry matter (g), increase in leaf number and leaf area (cm^2) of olive trees grown in a greenhouse under optimal irrigation (100% irrigation) and limited water availability (50% irrigation) treated with six different biostimulants: C = control, TR = Trichoderma, M = Micro-Algae, P = Seaweed mix, K = Kaolin, B = Glycine betaine, V = Pinolene.

Treatments	Leaf Dry Matter (g)	Increase Leaves Number	Leaf Area (cm^2)
	100%		
C	16.97 ± 0.61 a	83.70 ± 7.01 abc	770.29 ± 25.56 a
TR	18.37 ± 0.78 a	88.70 ± 5.29 abc	729.59 ± 19.18 a
M	18.06 ± 0.58 a	86.70 ± 8.03 abc	758.90 ± 27.87 a
P	17.20 ± 0.97 a	82.80 ± 6.93 abc	704.16 ± 25.26 a
B	16.38 ± 0.53 a	102.00 ± 8.17 a	736.15 ± 25.96 a
K	17.93 ± 0.71 a	77.50 ± 7.26 bc	759.66 ± 41.60 a
V	18.57 ± 0.81 a	94.50 ± 4.81 ab	723.25 ± 28.17 a
	50%		
C	10.20 ± 0.53 bcd	49.40 ± 5.47 ef	361.81 ± 16.27 ef
TR	8.74 ± 0.63 d	39.70 ± 5.48 ef	330.14 ± 11.49 f
M	9.87 ± 0.69 cd	49.20 ± 10.68 ef	454.09 ± 30.06 cd
P	11.61 ± 0.85 bc	45.20 ± 4.88 ef	522.28 ± 26.05 bc
B	10.77 ± 0.53 bcd	70.90 ± 3.76 cd	562.84 ± 34.43 b
K	12.08 ± 0.52 b	30.40 ± 5.46 f	437.72 ± 19.46 cde
V	12.32 ± 0.69 b	54.20 ± 10.76 de	515.79 ± 34.02 cde
	Significance		
W	***	***	***
T	ns	*	ns
W × T	***	***	***

Values are mean standard error. Asterisks indicate significant effect of limited water availability (W), bios- timulants treatments (T) and their interaction (WxT) according to ANOVA (ns = not significant; * = $p < 0.05$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p = 0.05$).

The eco-physiological parameters (stomatal conductance, SPAD and RWC) measured at the beginning (1 month following the first biostimulant application) and at the end of the growing season are reported in Table 2. For each of the three parameters, significant interactions emerged among the experimental factors W × T × S (water regime × treatments × measurement time).

Table 2. Stomatal conductance (mol m^{-2} s^{-1}), SPAD and RWC (%) values in leaves of olive trees grown in a greenhouse under optimal irrigation (100% irrigation) and limited water availability (50% irrigation) treated with several biostimulants: C = control, TR = Trichoderma, M = Micro-Algae, P = Seaweed mix, K = Kaolin, B = Glycine betaine, V = Pinolene; at the start (Time 1: T1; 1 month after the first biostimulant application) and at the end (Time 2: T2) of the experiment.

	Time 1		Time 2	
Treatments	**100%**	**50%**	**100%**	**50%**
		Stomatal conductance (mol m^{-2} s^{-1})		
C	0.534 ± 0.01 bc	0.165 ± 0.00 efgh	0.148 ± 0.02 efgh	0.064 ± 0.00 fgh
TR	0.538 ± 0.04 bc	0.120 ± 0.02 efgh	0.253 ± 0.04 def	0.036 ± 0.01 gh
M	0.233 ± 0.00 def	0.686 ± 0.10 b	0.616 ± 0.12 b	0.257 ± 0.02 de
P	0.276 ± 0.02 def	0.118 ± 0.01 efgh	0.389 ± 0.19 cd	0.030 ± 0.01 h
B	0.875 ± 0.01 a	0.539 ± 0.04 bc	0.557 ± 0.06 b	0.271 ± 0.03 de
K	0.193 ± 0.03 efgh	0.231 ± 0.01 def	0.054 ± 0.00 fgh	0.057 ± 0.00 fgh
V	0.195 ± 0.05 efgh	0.211 ± 0.03 efg	0.149 ± 0.00 efgh	0.221 ± 0.03 def
		SPAD		
C	75.57 ± 1.20 ghij	76.29 ± 0.77 fghi	73.06 ± 1.27 j	76.50 ± 0.74 fghi
TR	74.39 ± 1.35 hij	75.23 ± 0.82 ghij	74.20 ± 1.52 hij	77.05 ± 1.13 efghi
M	80.88 ± 0.83 bc	76.82 ± 0.99 efghi	78.93 ± 0.72 cdef	80.00 ± 0.95 bcde
P	78.23 ± 1.00 cdefg	77.83 ± 0.81 cdefg	75.68 ± 0.85 fghij	79.79 ± 1.02 bcde
B	80.52 ± 1.08 bcd	82.63 ± 0.73 ab	80.54 ± 0.85 bcd	78.87 ± 0.75 cdef
K	76.08 ± 1.05 fghij	74.41 ± 1.17 hij	78.03 ± 0.65 cdefg	84.42 ± 0.64 a
V	78.00 ± 0.85 cdefg	74.04 ± 0.89 ij	77.45 ± 1.20 defgh	76.93 ± 0.73 efghi
		RWC%		
C	79.87 ± 2.60 ab	75.14 ± 4.20 abcd	60.87 ± 3.46 fgh	55.24 ± 1.71 ghi
TR	79.90 ± 1.92 ab	76.33 ± 3.22 abc	65.56 ± 1.64 def	68.56 ± 6.69 cdef
M	81.09 ± 1.45 a	73.82 ± 2.49 abcd	63.11 ± 4.32 efg	50.95 ± 1.06 i
P	75.67 ± 3.81 abcd	78.15 ± 3.18 abc	62.45 ± 1.82 fgh	73.32 ± 4.32 abcd
B	74.51 ± 2.83 abcd	80.56 ± 3.20 ab	72.96 ± 1.71 abcde	61.22 ± 2.18 fgh
K	74.87 ± 0.72 abcd	77.82 ± 1.11 abc	67.82 ± 2.48 cdef	55.54 ± 0.92 ghi
V	74.25 ± 4.94 abcd	83.66 ± 1.69 a	70.06 ± 4.08 bcdef	52.55 ± 4.62 hi
	Stomatal conductance (mol m^{-2} s^{-1})		**SPAD**	**RWC (%)**
W	Ns		ns	ns
T	***		***	ns
S	Ns		ns	***
W × T × S	***		***	***

Values are means ± standard error. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (T), time of measurements (S) and their interaction (W × T × S) according to ANOVA (ns = not significant; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p = 0.05$).

The highest stomatal conductance value (Table 2) was recorded at Time 1 in plants at the 100% watering regime treated with glucine betaine (0.875 mol m^{-2} s^{-1}). At the same time, plants treated with glycine betaine (B) and with micro-algae (M) had the highest stomatal conductance values within the group grown at the 50% watering regime. Stomatal conductance of B and M treated plants at the 50% watering regime was comparable to control plants at the 100% watering regime. The effectiveness of the B an M treatments in

maintaining a high stomatal conductance was confirmed at the second measurement (Time 2), when the measured values were 0.557 and 0.616 mol m^{-2} s^{-1} for the B and the M treatments, respectively, at the 100% watering regime. Stomatal conductance of B and M treated plants was significantly higher than the control (0.148 mol m^{-2} s^{-1}). A similar effect was recorded in plants grown at the 50% watering regime, as plants treated with B and M biostimulants maintained a higher stomatal conductance compared with the control. Plants treated with the B and the M biostimulants also had significantly higher SPAD index values compared with the control, both at Time 1 and at Time 2 in the case of 100% watering regime. The SPAD index of M treated plants was 7% and 8% higher than Control at Time 1 and at Time 2, respectively, while B treated plants had SPAD index 7% and 10% higher than Control at Time 1 and Time 2, respectively. Among plants at the 100% watering regime, K and V treated plants at Time 2 also had 7% and 6% higher SPAD index than the control, respectively. Among plants grown in water deficit conditions (50% watering regime), the SPAD index for B treated plants was 8% higher than the control at Time 1, while M, P and K treated plants had a SPAD index 5%, 4% and 10% higher than the control, respectively, at Time 2. Our results are in agreement with previous studies reporting that the application of seaweed extracts increased chlorophyll content in leaves [33–36]. Moreover, treatments with seaweed extracts were also reported increase the leaf area as demonstrated in our study. Interestingly, the M and B treatments significantly increased the SPAD index: this may result from a protective effect of seaweed extracts and betaines on chlorophyll as in the reported case of glycine betaine delaying the loss of photosynthetic activity of isolated chloroplasts [37].

The leaf RWC values (Table 2) reflect the water status of the plant: accordingly, higher RWC values were measured at the beginning of the experiments (Time 1) both at 100% and at 50% watering regimes, while lower values were measured after prolonged stress at the end of summer (Time 2). At Time 1, no significant differences emerged between the control and the different biostimulant treatments at either 100% or 50%. Contrastingly, at Time 2 among the 100% watering regime group only plants treated with glycine betaine (B) had a significantly higher (+20%) RWC compared to the control, while in the case of plants at the

50% watering regime, TR and P treated plants had 24% and 33% higher RWC than the control, respectively. The reported effect of the TR treatment in maintaining the plant water status is in agreement with Shukla et al. [38], who showed a significant decrease in the RWC in response to drought stress in untreated *Triticum aestivum* plants, while colonized plants with drought-tolerant *Trichoderma* isolates were able to retain water. However, osmotic adjustment was higher in *Trichoderma*-colonized wheat plants compared to an untreated control and the degree of osmotic adjustment increased with the intensity of drought. Further, another study found that in *P. eugenioides*, the application of seaweed extracts under 100% ET irrigation conditions had no significant effects on improving RWC mean values; however, under water stress conditions (50% ET) the RWC remained significantly higher than the untreated control [39]. In agreement with our results, when exposed to water deficit plants lose water over time with a gradual reduction in transpiration rate [40]. Our results are in line with a previous study [41] showing that a treatment with glycine betaine increased RWC in stressed plants. These data suggested that glycine betaine could increase the plant hydraulic conductivity, enhancing the water flow from roots to shoots and eventually increasing RWC and transpiration rate under stress conditions [41].

2.3.2. Polyphenolic Compounds Analysis by UHPLC-Q-Orbitrap HRMS

The phenolic composition (12 phenolic compounds and their formula) gathered from the UHPLC-HRMS analysis are presented in Table S1, whereas a typical full-scan MS chromatogram of olive leaves extract is reported in Supplementary Figure S1. Table 3 shows the quali-quantitative polyphenolic profile of olive leaves in control and bios- timulant treated samples at two different water regimes (100% and 50% of evapotraspiration).

Table 3. Phenolic profiles and total phenolic composition in leaves of olive tree grown in a greenhouse under optimal irrigation (100% irrigation) and limited water availability (50% irrigation) treated with several biostimulants: C = control, TR = *Trichoderma*, M = Micro-Algae, P = Seaweed mix, K = Kaolin, B = Glycine betaine, V = Pinolene. Concentrations were expressed as mg/g dw.

Polyphenols	C		TR		M		P	
	100%	50%	100%	50%	100%	50%	100%	50%
hydroxytirosol glucoside	2183.11 e	1017.86 g	4978.55 d	1905.95 ef	1959.59 ef	1601.89 efg	5630.96 c	1664.50 efg
vanillic acid	5.78 bcde	3.85 g	7.01 b	5.41 cdef	6.03 bcd	4.89 defg	6.53 bc	4.26 fg

coumaric acid	4.82 b	2.24 ef	3.14 cd	3.39 c	2.49 def	2.56 def	4.86 b	2.04 f
ferulic acid	3.91 c	1.43 fgh	4.84 b	1.82 efg	2.28 e	1.42 fgh	4.15 c	1.31 gh
luteolin rutinoside	7.37 de	8.62 bc	8.05 cd	9.01 bc	7.33 de	9.16 b	7.23 de	7.57 de
verbascoside	156.91 e	118.14 f	504.48 b	715.50 a	403.05 d	472.92 c	28.96 g	25.11 g
oleuropein	629.34 gh	750.22 fg	966.74 d	1274.20 c	1058.80 d	813.96 ef	912.37 de	579.43 h
ligstroside	54.01 g	58.26 g	77.32 f	108.24 cd	67.40 fg	91.11 e	113.72 c	102.90 cde
pinoresinol	0.39 hi	0.34 i	0.54 ef	0.52 ef	0.71 d	0.80 c	0.44 gh	0.33 i
luteolin	205.00 cde	157.81 h	163.89 gh	175.82 fg	199.02 cde	195.64 de	201.15 cde	211.23 cd
oleuropein aglycone	54.67 h	38.27 i	67.72 g	95.61 e	82.34 f	83.22 f	23.06 l	27.80 l
secologanoside	15.08 f	23.88 de	45.76 b	58.54 a	20.16 def	21.27 def	17.37 ef	15.61 f
Total polyphenols	3322.40 de	2180.93 f	6828.03 b	4354.01 c	3809.17 cd	3298.83 de	6950.79 b	2642.09 ef

Polyphenols	**K**		**B**		**V**		**Significance**		
	100%	**50%**	**100%**	**50%**	**100%**	**50%**	**T**	**W**	**W × T**
hydroxytirosol glucoside	8215.27 b	8915.23 a	1540.62 efg	1562.68 efg	980.91 g	1251.98 fg	***	ns	***
vanillic acid	9.06 a	5.30 def	8.68 a	6.42 bc	4.59 efg	3.67 g	***	***	***
coumaric acid	5.70 a	3.51 c	2.08 f	2.05 f	3.51 c	2.91 cde	**	***	***
ferulic acid	7.19 a	1.79 efgh	1.29 gh	1.19 h	1.98 ef	3.06 d	*	***	***
luteolin rutinoside	10.19 a	10.40 a	6.92 e	8.66 bc	7.25 de	8.97 bc	***	***	***
verbascoside	381.39 d	472.28 c	23.28 g	31.35 g	31.51 g	35.97 g	***	ns	***
oleuropein	1868.74 a	1657.18 b	912.29 de	1006.04 d	575.93 h	955.20 de	***	ns	***
ligstroside	266.16 a	241.43 b	103.29 cde	94.61 de	67.79 fg	96.12 de	***	ns	***
pinoresinol	0.57 e	0.64 d	1.19 b	1.43 a	0.46 fg	0.54 e	***	ns	***
luteolin	217.60 c	258.80 b	315.03 a	324.19 a	191.02 ef	176.11 fg	***	ns	***
oleuropein aglycone	230.89 b	242.61 a	106.02 d	122.09 c	45.91 hi	49.40 h	***	ns	***
secologanoside	34.06 c	39.67 bc	13.83 f	14.12 f	13.36 f	25.23 d	***	ns	***
Total polyphenols	11246.82 a	11848.83 a	3034.54 de	3174.82 de	1924.22 f	2609.15 ef	***	ns	***

Values are mean and asterisks indicate significant effect of limited water availability (W), biostimulants treatments (T) and their interaction (WxT) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p = 0.05$).

2.3.3. Antioxidant Activity of Polyphenolics Extracts

Oleuropein and hydroxytirosol glucoside were the two main phenolic compounds detected. By contrast, the presence of tirosol was not observed. Hydroxytyrosol glucoside represented the main component in the olive leaves of well-watered and stressed plants, reaching an average 58.6% on the sum of all polyphenolic compounds. It was also note- worthy that the concentrations of this compound referred to in this work were in general higher than those illustrated by other researchers [20]; nonetheless, these latters used differ- ent cultivars, the sampling conditions reported in those works were different, and these factors could considerably alter levels of this metabolite. According to the literature data, oleuropein was

1123 also well represented in all leaf samples, representing on average 24.5% of the total
1124 compound concentration.
1125 Verbascoside, ligstroside and oleuropein aglycone were also present at significant con-
1126 centrations, ranging between 16.43–0.42%, 3.90–1.13% and 3.49–9.33% of total polyphenolic
1127 compounds, respectively. Among flavonoids, luteolin was the most abundant compound
1128 exhibiting levels between 10.38 and 1.93% of total polyphenols. Coumaric, ferulic and vanil-
1129 lic acid were found in minor concentrations as well as luteolin rutinoside, pinoresinol and
1130 secologanoside. In accordance with literature data [42] the concentration of hydroxytirosol
1131 glucoside showed higher values in leaves from fully irrigated plants compared to water
1132 stressed leaves for untreated leaves and for all other treatments with the exception of Kaolin
1133 and Pinolene, which generated in the leaves a higher concentration of this compound under
1134 water stress conditions. The concentration of oleuropein was shown to be dependent on the
1135 type of biostimu- lant treatment. In fact, a higher level this metabolite was observed in the
1136 leaves of plants subjected to water stress in the case of the untreated samples and after foliar
1137 the application of *Trichoderma*, glycine betaine and pinolene. Higher levels of oleuropein
1138 were found in the leaves of fully irrigated plants following treatment with micro-algae,
1139 seaweed mix and kaolin. An increased concentration of oleuropein in olive leaves subjected
1140 to drought stress was reported by Petridis et al. [2] and by Talhaoui et al. [20], especially in
1141 drought stress of control trees.
1142 The water stress condition resulted in an increase in verbascoside when *Trichoderma*,
1143 seaweed mix, and kaolin were applied to the plants, and a decrease in hydroxycinnamic
1144 acids in all the examined treatments. Water deficit, however, did not affect minor polyphe-
1145 nolic compounds. Overall, leaf polyphenol content decreased as a consequence of water
1146 deficit, while no decrease was observed in plants treated with kaolin, glycine betaine and
1147 pinolene (Table 3). In accordance with literature data [42] the water deficit induced by the
1148 50% the watering regime caused a reduction in polyphenol content, especially in the case of
1149 treatment with seaweed extracts (P treatment) which led to a reduction in polyphenol
1150 content of about 61.98%. A slight decrease of polyphenolic content was observed with
1151 micro-algae treatment (about 13.49%), while in the control and *Trichoderma* treatments, a

similar decrease of about 34.35% and 36.23%, respectively, was found. Plant stress response is related to bioactive metabolite arrangements which are dependent both on the plant (species and cultivar) and on the nature and intensity of the stress factor. Polyphenolic compounds such as flavonoids, secoiridoids and hydroxycinnamic acid derivatives are involved in the plant stress defense as they act as antioxidants useful to counteract oxidative stress [42]. Therefore, the decrease of such compounds under stress conditions may be related to the defense-related functions of phenolic compounds. Interestingly, in the case of treatments with kaolin, glycine betaine and pinolene, no significant differences between irrigation regimes were observed. Therefore, these last treatments support the implementation of agronomic practices to mitigate the negative consequences of water stress. In addition, the positive effects of the kaolin treatment on polyphenols biosynthesis could be related to the up regulation of gene transcription encoding chalcone synthase and phenylalanine ammonia lyase (PAL), as previously described in grape berries subjected to drought and heat stress [43]. On the other hand, Denaxa et al. [44] reported that water-stressed olive leaves treated with kaolin exhibited similar lipid oxidation, evaluated by measuring thiobarbituric acid reactive substances (TBARS), to those under fully irrigated conditions. This suggests that antioxidant defense systems under drought were sufficient and effective to counteract ROS production. Moreover, Brito et al. [6], reported that the attenuation of abiotic stress related to the use of kaolin causes the change of important physiological, morphological and biochemical mechanisms.

Disregarding water stress, olive leaves treated with kaolin had the highest level of total polyphenol detected on average (11,246.82 μg g^{-1} dw) followed by leaves treated with seaweed mix (6950.79 μg g^{-1} dw) and *Trichoderma* based product (6828.03 μg g^{-1} dw) (Table 3). In general, without considering the water stress, all treatments caused an increase in polyphenolic compounds compared to the control with the exception pinolene, which showed the lowest level of polyphenols. Treatment with pinolene provoked an inhibitory effect on PAL activity, hence reducing the concentration of polyphenolic compounds. In literature a similar effect was linked to the antioxidant 5-hydroxybenzimidazole foliar application on "Koroneiki" olive trees [44]. The results of the antioxidant activity essays,

carried out on the polyphenolics extracts of the olive leaves, were reported in Table 4 and expressed as TEAC (mmol Trolox kg−1 dw). As shown, a significant interaction was present between the two tested factors.

Table 4. Antioxidant activity and total polyphenols content in leaves of olive tree grown in a greenhouse under optimal irrigation (100% irrigation) and limited water availability (50% irrigation) treated with several biostimulants: C = control, TR = Trichoderma, M = Micro-Algae, P = Seaweed mix, K = Kaolin, B = Glycine betaine, V = Pinolene.

	DPPH	ABTS	FRAP	FOLIN
100%		**(mmol trolox/kg)**		**(mg/kg dw)**
TS	26.12 ± 0.44 defg	87.59 ± 1.18 c	95.74 ± 1.99 de	2620.16 ± 33.57 fg
M	27.47 ± 0.54 cde	86.47 ± 2.21 c	118.48 ± 0.23 c	3744.19 ± 581.40 e
P	29.17 ± 0.41 c	105.33 ± 0.98 b	138.01 ± 3.45 b	9538.76 ± 637.77 b
TR	28.76 ± 0.15 cd	70.54 ± 4.09 de	131.14 ± 2.23 bc	8337.21 ± 58.14 c
B	24.24 ± 0.44 gh	62.87 ± 2.19 ef	103.00 ± 5.16 d	3608.53 ± 134.27 e
V	26.32 ± 0.16 defg	64.33 ± 7.53 ef	96.84 ± 3.39 de	2484.50 ± 67.13 fg
C	36.43 ± 0.21 a	130.59 ± 4.60 a	166.62 ± 0.77 a	18375.97 ± 378.28 a
50%				
TS	23.22 ± 0.96 h	80.20 ± 2.44 cd	79.42 ± 1.87 fg	2426.36 ± 33.57 g
M	24.77 ± 0.59 fgh	80.11 ± 1.27 cd	85.04 ± 1.05 efg	2988.37 ± 209.63 f
P	20.65 ± 0.52 i	56.90 ± 2.42 f	82.46 ± 1.56 efg	2600.78 ± 33.57 fg
TR	25.98 ± 0.70 efg	74.83 ± 0.78 d	83.70 ± 0.86 def	5275.19 ± 412.48 d
B	22.59 ± 0.47 hi	62.61 ± 1.82 ef	80.95 ± 3.14 g	3511.63 ± 253.42 e
V	27.01 ± 0.56 cdef	78.82 ± 6.90 cd	101.84 ± 2.22 d	2620.16 ± 33.57 fg
C	33.57 ± 0.44 b	106.40 ± 1.04 b	138.64 ± 29.63 b	18686.05 ± 100.70 a
W	**	*	***	**
T	***	***	***	***
W × T	***	***	***	***

Values are means ± standard error of three biological and three technical replicates. Asterisks indicate significant effect of limited water availability (W), biostimulant treatment (T), time of measurements (S) and their interaction (WxTxS) according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Different letters indicate significant differences based on Duncan's test ($p = 0.05$).

According to DPPH data, all treatments induced an increase in compared to the untreated sample. In particular, the antioxidant activity measured with DPPH assay was between 24.24 mmol kg^{-1} (glycine betaine) to 36.43 (kaolin): leaves treated with kaolin showed the highest antioxidant activity, followed by pinolene, with both treatments being significantly higher than the control. In 50%, the values ranged from 20.65 mmol kg^{-1} (seaweed mix) to 33.57 mmol kg−1 (kaolin), whereas *Trichoderma*, pinolene and kaolin generated significantly

higher DPPH compared to the control. As for the ABTS test, the values obtained showed, at antioxidant activity compared to the untreated sample at 100% of evapotranspiration except for glycine betaine, for which a decrease in antioxidant activity was observed 100%, an improvement of antioxidant activity in correspondence with kaolin (+49.09%) and with seaweed mix treatment, (+20.25%), while a decrease in antioxidant activity of 19.47%, 28.22% and 26.56% was found, respectively, in the case of foliar application of *Trichoderma*, glycine betaine and pinolene. However, the treatment with micro-algae did not induce significant changes. For the water regime reduced by 50%, the values ranged from 56.90 mmol kg^{-1} to 106.40 mmol kg^{-1}, and the lower value was referred to seaweed mix treatment. In fact, this last treatment caused a decrease in antioxidant activity compared to untreated leaves equal to 29.08%, while for treatment with glycine betaine the reduction observed was –21.93%. Once again, an increase in antioxidant activity was observed with the kaolin treatment (+32.64%), while insignificant changes were measured for micro-algae, *Trichoderma* and pinolene treatments. In the case of FRAP, at 100%, the majority of the treatments exhibited an improvement in terms of antioxidant activity compared to untreated samples, expect for glycine betaine and pinolene. There was an average increase of 44.72% and kaolin treatment was the one that showed the highest increase (+74.03%). Similar results were recorded at 50% with kaolin, which showed to be the most effective treatment leading to the highest increase (+74.57%) followed by pinolene (+28.23%).

Folin results were in line with those of mass spectrometry investigations and with those of the antioxidant activity evaluated with the FRAP method, showing both in well- irrigated samples and in that water stressed an overall improvement of the antioxidant performances of all plants treated with biostimulant compared to untreated control.

In general, water stress reduced both the antioxidant activity and the level of polyphe- nolic compounds. This decrease may be associated with the defence-related functions of polyphenolic compounds [45]. In literature it is reported that total phenolic and flavonoid contents in leaves of two olive cultivars (Gemlik and Kilis Yaglik) were significantly affected by irrigation treatments, with a cultivar dependent response [45]. In line with our results, Dias et al. [42] observed a reduction in the flavonoid pool after a water deficit of thirty days

and attributed this development to the ROS scavenger capacity of flavonoids. Moreover, Dias et al. [42] reported that independently of the treatment, olive leaves are rich in the *o*-dihydroxy B-ring-substituted flavonoids such as luteolin-7-*O*-glucoside that could assist to this species' high-stress tolerance.

As regards biostimulant treatments, it is interesting to underline the remarkable effect of the kaolin that was responsible for both increased antioxidant activity and polyphenol content in both water regimes investigated compared to untreated control. This result could be due to the upregulation of the gene transcription encoding chalcone synthase and PAL, as antecedently stated for water stressed grape berries [43]. The results obtained showed that the different biostimulants differentially modulated olive leaves phenolic compounds content and antioxidant activity. Denaxa et al. [44] reported that kaolin engendered the highest total phenols concentration, whilst drought-stressed plants exhibited higher total flavonoids concentration when compared to the irrigated plants. However, in contrast to our data, the latter authors noted that glycinebetaine treated trees presented the highest oleuropein content just after foliar application, whereas kaolin treated trees presented the lowest one. In this same study it was featured that kaolin application engendered high activities of antioxidant enzymes such as glutathione reductase, peroxidase and superoxide dismutase under water deficit, compared to other alleviating products that were investigated. In our previous study we reported that *Trichoderma* strains may boost phenolic compounds concentrations, incrementing the plant nutrient uptake mechanism and meliorating plant nitrogen use efficiency, concomitantly with a positive influence on the antioxidant activity [19]. Similar effects were also reported, where the application of *Trichoderma harzianum* caused a significant increase in tomato fruit quality in terms of total soluble sugars, carotenoinds, antioxidant capacity, and polyphenolic content [46]. Pinolene, in agriculture, is used as film-forming antitranspirant that can prevent water loss from the arial part of a plant [47,48]. Several studies have highlighted the profitable effect of film-forming compounds, especially in horticultural crops [43–45]. Brillante et al. [8] reported that pinolene treatment on grape caused a decline in sugar content and anthocyanin level when compared to a control. Moreover, seaweed extracts are biostimulants traditionally

used as soil conditioners as a scope to improve the growth of agricultural crops [49]. The effects of foliar application of algal extracts on the polyphenolic quali-quantitative profile of plants were reported in the literature [50–52] and showed the ability of these extracts to stimulate primary and secondary metabolism by improving nutrient uptake and assimilation, as well as favoring the synthesis and accumulation of phytochemicals which are important for human diet.

2.3.4. Correlation between Total Phenolic Contents and Each Antioxidant Assays

In Figure 2, the correlation between the total phenolic content (Folin) and each of the antioxidant capacity methods in both water regimes is shown.

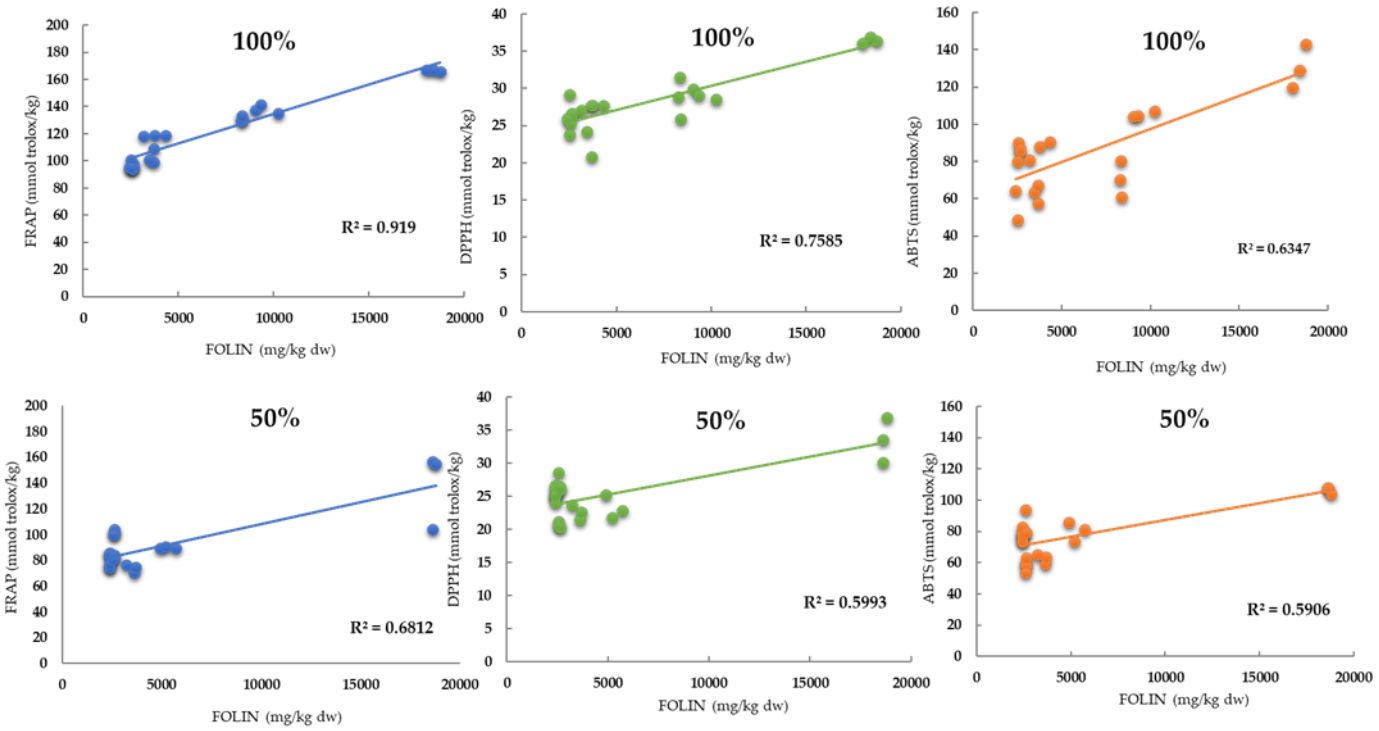

Figure 1. Correlation between the total phenolic content (Folin) and antioxidant capacities of leaves (FRAP, DPPH and ABTS assay) under optimal irrigation (100% irrigation) and limited water availability (50% irrigation).

In the 100% water regime there was a high and positive correlation between Folin and FRAP (r^2 = 0.919), Folin and DPPH (r^2 = 0.758), Folin and ABTS (r^2 = 0.635), while lower values of correlations were shown in 50% water regime with values of r2 respectively equal to 0.681 (Folin-FRAP), 0.599 (Folin-DPPH) and 0.591 (Folin-ABTS). These results are in agreement

with other studies, where a relationship was observed between the potential antioxidant activity, total phenolic and flavonoid levels of the extract in olive leaves [53]. There are some literature data revealing a strong correlation between the total number and content of phenolics and the antioxidant activity of food, medicinal, plants, fruits, or veg- etables [54–56]. The weaker correlation between total polyphenol content and antioxidant activity, observed in 50% water regime could be attributed to the modification of quan- titative polyphenolic pattern under stressful conditions. This can cause low correlations between different methods, taking into consideration that polyphenolic compounds have multiple activities and can scavenge radicals by different mechanisms [57].

2.3.5. Principal Component Analysis (PCA)

A principal component analysis (PCA) was done to feature the repercussion of the biostimulant treatments on the biometric, eco-physiological and qualitative parameters analyzed above. The first two principal components (PCs) disclosed 74.22% of the cumulative variance (Figure 3), with PC1 detailing for 41.86% and PC2 for 32.36%.

The PC1 was positively correlated with vegetative and nutraceutical parameters, while PC2 was positively correlated with eco-physiological parameters. PCA is effective in plotting the physiological, vegetative and nutraceutical parameters of the young olive trees in affiliation of the different biostimulant treatments and their utility. Kaolin treatment, in both water regimes 100 and 50%, was positioned in the downright quadrant of the PCA score plot, since it engendered the highest value of total polyphenols and antioxidant activity. In water regime 100%, all biostimulants were positioned in the upper right quadrant of PCA score plot showing positive correlations with RWC, stomatal conductance, leaves number, leaf area, and leaf dry matter.

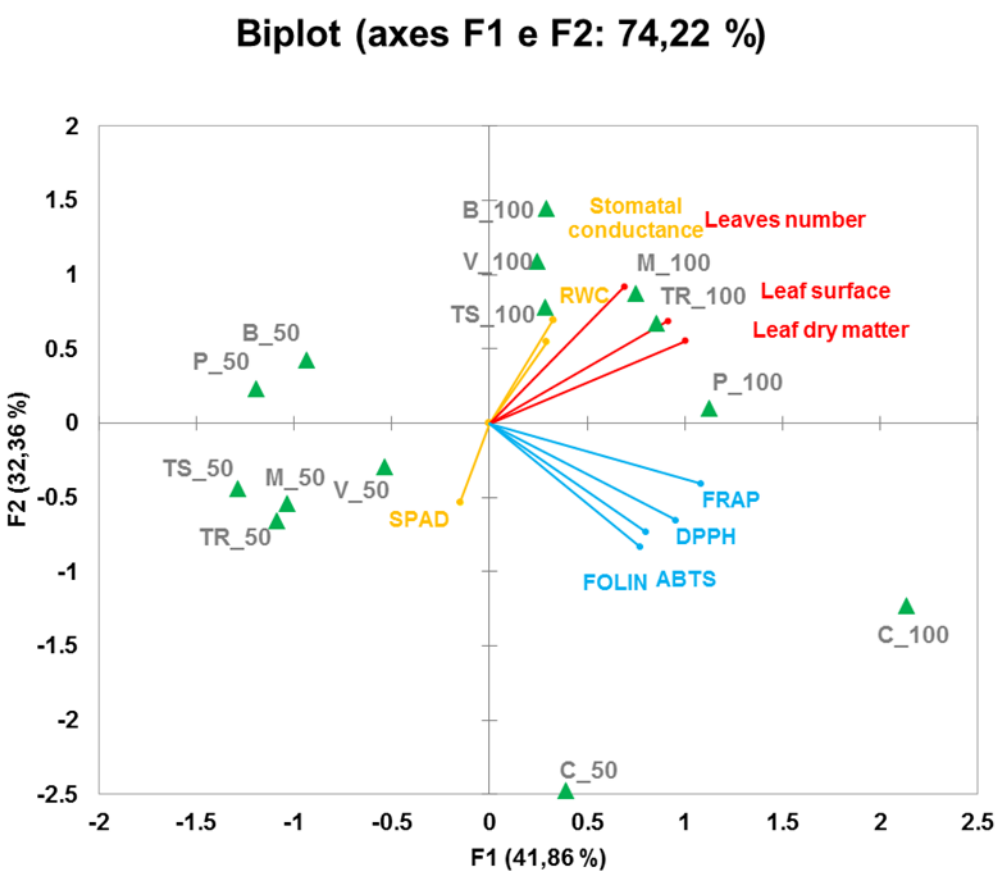

Figure 3. Principal component analysis (PCA) of vegetative parameters (leaf area, leaf dry weight and leaf number), eco-physiological parameters at the end of growing season (RWC, SPAD and stomatal conductance) and nutraceutical parameters (Folin, ABTS, DPPH and FRAP) in leaves of olive tree grown in a greenhouse under optimal irrigation (100% irrigation) and limited water availability (50% irrigation) treated with several biostimulants: C = control, TR = *Trichoderma*, M = Micro-Algae, P = Seaweed mix, K = Kaolin, B = Glycine betaine, V = Pinolene.

To conclude, we list in Table 5 a summary of the composition, the application proce- dure, application time and effects of the individual biostimulants that were used in this test on the leaves of young olive plants.

Table 5. Summary of the composition, the application procedure of the biostimulants and the effects on the olive leaves.

Treatments	Composition	Application Procedure	Effects
Trichoderma (TR)	1% *w/w Trichoderma harzianum*, strain T-22 spores (1×10^9 spores/g) and 99% *w/w* inert ingredients	Drench application—6.67 g/L of water	Improves RWC values and total polyphenols content
Micro-Algae (M)	Free L-amino acids (4.1% *w/w*), total nitrogen (7% *w/w*), organic nitrogeno (5.6% *w/w*), nitric nitrogen (5.6% *w/w*), P_2O_5 (0.5% *w/w*), K_2O (6.7% *w/w*)	Drench application—6.67 g/L of water	Improves SPAD values and stomatal conductance
Seaweed mix (P)	Organic carbon C (6%), mannitol 9 g/L	Drench application—4 ml/L of water	Improves SPAD and RWC values
Glycine betaine (B)	Glycine betaine, trehalose, plant extracts containing zeatin.	Drench application—10 g/L of water	Improves vegetative activity and eco-physiological parameters in the leaves
Kaolin (K)	Copper (Cu) totale 5%	Foliar application—40 g/L of water	Improves the polyphenol content and antioxidant activity in the leaves
Pinolene (V)	di-1-p-menthene (96%), coformulants, inert emulsifiers (4%)	Foliar application—10 mL/L of water	Improves vegetative activity and RWC values

2.4. Conclusions

The results of this study highlight the importance of biostimulants' application to mitigate the effects of abiotic stresses (high temperatures and drought), with different effects based on the product used. Regarding the vegetative parameters, significant differences were shown between the two watering regimes (100% and 50%), with higher values registered at 100%. Biostimulants' effects were evident in conditions of water stress, and glycine betaine treatment and algae products (micro-algae and seaweed mix) reported higher values in the increase in the number of leaves and leaf area; these same treatments showed positively significant values also regarding the eco-physiological parameters in both water regimes. Particularly interesting results were obtained with kaolin applications that caused a considerable two-fold increase in the total polyphenols content compared to the control, and a significant increase as well in the antioxidant activity. These results are interesting for improving the quality of olive oils characterized with low phenolic and antioxidant components, with the addition of leaves rich in polyphenols that can be used equally for pharmaceutical purposes. Future studies in the open field and on olive trees in full production will be necessary to evaluate the efficiency of biostimulants in mitigating damage from abiotic stress and to evaluate the effect on drupes and oil parameters.

Supplementary material

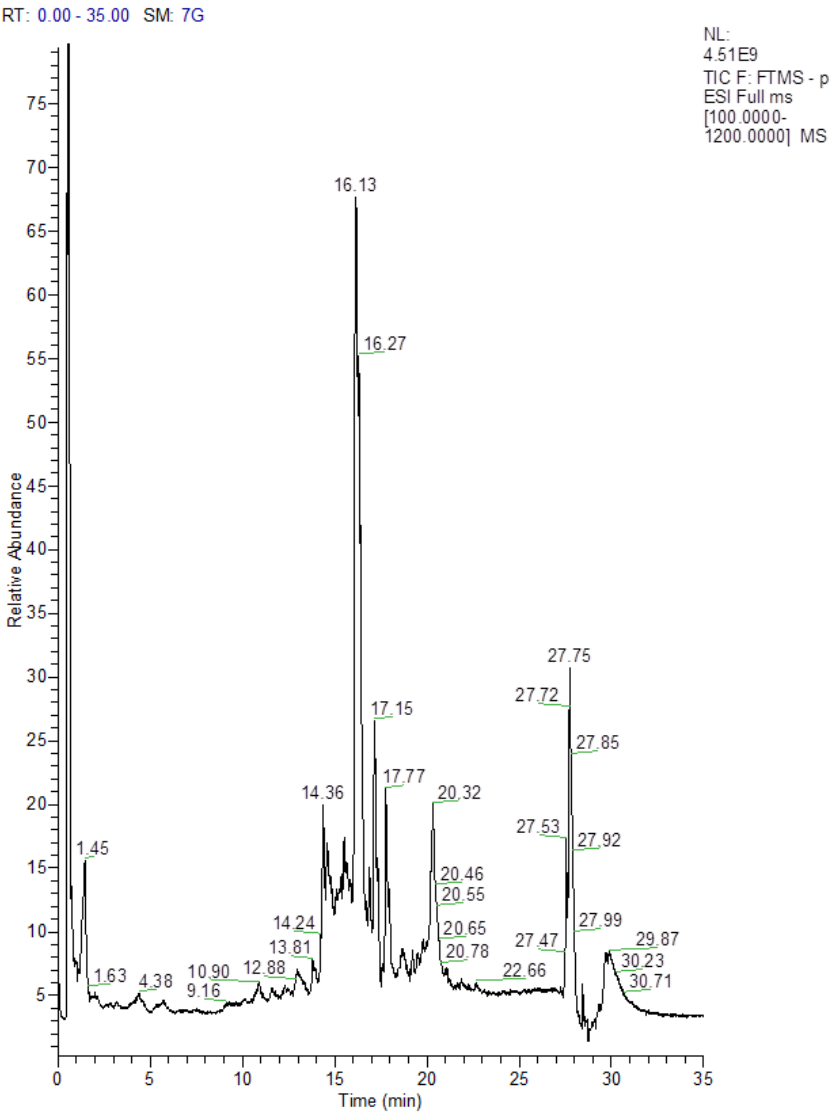

Figure S1: Typical chromatograms observed for the extracts of olive leaves analyzed in this study and the mass specifications of the compounds of interest relative to phenolic compounds (separation via UHPLC).

Table S1: Retention time and exact mass spectra data of apple polyphenols investigated by UHPLC-HRMS Orbitrap

		Theorical mass	Experimental mass	Error
Compound	**Formula**	**[M-H]⁻**		**Δ ppm**
ligstroside	C25H32O12	523.18210	523.18079	−2.50
oleuropein aglycone	C19H22O8	377.12419	377.12442	0.61
verbascoside	C29H36O15	623.19814	623.19952	2.21

oleuropein	C25H32O13	539.17701	539.17792	1.69
OH-tyrosol-glucoside	C13H18O8	301.09289	301.09329	−1.85
pinoresinol	C20H22O6	357.13436	357.1337	−1.85
vanillic acid	C8H8O4	167.03498	167.03426	−4.31
ferulic acid	C10H10O4	193.05063	193.04971	−4.77
coumaric acid	C9H8O3	163.03917	163.03931	0.86
luteolin rutinoside	C27H30O15	593.15119	593.15222	1.74
secologanoside	C16H22O11	389.10893	389.10837	−1.44
luteolin	C15H10O6	285.04062	285.04083	0.74

2.5. References

1. Mechri, B.; Tekaya, M.; Attia, F.; Hammami, M.; Chehab, H. Drought Stress Improved the Capacity of Rhizophagus irregularis for Inducing the Accumulation of Oleuropein and Mannitol in Olive (*Olea europaea*) Roots. Plant Physiol. Biochem. 2020, 156, 178–191.
2. Petridis, A.; Therios, I.; Samouris, G.; Koundouras, S.; Giannakoula, A. Effect of Water Deficit on Leaf Phenolic Composition, Gas Exchange, Oxidative Damage and Antioxidant Activity of Four Greek Olive (*Olea europaea* L.) Cultivars. Plant Physiol. Biochem. 2012, 60, 1–11.
3. Drobek, M.; Fra¸c, M.; Cybulska, J. Plant Biostimulants: Importance of the Quality and Yield of Horticultural Crops and the Improvement of Plant Tolerance to Abiotic Stress—A Review. Agronomy 2019, 9, 335.
4. Regni, L.; Del Buono, D.; Miras-Moreno, B.; Senizza, B.; Lucini, L.; Trevisan, M.; Morelli Venturi, D.; Costantino, F.; Proietti, P. Biostimulant Effects of an Aqueous Extract of Duckweed (Lemna minor L.) on Physiological and Biochemical Traits in the Olive Tree. Agriculture 2021, 11, 1299.
5. Prajapat, P.; Singh, D.; Tripathi, S.; Patel, K.; Abbas, H.; Patel, A. Effect ofWater Stress on Antioxidative Enzymes and Glycine Betaine Content in Drought Tolerant and Drought Susceptible Cotton (Gossypium hirsutum L.) Genotypes. Indian J. Biochem. Biophys. 2018, 55, 198–204.

6. Brito, C.; Dinis, L.-T.; Moutinho-Pereira, J.; Correia, C. Kaolin, an Emerging Tool to Alleviate the Effects of Abiotic Stresses on Crop Performance. Sci. Hortic. 2019, 250, 310–316.
7. Cirillo, A.; Conti, S.; Graziani, G.; El-Nakhel, C.; Rouphael, Y.; Ritieni, A.; Di Vaio, C. Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters. Agronomy 2021, 11, 1884.
8. Brillante, L.; Belfiore, N.; Gaiotti, F.; Lovat, L.; Sansone, L.; Poni, S.; Tomasi, D. Comparing Kaolin and Pinolene to Improve Sustainable Grapevine Production during Drought. PLoS ONE 2016, 11, e0156631.
9. Tosin, R.; Pôças, I.; Cunha, M. Spectral and Thermal Data as a Proxy for Leaf Protective Energy Dissipation under Kaolin Application in Grapevine Cultivars. Open Agric. 2019, 4, 294–304.
10. Palliotti, A.; Poni, S.; Berrios, J.G.; Bernizzoni, F. Vine Performance and Grape Composition as Affected by Early-Season Source Limitation Induced with Anti-Transpirants in Two Red Vitis vinifera L. Cultivars: Crop Regulation and Antitranspirants. Aust. J. Grape Wine Res. 2010, 16, 426–433.
11. Palliotti, A.; Panara, F.; Famiani, F.; Sabbatini, P.; Howell, G.S.; Silvestroni, O.; Poni, S. Postveraison Application of Antitranspirant Di-1-p-Menthene to Control Sugar Accumulation in Sangiovese Grapevines. Am. J. Enol. Vitic. 2013, 64, 378–385.
12. Di Vaio, C.; Villano, C.; Lisanti, M.T.; Marallo, N.; Cirillo, A.; Di Lorenzo, R.; Pisciotta, A. Application of Anti-Transpirant to Control Sugar Accumulation in Grape Berries and Alcohol Degree in Wines Obtained from Thinned and Unthinned Vines of Cv. Falanghina (Vitis vinifera L.). Agronomy 2020, 10, 345.
13. Di Vaio, C.; Marallo, N.; Lorenzo, R.D.; Pisciotta, A. Anti-Transpirant Effects on Vine Physiology, Berry and Wine Composition of Cv. Aglianico (Vitis vinifera L.) Grown in South Italy. Agronomy 2019, 9, 244.

14. Graziani, G.; Ritieni, A.; Cirillo, A.; Cice, D.; Di Vaio, C. Effects of Biostimulants on Annurca Fruit Quality and Potential Nutraceutical Compounds at Harvest and during Storage. Plants 2020, 9, 775.
15. Kusvuran, S. Microalgae (Chlorella vulgaris Beijerinck) Alleviates Drought Stress of Broccoli Plants by Improving Nutrient Uptake, Secondary Metabolites, and Antioxidative Defense System. Hortic. Plant J. 2021, 7, 221–231.CrossRef]
16. Rudresh, D.L.; Shivaprakash, M.K.; Prasad, R.D. Effect of Combined Application of Rhizobium, Phosphate Solubilizing Bacterium and Trichoderma spp. on Growth, Nutrient Uptake and Yield of Chickpea (Cicer aritenium L.). Appl. Soil Ecol. 2005, 28, 139–146.
17. Contreras-Cornejo, H.A.; Macías-Rodríguez, L.; Alfaro-Cuevas, R.; López-Bucio, J. Trichoderma spp. Improve Growth of Arabidopsis Seedlings Under Salt Stress Through Enhanced Root Development, Osmolite Production, and Na+ Elimination Through Root Exudates. MPMI 2014, 27, 503–514.
18. Hashem, A.; Abd_Allah, E.F.; Alqarawi, A.A.; Al Huqail, A.A.; Egamberdieva, D. Alleviation of Abiotic Salt Stress in Ochradenus Baccatus (Del.) by Trichoderma Hamatum (Bonord.) Bainier. J. Plant Interact. 2014, 9, 857–868.
19. Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A. Effects of Trichoderma Biostimulation on the Phenolic Profile of Extra-Virgin Olive Oil and Olive Oil By-Products. Antioxidants 2020, 9, 284.
20. Talhaoui, N.; Gómez-Caravaca, A.M.; León, L.; De la Rosa, R.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Determination of Phenolic Compounds of 'Sikitita' Olive Leaves by HPLC-DAD-TOF-MS. Comparison with Its Parents 'Arbequina' and 'Picual' Olive Leaves. LWT-Food Sci. Technol. 2014, 58, 28–34.
21. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic. Biol. Med. 1999, 26, 1231–1237.
22. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. LWT-Food Sci. Technol. 1995, 28, 25–30.

23. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of Antioxidant Power: The FRAP Assay. Anal. Biochem. 1996, 239, 70–76.
24. Formisano, L.; Ciriello, M.; El-Nakhel, C.; Poledica, M.; Starace, G.; Graziani, G.; Ritieni, A.; De Pascale, S.; Rouphael, Y. Pearl Grey Shading Net Boosts the Accumulation of Total Carotenoids and Phenolic Compounds That Accentuate the Antioxidant Activity of Processing Tomato. Antioxidants 2021, 10, 1999.
25. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. In Methods in Enzymology; Elsevier: Amsterdam, The Netherlands, 1999; Volume 299, pp. 152–178. ISBN 978-0-12-182200-2.
26. Newman, J.E. Symposium: Responses of Field Crops to Environmental Factors Summary Statements. Agron. J. 1963, 55, 31.
27. Boutraa, T.; Akhkha, A.; Al-Shoaibi, A.A.; Alhejeli, A.M. Effect ofWater Stress on Growth andWater Use Efficiency (WUE) of Some Wheat Cultivars (Triticum durum) Grown in Saudi Arabia. J. Taibah Univ. Sci. 2010, 3, 39–48.
28. Sakamoto, A.; Murata, N. The Role of Glycine Betaine in the Protection of Plants from Stress: Clues from Transgenic Plants: Role of Betaine in Stress Tolerance. Plant Cell Environ. 2002, 25, 163–171.
29. Alia; Hayashi, H.; Sakamoto, A.; Murata, N. Enhancement of the Tolerance of Arabidopsis to High Temperatures by Genetic Engineering of the Synthesis of Glycinebetaine. Plant J. 1998, 16, 155–161.
30. Ashraf, M.; Foolad, M.R. Roles of Glycine Betaine and Proline in Improving Plant Abiotic Stress Resistance. Environ. Exp. Bot. 2007, 59, 206–216.
31. Diaz-Zorita, M.; Fernandez-Canigia, M.V.; Grosso, G.A. Applications of Foliar Fertilizers Containing Glycinebetaine Improve Wheat Yields. J. Agron. Crop. Sci. 2001, 186, 209–215.
32. Mukherjee, A.; Patel, J.S. Seaweed Extract: Biostimulator of Plant Defense and Plant Productivity. Int. J. Environ. Sci. Technol. 2020, 17, 553–558.

33. Mansori, M.; Chernane, H.; Latique, S.; Benaliat, A.; Hsissou, D.; El Kaoua, M. Seaweed Extract Effect on Water Deficit and Antioxidative Mechanisms in Bean Plants (Phaseolus Vulgaris L.). J. Appl. Phycol. 2015, 27, 1689–1698.
34. Krajnc, A.U.; Ivanuš, A.; Kristl, J.; Šušek, A. Seaweed Extract Elicits the Metabolic Responses in Leaves and Enhances Growth of Pelargonium Cuttings. Eur. J. Hortic. Sci. 2012, 77, 170–181.
35. Spinelli, F.; Fiori, G.; Noferini, M.; Sprocatti, M.; Costa, G. A Novel Type of Seaweed Extract as a Natural Alternative to the Use of Iron Chelates in Strawberry Production. Sci. Hortic. 2010, 125, 263–269.
36. Whapham, C.A.; Blunden, G.; Jenkins, T.; Hankins, S.D. Significance of Betaines in the Increased Chlorophyll Content of Plants Treated with Seaweed Extract. J. Appl. Phycol. 1993, 5, 231–234.
37. Blunden, G.; Jenkins, T.; Liu, Y.-W. Enhanced Leaf Chlorophyll Levels in Plants Treated with Seaweed Extract. J. Appl. Phycol. 1996, 8, 535–543.
38. Shukla, N.; Awasthi, R.P.; Rawat, L.; Kumar, J. Seed Biopriming with Drought Tolerant Isolates of Trichoderma Harzianum Promote Growth and Drought Tolerance in Triticum Aestivum: Trichoderma Mediated Drought Responses in Bioprimed Wheat Plants. Ann. Appl. Biol. 2015, 166, 171–182.
39. Elansary, H.O.; Skalicka-Wo´zniak, K.; King, I.W. Enhancing Stress Growth Traits as Well as Phytochemical and Antioxidant Contents of Spiraea and Pittosporum under Seaweed Extract Treatments. Plant Physiol. Biochem. 2016, 105, 310–320.
40. Zegaoui, Z.; Planchais, S.; Cabassa, C.; Djebbar, R.; Belbachir, O.A.; Carol, P. Variation in Relative Water Content, Proline Accumulation and Stress Gene Expression in Two Cowpea Landraces under Drought. J. Plant Physiol. 2017, 218, 26–34.
41. Hu, L.; Hu, T.; Zhang, X.; Pang, H.; Fu, J. Exogenous Glycine Betaine Ameliorates the Adverse Effect of Salt Stress on Perennial Ryegrass. J. Am. Soc. Hortic. Sci. 2012, 137, 38–46.

42. Dias, M.C.; Pinto, D.C.G.A.; Figueiredo, C.; Santos, C.; Silva, A.M.S. Phenolic and Lipophilic Metabolite Adjustments in *Olea europaea* (Olive) Trees during Drought Stress and Recovery. Phytochemistry 2021, 185, 112695.
43. Dinis, L.-T.; Bernardo, S.; Conde, A.; Pimentel, D.; Ferreira, H.; Félix, L.; Gerós, H.; Correia, C.M.; Moutinho-Pereira, J. Kaolin Exogenous Application Boosts Antioxidant Capacity and Phenolic Content in Berries and Leaves of Grapevine under Summer Stress. J. Plant Physiol. 2016, 191, 45–53.
44. Denaxa, N.-K.; Roussos, P.A.; Damvakaris, T.; Stournaras, V. Comparative Effects of Exogenous Glycine Betaine, Kaolin Clay Particles and Ambiol on Photosynthesis, Leaf Sclerophylly Indexes and Heat Load of Olive Cv. Chondrolia Chalkidikis under Drought. Sci. Hortic. 2012, 137, 87–94.
45. Cetinkaya, H.; Koc, M.; Kulak, M. Monitoring of Mineral and Polyphenol Content in Olive Leaves under Drought Conditions: Application Chemometric Techniques. Ind. Crop. Prod. 2016, 88, 78–84.
46. Rouphael, Y.; Carillo, P.; Colla, G.; Fiorentino, N.; Sabatino, L.; El-Nakhel, C.; Giordano, M.; Pannico, A.; Cirillo, V.; Shabani, E.; et al. Appraisal of Combined Applications of Trichoderma Virens and a Biopolymer-Based Biostimulant on Lettuce Agronomical, Physiological, and Qualitative Properties under Variable N Regimes. Agronomy 2020, 10, 196.
47. Pandey, P.P.; Sharma, R.; Neelkanthe, S.S. Climate change: Combating drought with antitranspirants and super absorbent. Plant Archive 2017, 17, 1146–1156.
48. Del Amor, F.M.; Cuadra-Crespo, P.; Walker, D.J.; Cámara, J.M.; Madrid, R. Effect of Foliar Application of Antitranspirant on Photosynthesis and Water Relations of Pepper Plants under Different Levels of CO2 and Water Stress. J. Plant Physiol. 2010, 167, 1232–1238.
49. Hurtado, A.Q.; Yunque, D.A.; Tibubos, K.; Critchley, A.T. Use of Acadian Marine Plant Extract Powder from Ascophyllum nodosum in Tissue Culture of Kappaphycus Varieties. J. Appl. Phycol. 2009, 21, 633–639.

50. Lola-Luz, T.; Hennequart, F.; Gaffney, M. Enhancement of Phenolic and Flavonoid Compounds in Cabbage (*Brassica oleraceae*) Following Application of Commercial Seaweed Extracts of the Brown Seaweed, (*Ascophyllum nodosum*). Agric. Food Sci. 2013, 22, 288–295.
51. Rouphael, Y.; Colla, G.; Giordano, M.; El-Nakhel, C.; Kyriacou, M.C.; De Pascale, S. Foliar Applications of a Legume-Derived Protein Hydrolysate Elicit Dose-Dependent Increases of Growth, Leaf Mineral Composition, Yield and Fruit Quality in Two Greenhouse Tomato Cultivars. Sci. Hortic. 2017, 226, 353–360.
52. Rouphael, Y.; Giordano, M.; Cardarelli, M.; Cozzolino, E.; Mori, M.; Kyriacou, M.; Bonini, P.; Colla, G. Plant- and Seaweed-Based Extracts Increase Yield but Differentially Modulate Nutritional Quality of Greenhouse Spinach through Biostimulant Action. Agronomy 2018, 8, 126.
53. Brahmi, F.; Mechri, B.; Dabbou, S.; Dhibi, M.; Hammami, M. The Efficacy of Phenolics Compounds with Different Polarities as Antioxidants from Olive Leaves Depending on Seasonal Variations. Ind. Crop. Prod. 2012, 38, 146–152.
54. Vinson, J.A.; Hao, Y.; Su, X.; Zubik, L. Phenol Antioxidant Quantity and Quality in Foods: Vegetables. J. Agric. Food Chem. 1998, 46, 3630–3634.
55. Velioglu, Y.S.; Mazza, G.; Gao, L.; Oomah, B.D. Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. J. Agric. Food Chem. 1998, 46, 4113–4117.
56. Pellati, F.; Benvenuti, S.; Magro, L.; Melegari, M.; Soragni, F. Analysis of Phenolic Compounds and Radical Scavenging Activity of Echinacea spp. J. Pharm. Biomed. Anal. 2004, 35, 289–301.
57. Mohtar, L.G.; Messina, G.A.; Bertolino, F.A.; Pereira, S.V.; Raba, J.; Nazareno, M.A. Comparative Study of Different Methodologies for the Determination the Antioxidant Activity of Venezuelan Propolis. Microchem. J. 2020, 158, 105244.

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Biostimulants Improve Plant Growth and Bioactive Compounds of Young Olive Trees under Abiotic Stress Conditions

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Abstract: The negative impacts of extreme heat and drought on olive plants have driven the quest for mitigation approaches based on the use of biostimulants, which have proved to be effective in contrasting environmental stresses. The aim of our study was to evaluate the effectiveness of six biostimulants in mitigating high temperature and water stress in young olive trees in terms of vegetative and eco-physiological parameters as well as bioactive compound content. Biostimulants based on glycine betaine and macro- and micro-algae effectively protected the plants from abiotic stress by improving their eco-physiological and vegetative parameters. At the end of the growing season, olive plants were experiencing water deficit which had built up through the summer months. At this time, the glycine betaine-treated plants had a three-fold higher stomatal conductance compared with the control, while plants sprayed with the seaweed mix had a relative water content 33% higher than the control. The kaolin treatment resulted in higher total phenolics and antioxidant activities (DPPH, FRAP and ABTS) in water stress conditions and caused an increase of 238.53 and 443.49% in leaves total polyphenols content in 100% and 50% water regime, respectively. This study showed the effectiveness of biostimulants in mitigating the damage from abiotic stress on young olive trees, by improving some vegetative, eco-physiological and leaf nutraceutical parameters. Further studies are needed to test the efficiency of these biostimulants in open field conditions on olive trees in full production.

Keywords: *Olea europaea* L.; *Trichoderma*; *Ascophyllum nodosum*; *Laminaria digitata*; pinolene; phenolic profile; high resolution mass spectrometry; antioxidant activity

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1. Introduction

Olive (*Olea europaea* L.) is one of the most important fruit trees of the Mediterranean region with an enormous economic and ecological value. In its climatic region, olive is often subject to drought periods during the warm season; nevertheless, it is characterized by high morphological and physiological adaptation capacities [1]. Water stress was reported to induce in olive plants the activation of antioxidant enzyme systems such as ascorbate peroxidase, catalase and superoxide dismutase [2]. Moreover, the activation of the phenylpropanoid biosynthetic pathway leading to the accumulation of phenolic compounds is a well-known metabolic response to water deficit as well as to other environmental stresses [2]. Such metabolic responses of the plants to unfavorable environmental conditions play a key role in preventing cellular damage caused by oxidative stress. However, the constitutive tolerance to water deficit alone is not sufficient to protect olive trees from the combined effects of extreme heat waves, water stress and high irradiance, which are all linked to

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Chapter 3. Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters

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Chapter 3. Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters

Abstract: Various products are used to mitigate the negative effects of abiotic stress in olive trees. The aim of the research was to examine an anti-transpirant product (Vapor Gard®, V) and a kaolin-based product (Manisol, K) effect on the growth of two-year-old olive tree seedlings under high temperature. The study was conducted in a greenhouse on trees of a native cultivar of Campania (cv. Salella) grown in pot during the growing season from May to September 2020. The experimental design included two products: di-1-p-menthene (product V) and kaolin (product K), applied five times at 20-day intervals compared with a control. The following biometric, physiological, and nutraceutical parameters were evaluated: stomatal conductance, chlorophyll a fluorescence, Soil Plant Analysis Development (SPAD) index, relative water content (RWC), shoots growth, total leaf area per plant, trunk cross-sectional area, dry matter partitioning, total polyphenols, and antioxidant activity. The results obtained showed that the application of di-1-p-menthene (V) was able to induce a significant improvement of shoots growth (+37.22%) and trunk cross-sectional area (+46.60%) and a reduction of the stomatal conductance and an increase of leaf RWC values. Application with kaolin had positive effects on the total polyphenol content, with an increase over the control of 240.33% and higher antioxidant activity values. Further studies are necessary to determine the effect of these products on the biometric, physiological and nutraceutical parameters of mature olive trees cultivated in open field conditions.

3.1 Introduction

The olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean

basin, and its growth and development are mainly controlled by atmospheric conditions [1,2]. In recent years, with the scenario of climate change, this cultivar is strongly exposed to thermal and hydric stresses during the growing season, mostly during summer and in the innermost areas of Europe [3]. These changes in temperature and precipitation along with a greater frequency of extreme weather have reduced agricultural yield [4,5]. Temperature affects most plant physiological processes, including photosynthesis and transpiration, which are both regulated by stomatal conductance, and which mutually affect each other [6]. The atmospheric CO^2 concentration is increasing, and in addition to its direct effects on plant growth, this change is expected to raise the global mean surface temperature and result in an increase in the severity of summer drought [7]. The olive tree is considered to be a sclerophyllous, high-temperature, and drought-tolerant species with a photosynthetic apparatus resistant enough to moderate water stress, while the stomata are the main limiting factors to carbon assimilation [8]. In conditions of low available water and high temperatures, the olive tree undergoes significant stress, resulting in reduced productivity [9–11]; one of the immediate responses is the reduction of stomata openings in order to decrease the transpiration rate and maintain positive turgor pressure of the cells [12–14]. In olive trees, the recovery after stress is a complex process; the trees restore the water status rapidly along with a slow recovery of stomatal conductance [15]. A conservative bearing after re-watering has been identified, as trees rapidly restore the water status along with a slow recovery of stomatal conductance [16]. Although olive trees adapt well to stressful conditions, high expenditure of energy is required by the plant to withstand stressful conditions [10]. Water deficit, commonly associated to heat and high irradiance stresses, impairs plant water status, drives stomatal closure, mesophyll compactness, and photoinhibition, compromising photosynthetic capacity [11,17,18]. Photosynthesis is the primary factor of plant growth [19], and all stress factors affecting the efficiency of photosynthesis lead to reduced plant biomass and growth [20]. The measurement of Chlorophyll a (Chl a) fluorescence [21,22] is an important tool for studying the photosynthetic metabolism. It is important to adopt agronomic practices that allow a better adaptability for drought and high temperature, and therefore the capacity to integrate both

tolerance and recovery capacity [15] of olive orchards. One neglected agronomic technique that has the potential to significantly contribute to abiotic stress amelioration in food crop production is the use of anti-transpirants. These latter are substances that are applied on leaves to reduce transpiration, and hence improve plant water potential [23]. Originally, kaolin was developed for the suppression of pests in many crops [24], but later, it has been demonstrated that the white kaolin film formed on the leaf surface increases the reflection of incoming solar radiation. Kaolin has a reflective power and redistributes the radiation throughout the plant, reaching both leaf surfaces and shaded plant canopy areas [25,26]. According to the above, kaolin also has an anti-transpirant effect: reducing heat stress and solar injury to the entire tree canopy, leaf, and fruit and, in particular, their temperature [25]. However, products based on 1-p-menthene (pinolene), which is an emulsifiable terpenic polymer distilled from conifer resins, create a thin, similar to a physical barrier, transparent and flexible coat that stops water dispersion [27], decreases the stomatal conductance, and consequently reduces transpirational losses, wilting, and leaf abscission, and favors improving plant water status [28]. In an agronomic scenario in which the damage from high temperatures caused by climate change is increasingly widespread, the aim of our preliminary study was to evaluate the effects of two anti-transpirants products on the improvement of growth and physiological status of young olive trees, grown in greenhouses under high temperatures, and the effects on bioactive components in their leaves.

3.2. Materials and Methods

3.2.1. Growth Conditions, Experimental Design and Products Applications

The trial was conducted in a greenhouse at the University of Naples "Federico II"-Department of Agriculture, situated in Portici (Province of Naples, Italy; 40_480 N,14_200 E, m.s.l) between the end of May and the end of September 2020, adopting 2-year-old potted olive trees cv. Salella. Temperatures were recorded continuously using Tinytag data loggers (Gemini data loggers, Chichester, UK) placed at canopy level of the trees. At the beginning of the trial, all plants were characterized by homogeneous growing characteristics; and 0.05

kg/tree of Nitrophoska Gold (Compo Expert, Cesano Maderno, MB, Italy) was added to the substrate. The study included two spray treatments, conducted five times during the growing season: 25 May, 15 June, 06 July, 27 July and 24 August, compared with a control thesis:

(1) Control (C) plants treated only with water;

(2) Kaolin (K), the product used was Manisol (Kaolin + Copper 5%) from Manica S.p.a (Rovereto, Italy), which was applied by foliar application with 80 g in 2 L of water;

(3) Di-1-p-menthene (C20H34) (V), a terpenic polymer also known as pinolene, in the form of a water emulsifiable organic concentrate. The product used was Vapor Gard® from Biogard® (Bergamo, Italy), which was applied by foliar application with 20 ml in 2 L of water.

The two treatments were sprayed using a portable pump. Plants were irrigated with water equal to 100% of the evapotranspiration (ET), calculated by weight. A drip irrigation water was considered by placing two drippers (2 L/h) per plant. The system was fed by electric pumps, and irrigation was automatized due to a timer. For each treatment, ten technical replicates were utilized, each consisting of a single tree.

3.2.2 Biometric and Physiological Analysis

During the growing season, the following measurements were performed: (i) shoots growth with a digital caliber (Borletti CDJB20 accuracy 0.03 mm, Antegnate (BG), Italy), (ii) the circumference of the trunk at 3 cm above the soil, in order to determine the increase in the trunk cross-sectional area (TCSA) calculated by standard formula ($girth^2/4\pi$), and (iii) leaf number per plant to determine leaf area per plant using imageJ software version 1.50 (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). On fully developed leaves, the stomatal conductance was measured using a Porometer (Li-1600 Steady State Porometer, Lincoln, NE, USA) taking 6 measurements per treatments; leaf soil plant development (SPAD) index was assessed by taking 20 measurements per treatments using a chlorophyll meter SPAD-502 (Konica-Minolta, Osaka, Japan). Plant water status was assessed by calculating the leaf relative water content (RWC) as follows. Immediately after sampling, leaves were placed in plastic bags inside a cooler box and transferred to the

laboratory where the fresh weight (fw) was recorded. Due to the thick cuticle/epidermal layers, the sampled leaves were then rehydrated for 48 h in distilled water at 4 °C in the dark prior to measuring the rehydrated weight (rw). Subsequently, the same leaves were dehydrated for at least 48 hours at 80 °C until constant weight was reached, before their dry weight (dw) was measured. RWC (%) was calculated as:

$$RWC\ (\%) = ([fw - dw]/[rw - dw]) * 100$$

These eco-physiological measurements were performed three times during the trial: on 29 June, 21 July, and 25 August. Before the end of the experiment, chlorophyll a fluorescence measurements were recorded in the field on 30 min dark-adapted leaves using a PAR-FluorPen FP 110/D portable fluorimeter (Photon Systems Instruments, Drásov, Czech Republic) equipped with detachable leaf-clip. Measurements occurred at 9 a.m. and 1:30 p.m., for a one hour lap each, according to the procedure reported by Mola et al. [29]. Fluorescence data were analyzed by the FluorPen software ver. 1.1 (Photon Systems Instruments, Drásov, Czech Republic), and the Fv/Fm parameter was calculated as follows:

$$Fv/Fm = (Fm - F0)/Fm$$

where F0 is the basal fluorescence recorded at 40 μs and Fm is the peak of the fast fluorescence rise following illumination of the dark-adapted leaves with a saturating flash of light. At the end of the growing season, the plants were harvested to determine the dry matter partitioning of the various components of the trees (roots, leaves, wood and total). Then, the root/canopy ratio of the individual plants was calculated. At the end of the season, six samples of leaves per treatment were frozen and freeze-dried to determine the polyphenol content and antioxidant activity.

3.2.3. Determination of Leaf Polyphenols

3.2.3.1. Chemicals

All the standards for the analyses were the same used in a previous study conducted by Di Vaio et al. [30] on a minor autochthonous cultivar of olive of the Campania Region.

3.2.3.2. Ultrasound-Assisted Extraction of Polyphenolic Compounds

Lyophilized samples were extracted using the method reported in the literature [31] with few modifications. In particular, 0.3 g of dried sample was extracted with 15 mL of

methanol/water (80:20 v/v, 0.1% formic acid) by sonication at room temperature for 15 min. Samples were centrifuged to 4000 rpm at 4 °C for 10 min, and the pellets were extracted again in the same way. The supernatants were collected, filtered through 0.45 mm nylon syringe membranes, and then used for high-resolution mass spectrometry analysis and antioxidant activity assay.

3.2.3.3. UHPLC-HRMS Analysis of Polyphenolic Compounds

An Ultra-High-Pressure Liquid Chromatograph (UHPLC, Dionex UltiMate 3000, Thermo Fisher Scientific,Waltham, MA, USA) coupled with a Q-Exactive Orbitrap mass spectrometer (UHPLC, Thermo Fischer Scientific, MA, USA) was used to investigate the quali-quantitative profile of polyphenolic compounds, by applying the conditions reported in our previous work [32]. All results are expressed as _g/g dw of olive leaves.

3.2.4. Antioxidant Activity: ABTS Assay

Determination of the ABTS free radical scavenging activity was conducted following the method described by Re et al. [33].

3.2.5. Antioxidant Activity: DPPH Assay

The DPPH assay was conducted according to the procedure reported by Brand-Williams et al. (1995) [34] with minor modifications. Briefly, methanolic DPPH radical working solution was prepared diluting methanolic DPPH (4 mg in 10 mL) with methanol until an absorbance value of 0.900±0.020 at 517 nm. For the assay, 200 μL of sample were added to 1 mL of radical working solution, and the absorbance value was monitored after 10 min. The results were expressed as Trolox equivalents antioxidant capacity (TEAC, mmol Trolox equivalent kg◎1 of dry weight of sample). All determinations were performed in triplicate.

3.2.6. Statistical Analysis

Analysis of variance (two-way ANOVA) was applied to analyze the group means. Duncan's multiple range test was (DMRT) performed for mean separation of each of the significant ($p < 0.05$) measured variables. A principal component analysis (PCA) was executed on biometric, physiological and nutraceutical parameters at the end of growing season, to

detect the interrelationship. The statistical package XLSTAT Version 2013 (New York, NY, USA) was implemented for all the analyses.

3.3. Results and Discussion

3.3.1. Implications of Kaolin and Pinolene for Biometric and Physiological Parameters

Mean, maximum and minimum air temperature inside the greenhouse during the cultivation cycle are presented in Figure 1. The maximum temperatures of 51.74 °C in July and 49.90 °C in August were recorded, while the minimum temperature was 23.42 °C in June (Figure 1). Figure 2 shows the effect of Kaolin (K) and di-1-p-menthene (V) products on shoots growth of olive plants compared with control (C) during the growing season. No significant difference was shown between the various treatments; on the contrary, a significant difference was highlighted during the days of growing season for the shoots growth. The interaction between treatments and days of measurements (TxD) was found to be significant with $p < 0.001$. At the end of the growing season, V applications improved the growth of the shoots compared to C by about + 46.60%. On the contrary, K application did not significantly improve the performance of this parameter with respect to the C (+ 10.70%).

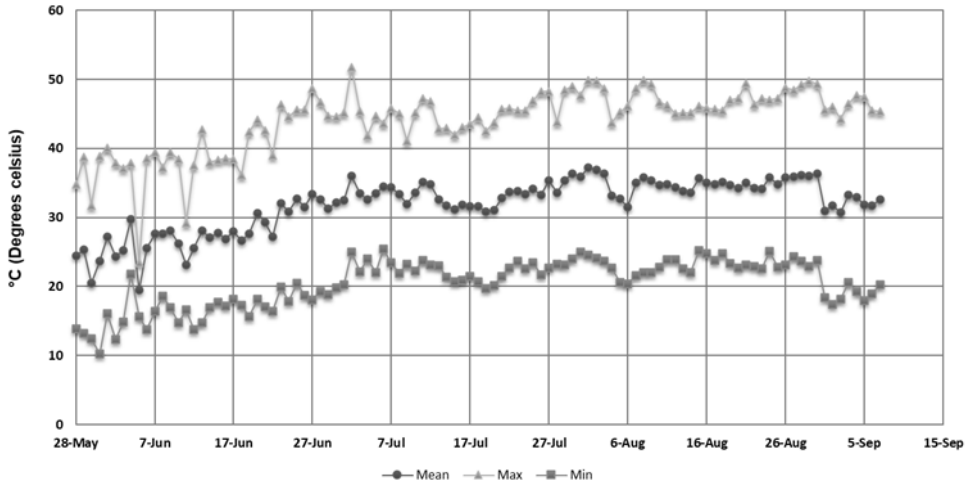

Figure 1. Monthly temperature trend: maximum, minimum, and mean temperature (°C) recorded during the growing season under the greenhouse.

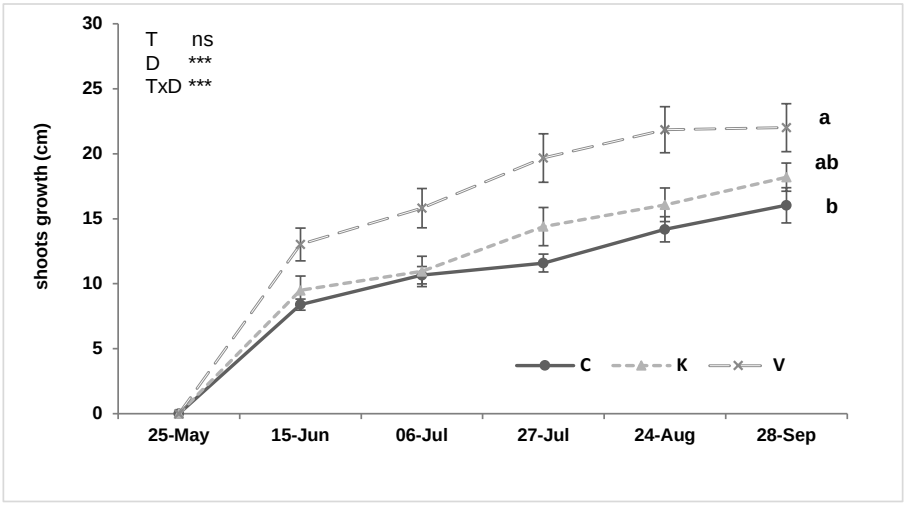

Figure 2. Effects of kaolin (K), di-1-p-menthene (V) on shoots growth during the growing season. C: control. Different letters indicate significant differences according to Duncan's multiple-range test ($p = 0.05$). * indicates the first application of the anti-transpirants. Vertical bars indicate ± SE of the means, n = 10 plants for treatments. Asterisks indicate significant effect of biostimulant treatment (T), days of measurements (D), and their interaction (T × D), according to two-way ANOVA (ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively). At the end of the growing season, V treatment resulted in significantly higher values of TCSA, in comparison with C (+34.7%) and K treatment (+41.7) (Table 1), in addition to shoots growth being only significantly higher under V treatment, 37.2% compared to C. Meanwhile, leaf area per plant showed significant differences when plants were treated with K and V with respect to C, registering an increase of about +10.2% and +9.4% respectively. Our results are consistent with those reported previously by Roussos et al. (2010) [35], where plant height in olive trees with kaolin applications did not report significant differences with respect to control. Similar results to ours were also reported by MI et al. [36], where in a test on Ficus carica L., the use of two anti-transpirant types, namely "Folicote" and Vaporgard, increased growth parameters, yield and fruit quality in the first season. The anti-transpirants products are expected to reduce photosynthesis and hence the growth of the plants. However, since the anti-transpirants close the stomata, the plant water potential increases, and it is possible to induce an increase in growth, despite reduced photosynthesis [37].

Table 1. Effect of kaolin (K) and di-1-p-menthene (V) on shoots growth, trunk cross sectional area (TCSA) and leaf area per plant at the end of growing season. C: control.

Treatments	Shoots Growth (cm)	TCSA (cm²)	Leaf Area per Plant (cm²)
C	14.44 ± 1.55b	17.05 ± 1.78 b	682.02 ± 16.00 b
K	18.20 ± 1.08 ab	16.21 ± 1.51 b	759.03 ± 25.94 a
V	21.17 ± 1.84 a	22.97 ± 2.09 a	746.30 ± 23.88 a
Significance	***	*	*

Different letters indicate significant differences according to Duncan's multiple-range test ($p = 0.05$). Means ± SE (standard error). Significance levels of parameters in ANOVA test: * $p < 0.05$; *** $p < 0.001$. The effect of kaolin (K) and 1-p-menthene (V) applications on eco-physiological parameters are reported in Table 3, after one (29-Jun), two (21-Jul) and three (25 Aug) months of the first application (25 May). Figure 3a shows the stomatal conductance values, where already after one month from the first application, the effects of the treatments were evident.

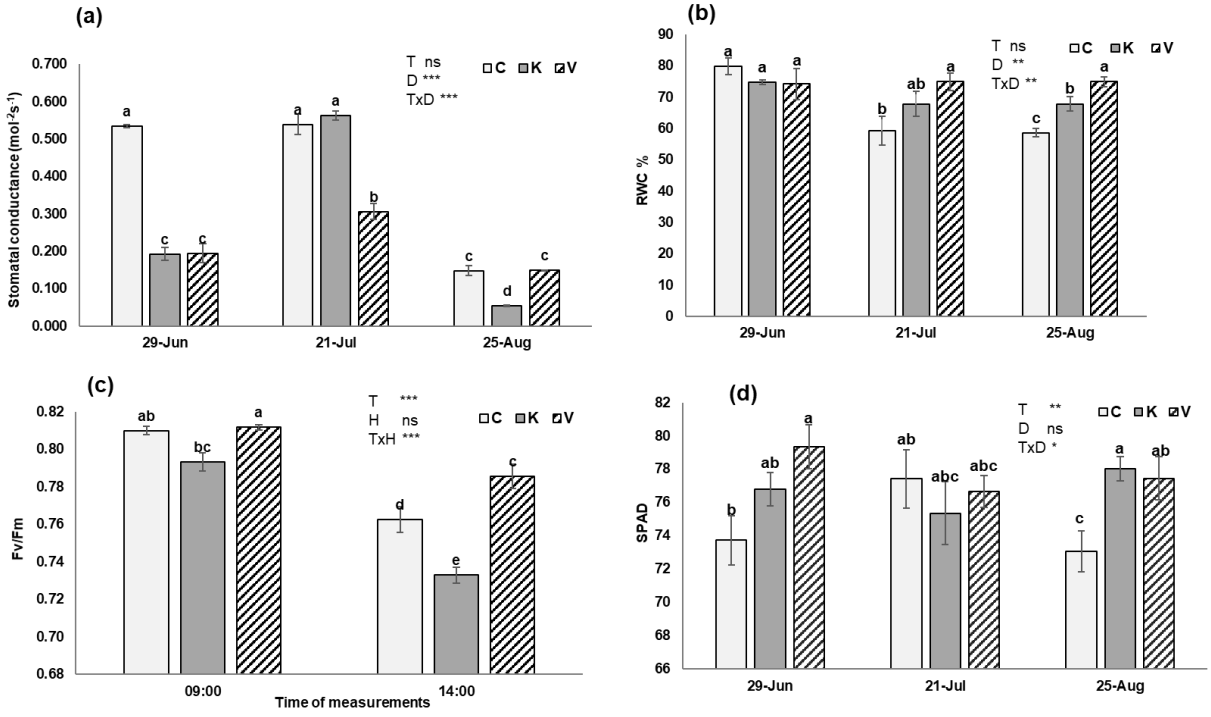

Figure 3. Effect of kaolin (K) and 1-p-menthene (V) after one (29-Jun), two (21-Jul) and three (25 Aug) months by the first application on: stomatal conductance (**a**), RWC (relative water content) (**b**), fluorescence (Fv/Fm) measured at different temperature condition at 9:00 h and at 14:00 h (**c**). SPAD (soil plant development) index (**d**), versus C: control. Vertical bars indicate ± SE (standard error) of the means. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Asterisks indicate significant effect of biostimulant treatment (T), days of measurements (D) and their interaction (TxD) according to two-way ANOVA (ns not-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

No significant difference was shown between the various treatments; on the contrary, a significant difference was highlighted during the days of growing season for the stomatal conductance. The interaction between treatments and days of measurements (T×D) was found to be significant with $p < 0.001$; conductance values with C applications were equal to 0.53 $mol^{-2}s^{-1}$, significantly higher than the treated plants. During the vegetative season in July, K applications showed an increase in stomatal conductance (0.56 $mol^{-2}s^{-1}$), but at the end of the vegetative season, all three treatments showed low conductance values, in particular K, which was statistically different from C with a reduction of 3.70%. Similar results have also been observed by Di Vaio et al. [38,39] on "Aglianico" and "Falanghina" (*Vitis Vinifera* L.), where a product based on 1-p-menthene significantly reduced the stomatal conductance and assimilation rate. Stomatal conductance usually decreases at high temperatures; however, this result is probably due to leaf water deficit or large leaf-to-air water vapor concentration differences generated by high temperature [40,41].

Another parameter used for evaluating the state of plants was the relative water content index (RWC) (Figure 3b); in this case, no significant difference was shown between the various treatments, but a significant difference was highlighted during the days of growing season. The interaction between treatments and days of measurements (T x D) was found to be significant with $p < 0.01$. In particular, in June, no statistically significant differences were recorded, as the plants had not yet undergone prolonged thermal stress; in July, only treatment with V reported significant different values, about +26.39% higher than C. In August, the effect of stomatal closure of the two spray products was clearly highlighted, as they showed significantly higher values of RWC, about +15.8% in K and +27.87% in V compared to C. Anti-transpirants are chemical compounds used to limit the transpiration process and to maintain the advantageous parameters of the water balance of plants [42], and they are commonly used to reduce leaf water loss. For instance, anti-transpirant products increased the relative water content of Morus alba leaves at different irrigation levels [43]. They may allow a reduction in water transpiration without greatly affecting photosynthetic activity [44]. In addition to the beneficial effect of elevated CO2

concentration on drought stress, anti-transpirants can significantly improve drought tolerance [45]. Our results are in agreement with Mikiciuk et al. [46], where the antitranspirant used product increased the RWC in leaves of the tested strawberry cultivar by 4.4%. According to Abdel-Fattah [47], anti-transpirants, which form a film on the surface of the plants, increase the RWC in leaves. Plants treated with both products had an Fv/Fm of 0.81 at 09:00 a.m., corresponding to the optimum value for healthy, non-stressed plants [48], while plants treated with K showed an Fv/Fm of 0.79 always at 09:00 a.m. (Figure 3c). Significant differences were shown between the various treatments and during the time of measurements. The interaction between temperature and time of measurements (T x H) was found to be significant with $p < 0.001$. The Fv/Fm decreased gradually and at the mid-day measurement; it was significantly lower than that registered at 09:00 a.m. thus evidencing the daily fluctuation in the maximum quantum yield of PSII photochemistry. At 02:00 p.m., significant differences emerged between C (Fv/Fm = 0.76), K (Fv/Fm = 0.73) and V (Fv/Fm = 0.79) plants. The V treatment reduced the mid-day depression in photosynthetic efficiency by narrowing the daily fluctuation in Fv/Fm, while the K treatment increased this depression. Overall, this positive effect of V application on the plant photosynthetic metabolism may explain the higher growth parameters recorded for plants treated with this anti-transpirant. Our Fv/Fm results with K applications are in contrast with Segura- Monroy et al. [49] findings. The authors noted significant differences in Fv/Fm ratio due to kaolin treatments in Physalis peruviana L., with untreated plants having reduced quantum efficiency (with kaolin (0.83) vs. without kaolin (0.79)). Our results are consistent with those reported previously by Latocha et al. [50], where anti-transpirant product application increased the chlorophyll content in leaves at the beginning of the experiments, and it enhanced the efficiency of the photosynthetic apparatus during almost the whole experimental period on Actinidia arguta. Data on the degradation of the leaf chlorophyll molecule after thermal stress conditions were recorded using a Minolta chlorophyll meter SPAD during the vegetative season, showing a significant difference between the various treatments. The interaction between treatments and days of measurements (T x D) was found to be significant with $p < 0.05$. Our results showed, at the end of the test, positive

effects of K and V products in maintaining higher levels of leaf SPAD than C (Figure 3d). K induced an increase in SPAD index equal to +6.8% and V equal to +6% compared to C. Higher Fv/Fm chlorophyll fluorescence emissions and SPAD index values on anti-transpirant trees compared with C, indicating less damage of the photosynthetic apparatus and higher leaf chlorophyll content. As chlorophyll is the major component of photosynthetic activity [51], it may explain yield increase at harvest in polymer-treated trees due to the improvements in these two measurements. Nevertheless, not all studies are consistent with these findings, since Percival et al. [52] on Aesculus hippocasta-num L. and Quercus robur L showed no significant effects of film-forming polymers on leaf Fv/Fm values and SPAD index values compared with C.

However, our findings are confirmed by other studies where the SPAD index reading was greater in leaves treated with kaolin [51]; this positive effect on SPAD index was also shown by Lombardini et al. [25] after kaolin treatment. A possible explanation for the increase of this parameter may be due to the fact that leaves not treated with kaolin can show a lower light reflectance, suggesting an increase of degradation of the photosynthetic pigments [53]. In September, at the end of the test, the plants were sampled to determine the dry matter (Figure 4). An increase in both roots and total dry matter with V application compared to C was highlighted, equal to +17.43% and +12.07%, respectively, while K applications caused a reduction in total dry matter compared to C, equal to - 23.63%. Our findings concerning K application are consistent with those reported previously by Cantore et al. [24], where the use of kaolin did not affect fruit dry matter. Similar results have also been observed in other previous studies on anti-transpirant products that influenced plant height and total dry matter [42], where the results obtained in greenhouse and field trials on unirrigated sweet corn indicated that significant increases in growth and of dry matter/yield occur in response to Vapor Gard (6%) treatment. Positive effect of 1-p-menthene-based product on vegetative growth and development may be due to improved plant water status related to lower transpiration [54].

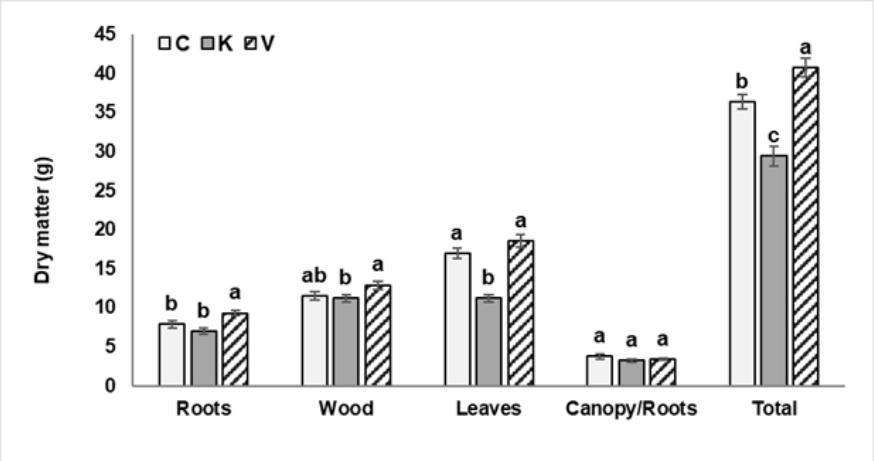

Figure 4. Effect of kaolin (K) and 1-p-menthene (V) on dry matter of roots, wood, leaves, canopy/roots ratio and total dry matter at the end of growing season. C: control. Vertical bars indicate ± SE (standard error) of the means. Different letters indicate significant differences according to Duncan's multiple-range test ($p = 0.05$).

3.3.2. Analysis of Polyphenols by UHPLC-Q-Orbitrap HRMS

Table 2 reports the phenolic composition obtained by UHPLC-HRMS analysis and Table 3 shows the quali-quantitative polyphenolic profile of olive leaves in control and treated plants. Single phenolic compounds were identified and quantified using calibration curves built with appropriate reference compounds. As certain standards were not available the case of secoiridoids), quantification was calculated employing calibration curves of oleuropein, while for the identification, MS/MS experiments had to be used.

Table 2. Exact mass spectra data (molecular formula, deprotonated molecular ion ($[M-H]^-$) and accuracy (Δ ppm)) of olive leaves polyphenols investigated UHPLC-HRMS Orbitrap.

		Theorical Mass	Experimental Mass	Error
Compound	Formula	$[M-H]^-$		Δ ppm
Ligstroside	C25H32O12	523.18210	523.18079	−2.50
Oleuropein aglycone	C19H22O8	377.12419	377.12442	0.61
Verbascoside	C29H36O15	623.19814	623.19952	2.21
Oleuropein	C25H32O13	539.17701	539.17792	1.69
OH-tyrosol-glucoside	C13H18O8	301.09289	301.09329	−1.85
Luteolin	C15H10O6	285.04062	285.04083	0.74

Table 3. Effect of kaolin (K) and 1-p-menthene (V) on phenolic compounds content in leaves of olive trees at the end of growing season. C: control. OH: hydroxy

Polyphenols	Treatments			Significance
	C	K	V	T
Ligstroside	54.01 b	266.16 a	67.79 b	***

Oleuropein aglycone	54.67 b	230.89 a	45.90 c	***
Verbascoside	156.91 b	381.39 a	31.51 c	***
Oleuropein	629.34 b	1868.74 a	575.93 b	***
OH-tyrosol glucoside	2185.11 b	8215.27 a	980.91 c	***
Luteolin	205.00 ab	217.59 a	191.02 b	ns
Total polyphenols	3285.04 b	11180.05 a	1893.07 c	***

Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Significance level of total polyphenols in leaves in ANOVA test: ns not significant; *** p < 0.001. All results are expressed as μg /g dw of olive leaves.

The Q Exactive Orbitrap LC-MS/MS analysis allowed the separation and identification of six phenolic compounds, already reported in olive leaves. They were identified by comparison of their molecular formula, the fragments obtained, and the order of elution in the literature [32]. The chromatographic separation and qualitative analysis highlighted a nonsubstantial difference in the phenolic composition between the different samples investigated. The two main phenolic compounds detected were oleuropein and tyrosol glucoside in all three treatments. K treatments showed significantly higher values of ydroxytyrosol glucoside with an increase of +276.00% compared to C. On the contrary, V application caused significantly lower values than that of C (- 55.11%). This trend is also confirmed for oleuropein, where the highest concentration is shown with K application, which showed an increase compared to C of +196.94%; on the contrary, V application did not show significant differences compared to C. K application also positively influenced the total polyphenols content; an increase of +240.33% (11,180.05 μg g^{-1}) was recorded with respect to C (3285.04 μg g^{-1}), while V treatment induced the lowest accumulation of polyphenols content (1893.07 μg g^{-1}). Treatment with pinolene caused an inhibitory effect on PAL activity, thus reducing the concentration of polyphenolic compounds. In iterature, a similar effect was associated with the foliar application of the antioxidant 5-ydroxybenzi midazole on "Koroneiki" olive trees [55].

3.3.3. Antioxidant Activity of Polyphenolics Extracts

The results of the antioxidant activity assays, conducted on the polyphenolics extracts of the olive leaves, were reported in Figure 5 and expressed as TEAC (mmol Trolox kg^{-1} dw). According to ABTS test, the values obtained showed an improvement of antioxidant activity with K treatment (+49.09%) and a reduction with V treatment (36.16%) with respect to C. As for the DPPH test, the obtained values showed an improvement of antioxidant activity only with K treatments, with an increase of +39.47% with respect to C. Our results are in agreement with Denaxa et al. [55], who reported that kaolin clay particles resulted in the highest total phenol concentration in young olive plants. In this same study, it was highlighted that Kaolin application resulted in high activities of antioxidant enzymes such as superoxide dismutase, peroxidase and glutathione reductase under water deficit compared to other alleviating products in investigation. Brillante et al. [56] reported that pinolene treatment on grapes caused a decrease in sugar content and anthocyanin level compared to control. In addition, recent studies on Vitis Vinifera showed that treatments with kaolin have highlighted positive effects on reducing canopy temperatures, enhancing the accumulation of anthocyanins [57].

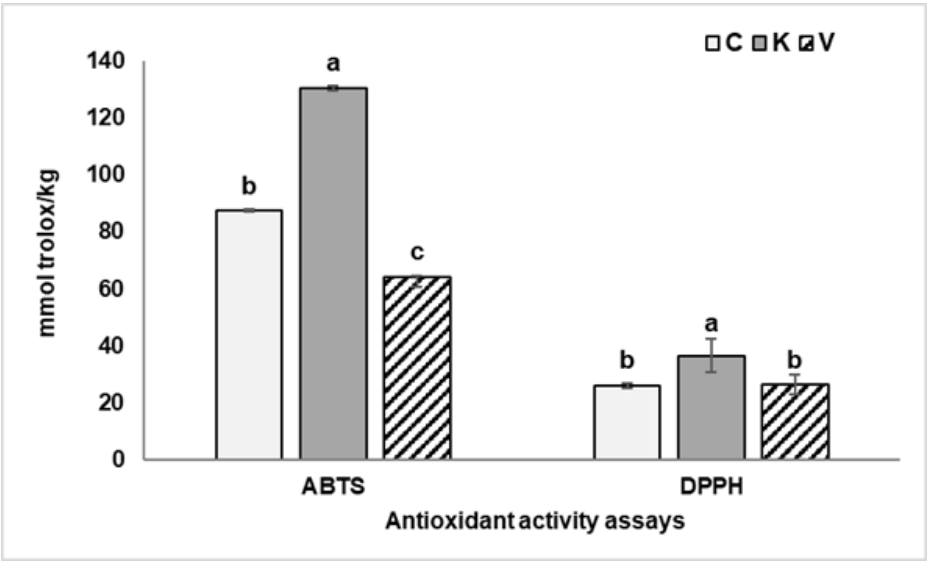

Figure 5. Effect of kaolin (K) and 1-p-menthene (V) on antioxidant activity (ABTS and DPPH) in leaves olive trees at the end of growing season. C: control. Vertical bars indicate ± SE (standard error) of the means. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05).

3.3.4. Principal Component Analysis (PCA)

A principal component analysis (PCA) was conducted to highlight the effects of the treatments on all the parameters analyzed above. The first two principal components (PCs) explained 100% of the cumulative variance (Figure 6), with PC1 accounting for 76.71% and PC2 for 23.29%. The PC1 was positively correlated with the nutraceutical compounds: OH-tyrosol glucoside, verbascoside, oleuropein, ligstroside, luteolin, oleuropein aglycone, total polyphenols and with antioxidant activity assays (ABTS and DPPH), and negatively correlated with the stomatal conductance, fluorescence (Fv/Fm), TSCA and dry matter of roots, leaves and total. Moreover, PC2 was positively correlated with shoots growth, leaf area, RWC and SPAD index. PCA is effective in plotting the physiological, biometric, and nutraceutical parameters of the young olive trees in relation to the different treatments and their usefulness. In particular, the Kaolin (K) treatment was positioned in the upper right quadrant of the PCA score plot, as it delivered the highest value of total polyphenols and all single polyphenols analyzed except for luteolin and verbascoside, and it showed the highest value of DPPH and SPAD. The pinolene (V) treatment was positioned in the upper left quadrant of PCA score, as it delivered the highest value of RWC, Fv/Fm, shoots growth, TCSA and dry matter of roots, wood and total dry matter, whereas control was located in the lower left quadrant.

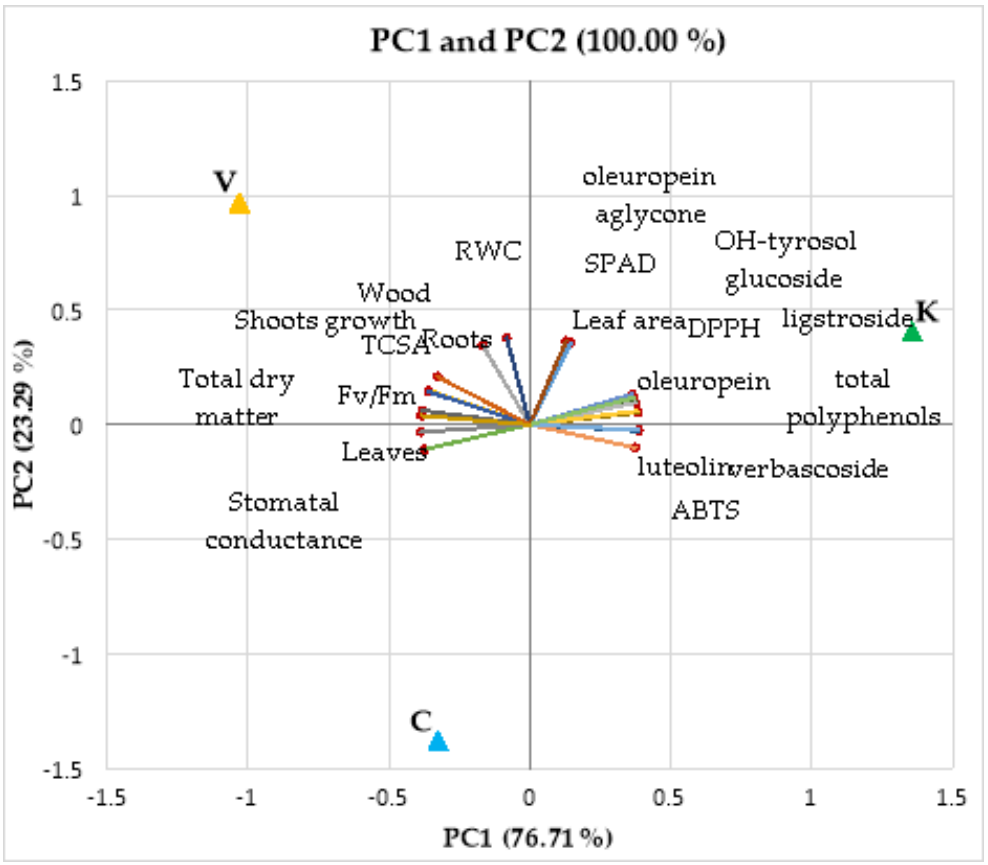

Figure 6. Principal component analysis (PCA) of biometric parameters (shoots growth, TCSA (trunk cross-sectional area), leaf area and dry matter of roots, wood, leaves and total dry matter), physiological parameters (stomatal conductance, RWC, SPAD and F_v/F_m), nutraceutical parameters (OH-tyrosol glucoside, verbascoside, oleuropein, ligstroside, luteolin, oleuropein aglycone, total polyphenols, ABTS and DPPH), under the application of two anti-transpirants K: kaoline and V: di-1-p-menthene in comparison to C: control. For the PCA, the parameters of stomatal conductance, RWC and SPAD analyzed were those of August, and for F_v/F_mthose of 2:00 pm (the values obtained after prolonged thermal stress were considered).

3.5. Conclusions

This preliminary study emphasizes the importance of using products based on kaolin and pinolene to improve the biometric, physiological and nutraceutical characteristics of young olive trees subjected to high thermal stress. As tentative results, foliar application of the anti-transpirant pinolene-based product improved the biometric parameters of the plants, in particular the shoots growth, TCSA, and total dry matter. Plants sprayed with the tested preparation were characterized by a higher relative water content (RWC) in leaves and a

higher value of efficiency of photosystem II (PSII); this positive effect of 1-p-menthene on vegetative growth and development may be due to improved plant water status related to lower transpiration. Kaolin application also reduced stomatal conductance and similar to the V application, recorded higher values of leaf chlorophyll concentrations (SPAD) at the end of the vegetative season. In particular, interesting effects were recorded with the use of kaolin on the nutraceutical compounds of olive leaves; high contents of total polyphenols were recorded compared to control plants and this product showed a higher value of the antioxidant activity.

It is important to underline that further studies are necessary to determine the effect of these products on the biometric, physiological and nutraceutical parameters of mature olive trees cultivated in open field conditions in consecutive years and on the quality of the produce.

3.6 References

1. Moriondo, M. Modelling Olive Trees and Grapevines in a Changing Climate. Environ. Model. 2015, 72, 387–401.
2. Orlandi, F.; Avolio, E.; Bonofiglio, T.; Federico, S.; Romano, B.; Fornaciari, M. Potential Shifts in Olive Flowering According to Climate Variations in Southern Italy. Meteorol. Appl. 2012, 20, 497–503.
3. Fraga, H.; Pinto, J.G.; Viola, F.; Santos, J.A. Climate Change Projections for Olive Yields in the Mediterranean Basin. Int. J. Climatol. 2020, 40, 769–781.
4. Mearns, L.O.; Rosenzweig, C.; Goldberg, R. Mean and variance change in climate scenarios: Methods, agricultural applications, and measures of uncertainty. Clim. Chang. 1997, 35, 367–396.
5. Orlandi, F.; Ruga, L.; Romano, B.; Fornaciari, M. Olive flowering as an indicator of local climatic changes. Theor. Appl. Climatol. 2005, 81, 169–176.
6. Urban, J.; Ingwers, M.; McGuire, M.A.; Teskey, R.O. Stomatal conductance increases with rising temperature. Plant Signal. Behav. 2017, 12, e1356534.
7. Gregory, J.M.; Mitchell, J.F.B.; Brady, A.J. Summer Drought in Northern Midlatitudes in a Time-Dependent CO2 Climate Experiment. J. Clim. 1997, 10, 25.

8. Di Vaio, C.; Marallo, N.; Marino, G.; Caruso, T. Effect of Water Stress on Dry Matter Accumulation and Partitioning in Pot-Grown Olive Trees (Cv Leccino and Racioppella). Sci. Hortic. 2013, 164, 155–159.
9. Angelopoulos, K.; Dichio, B.; Xiloyannis, C. Inhibition of photosynthesis in olive trees (*Olea europaea* L.) during water stress and rewatering. Exp. Bot. J. 1996, 47, 1093–1100.
10. Fernández, J.E.; Moreno, F.; Girón, I.F.; Blázquez, O.M. Stomatal control of water use in olive tree leaves. Plant Soil 1997, 190, 179–192.
11. Bacelar, E.A.; Santos, D.L.; Moutinho-Pereira, J.M.; Goncalves, B.C.; Ferreira, H.F.; Correia, C.M. Immediate Responses and Adaptative Strategies of Three Olive Cultivars under ContrastingWater Availability Regimes: Changes on Structure and Chemical Composition of Foliage and Oxidative Damage. Plant Sci. 2006, 170, 596–605.
12. Bosabalidis, A.M.; Kofidis, G. Comparative Effects of Drought Stress on Leaf Anatomy of Two Olive Cultivars. Plant Sci. 2002, 163, 375–379.
13. Boughalleb, F.; Hajlaoui, H. Physiological and Anatomical Changes Induced by Drought in Two Olive Cultivars (Cv Zalmati and Chemlali). Acta Physiol. Plant. 2011, 33, 53–65.
14. Denaxa, N.-K. Comparative Effects of Exogenous Glycine Betaine, Kaolin Clay Particles and Ambiol on Photosynthesis, Leaf Sclerophylly Indexes and Heat Load of Olive Cv. Chondrolia Chalkidikis under Drought. Sci. Hortic. 2012, 137, 87–94.
15. Wang, S. Genotypic Variation in Growth and Physiological Response to Drought Stress and Re-Watering Reveals the Critical Role of Recovery in Drought Adaptation in Maize Seedlings. Front. Plant Sci. 2016, 6, 15.
16. Perez-Martin, A.; Torres-Ruiz, J.M.; Fernández, J.E.; Diaz-Espejo, A.; Flexas, J.; Michelazzo, C.; Sebastiani, L. Physiological and genetic response of olive leaves to water stress and recovery: Implications of mesophyll conductance and genetic expression of aquaporins and carbonic anhydrase. In Proceedings of the XXVIII International Horticultural Congress on Science and Horticulture for People (IHC2010): International Symposium on CLIMWATER 2010: Horticultural Use of

Water in a Changing Climate, Lisbon, Portugal, 22–27 August 2010; Fernández, J.E., Ferreira, M.I., Eds.; ISHS Acta Horticulturae: Leuven, Belgium, 2010; Volume 922, pp. 99–105.

17. Bacelar, E.A.; Santos, D.L.; Moutinho-Pereira, J.M. Physiological Behaviour, Oxidative Damage and Antioxidative Protection of22:00 Olive Trees Grown under Different Irrigation Regimes. Plant Soil. 2007, 292, 1.
18. Petridis, A. Effect of Water Deficit on Leaf Phenolic Composition, Gas Exchange, Oxidative Damage and Antioxidant Activity of Four Greek Olive (*Olea europaea* L.) Cultivars. Plant Physiol. Biochem. 2012, 60, 1–11.
19. Wungrampha, S.; Joshi, R.; Singla-Pareek, S.L.; Pareek, A. Photosynthesis and salinity: Are these mutually exclusive? Photosynthetica 2018, 56, 366–381.
20. Munns, R.; Gilliham, M. Salinity Tolerance of Crops—What Is the Cost? New Phytol. 2015, 208, 668–673.
21. Maxwell, K.; Johnson, G.N. Chlorophyll fluorescence—A practical guide. J. Exp. Bot. 2000, 51, 659–668.
22. Roháˇcek, K.; Soukupová, J.; Barták, M. Chlorophyll fluorescence: A wonderful tool to study plant physiology and plant stress. Plant Cell Compart.-Sel. Top. Res. Signpost Kerala India 2008, 37, 41–104.
23. Del Amor, F.M. Growth, Photosynthesis and Chlorophyll Fluorescence of Sweet Pepper Plants as Affected by the Cultivation Plant Method. Ann. Appl. Biol. 2006, 148, 133–139.
24. Cantore, V.; Pace, B.; Albrizio, R. Kaolin-Based Particle Film Technology Affects Tomato Physiology, Yield and Quality. Environ. Exp. Bot. 2009, 66, 279–288.
25. Lombardini, L.; Harris, M.K.; Glenn, D.M. Effects of particle film application on leaf gas exchange, water relations, nut yield, and insect populations in mature pecan trees. HortScience 2005, 40, 1376–1380.
26. Rosati, A.; Metcalf, S.G.; Buchner, R.P.; Fulton, A.E.; Lampinen, B.D. Effects of kaolin application on light absorption and distribution, radiation use efficiency and photosynthesis of almond and walnut canopies. Ann. Bot. 2007, 99, 255–263.

27. Palliotti, A.; Poni, S.; Berrios, J.G.; Bernizzoni, F. Vine performance and grape composition as affected by early-season source limitation induced with anti-transpirants in two red Vitis vinifera L. cultivars. Aust. J. GrapeWine Res. 2010, 16, 426–433.
28. Gu, S.; Fuchigami, L.H.; Cheng, L.; Guak, S.H.; Shin, C. Effects of antitranspirant and leaching on medium solution osmotic potential, leaf stomatal status, transpiration, abscisic acid content and plant growth in'Early Girl'tomato plants (*Lycopersicon esculentum*). J. Hortic. Sci. 1998, 73, 473–477.
29. Mola, I.D.; Conti, S.; Cozzolino, E.; Melchionna, G.; Ottaiano, L.; Testa, A.; Sabatino, A.; Rouphae, Y.; Mori, M. Plant-Based Protein Hydrolysate Improves Salinity Tolerance in Hemp: Agronomical and Physiological Aspects. Agronomy 2021, 11, 342.
30. Di Vaio, C.; Graziani, G.; Gaspari, A.; De Luca, L.; Aiello, A.; Cirillo, A.; Bruno, A.; Romano, R.; Ritieni, A. Drupe Characters, Fatty Acids, Polyphenolic and Aromatic Profile of Olive Oil Obtained from "Oliva Bianca", Minor Autochthonous Cultivar of Campania. Plants 2021, 10, 1119.
31. Talhaoui, N.; Gómez-Caravaca, A.M.; León, L.; De la Rosa, R.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Determination of Phenolic Compounds of 'Sikitita' Olive Leaves by HPLC-DAD-TOF-MS. Comparison with Its Parents 'Arbequina' and 'Picual' Olive Leaves. LWT 2014, 58, 28–34.
32. Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A. Effects of Trichoderma Biostimulation on the Phenolic Profile of Extra-Virgin Olive Oil and Olive Oil By-Products. Antioxidants 2020, 9, 284.
33. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic. Biol. Med. 1999, 26, 1231–1237.
34. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. LWT 1995, 28, 25–30.

35. Roussos, P.A.; Denaxa, N.-K.; Damvakaris, T.; Stournaras, V.; Argyrokastritis, I. Effect of Alleviating Products with Different Mode of Action on Physiology and Yield of Olive under Drought. Sci. Hortic. 2010, 125, 700–711.
36. MI, A.D.; Abd El-Rhman, I.E.; Sahar, A.F. Effect of Some Antitranspirants and Supplementery Irrigation on Growth, Yield and Fruit Quality of Sultani Fig (Ficus carica) Grown in the Egyption Western Coastal Zone under Rainfed Conditions. AGBR 2009, 5, 899–908.
37. Davenport, D.C.; Fisher, M.A.; Hagan, R.M. Some counteractive effects of antitranspirants. Plant Physiol. 1972, 49, 722–724.
38. Di Vaio, C.; Marallo, N.; Di Lorenzo, R.; Pisciotta, A. Anti-transpirant effects on vine physiology, berry and wine composition of cv. Aglianico (Vitis vinifera L.) grown in south Italy. Agronomy 2019, 9, 244.
39. Di Vaio, C.; Villano, C.; Lisanti, M.T.; Marallo, N.; Cirillo, A.; Di Lorenzo, R.; Pisciotta, A. Application of Anti-Transpirant to Control Sugar Accumulation in Grape Berries and Alcohol Degree in Wines Obtained from Thinned and Unthinned Vines of cv. Falanghina (Vitis vinifera L.). Agronomy 2020, 10, 345.
40. Nielson, R.E.; Jarvis, P.G. Photosynthesis in Sitka Spruce (Picea sitchensis (Bong.) Carr.): VI. Response of Stomata to Temperature. J. Appl. Ecol. 1975, 12, 879–891.
41. Turner, N.C. Concurrent comparisons of stomatal behavior, water status, and evaporation of maize in soil at high or low water potential. Plant Physiol. 1975, 55, 932–936.
42. Song, X.; Wang, W.; Zhang, C.; Ma, Q.; Li, Y. Postharvest physiochemical responses of cut rose (Rosa hybrida L.) to antitranspirant and vacuum cooling. Philipp. Agric. Sci. 2011, 94, 368–374.
43. Misra, A.K.; Das, B.K.; Datta, J.K.; De, G.C. Effect of antitranspirants on water status and growth pattern of Mulberry (Morus alba L.) under two levels of irrigation. Indian J. Agric. Res. 2009, 43, 307–310.

44. Glenn, D.M. The mechanisms of plant stress mitigation by kaolin-based particle films and applications in horticultural and agricultural crops. HortScience 2012, 47, 710–711.
45. Del Amor, F.M.; Cuadra-Crespo, P.; Walker, D.J.; Cámara, J.M.; Madrid, R. Effect of foliar application of antitranspirant on photosynthesis and water relations of pepper plants under different levels of CO2 and water stress. J. Plant Physiol. 2010, 167, 1232–1238.
46. Mikiciuk, G.; Mikiciuk, M.; Ptak, P. The effects of anitranspirant di-1-p-menthene on some physiological traits of strawberry. J. Ecol. Eng. 2015, 16, 161–167.
47. Abdel-Fattah, G.H. Response of water-stressed rose of China (Hibiscus rosa-sinensis L.) plant to treatment with calcium carbonate and vapor gard antitranspirants. J. Appl. Sci. Res. 2013, 9, 3566–3572.
48. Björkman, O.; Demmig, B. Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta 1987, 170, 489–504.
49. Segura-Monroy, S.; Uribe-Vallejo, A.; Ramirez-Godoy, A.; Restrepo-Diaz, H. Effect of kaolin application on growth, water use efficiency, and leaf epidermis characteristics of Physallis peruviana seedlings under two irrigation regimes. J. Agric. Sci. Technol. 2015, 17, 1585–1596.
50. Latocha, P.; Ska, M.C.; Pietkiewicz, S.; Kalaji, M.H. Preliminary Assessment of Antitranspirant Vapor Gard® Influence on Actinidia Arguta Growing under Drought Stress Conditions. Ann. Wars. Univ. Life Sci. 2009, 30, 149–150.
51. Lawlor, D.W. Limitation to Photosynthesis in Water-Stressed Leaves: Stomata vs. Metabolism and the Role of ATP. Ann. Bot. 2002, 89, 871–885.

52. Percival, G.C.; Keary, I.P.; Marshall, K. The use of film-forming polymers to control Guignardia leaf blotch and powdery mildew on Aesculus hippocastanum L. and Quercus robur L. Arboric. Urban For. 2006, 32, 100.

53. Anderson, J.M. Photoregulation of the composition, function, and structure of thylakoid membranes. Annu. Rev. Plant Physiol. 1986, 37, 93–136.
54. Shekour, G.M.; Brathwaite, R.A.I.; McDavid, C.R. Dry Season Sweet Corn Response to Mulching and Antitranspirants1. J. Agron. 1987, 79, 629–631.
55. Denaxa, N.-K.; Damvakaris, T.; Roussos, P.A. Antioxidant Defense System in Young Olive Plants against Drought Stress and Mitigation of Adverse Effects through External Application of Alleviating Products. Sci. Hortic. 2020, 259, 108812.
56. Brillante, L.; Belfiore, N.; Gaiotti, F.; Lovat, L.; Sansone, L.; Poni, S.; Tomasi, D. Comparing Kaolin and Pinolene to Improve Sustainable Grapevine Production during Drought. PLoS ONE 2016, 11, e0156631.
57. Valentini, G.; Pastore, C.; Allegro, G.; Muzzi, E.; Seghetti, L.; Filippetti, I. Application of Kaolin and Italian Natural Chabasite-Rich Zeolitite to Mitigate the Effect of GlobalWarming in Vitis vinifera L. cv. Sangiovese. Agronomy 2021, 11, 1035.

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Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters

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Abstract: Various products are used to mitigate the negative effects of abiotic stress in olive trees. The aim of the research was to examine an anti-transpirant product (Vapor Gard®, V) and a kaolin-based product (Manisol, K) effect on the growth of two-year-old olive tree seedlings under high temperature. The study was conducted in a greenhouse on trees of a native cultivar of Campania (cv. Salella) grown in pot during the growing season from May to September 2020. The experimental design included two products: di-1-p-menthene (product V) and kaolin (product K), applied five times at 20 day intervals compared with a control. The following biometric, physiological, and nutraceutical parameters were evaluated: stomatal conductance, chlorophyll *a* fluorescence, Soil Plant Analysis Development (SPAD) index, relative water content (RWC), shoots growth, total leaf area per plant, trunk cross-sectional area, dry matter partitioning, total polyphenols, and antioxidant activity. The results obtained showed that the application of di-1-p-menthene (V) was able to induce a significant improvement of shoots growth (+37.22%) and trunk cross-sectional area (+46.60%) and a reduction of the stomatal conductance and an increase of leaf RWC values. Application with kaolin had positive effects on the total polyphenol content, with an increase over the control of 240.33% and higher antioxidant activity values. Further studies are necessary to determine the effect of these products on the biometric, physiological and nutraceutical parameters of mature olive trees cultivated in open field conditions.

Keywords: anti-transpirant; stomatal conductance; chlorophyll *a* fluorescence; RWC; total polyphenols; antioxidant activity

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1. Introduction

The olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean basin, and its growth and development are mainly controlled by atmospheric conditions [1,2]. In recent years, with the scenario of climate change, this cultivar is strongly exposed to thermal and hydric stresses during the growing season, mostly during summer and in the innermost areas of Europe [3]. These changes in temperature and precipitation along with a greater frequency of extreme weather have reduced agricultural yield [4,5]. Temperature affects most plant physiological processes, including photosynthesis and transpiration, which are both regulated by stomatal conductance and which mutually affect each other [6]. The atmospheric CO_2 concentration is increasing, and in addition to its direct effects on plant growth, this change is expected to raise the global mean surface temperature and result in an increase in the severity of summer drought [7]. The olive tree

Chapter 4. Biostimulants Application on *Olea europaea* L. in Mediterranean Conditions Increase the Production and Bioactive Compounds of Drupes and Oil

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Chapter 4. Biostimulants Application on *Olea europaea* L. in Mediterranean Conditions Increase the Production and Bioactive Compounds of Drupes and Oil

Abstract: Over the years, the use of biostimulants has become increasingly widespread due to their proven efficiency in improving plant productivity and quality of fruits and mitigating the effects related to environmental stress. The aim of the present study was to evaluate the effect of three biostimulants on oil yield, production of drupes per plant, and nutraceutical components of olive drupes and oil (total polyphenols, anthocyanins, and fatty acids %) for "Racioppella" cultivar trees growing in South Italy (May–October 2021). The biostimulants used were: a tropical plants extract (A) containing amino acids, vitamins, enzymes, phytochelatins, macro- and microelements, a glycine betaine-based product (B), and a *Trichoderma* spp.-based biostimulant (T). The three biostimulants were compared with a control thesis (C) treated only with water. T treatment increased the polyphenols content of olive drupes by 41.04% compared to C. A and B treatments increased polyphenols content by 21.87% on average compared to C. All three biostimulants showed positive effect by increasing the amount of polyphenols in olive oil compared to C:T showed an increase of 32.19%, B 7.76%, and A 19.78%. Biostimulant application proved useful in boosting fundamental parameters that determine better drupe and oil in terms of antioxidant capacity and nutraceutical potential, other than an increased production.

4.1. Introduction

Global warming is predicted to have a generally negative effect on plant growth due to the damaging effect of high temperatures on their development. The increasing threat of extreme climatic events, including high temperatures might lead to lower crop productivity and quality loss [1]. Abiotic stresses cause morphological, physiological, biochemical, and molecular changes that negatively impact plant growth and yield. The rising threat of climate change is already having a substantial impact on agricultural production

worldwide, as heat waves can cause significant yield losses threatening future global food security [2]. Moreover, the environmental conditions of the Mediterranean basin are expected to change soon [3]. In particular, the mean air temperature is projected to rise drastically in the range of 2–5 °C in the next 30 years [3–5]. Olive trees are considered one of the most suitable and best-adapted species to the Mediterranean-type climate [6], characterized with long, warm, and dry summers, with mild and wet winters [7]. In addition, olive orchards in the Mediterranean basin are normally subjected to high levels of solar radiation, especially in spring and summer seasons. Nowadays, olive trees face new challenges and threats, namely related to climate change. In fact, increased temperatures and drought and a frequent occurrence of extreme weather events such as heatwaves, are among the problems that growers will have to deal with in the upcoming decades [8], as high temperatures influence some parameters such as olive oil and fatty acid content [9]. To mitigate the damages caused by high temperatures, it is important to adopt agronomic practices that allow a better adaptability for drought and high temperature, and therefore the capacity to integrate both tolerance and recovery of olive orchards capacity [10]. The plants defense strategies can be enhanced using various approaches such as the use of biostimulants [11,12]. The effect of biostimulant stems from a variety of factors, starting from the source materials and the production methods [13]. Several studies highlighted the vital role of biostimulants in improving the efficiency of plant's metabolism, increasing plant tolerance and recovery from abiotic stresses, facilitating nutrient assimilation, translocation and use, and enhancing quality attributes of the produce; by including sugar content, color, phenols, antioxidant activity and particularly by fostering the development of complementary soil micro-organisms [14]. In the literature, various studies about the effect of biostimulants on plant growth and production are present [15–18], but very few studies have evaluated the use of biostimulants on olive trees [17,19–21].

The bioactive compounds/secondary metabolites in plants are a wide range of molecules that are generated in suboptimal growing conditions. The biosynthesis of these molecules is intended to enhance crop tolerance to abiotic and biotic stresses and other stressful conditions or avoid attacks from pathogens or animals [22]. Olives and oil are an important

valuable source of natural phenolic antioxidants [23] and fatty acids content that have a benefic role on human health [24]. In fact, an increasing number of epidemiologic and experimental studies report that olive oil may have a role in the prevention of different pathology [25,26]. Several studies demonstrated the effect of various biostimulants based on protein hydrolysates, tropical plants extract, and *Trichoderma* strains in increasing the content of secondary metabolites, improving fruit yield components and fruit qualitative traits in different crops [27], fruit trees [28], and on olive drupes [29]. In this respect, the aim of our study was to evaluate the effect of these different categories of biostimulants on agronomic parameters and on bioactive compounds and fatty acids of *Olea Europaea* L. drupes and oil, in a scenario of Mediterranean temperatures. Therefore, based on the above mentioned, this study was designed to better understand and broaden our knowledge on the effect of different categories of biostimulants in modulating the yield of olive trees and the bioactive compounds of olive drupes and oil in Mediterranean conditions.

4.2. Materials and Methods

4.2.1. Plant Material, Biostimulants Treatments, and Experimental Design

The trial was conducted in an olive orchard in Castelvenere, in the province of Benevento, Southern Italy (41.23488° N. 14.547609° E) at an altitude of 140 m msl, during the growing season from May to October 2021. The experiment was carried out on sixteen years old olive trees in production belonging to the cultivar "Racioppella". The plants were trained to open vase system and planted with an inter-row spacing of 6 m, and an intra-row spacing of 3 m. The olive field received standard horticultural cares, and the treatments against the main parasites were applied according to the regulation of integrated production. The experiment set up was organized as a completely randomized block design with ten trees/replicates per treatment. Ten untreated trees were adopted between the different treatments to avoid any interference from the foliar treatments. The trees were selected according to the uniformity of vegetative and productive status. For foliar treatments, an atomizer was used, and for the radical treatment a ground injector was used. The

experimental design was based on three commercial biostimulants treatments compared with a control:

(1) Auxym (A) product derived from tropical plants extracts by Hello Nature® (Rivoli Veronese, VR, Italy). The product was used as foliar application at the dose of 1.5 L ha^{-1}.

(2) Biohelp (B) glycine betaine-based product by Biolchim SPA (Bologna, BO, Italy), a bio-promoter of resistance to environmental stress. The product was used as foliar application at the dose of 10 kg ha^{-1}.

(3) Trianum-P (T) a product based on *Trichoderma* by Koppert Biological Systems (Bussolengo, VR, Italy), with active ingredient *Trichoderma harzianum* Rifai strain T-22 (also known as KRL-AG2*). The product was used both as foliar application and radical application at the dose of 2.5 kg ha^{-1}.

(4) Control (C) plants were only treated with water.

All biostimulants were applied five times during the growing season at 30 days intervals, at the phenological stage 53, 55, 71, 79, and 81 according to the BBCH scale [30]. The biostimulants were applied adopting a concentration recommended by the producers.

The minimum, maximum, and average temperature data recorded during the growing season were downloaded from the meteorological station of Castelvenere (BN) Italy, where the study was conducted.

4.2.2. Harvest Time, Production Plants^{-1}, Maturation Index and Oil Extraction

The evaluation of the ripening of drupes was done according to the pigmentation of the olives (Jaen index 0–7). The olive trees cv. "Racioppella" were harvested on 26 October 2021 by a vibrating comb, when 50% of the drupes reached a red-mahogany or darker skin color, with a Jaen index of about 3. Fruits were weighed to determine the yield per plant by a digital dynamometer (Kern & Sohn, Germany). Immediately after harvesting, the olives were transported to a crusher where they were processed with a 3-phase continuous malaxing machine (Pieralisi F.lli S.p.A., Ancona, Italy).

4.2.3. Carotenoids Determination of the Drupes

The carotenoids determination of drupes was done based on a spectrophotometric method described by Aiello et al. [31], with slight modifications. The sample (100 mg) was dissolved in 5 mL of ethyl ether, then placed in an ultrasonic bath for 1 min and vortexed for 30 s. The absorbance measurement was carried out using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 470 nm. The results were expressed as mg kg^{-1}.

4.2.4. Anthocyanins Determination of the Drupes

The anthocyanins determination of drupes was done based on the method of Raj and Ahmad [32], with slight modifications. Briefly, 2 g of fruit epicarp was macerated in 20 mL of 5% acidified methanol using a mortar and pestle. The extraction was repeated three times. The extracts were collected and centrifuged at 6500 rpm for 10 min. The supernatant was kept at dark overnight. Finally, the absorbance was measured at 520 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The anthocyanins content was expressed as cyanidin-3-glucoside equivalent since that is the most abundant anthocyanin in nature [33]. The total anthocyanin was reported as mg cyanidin-3-glucoside equivalent kg^{-1}.

4.2.5. Fat Extraction by Drupes

The fat content of drupes was assessed based on the method of Gonçalves et al. [34], with slight modifications. Briefly, 4 g of olive was added to 100 mL solution of chloroform/methanol (2:1; *v*/*v*) (Carlo Erba reagents, Milan, Italy) and 100 mg L^{-1} of butylated hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA), then a mechanical homogenization was performed using an ultra-turrax (Janke and Kunkel, Germany, type TP 18/10) for 6 min on ice. The extract was filtered and added to a separating funnel and the procedure was repeated twice. The obtained volume was adjusted to 150 mL with chloroform/methanol (2:1; *v*/*v*), and then 37.5 mL of sodium chloride (0.73%) was added. After mixing, it was left to rest for 20 min. Then the lipidic extract was recovered and filtered with sodium sulphate anhydrous (Na_2SO_4) (Sigma-Aldrich, St. Louis, MO, USA). The lower phase was collected to previously weighted glass flasks and the solvent was evaporated using a rotary evaporator.

4.2.6. Chemicals, Reagents, and Material

Phenolic standards were purchased from Sigma Aldrich (St. Louis, MO, USA), whereas hydroxytyrosol was obtained from Indofine (Hillsborough, NJ, USA), secologanoside from ChemFaces Biochemical Co., Ltd. (Wuhan, China) and oleuropein form Extrasynthese (France). The standard stock solutions at 1 mg mL^{-1} in methanol were stored at −20 °C for a period of 1 month. Mix stock solution was prepared using the individual stock solutions, then working mix solutions were prepared by diluting the stocks in methanol in order to build calibration curves in the range of 0.02–5 mg mL^{-1}. Methanol, hexane, and formic acid (LC-MS grade) were obtained from Carlo Erba reagents (Milan, Italy), while acetic acid (98–100%) was acquired from Fluka (Milan, Italy).

4.2.7. Ultrasound-Assisted Extraction of Polyphenolic Compounds of the Drupes

Based on the method of Talhaoui et al. [35], the extraction of the lyophilized samples was done with few modifications. About 0.2 g of lyophilized sample was extracted and centrifuged at 4000 rpm. The supernatants were collected and filtered (0.45 mm nylon syringe membranes). Finally, the extract was dried under nitrogen flow and then solubilized in 1 mL of methanol before high-resolution mass spectrometry analysis and antioxidant activity tests.

4.2.8. UHPLC-HRMS Analysis of Polyphenolic Compounds of the Drupes

Polyphenolic compounds were quantified and separated using an UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectrometry analysis was performed by a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). According to Dini et al. [36], where the analytical method is fully detailed, the polyphenolic compounds were acquired.

4.2.9. Antioxidant Activity Evaluation of the Drupes

The free radical scavenging activity was carried out with a 2,2-diphenyl-1-picrylhydrazyl (DPPH)-based assay using the procedure reported by Brand-Williams et al. [37]. The ferric reducing antioxidant activity was measured using the FRAP assay [38], with few adaptations. The ABTS-scavenging activity was evaluated according to the previously

published procedures with minor modifications [39]. All the determinations were performed in triplicates, and the values were expressed as mmol Trolox equi. kg^{-1} dw.

4.2.10. Quality Indices of Olive Oil

Acidity (% oleic acid 100 g^{-1} oil), peroxide value (meq O_2 kg^{-1} oil), and spectrophotometric indices (K232, K270, and ΔK) were determined according to the official method (EC Reg. 2568/1991 and International Olive Council (IOC) methods). The sensory analysis was carried out by eight well-trained assessors for the evaluation of extra-virgin olive oil (EVOO) according to the official methods of the IOC (1996) and EC Reg. 1604/2019. The panel test was performed using the evaluation form regulated by EC Reg. 640/2008.

4.2.11. Fatty Acid Profile of Olive oil

The determination of fatty acid profile was determined by analyzing the fatty acid methyl esters (FAMEs) obtained after trans-esterification as mentioned in detail by Di Vaio et al. [15]. The results were expressed as % *w*/*w*.

4.2.12. Total Polyphenols Content of the Oil

The total phenolic compounds (TPC) quantification was carried out according to the Folin–Ciocalteau colorimetric method. The phenolic compounds extraction was performed according to Genovese et al. [40], with modifications. The oil (300 mg) was added to 300 μL of hexane, and the mixture was vortexed for 30 s. Subsequently, 1.5 mL of methanol:water (60/40 *v*/*v*) was added to the sample and the obtained mixture was vortexed for 1 min, then the sample was centrifuged at 4000 rpm for 10 min. This procedure was repeated twice. The extract (100 μL) was added to 400 μL of water, 800 μL of 7.5% sodium carbonate (Na_2CO_3), and 100 μL of Folin–Ciocalteau (2 N). The samples were left for 30 min in the dark at room temperature. For each extraction, the analyses were executed in triplicates.

4.2.13. Polyphenols Determination by HPLC of Olive oil

To determine the individual polyphenols concentration, 150 mg of sample was dissolved with 3 mL of methanol. The mixture was shaken for 30 s and it was sonicated for 20 min and filtered with a 0.22-μm PES filter before injection into the HPLC system. HPLC analysis was performed following the method of Romano et al. [41]. The results were expressed as mg kg^{-1} of oil.

4.2.14. Statistical Analysis

Analysis of variance (two-way ANOVA) was applied to analyze the group means. Duncan's multiple range test (DMRT) was performed for means separation of each of the significant ($p < 0.05$) measured variables. Principal component analyses (PCA) was executed with a custom python script using scikit-learn 1.1.3, matplotlib, and pandas dataframe libraries.

4.3. Results and Discussion

4.3.1. Effects of Biostimulants on Production/Plant and Drupe Characteristics

Data of minimum, maximum, and mean air temperature (°C) were recorded throughout the experimental period at the agro-meteorological station located at the "Castelvenere" (BN) city (Figure 1). The maximum temperature was recorded in August (41.7 °C), while the minimum temperature was recorded in October (6.5 °C). On the other hand, higher average temperatures were recorded in August with values of around 30 °C.

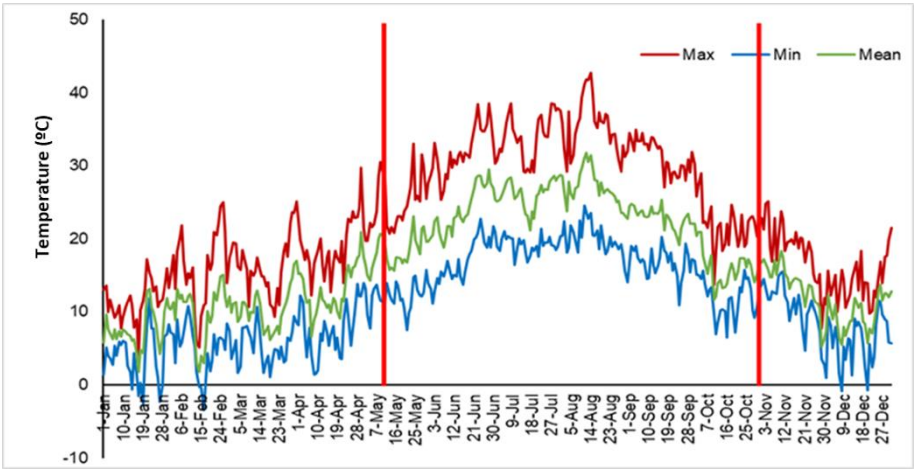

Figure 1. Temperature trend: maximum, minimum, and mean temperature (°C) recorded during the growing season in the open field. The vertical red lines indicate the start and end of the trial period.

The "Racioppella" olives were harvested when the Jaen index conditions were between 2.87 and 2.99. As shown in Table 1, there are no major differences between the various treatments in determining the fruit epicarp color. The maturation stage of collected olive fruits samples is a very important parameter, as it sets olive oil quality, stability, and composition [42]; even if further parameters are needed to determine the exact ripening period of the olives [43]. Several studies were reported on the efficiency of biostimulants in increasing

production especially in horticultural plants [44]. Instead, a smaller number of studies have been conducted on fruit plants demonstrating the efficiency of biostimulants in increasing production, for example as reported in our previous study on "Annurca" apple [45], and on other species as reported by Colla et al. [46]. Table 1 shows the production values of olive trees, and an increase is highlighted following the application of biostimulants. In particular, T treatment recorded an increase in the production by 71%, compared to the control, while the other two biostimulants A and B reported a significant increase of 37.76% and 28% respectively. It is well-known that *Trichoderma* strains can improve plant fitness especially in suboptimal growth conditions in the field, where the fungus has a direct positive influence on plant growth other than alleviating the effects of biotic or abiotic stresses that may naturally occur [47,48]. Our findings are comparable to those of Harman et al. [47], which reported an enhancement of corn yield in several trials. The plant growth promotion induced by *Trichoderma* can be explained by an upregulation of photosynthesis-related proteins and a higher photosynthetic efficiency, as well as a direct effect of an increased root and foliar systems [48]. In the literature, it was reported that glycine betaine enhances the endogenous levels of both GB and proline in many plant species, suggesting the positive role of this chemical compound in enhancing drought stress tolerance by upregulating the mechanisms involved in growth and yield production under stress conditions [49]. The positive effect of Auxym (A) was also demonstrated by Carillo et al. [50] in a study on jute plants, where weekly foliar application of the commercial tropical plants extract, improved fresh yield under sub-optimal nutrient regimens, compared to control treatment. In Table 1, the oil (%), anthocyanins, and carotenoids contents of drupes are shown. The oil content of fruit ranged between 14.11% in B treatment and 14.64% in T treatment, showing a positive effect on oil content, such as reported by Ahmad et al. [51] in seeds of Indian mustard. The anthocyanins content ranged from 407.96 mg kg^{-1} in T treatment to 451.16 mg kg^{-1} in A treatment. These values were higher than those showed by Di Vaio et al. [15], which reported a concentration of 116.10 mg kg^{-1} in drupes of "Oliva Bianca" cultivar, while they were similar to those of Fourati et al. [52], which reported values ranging from 331 mg kg^{-1} to 660 mg kg^{-1}, depending on sampling time and treatment. Furthermore, the use of

Trichoderma reduced the anthocyanins content of drupes, similar to do Rêgo Meneses et al. [53], who showed a reduction of these compounds in maize treated with *Trichoderma asperelloides*. The carotenoids concentration ranged between 5.42 mg kg^{-1} in B and 7.57 in T. All values were higher than that reported by Di Vaio et al. [15], that showed a concentration of 2.10 mg kg^{-1} in drupes of "Oliva Bianca" cultivar, while Motilva and Romero [54] showed a concentration that ranged between 1.8 mg kg^{-1} dw and 70 mg kg^{-1} dw, depending on the maturity time. Yorulmaz et al. [55] showed a carotenoids concentration in the range of 1.19 mg kg^{-1} to 12.87 mg kg^{-1} in olive oil, depending on the cultivar and maturation time. As well, a positive effect of *Trichoderma* on carotenoids was shown by Ahmad et al. [51] in Indian mustard.

Table 1. Production/plant, Jaen index, oil content, anthocyanins, and carotenoids at the time of drupe harvest of olive "Racioppella" cv. treated with three biostimulants: A (tropical plants extract), B (glycine betaine) and T (*Trichoderma*), all compared with C (control).

	A	B	C	T	Significance
Jaen index	2.99	2.89	2.87	2.9	
Production $plant^{-1}$ (kg)	34.44 ± 2.51 b	32.00 ± 1.96 b	25.00 ± 0.95 c	42.73 ± 2.43 a	***
Oil content drupe (%)	14.3 ± 0.10 b	14.1 ± 0.07 b	14.3 ± 0.14 b	14.7 ± 0.10 a	*
Anthocyanins (mgCGE/kg)	451.16 ± 1.35 a	445.65 ± 2.47 a	428.03 ± 4.57 b	407.96 ± 2.72 c	***
Carotenoids (mg/kg)	5.97 ± 0.09 c	5.42 ± 0.14 d	6.78 ± 0.08 b	7.57 ± 0.16 a	***

Values are mean ± standard error. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Asterisks indicate significant effect of biostimulants treatments according to ANOVA (ns = not significant; * = $p < 0.05$; *** = $p < 0.001$).

4.3.2. Polyphenolic Compounds Analysis by UHPLC-Q-Orbitrap HRMS of Olive Drupes

The influence of three different commercially available biostimulants, including Auxym, Biohelp, and *Trichoderma* on the qualitative and quantitative profile of polyphenolic compounds of olive drupes is included in Table 2. Olive drupes were collected from the control and treated plants and a polyphenolic profiling was performed by UHPLC-HRMS Orbitrap. A total of 16 metabolites were detected and identified by high resolution mass spectrometry comprising phenolic acids, flavonoids, phenolic alcohols, and secoiridoids. Some authors reported the use of the foliar product and its effect on olive oil quality [56,57].

Some authors have reported that foliar sprays of biostimulants on olive tree improved oil quality characteristics [21], mineral content [58], and fruit yield [21].

Table 2. Phenolic profiles and total phenolic composition in drupes treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control). Concentrations were expressed as μg g^{-1} dw.

	A	B	C	T	Significance
Hydroxytyrosol glucoside	17.10 ± 2.29 a	12.42 ± 1.31 b	4.85 ± 0.47 c	5.92 ± 0.96 c	***
Hydroxytyrosol (3,4-DHPEA)	119.88 ± 52.19 a	116.97 ± 35.11 a	73.18 ± 7.10 b	148.30 ± 22.15 a	***
Tyrosol (4-HPEA)	13.63 ± 0.88 bc	17.76 ± 1.39 b	8.26 ± 1.12 c	49.16 ± 3.95 a	***
Vanillic acid	17.68 ± 0.61 a	18.21 ± 2.08 a	7.52 ± 1.03 b	11.82 ± 2.75 b	**
Rutin	81.62 ± 32.27 a	91.88 ± 33.00 a	77.36 ± 15.82 a	88.65 ± 23.75 a	ns
Elenolic acid	25.69 ± 1.92 a	19.33 ± 1.40 a	20.25 ± 2.17 a	22.17 ± 3.68 a	ns
Verbascoside	8274.50 ± 708.05 ab	8486.11 ± 468.73 ab	7078.61 ± 618.05 b	9420.64 ± 477.80 a	ns
3,4-DHPEA-EDA	518.22 ± 148.5 a	308.79 ± 124.3 a	391.49 ± 181.3 a	615.32 ± 38.6 a	ns
Ligstroside	13.31 ± 12.39 c	20.54 ± 16.86 bc	47.00 ± 23.97 ab	66.00 ± 31.76 a	**
Oleuropein	473.92 ± 80.91 a	610.51 ± 119.94 b	582.78 ± 64.24 b	532.67 ± 122.40 ab	*
p HPEA-EDA	10.10 ± 0.95 b	14.09 ± 0.73 a	10.51 ± 1.35 b	15.04 ± 4.83 a	ns
Hydroxy-Oleuropein-aglycon	8.57 ± 2.45 ab	9.73 ± 1.77 a	5.52 ± 1.66 b	8.50 ± 4.52 ab	ns
Luteolin	20.49 ± 1.61 bc	31.31 ± 10.39 b	14.92 ± 2.23 c	67.20 ± 22.79 a	***
3,4-DHPEA-AC	56.59 ± 44.83 a	42.92 ± 33.15 ab	12.27 ± 1.84 b	65.38 ± 33.09 a	*
DHPEA-EA	412.82 ± 147.25 bc	225.98 ± 94.42 ab	193.58 ± 109.45 a	478.94 ± 89.65 c	**
p-HPEA-EA	17.04 ± 3.16 a	17.84 ± 5.95 a	8.37 ± 0.95 b	17.14 ± 1.57 a	*
Total polyphenols	10,081.16 ± 812.41 ab	10,044.36 ± 544.34 ab	8535.46 ± 698.45 b	11,612.85 ± 534.10 a	**

Values are mean ± standard error. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Asterisks indicate significant effect of biostimulants treatments according to ANOVA (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

The content of polyphenolic compounds is an important parameter in olive oil quality due to their high antioxidant effects. In our research, the studied biostimulant treatments, showed significant differences in phenolic compounds content, with *Trichoderma* treatment having the highest total phenolic content and reaching a value of 11,612.85 μg g^{-1} dw. The treatment with *Trichoderma* influenced the metabolic response in drupes, in terms of polyphenol biosynthesis, compared to the control, showing a significant increase in the concentration of these compounds of about 41.04%. On the other hand, the treatment with Auxym and Biohelp also had a positive effect on the content of polyphenols in the olive

drupes reaching in both cases an increase of about 21%, but not significantly different from the Control and T treatment.

Our results are consistent with those reported previously by Dini et al. [29], who mentioned that the treatment of olive plants with *Trichoderma* strains promoted the activation of plant defense mechanisms, including the production of secondary metabolites such as phenolic compounds. In addition, in our previous work [20] it was reported the effect of some agronomic practices such as the application of antitranspirants on biometric, eco-physiological, and nutraceutical parameters in young olive trees. Rouphael et al. [59] highlighted the importance of biostimulants in increasing biosynthesis of primary and secondary metabolites, including carotenoids, polyphenols, and ascorbic acid, thus improving the nutritional and nutraceutical quality of the edible products. As for olives, in line with our results, Lobianco and Massenti [60] reported that the foliar application of SUNRED® Biostimulant containing phenylalanine, methionine, monosaccharides, and oxylipins, lead to an increase in oleocanthal and 3,4-DHPEA-EDA in olives.

3.3. Antioxidant Activity of Extracts of Olive Drupes

The antioxidant activity of the drupes is illustrated in Figure 2. FRAP and ABTS showed a similar trend, where A treatment engendered a lower antioxidant activity compared to C. B treatment engendered significantly lower antioxidant activity based on the ABTS assay, whereas T treatment caused the opposite trend. Instead, the DPPH method highlighted the efficiency of all biostmulants in significantly increasing the antioxidant activity of olive drupes. Once again, better results were obtained with T application, which caused an increase of 42.23% compared to the control, while A and B showed an increase of 18.95% and 17.17% respectively. In literature, Dini et al. [36] reported a similar trend for antioxidant activity measured with DPPH assay, highlighting that the Biostimulant treatment on olive trees had a positive effect on the antioxidant activity of the olive leaf samples and of the EVOO samples obtained from the olive trees treated with *Trichoderma harzianum* (strain M10). On the other hand, Del Buono et al. [61] reported that the application of Megafol, a commercial plant biostimulant, on olive plants subjected to severe saline stress caused an

increase of the activity of some key antioxidant enzymes, thus avoiding the accumulation of hydrogen peroxide and lipid peroxidation.

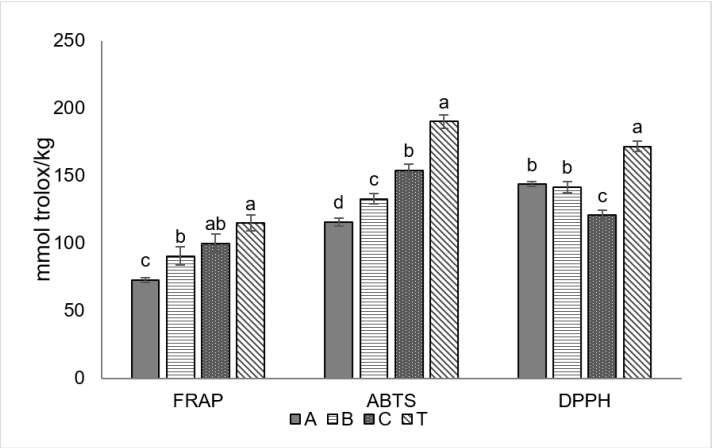

Figure 2. Antioxidant activity (FRAP, ABTS, and DPPH) of olive drupe treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control). Values are mean ± standard error and different letters indicate significant differences based on Duncan's test ($p = 0.05$).

4. *3.4. Quality Indices of Olive Oil*

In Table 3, the quality indices of olive oil are presented. Free acidity ranged from 0.28% in C to 0.31% oleic acid 100 g^{-1} both in A and in B, while peroxide value ranged from 5.34 meq O_2/kg oil in C to 9.08 meq O_2/kg in A. K_{232} ranged from 1.30 in B to 1.76 in T, while ΔK was less than 0.01 in all analyzed sample and K_{270} ranged from 0.17 both in A and B to 0.25 in T. So, all the analyzed samples of olive oil quality indices (free acidity, peroxide value and K_{232}, K_{270} and ΔK index), were within the range for characterizing the oil as "extra virgin" (EEC regulation no. 2019/1604). Furthermore, among the used biostimulants, the treatment with *Trichoderma* resulted more similar to control and so the value of acidity and peroxide both resulted lower than the treatment used with the other biostimulants.

Table 3. Quality indices of analyzed oils treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control).

Oil Quality Index	A	B	C	T
Acidity (% oleic acid 100 g^{-1} oil)	0.31 ± 0.01 a	0.31 ± 0.01 a	0.28 ± 0.01 b	0.29 ± 0.01 b
Peroxide value (meqO_2 kg^{-1})	9.08 ± 0.04 a	7.4 ± 0.04 b	5.34 ± 0.03 d	7.01 ± 0.03 c
K_{270}	0.17 ± 0.00 c	0.17 ± 0.00 c	0.20 ± 0.00 b	0.25 ± 0.00 a
K_{232}	1.61 ± 0.01 b	1.30 ± 0.01 d	1.46 ± 0.00 c	1.76 ± 0.00 a
Delta K	−0.01 ± 0.00 b	0.00 ± 0.00 a	0.00 ± 0.00 a	−0.01 ± 0.00 b

Values are mean ± standard error. Different letters indicate significant differences according to Duncan's multiple-range test ($p = 0.05$).

4.3.5. Oil Sensorial Analysis

In Table 4, the results about the panel test are presented. The sensory analysis carried out by the panel did not report defects in all analyzed oil. The oil obtained with *Trichoderma* treatment resulted the most pungent and the less fruity with value pungency of 6.2, while the most bitter oil was the oil obtained by drupes treated with biostimulant of tropical plants extract (sample A) with a value of 6.4. The taste of bitterness and pungency was reported to be related to the phenols content [62] and in this way, both T and A had shown a higher TPC compared to C (529.81 mg GAE 100 g^{-1} in A and 584.69 mg GAE 100 g^{-1} in T). Furthermore, Servili et al. [63] reported that both bitterness and pungency of Italian oils were correlated with TPC, and in particular low perception was found with 50–200 mg GAE kg^{-1}, medium with 200–500 mg GAE kg^{-1}, and high with 500–1000 mg GAE kg^{-1}.

Table 4. Panel test of oils treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control).

Panel Test	A	B	C	T
Fruity	6.6	5.4	7.2	3.6
Bitterness	6	5.4	3.6	5.8
Pungency	6.4	4	4.6	6.2
Heating/Sludge	0	0	0	0
Winey/Acid/acidic/sour	0	0	0	0
Rancid	0	0	0	0
Mold/moisture/ground	0	0	0	0
Frozen olive	0	0	0	0

2556 *4.3.6. Fatty Acids Composition of Oil*

2557 In Table 5, the fatty acids composition of different oils is presented. The most present fatty
2558 acids in olive oil were palmitic, oleic acid, linoleic, and stearic acid, similar to what is
2559 reported by Noorali et al. [64] and Di Vaio et al. [15]. The fatty acids composition was
2560 influenced by plant treatments, except for behenic acid. In particular, the concentration of
2561 palmitic acid was the lowest in T (12.66%) and the highest in A (14.00%). The palmitic acid
2562 content, in all analyzed samples, was in line with Noorali et al. [64] findings, that reported
2563 a range from 7.5% to 20% depending on the analyzed cultivar. Interestingly, the use of
2564 biostimulants increased the oleic acid content with the highest concentration under T
2565 treatment (71.53%) suggesting an 8% increase compared to C. Moreover, the linoleic acid
2566 concentration decreased in samples treated with biostimulants compared to C, with the
2567 highest decrease in T (-4%). A similar result was obtained by Marra et al. [65] on soybean
2568 seeds that under *Trichoderma* (T22) treatment produced soybean seeds richer in oleic acid
2569 (C18:1) and a reduced content of linoleic acid (C18:2) compared to the control. Furthermore,
2570 Chouliaras et al. [21] found an increase in oleic acid and a reduction in linoleic acid in olive
2571 oil sample treated with seaweed extract applied foliarly, in addition to soil application of
2572 nitrogen and boron fertilizers. On the other hand, Hernández-Hernandez et al. [66] did not
2573 find any significant difference in fatty acids composition of olive oil obtained from plants
2574 treated with biostimulants, maybe due to the high density growing of olive plants. So, the
2575 environmental conditions such as soil type and climate as well as the use of biostimulants
2576 may have influenced the results. Anyway, to the best of our knowledge, this is the first study
2577 reporting the ability of *Trichoderma* strains to influence lipid content of drupes and modify
2578 the fatty acid profile of olive oil.

2579 The oleic/linoleic ratio ranged from 4.71 in C to 6.73 in T, and this value increased with
2580 biostimulant treatment, as well as total MUFA; whereas total PUFA was reduced. The
2581 oleic/linoleic acid ratio was similar to that obtained in "Oblica" cultivar that ranged from
2582 4.17 to 4.96 depending on the production year. While it was lower than the olive oil obtained
2583 by "Leccino" cultivar that ranged from 9.66 to 11.59 depending on the production year [67].

The MUFA/PUFA ratio ranged from 4.38 in C to 6.16 in T. This ratio increased equally under all the other biostimulants, indicating a possible reduction of oxidative susceptibility of oil [68]. The value was similar to that stated by El Qarnifa et al. [69], which showed a range from 4.36 to 10.82. Similarly, the ratio MUFA/SFA increased with biostimulants treatments, and this ratio ranged from 3.78 in C to 4.46 in T treatment. These values are similar to those obtained in "Oblica" and "Leccino" cultivars in different production years (range 4.17–4.96 and 4.14–4.41, respectively) [67]. Finally, the MUFA/SFA ratio and the MUFA/PUFA ratio were relatively low; but the high phenol content could indicate that oil quality was preserved without lipid deterioration, in harmony with the report of Pinelli et al. [70].

Table 5. Fatty acid composition of analyzed oils treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control).

% Fatty Acids	A	B	C	T	Significance
Palimitic (C16)	14.00 ± 0.04 a	13.67 ± 0.03 b	13.90 ± 0.06 a	12.66 ± 0.02 c	***
Palmitoleic (C16:1)	1.43 ± 0.01 a	1.28 ± 0.02 b	1.10 ± 0.01 c	0.82 ± 0.00 d	***
Heptadecanoic (C17)	0.06 ± 0.00 c	0.19 ± 0.01 a	0.20 ± 0.00 a	0.11 ± 0.00 b	***
Stearic (C18)	2.14 ± 0.01 c	2.77 ± 0.02 b	2.90 ± 0.02 a	2.72 ± 0.01 b	***
Oleic (C18.1n9c)	68.81 ± 0.04 b	67.15 ± 0.01 c	66.23 ± 0.00 d	71.53 ± 0.03 a	***
Linoleic (C18:2 Z 9, 12)	12.15 ± 0.04 c	13.34 ± 0.05 b	14.06 ± 0.04 a	10.63 ± 0.01 d	***
Arachidic (C20)	0.33 ± 0.01 d	0.36 ± 0.01 c	0.39 ± 0.00 b	0.41 ± 0.00 a	***
Linolenic (C18:3n3)	0.96 ± 0.02 b	1.12 ± 0.02 a	1.08 ± 0.00 a	0.98 ± 0.01 b	***
Behenic (C22)	0.11 ± 0.00 a	0.14 ± 0.00 a	0.14 ± 0.00 a	0.14 ± 0.01 a	ns
MUFA	70.24 ± 0.03 b	68.43 ± 0.03 c	67.34 ± 0.01 d	72.35 ± 0.03 a	***
PUFA	13.12 ± 0.02 c	14.46 ± 0.03 b	15.14 ± 0.04 a	11.61 ± 0.01 d	***
SFA	16.65 ± 0.05 c	17.11 ± 0.00 b	17.53 ± 0.04 a	16.04 ± 0.03 d	***
MUFA/PUFA	5.35 ± 0.01 b	4.64 ± 0.01 c	4.38 ± 0.01 d	6.16 ± 0.00 a	***
MUFA/SFA	4.22 ± 0.01 b	3.92 ± 0.00 c	3.78 ± 0.01 d	4.46 ± 0.01 a	***
Oleic/linoleic	5.66 ± 0.01 b	5.03 ± 0.02 c	4.71 ± 0.01 d	6.73 ± 0.00 a	***

Values are mean ± standard error. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Asterisks indicate significant effect of biostimulant treatments according to ANOVA (ns = not significant; *** = $p < 0.001$).

4.3.7. Polyphenols Content of Olive Oil

In Table 6, the individual polyphenol content and total polyphenol content are presented. Regarding the phenolic compounds, tyrosol, p-coumaric, ferulic, and vanillic acid were found in all the analyzed samples. The tyrosol content ranged from 8.17 mg kg^{-1} in C to 9.43 in B mg kg^{-1}. The tyrosol content was in harmony with Toric et al.'s [71] study which showed a range from 4.57 mg kg^{-1} to 9.26 mg kg^{-1} depending on the analyzed cultivar, and with Palla

et al.'s [72] study, which showed a concentration that ranged from 4.1 mg kg^{-1} to 86.7 mg kg^{-1} depending on the storage time. The use of biostimulants increased the phenols content in olive oil. In particular, tyrosol and vanillic acid concentrations were the highest in T, while p-coumaric and ferulic acid were highest in B. The tyrosol concentration was higher than that stated by Tovar et al. [73], who showed a concentration ranging between 0.23 mg kg^{-1} and 0.37 mg kg^{-1} depending on the irrigation method. Similarly, Gomez-Alonso et al. [74] showed a concentration ranging from 0.2 mg kg^{-1} to 6.1 mg kg^{-1}. The results are partially in accordance with Dini et al.'s [75], which showed a positive effect of the biostimulants on vanillic, ferulic, and p-coumaric contents, while the tyrosol concentration was reduced in their case. While Dini et al. [75] showed an increase of tyrosol concentration in olive oil of "*Leccino"* and "*Carolea" cultivar* after the treatment with *Trichoderma.*

The second most present compound was p-coumaric acid with a concentration ranging between 4.31 mg kg^{-1} with C treatment and 4.70 mg kg^{-1} with B treatment. The concentration was in line with El Riachy et al.'s [75] study, that showed a range between 1.87 mg kg^{-1} and 6.04 mg kg^{-1}, depending on progenies from crosses of olive. Ferulic and vanillic acid were present at concentrations <1.0 mg kg^{-1}. The polyphenols content of oil is influenced also by region growth, olive tree age, olive maturation, and processing of olive fruit and oil [76].

The TPC ranged from 442.31 mg kg^{-1} in C to 584.69 mg kg^{-1} in T. These values were higher than that of Baiano et al.'s [73] study, that showed a range from 26.53 mg GAE kg^{-1} to 322.18 mg GAE kg^{-1} depending on both the cultivation zone and storage time, and higher than those showed by Mafrica et al.'s [77] that reported a maximum concentration of 457.90 mg GAE kg^{-1}. The use of biostimulants increased the TPC in all sample compared to the control and the increase was in the range of 7.76% in B to 32.19% in T samples. Moreover, Dini et al. [78] and Leogrande et al. [79] showed an increase in TPC in oil obtained by olive treated with biostimulants. The increase of TPC with biostimulants treatment and the reduction of linoleic acid could increase the oxidative stability of oil.

Table 6. Polyphenols content of analyzed oils treated with three biostimulants: A (tropical plants extract), B (glycine betaine), and T (*Trichoderma*) all compared with C (control).

Compounds (mg/kg)	A	B	C	T	Significance
Tyrosol	8.52 ± 0.06 b	9.43 ± 0.13 a	8.17 ± 0.11 c	9.25 ± 0.09 a	***
p-coumaric acid	4.53 ± 0.00 b	4.70 ± 0.01 a	4.31 ± 0.01 d	4.43 ± 0.01 c	***
Ferulic acid	0.76 ± 0.01 c	0.87 ± 0.01 a	0.81 ± 0.01 b	0.70 ± 0.00 d	***
Vanillic acid	0.40 ± 0.00 bc	0.38 ± 0.00 c	0.42 ± 0.01 b	0.58 ± 0.00 a	***
Oleuropein	<LOD	<LOD	<LOD	<LOD	
Total polyphenols	529.81 ± 7.52 b	476.64 ± 11.93 c	442.31 ± 10.77 d	584.69 ± 3.93 a	***

Values are mean ± standard error. Different letters indicate significant differences according to Duncan's multiple-range test (p = 0.05). Asterisks indicate significant effect of biostimulants treatments according to ANOVA (ns = not significant; *** = $p < 0.001$). LOD: limit of detection.

4.3.8. Principal Component Analysis (PCA)

To obtain a broad overview of the drupes and oil parameters characterizing "Racioppella" cultivar following the biostimulants treatments, two principal component analyses were conducted. The Figure 3 shows a principal component analysis of all the analyzed parameters of the drupes. The first two principal components (PCs) disclosed 88.56% of the cumulative variance, with PC1 detailing for 59.82% and PC2 for 28.74%. The figure clearly shows the correlation between the three biostimulants and the individual parameters analyzed. PC1 was positively correlated with hydroxytyrosol glucoside, hydroxytyrosol (3,4-DHPEA), tyrosol (4-HPEA), vanillic acid, rutin, elenolic acid, verbascoside, DHPEA-EDA, ligstroside, oleuropeina, p HPEA-EDA, hydroxy-oleuropein-aglycon, luteolin, 3,4-DHPEA-AC, DHPEA-EA, p-HPEA-EA, and total polyphenols, while it was negatively correlated with ligstroside, it instead shows a positive correlation with PC2. The control is placed in the upper left quadrant, and it is correlated only with ligstroside, T is placed in the upper right quadrant and it is correlated with verbascoside,l uteolin, tyrosol, 3,4-DHPEA-EDA, Hydroxytyrosol (3.4-DHPEA),DHPEA-EA, p HPEA-EDA, 3.4 DHPEA-AC; while A and B are in the same lower right quadrant and they are correlated with elenolic acid, rutin, oleuropeina, p-HPEA-EA, vanillic acid, hydroxytyrosol glucoside, hydroxy-oleuropein-aglycon.

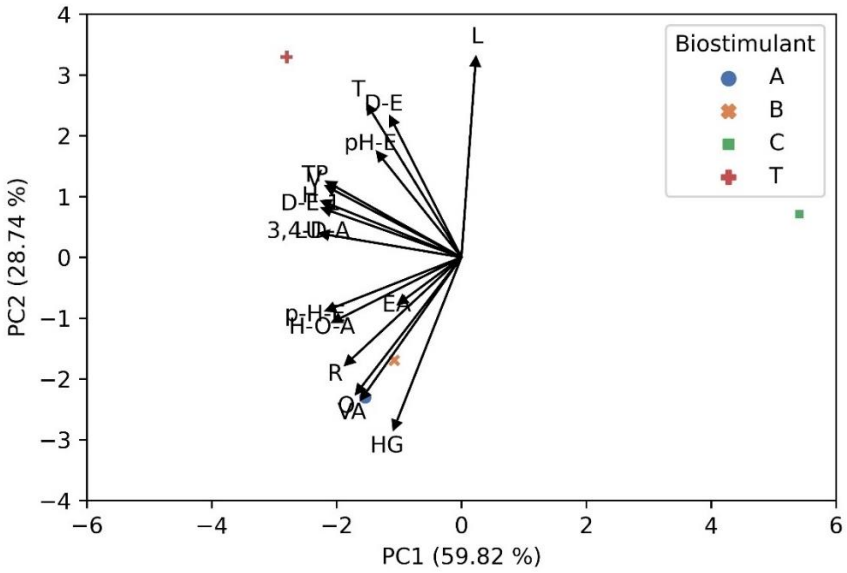

Figure 3. Principal component analysis (PCA) of drupe parameters: hydroxytyrosol glucoside (HG), hydroxytyrosol (3,4-DHPEA) (H), tyrosol (4-HPEA) (T), vanillic acid (VA), rutin (R), elenolic acid (EA), verbascoside (V), DHPEA-EDA (DE), ligstroside (L), oleuropeina (O), p HPEA-EDA (pH-E), hydroxy-oleuropein-aglycon (H-O-A), luteolin (LU), 3,4-DHPEA-AC (3,4-D-A), DHPEA-EA (D-E), p-HPEA-EA (p-H-E), and total polyphenols (TP). C = control, T = *trichoderma*, B = glycine betaine, A = tropical plants extract.

Figure 4 shows the principal component analysis of all the analyzed parameters of the oil. PCA was performed on all the analytical data to examine differences between oils. These two principal components account for 88.4% of the variance among the four oil samples, with PC1 and PC2 accounting for 64.61% and 23.79%, respectively. The differences between oil samples suggested that the type of biostimulant treatment has significant influence on oil composition. Indeed, the T sample, showed the highest content of oleic acid, arachidic acid (Table 5), tyrosol, vanillic acid, and total polyphenols (Table 6). C and B samples were present in the same quadrant and C was positively correlated with heptadecanoic, stearic and behenic, linolenic, linoleic acids, PUFA, and SFA (Table 5), while sample B was positively correlated also with ferulic acid (Table 6). In addition, samples belonging to A treatment, forms a distinct cluster in the lower right quadrant.

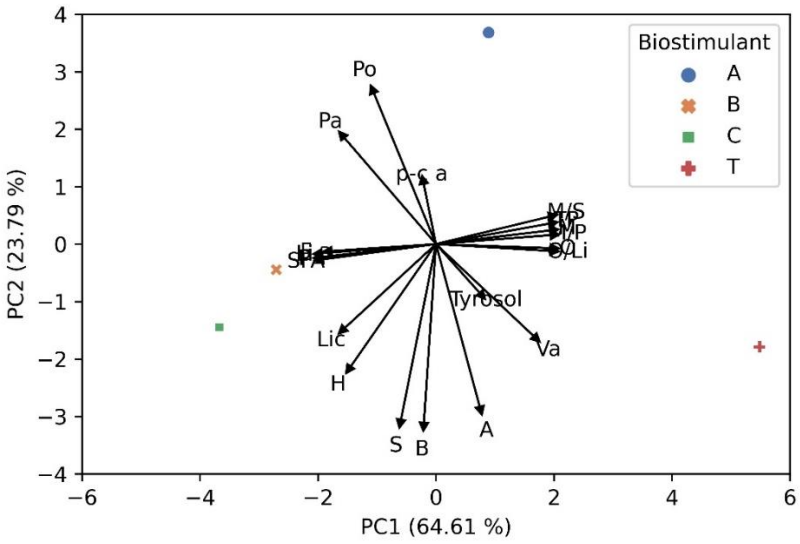

Figure 4. Principal component analysis (PCA) of oil parameters: linolenic (Lic), PUFA (P), SFA, linoleic (Li), ferulic acid (Fa), behenic (B), stearic (S), heptadecanoic (H), p-coumaric acid (p-c a), palmitic (Pa), palmitoleic (Po), arachidic (A), vanillic acid (Va), tyrosol, oleic acid, oleic/linoleic (O/Li), MUFA (M), MUFA/SFA (M/S), MUFA/PUFA (M/P), total polyphenols (TP). C = control, T = *trichoderma*, B = glycine betaine, A = tropical plants extract.

4.4. Conclusions

The Mediterranean area is increasingly affected by a climate change scenario where high temperatures compromise the yield and quality of agricultural products, including olives and olive oil. In our study we evaluated the impact of different biostimulants to reduce climate damage and improve the performance of plants and the quality of the derived product. In this study, the biostimulants increased the production per plant, in particular the *Trichoderma* by about 70%, which positively influenced the carotenoids content and polyphenols biosynthesis in the drupes as well. All the oils analyzed showed quality parameters that fell within the parameters of an extra virgin olive oil. Biostimulants based on tropical plant extracts and *trichoderma* reported changes to the flavor (bitter and spicy), due to an increase in the total polyphenol content in the oil by 32.1% and 19.8% respectively. All biostimulants influenced the oil fatty acid content. In conclusion, our study demonstrated that it is possible to state that biostimulants affect some qualitative-quantitative aspects of both the oil and the drupes, improving, in some cases, fundamental

parameters that determine the consumer satisfaction of a good product, as well as their antioxidant capacity and nutraceutical potential. A second year of testing is currently underway in order to confirm the results obtained in the first year.

4.5 References

1. Bita, C.E.; Gerats, T. Plant Tolerance to High Temperature in a Changing Environment: Scientific Fundamentals and Production of Heat Stress-Tolerant Crops. Front. Plant Sci. 2013, 4, 273.
2. Christensen, J.H.; Christensen, O.B. A Summary of the PRUDENCE Model Projections of Changes in European Climate by the End of This Century. Clim. Chang. 2007, 81, 7–30.
3. Giorgi, F. Climate Change Hot-Spots. Geophys. Res. Lett. 2006, 33, L08707.
4. Giannakopoulos, C.; Le Sager, P.; Bindi, M.; Moriondo, M.; Kostopoulou, E.; Goodess, C.M. Climatic Changes and Associated Impacts in the Mediterranean Resulting from a 2 _C Global Warming. Glob. Planet. Chang. 2009, 68, 209–224.
5. Gualdi, S.; Somot, S.; Li, L.; Artale, V.; Adani, M.; Bellucci, A.; Braun, A.; Calmanti, S.; Carillo, A.; Dell'Aquila, A.; et al. The CIRCE Simulations: Regional Climate Change Projections with Realistic Representation of the Mediterranean Sea. Bull. Am. Meteorol. Soc. 2013, 94, 65–81.
6. Orlandi, F.; Avolio, E.; Bonofiglio, T.; Federico, S.; Romano, B.; Fornaciari, M. Potential Shifts in Olive Flowering According Variations in Southern Italy. Meteorol. Appl. 2012, 7, 497–503.
7. Kottek, M.; Grieser, J.; Beck, C.; Rudolf, B.; Rubel, F. World Map of the Köppen-Geiger Climate Classification Updated. Metz 2006, 15, 259–263.
8. Fraga, H.; Pinto, J.G.; Viola, F.; Santos, J.A. Climate Change Projections for Olive Yields in the Mediterranean Basin. Int. J. Climatol. 2020, 40, 769–781.
9. García-Inza, G.P.; Castro, D.N.; Hall, A.J.; Rousseaux, M.C. Opposite Oleic Acid Responses to Temperature in Oils from the Seed and Mesocarp of the Olive Fruit. Eur. J. Agron. 2016, 76, 138–147.

10. Wang, S. Genotypic Variation in Growth and Physiological Response to Drought Stress and Re-Watering Reveals the Critical Role of Recovery in Drought Adaptation in Maize Seedlings. Front. Plant Sci. 2016, 6, 15. 11.
11. Graziani, G.; Cirillo, A.; Giannini, P.; Conti, S.; El-Nakhel, C.; Rouphael, Y.; Ritieni, A.; Di Vaio, C. Biostimulants Improve Plant 11:00 Growth and Bioactive Compounds of Young Olive Trees under Abiotic Stress Conditions. Agriculture 2022, 12, 227.
12. Mazeh, M.; Almadi, L.; Paoletti, A.; Cinosi, N.; Daher, E.; Tucci, M.; Lodolini, E.M.; Rosati, A.; Famiani, F. Use of an Organic Fertilizer Also Having a Biostimulant Action to Promote the Growth of Young Olive Trees. Agriculture 2021, 11, 593.
13. Cristofano, F.; El-Nakhel, C.; Rouphael, Y. Biostimulant Substances for Sustainable Agriculture: Origin, Operating Mechanisms and Effects on Cucurbits, Leafy Greens, and Nightshade Vegetables Species. Biomolecules 2021, 11, 1103.
14. Graziani, G.; Ritieni, A.; Cirillo, A.; Cice, D.; Di Vaio, C. Effects of Biostimulants on Annurca Fruit Quality and Potential Nutraceutical Compounds at Harvest and during Storage. Plants 2020, 9, 775.
15. Di Vaio, C.; Graziani, G.; Gaspari, A.; De Luca, L.; Aiello, A.; Cirillo, A.; Bruno, A.; Romano, R.; Ritieni, A. Drupe Characters, Fatty Acids, Polyphenolic and Aromatic Profile of Olive Oil Obtained from “Oliva Bianca”, Minor Autochthonous Cultivar of Campania. Plants 2021, 10, 1119.
16. Brown, P.; Saa, S. Biostimulants in Agriculture. Front. Plant Sci. 2015, 6, 671.
17. Ali, E.F.; Hassan, F.A.S.; Elgimabi, M. Improving the Growth, Yield and Volatile Oil Content of Pelargonium graveolens L. Herit by Foliar Application with Moringa Leaf Extract through Motivating Physiological and Biochemical Parameters. S. Afr. J. Bot. 2018, 119, 383–389.
18. Fleming, T.R.; Fleming, C.C.; Levy, C.C.B.; Repiso, C.; Hennequart, F.; Nolasco, J.B.; Liu, F. Biostimulants Enhance Growth and Drought Tolerance in Arabidopsis Thaliana and Exhibit Chemical Priming Action. Ann. Appl. Biol. 2019, 174, 153–165.

19. Almadi, L.; Paoletti, A.; Cinosi, N.; Daher, E.; Rosati, A.; Di Vaio, C.; Famiani, F. A Biostimulant Based on Protein Hydrolysates Promotes the Growth of Young Olive Trees. Agriculture 2020, 10, 618.
20. Cirillo, A.; Conti, S.; Graziani, G.; El-Nakhel, C.; Rouphael, Y.; Ritieni, A.; Di Vaio, C. Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters. Agronomy 2021, 11, 1884.
21. Chouliaras, V.; Therios, I.; Chatzissavvidis, C.; Kouropalatis, T.; Mantzoutsos, I. Organic farming fertilization treatments on productivity, skin carotenoids and nutritional status of 'kalamon' table olives. Olivebioteq 2011, 2, 465–470.
22. Franzoni, G.; Trivellini, A.; Bulgari, R.; Cocetta, G.; Ferrante, A. Bioactive Molecul es as Regulatory Signals in Plant Responses to Abiotic Stresses. In Plant Signaling Molecules; Elsevier: Amsterdam, The Netherlands, 2019; pp. 169–182. ISBN 978-0-12-816451-8.
23. Briante, R.; Febbraio, F.; Nucci, R. Antioxidant Properties of Low Molecular Weight Phenols Present in the Mediterranean Diet. J. Agric. Food Chem. 2003, 51, 6975–6981.
24. Calder, P.C. Functional Roles of Fatty Acids and Their Effects on Human Health. JPEN J. Parenter Enter. Nutr. 2015, 39, 18S–32S.
25. Scarmeas, N.; Stern, Y.; Tang, M.-X.; Mayeux, R.; Luchsinger, J.A. Mediterranean Diet and Risk for Alzheimer's Disease. Ann. Neurol. 2006, 59, 912–921.
26. Braga, C.; Vecchia, C.L.; Franceschi, S.; Negri, E.; Parpinel, M.; Decarli, A.; Giacosa, A.; Trichopoulos, D. Olive Oil, Other Seasoning Fats, and the Risk of Colorectal Carcinoma. Cancer Interdiscip. Int. J. Am. Cancer Soc. 1998, 82, 448–453.
27. Giordano, M.; El-Nakhel, C.; Carillo, P.; Colla, G.; Graziani, G.; Di Mola, I.; Mori, M.; Kyriacou, M.C.; Rouphael, Y.; Soteriou, G.A.; et al. Plant-Derived Biostimulants Differentially Modulate Primary and Secondary Metabolites and Improve the Yield Potential of Red and Green Lettuce Cultivars. Agronomy 2022, 12, 1361.

28. Woo, S.L.; Ruocco, M.; Vinale, F.; Nigro, M.; Marra, R.; Lombardi, N.; Pascale, A.; Lanzuise, S.; Manganiello, G.; Lorito, M. *Trichoderma*-based products and their widespread use in agriculture. Open Mycol. J. 2014, 8, 71–126.
29. Dini, I.; Pascale, M.; Staropoli, A.; Marra, R.; Vinale, F. Effect of Selected *Trichoderma* Strains and Metabolites on Olive Drupes. Appl. Sci. 2021, 11, 8710.
30. Sanz-Cortes, F.; Martinez-Calvo, J.; Badenes, M.L.; Bleiholder, H.; Hack, H.; Llacer, G.; Meier, U. Phenological Growth Stages of Olive Trees (*Olea europaea*). Ann. Appl. Biol. 2002, 140, 151–157.
31. Aiello, A.; Pizzolongo, F.; Scognamiglio, G.; Romano, A.; Masi, P.; Romano, R. Effects of Supercritical and Liquid Carbon Dioxide Extraction on Hemp (Cannabis Sativa L.) Seed Oil. Int. J. Food Sci. Technol. 2020, 55, 2472–2480.
32. Khan, W.; Rayirath, U.P.; Subramanian, S.; Jithesh, M.N.; Rayorath, P.; Hodges, D.M.; Critchley, A.T.; Craigie, J.S.; Norrie, J.; Prithiviraj, B. Seaweed Extracts as Biostimulants of Plant Growth and Development. J. Plant Growth Regul. 2009, 28, 386–399.
33. Francis, F.J.; Markakis, P.C. Food Colorants: Anthocyanins. Crit. Rev. Food Sci. Nutr. 1989, 28, 273–314.
34. Gonçalves, A.; Silva, E.; Brito, C.; Martins, S.; Pinto, L.; Dinis, L.; Luzio, A.; Martins-Gomes, C.; Fernandes-Silva, A.; Ribeiro, C.; et al. Olive Tree Physiology and Chemical Composition of Fruits Are Modulated by Different Deficit Irrigation Strategies. J. Sci. Food Agric. 2020, 100, 682–694.
35. Talhaoui, N.; Taamalli, A.; Gómez-Caravaca, A.M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Phenolic Compounds in Olive Leaves: Analytical Determination, Biotic and Abiotic Influence, and Health Benefits. Food Res. Int. 2015, 77, 92–108.
36. Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A. Effects of *Trichoderma* Biostimulation on the Phenolic Profile of Extra-Virgin Olive Oil and Olive Oil By-Products. Antioxidants 2020, 9, 284.

37. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. LWT Food Sci. Technol. 1995, 28, 25–30.
38. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239, 70–76.
39. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic. Biol. Med. 1999, 26, 1231–1237.
40. Genovese, A.; Caporaso, N.; De Luca, L.; Paduano, A.; Sacchi, R. Influence of Olive Oil Phenolic Compounds on Headspace Aroma Release by Interaction with Whey Proteins. J. Agric. Food Chem. 2015, 63, 3838–3850.
41. Romano, R.; De Luca, L.; Aiello, A.; Rossi, D.; Pizzolongo, F.; Masi, P. Bioactive Compounds Extracted by Liquid and Supercritical Carbon Dioxide from Citrus Peels. Int. J. Food Sci. Technol. 2022, 57, 3826–3837.
42. Bajoub, A.; Hurtado-Fernández, E.; Ajal, E.A.; Fernández-Gutiérrez, A.; Carrasco-Pancorbo, A.; Ouazzani, N. Quality and Chemical Profiles of Monovarietal North Moroccan Olive Oils from "Picholine marocaine" Cultivar: Registration Database Development and Geographical Discrimination. Food Chem. 2015, 179, 127–136.
43. Bouaziz, M.; Jemai, H.; Khabou,W.; Sayadi, S. Oil Content, Phenolic Profiling and Antioxidant Potential of Tunisian Olive Drupes. J. Sci. Food Agric. 2010, 90, 1750–1758.
44. Drobek, M.; Fra¸c, M.; Cybulska, J. Plant Biostimulants: Importance of the Quality and Yield of Horticultural Crops and the Improvement of Plant Tolerance to Abiotic Stress—A Review. Agronomy 2019, 9, 335.
45. Di-Vaio, C.; Cirillo, A.; Cice, D.; El-Nakhel, C.; Rouphael, Y. Biostimulant Application Improves Yield Parameters and Accentuates Fruit Color of Annurca Apples. Agronomy 2021, 11, 715.
46. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein Hydrolysates as Biostimulants in Horticulture. Sci. Hortic. 2015, 196, 28–38.

47. Harman, G.E. Myths and Dogmas of Biocontrol Changes in Perceptions Derived from Research on *Trichoderma harzinum* T-22. Plant Dis. 2000, 84, 377–393.
48. Shoresh, M.; Harman, G.E.; Mastouri, F. Induced Systemic Resistance and Plant Responses to Fungal Biocontrol Agents. Annu. Rev. Phytopathol. 2010, 48, 21–43.
49. Shafiq, S.; Akram, N.A.; Ashraf, M.; García-Caparrós, P.; Ali, O.M.; Latef, A.A.H.A. Influence of Glycine Betaine (Natural and Synthetic) on Growth, Metabolism and Yield Production of Drought-Stressed Maize (Zea mays L.) Plants. Plants 2021, 10, 2540.
50. Carillo, P.; Colla, G.; El-Nakhel, C.; Bonini, P.; D'Amelia, L.; Dell'Aversana, E.; Pannico, A.; Giordano, M.; Sifola, M.; Kyriacou, M.; et al. Biostimulant Application with a Tropical Plant Extract Enhances Corchorus Olitorius Adaptation to Sub-Optimal Nutrient Regimens by Improving Physiological Parameters. Agronomy 2019, 9, 249.
51. Ahmad, P.; Hashem, A.; Abd-Allah, E.F.; Alqarawi, A.A.; John, R.; Egamberdieva, D.; Gucel, S. Role of *Trichoderma* harzianum in Mitigating NaCl Stress in Indian Mustard (Brassica juncea L.) through Antioxidative Defense System. Front. Plant Sci. 2015, 6, 868.
52. Fourati, R.; Scopa, A.; Ben Ahmed, C.; Ben Abdallah, F.; Terzano, R.; Gattullo, C.E.; Allegretta, I.; Galgano, F.; Caruso, M.C.; Sofo, A. Leaf Biochemical Responses and Fruit Oil Quality Parameters in Olive Plants Subjected to Airborne Metal Pollution. Chemosphere 2017, 168, 514–522.
53. do Rêgo Meneses, F.J.; de Oliveira Lopes, Á.L.; Setubal, I.S.; da Costa Neto, V.P.; Bonifácio, A. Inoculation of *Trichoderma* asperelloides Ameliorates Aluminum Stress-Induced Damages by Improving Growth, Photosynthetic Pigments and Organic Solutes in Maize. 3 Biotech 2022, 12, 246.
54. Motilva, M.-J.; Romero, M.-P. The Effect of the Ripening Process of the Olive Fruit on the Chlorophyll and Carotenoid Fractions of Drupes and Virgin Oils. In Olives and Olive Oil in Health and Disease Prevention; Elsevier: Amsterdam, The Netherlands, 2010; pp. 59–68. ISBN 978-0-12-374420-3.

55. Yorulmaz, A.; Erinc, H.; Tekin, A. Changes in Olive and Olive Oil Characteristics During Maturation. J. Am. Oil Chem. Soc. 2013, 90, 647–658.
56. Tekaya, M.; Mechri, B.; Mbarki, N.; Cheheb, H.; Hammami, M.; Attia, F. Arbuscular Mycorrhizal Fungus Rhizophagus irregularis Influences Key Physiological Parameters of Olive Trees (*Olea europaea* L.) and Mineral Nutrient Profile. Photosynthetica 2017, 55,308–316.
57. Rizwan, M.; Ali, S.; Zaheer Akbar, M.; Shakoor, M.B.; Mahmood, A.; Ishaque, W.; Hussain, A. Foliar Application of Aspartic Acid Lowers Cadmium Uptake and Cd-Induced Oxidative Stress in Rice under Cd Stress. Environ. Sci. Pollut. Res. 2017, 24, 21938–21947.
58. Zouari, I.; Mechri, B.; Tekaya, M.; Dabbaghi, O.; Cheraief, I.; Mguidiche, A.; Annabi, K.; Laabidi, F.; Attia, F.; Hammami, M.; et al. Olive Oil Quality Influenced by Biostimulant Foliar Fertilizers. Braz. J. Biol. Sci. 2020, 7, 3–18.
59. Rouphael, Y.; Carillo, P.; Cristofano, F.; Cardarelli, M.; Colla, G. Effects of Vegetal- versus Animal-Derived Protein Hydrolysate on Sweet Basil Morpho-Physiological and Metabolic Traits. Sci. Hortic. 2021, 284, 110123.
60. Massenti, R.; Ciaccio, V.; Bianco, R.L. Foliar applications with sunred®biostimulant advance and uniform fruit ripening in orange and olive. Int. J. Plant Anim. Environ. Sci. 2015, 5, 227–232.
61. Del Buono, D.; Regni, L.; Del Pino, A.M.; Bartucca, M.L.; Palmerini, C.A.; Proietti, P. Effects of Megafol on the Olive Cultivar 'Arbequina' Grown Under Severe Saline Stress in Terms of Physiological Traits, Oxidative Stress, Antioxidant Defenses, and Cytosolic Ca2+. Front. Plant Sci. 2021, 11, 603576.
62. Bendini, A. Phenolic Molecules in Virgin Olive Oils: A Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade. Molecules 2007, 12, 1679–1719.
63. Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and Sensory Properties of Virgin Olive Oil Hydrophilic Phenols: Agronomic

and Technological Aspects of Production That Affect Their Occurrence in the Oil. J. Chromatogr.A 2004, 1054, 113–127.

64. Noorali, M.; Barzegar, M.; Sahari, M.A. Sterol and Fatty Acid Compositions of Olive Oil as an Indicator of Cultivar and Growing Area. J. Am. Oil Chem. Soc. 2014, 91, 1571–1581.

65. Marra, F.P.; Caruso, T.; Costa, F.; Di Vaio, C.; Mafrica, R.; Marchese, A. Genetic Relationships, Structure and Parentage Simulation among the Olive Tree (*Olea europaea* L. Subsp. europaea) Cultivated in Southern Italy Revealed by SSR Markers. Tree Genet. Genomes 2013, 9, 961–973.

66. Hernández-Hernandez, G.; Salazar, D.M.; Martínez-Tomé, J.; López-Cortés, I. The Use of Biostimulants in High-Density Olive Growing: Quality and Production. AJAAR 2019, 10, 1–14.

67. Juki´c Špika, M.; Perica, S.; Žaneti´c, M.; Škevin, D. Virgin Olive Oil Phenols, Fatty Acid Composition and Sensory Profile: Can Cultivar Overpower Environmental and Ripening Effect? Antioxidants 2021, 10, 689.

68. Bengana, M.; Bakhouche, A.; Lozano-Sánchez, J.; Amir, Y.; Youyou, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Influence of Olive Ripeness on Chemical Properties and Phenolic Composition of Chemlal Extra-Virgin Olive Oil. Food Res. Int. 2013, 54,1868–1875.

69. El Qarnifa, S.; El Antari, A.; Hafidi, A. Effect of Maturity and Environmental Conditions on Chemical Composition of Olive Oils of Introduced Cultivars in Morocco. J. Food Qual. 2019, 2019, 1854539.

70. Pinelli, P.; Galardi, C.; Mulinacci, N.; Vincieri, F.F.; Cimato, A.; Romani, A. Minor Polar Compound and Fatty Acid Analyses in Monocultivar Virgin Olive Oils from Tuscany. Food Chem. 2003, 80, 331–336.

71. Tori´c, J.; Brozovic, A.; Baus Lonˇcar, M.; Jakobuši´c Brala, C.; Karkovi´c Markovi´c, A.; Benˇci´c, Đ.; Barbari´c, M. Biological Activity of Phenolic Compounds in Extra Virgin Olive Oils through Their Phenolic Profile and Their Combination with

Anticancer Drugs Observed in Human Cervical Carcinoma and Colon Adenocarcinoma Cells. Antioxidants 2020, 9, 453.

72. Palla, M.; Digiacomo, M.; Cristani, C.; Bertini, S.; Giovannetti, M.; Macchia, M.; Manera, C.; Agnolucci, M. Composition of Health-Promoting Phenolic Compounds in Two Extra Virgin Olive Oils and Diversity of Associated Yeasts. J. Food Compos. Anal. 2018, 74, 27–33.
73. Tovar, M.J.; Motilva, M.J.; Romero, M.P. Changes in the Phenolic Composition of Virgin Olive Oil from Young Trees (*Olea europaea* L. cv. Arbequina) Grown under Linear Irrigation Strategies. J. Agric. Food Chem. 2001, 49, 5502–5508.
74. Gómez-Alonso, S.; Salvador, M.D.; Fregapane, G. Phenolic Compounds Profile of Cornicabra Virgin Olive Oil. J. Agric. Food Chem. 2002, 50, 6812–6817.
75. El Riachy, M.; Priego-Capote, F.; León, L.; Luque de Castro, M.D.; Rallo, L. Virgin Olive Oil Phenolic Profile and Variability in Progenies from Olive Crosses. J. Sci. Food Agric. 2012, 92, 2524–2533.
76. Cicerale, S.; Lucas, L.; Keast, R. Biological Activities of Phenolic Compounds Present in Virgin Olive Oil. Int. J. Mol. Sci. 2010, 11, 458–479.
77. Mafrica, R.; Piscopo, A.; De Bruno, A.; Poiana, M. Effects of Climate on Fruit Growth and Development on Olive Oil Quality in Cultivar Carolea. Agriculture 2021, 11, 147.
78. Dini, I.; Graziani, G.; Gaspari, A.; Fedele, F.L.; Sicari, A.; Vinale, F.; Cavallo, P.; Lorito, M.; Ritieni, A. New Strategies in the Cultivation of Olive Trees and Repercussions on the Nutritional Value of the Extra Virgin Olive Oil. Molecules 2020, 25, 2345.
79. Leogrande, R.; El Chami, D.; Fumarola, G.; Di Carolo, M.; Piegari, G.; Elefante, M.; Perrelli, D.; Dongiovanni, C. Biostimulants for Resilient Agriculture: A Preliminary Assessment in Italy. Sustainability 2022, 14, 6816.

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Biostimulants Application on *Olea europaea* L. in Mediterranean Conditions Increase the Production and Bioactive Compounds of Drupes and Oil

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Abstract: Over the years, the use of biostimulants has become increasingly widespread due to their proven efficiency in improving plant productivity and quality of fruits and mitigating the effects related to environmental stress. The aim of the present study was to evaluate the effect of three biostimulants on oil yield, production of drupes per plant, and nutraceutical components of olive drupes and oil (total polyphenols, anthocyanins, and fatty acids %) for "Racioppella" cultivar trees growing in South Italy (May–October 2021). The biostimulants used were: a tropical plants extract (A) containing amino acids, vitamins, enzymes, phytochelatins, macro- and microelements, a glycine betaine-based product (B), and a *Trichoderma* spp.-based biostimulant (T). The three biostimulants were compared with a control thesis (C) treated only with water. T treatment increased the polyphenols content of olive drupes by 41.04% compared to C. A and B treatments increased polyphenols content by 21.87% on average compared to C. All three biostimulants showed positive effect by increasing the amount of polyphenols in olive oil compared to C:T showed an increase of 32.19%, B 7.76%, and A 19.78%. Biostimulant application proved useful in boosting fundamental parameters that determine better drupe and oil in terms of antioxidant capacity and nutraceutical potential, other than an increased production.

Keywords: olive; yield; quality; nutraceutical compounds; panel test; thermal stress

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1. Introduction

Global warming is predicted to have a generally negative effect on plant growth due to the damaging effect of high temperatures on their development. The increasing threat of extreme climatic events, including high temperatures might lead to lower crop productivity and quality loss [1]. Abiotic stresses cause morphological, physiological, biochemical, and molecular changes that negatively impact plant growth and yield. The rising threat of climate change is already having a substantial impact on agricultural production worldwide, as heat waves can cause significant yield losses threatening future global food security [2]. Moreover, the environmental conditions of the Mediterranean basin are expected to change soon [3]. In particular, the mean air temperature is projected to rise drastically in the range of 2–5 °C in the next 30 years [3–5].

Olive trees are considered one of the most suitable and best-adapted species to the Mediterranean-type climate [6], characterized with long, warm, and dry summers, with mild and wet winters [7]. In addition, olive orchards in the Mediterranean basin are normally subjected to high levels of solar radiation, especially in spring and summer seasons. Nowadays, olive trees face new challenges and threats, namely related to climate change. In fact, increased temperatures and drought and a frequent occurrence of extreme weather events such as heatwaves, are among the problems that growers will have to deal with in the upcoming decades [8], as high temperatures influence some parameters such as

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Chapter 5. High Temperature and Humidity Affect Pollen Viability and Longevity in *Olea europaea* L.

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Chapter 5. High Temperature and Humidity Affect Pollen Viability and Longevity in *Olea europaea* L.

Abstract: *Olea europaea* L. is a crop typical of the Mediterranean area that has an important role in economy, society, and culture of this region. Climate change is expected to have significant impact on this crop, which is typically adapted to certain pedo-climatic characteristics of restricted geographic areas. In this scenario, the aim of this study was to evaluate the time-course response of pollen viability to different combinations of temperature and humidity. The study was performed comparing flowering time and pollen functionality of O. *europaea* from twelve cultivars growing at the same site belonging to the Campania olive collection in Italy. Pollen was incubated at 12°C, 22°C, and 36°C in combination with 50% RH or 100% RH treatments for 5 days. The results highlighted that a drastic loss of pollen viability occurs when pollen is subjected to a combination of high humidity and high temperature, whereas 50% RH had less impact on pollen thermotolerance, because most cultivars preserved a high pollen viability over time. In the ongoing climate change scenario, it is critical to assess the effect of increasing temperatures on sensitive reproductive traits such as pollen viability to predict possible reduction in crop yield. Moreover, the results highlighted that the effect viability of temperature increase on pollen thermotolerance should be evaluated in combination with other environmental factors such as humidity conditions. The screening of olive cultivars based on pollen environmental thermotolerance is critical in the ongoing climate change scenario, especially considering that the economic value of this species relies on successful fertilization and embryo development, and also that production cycle of *Olea europaea* can be longer than a hundred years.

5.1. Introduction

Climate change will severely impact the Mediterranean Basin with an expected rise in temperatures in the range of 2–5 °C [1–3]. Besides substantial warming, it has been estimated that climate change will result in a significant decrease in precipitation in this region [3,4], which might cause serious economic and ecological changes, influencing plant growth, the

attack of pests and weeds, and ultimately, crop yield [5]. The olive (*Olea europaea* L.) is one of the most characteristic crops of the Mediterranean Basin, having a remarkable economic, social, and cultural impact. This species is widely spread and well adapted to the environmental conditions of the Mediterranean Basin. However, the predicted increase in ambient temperature due to global warming may affect plant physiology, phenology, and reproductive biology of this crop, ultimately reducing its yield [6,7]. The Mediterranean region is characterized by a changeable climate, especially in spring season, when daily temperatures can vary considerably. Moreover, it has been shown that inter-annual climate variations can affect flowering time and pollen production [8]. Indeed, previous studies have shown that the flowering time of *O. europaea* is highly dependent on yearly spring temperatures, which are rising steadily over time due to global warming [9,10]. In this scenario, the olive phenology may provide useful indications to evaluate the influence of climate change on plant growth for the whole Mediterranean region, since the geographical limits of this cultivation approximately delimit the extent of the Mediterranean climate in Eurasia and North Africa [11,12]. Since the different olive varieties are adapted to specific climatic, edaphic, and lithological conditions, the possible variations occurring in a climate change scenario would have a significant impact on the distribution of these varieties and, consequently, on their growth and productivity [13,14]. This is especially expected for some old varieties cultivated in narrow geographic niches with specific micro-climatic characteristics [15]. Indeed, it has been shown that these varieties exhibit greater vulnerability to both short-term climate variability and long-term climate change [16]. The olive has significant phenotypic and genetic variability [17,18]. Factors including plant longevity, limited selection pressure, and limited replacement with new genotypes have reduced genetic erosion and favored the preservation of genetic diversity of olive varieties [19]. Italy has the richest olive collection, including about 700 different varieties. Among the Italian regions, Campania has one of the largest collections [20,21], and many of these varieties are characterized by an extensive morphological diversity and adaptation to local environmental conditions [22]. Olive plants can produce an abundant number of flowers, but generally, only a small percent (1–2%) of them set normal fruits that reach maturity

[23,24]. The success of a flower to become a fruit mainly depends on the pollination and fertilization processes. Previous studies reported that pollen germination and pollen tube growth are sensitive to elevated temperatures [25,26]. Moreover, it has been shown that the combination of relative humidity (RH) and temperature can affect pollen viability of other species [27–29]. Temperature also influences both drupe development and oil composition in olive. For example, in very hot sites, olives can show early pigmentation due to the rapid degradation of chlorophyll due to high temperatures [30], whereas in sites with lower temperatures, olive oil has a high content of unsaturated fatty acids [31]. Temperature can also influence the aromatic components of olive oil, reducing the content of volatile substances [32]. Hence, it is arguable that the growing environment is crucial in expressing the typical characteristics and quality of olive cultivars [33]. In most crop species, including *Olea europaea* L., the production of fruits and seeds relies on pollen functionality. Since pollen viability and germinability are both essential to ensure fertilization, the interaction of pollen with extreme weather events can significantly limit crops productivity in the current climate change scenario [34]. Among extreme weather events, heat waves during early stages of pollen development can reduce pollen functionality in *Solanum* lycopersicumL., resulting in a drastic loss of pollen germinability [35]. Most of the studies evaluating the effect of environmental factors on olive pollen mainly focused on temperature [26,36], whereas very few studies evaluated the combined effect of temperature and humidity [37]. Specifically, Koubouris et al. [37] studied the effect of pre-incubation temperature and humidity on olive pollen before in vitro germination. However, authors did not test the combined effect of high temperature and high humidity, which could severely affect pollen functionality. Among protocols to assess pollen functionality, the use of diaminobenzidine (DAB) reaction is an efficient method to assess pollen viability responses towards environmental factors [38,39]. Due to its ease of use, the DAB method can be useful for large screening of pollen viability, such as the case of studies with numerous cultivars. In the current climate change scenario, the aim of this study was to highlight possible differences among different olive cultivars in the time-course response of pollen viability to different combination of temperature and humidity treatments. We used pollen from 12 olive

cultivars belonging to germplasm of the Campania region in Southern Italy and growing at the same site. We hypothesized that both temperature and humidity would affect pollen viability with possible changes due to treatment duration and cultivars. Moreover, we hypothesized that high humidity would enhance the negative effect of high temperature on pollen viability and longevity.

5.2. Materials and Methods

5.2.1. Plant Material and Flowering Monitoring

The experiment was performed using pollen from 12 olive cultivars of Campania region. Plants belong to the open-field collection conserved at "Improsta" Regional Experimental Farm in Eboli (SA) (40°33′29 N; 14°58′28′′ E at 15 m.a.m.s.l.). We selected olive cultivars representative of 4 provinces of Campania region and covering the bioclimatic diversity of the whole area (Table 1).

Table 1. Cultivars of *Olea europaea* L. from 4 provinces of Campania region in Italy.

N	Province	Name
1	Avellino	Marinese
2		Ravece
3		Ogliarola
4	Benevento	Ortice
5		Ortolana
6		Racioppella
7		Femminella
8	Caserta	Caiazzana
9	Salerno	Biancolilla
10		Carpellese
11		Pisciottana
12		Salella

For each cultivar, the duration of flowering was determined by a procedure reported by Rapoport and Rallo 1991 [40] with some modifications: the flowering phenology of three different branches with south exposure and approximatively 100 flowers per branch was observed. We considered the first day of flowering to be when 10% of flowers per each branch were open, and the last day to be when 100% of flowers per branch were open. The duration of flowering (number of days) was then averaged based on measurements on the three

different branches. In the field, data of minimum, maximum, and medium temperature (°C) and RH (%) during May 2021 were recorded from the agro-meteorological regional station of Eboli (SA), located at "Improsta" Regional Experimental Farm. The collection of pollen samples was carried out from 18 to 28 May 2021. Pollen was shed from the inflorescences in Petri dishes using pollen vibrators. Sampling was performed in the morning, Petri dishes were placed inside a thermal bag at 5 °C and transported to the laboratory in few hours. For each cultivar, pollen was collected from branches with north, south, east, and west exposure from three different plants.

5.2.2. Temperature and Humidity Treatments

Pollen samples were incubated under six different combinations of temperature and humidity for a total of 5 days. According to the temperature measured during the flowering season at the experimental farm, three temperature treatments were tested: 12 °C, 24 °C and 36 °C. More specifically, 12 °C and 24 °C were tested to simulate the averaged minimum and maximum temperatures of May, whereas 36 °C was chosen to simulate a possible scenario of global warming and heat waves. In combination with temperature treatments, the pollen from the 12 cultivars was incubated at 50% RH and 100% RH to simulate the effect of dry-sunny and wet-rainy days occurring during the flowering season. Temperature treatments were performed using three separated incubators (VELP, FOC 200 IL) set with 12°C, 24°C, 36°C respectively. In each incubator, to achieve 50% or 100% RH conditions we enclosed the bulk samples of pollen in two separated plastic containers containing: a) a beaker with Mg (NO3)2 saturated solution to reproduce 50% RH, b) wet tissues to achieve 100% RH.

5.2.3 Analysis of pollen viability

Pollen thermotolerance and longevity of the 12 olive cultivars were assessed performing viability tests at 1-, 3-, and 5-days incubation. Pollen viability was assessed through diaminobenzidine (DAB) reaction [38,39]. Each pollen sample was gently collected with a brush from the petri dish and placed onto 10 μL droplet of water on a microscope slide. One droplet of 10 μL of DAB reagent was then added on each sample. Successively, the microscope slides were gently warmed on a heating plate (set at 50°C) and mounted with a

cover slip. The viability of pollen at sampling (T_0) was assessed to compare possible differences in initial pollen functionality among cultivars and to have a reference point for comparing the effect of temperature, RH, and their interaction throughout the incubation period. We scored as viable the pollen grains stained black/brown and as not viable the ones that remained faint/colourless. The percentage of pollen viability was measured at different incubation time, counting at least 100 pollen grains per microscope slide on a total amount of 6 slides per cultivar per treatment.

5.2.4 Data analyses

Data were analysed using Excel ver. 16 (Microsoft Corp., Redmond, USA) and SPSS Statistics ver. 21 (IBM Corp., Chicago, USA). Percentage data of pollen viability was preliminary converted with arcsine function. The Shapiro Wilk's and Levene's tests were used to assess the normality and homogeneity of variance, respectively. The influence of the different categorical independent variables (i.e., cultivar, time, temperature, and humidity), and their possible interactions on pollen viability was analyzed using the ANOVA. Pairwise comparisons were performed with Tukey's HSD test ($P > 0.05$) to identify differences among treatments and cultivars.

5.3. Results

5.3.1 Climatic parameters

Temperature and humidity data recorded in May 2021 at the experimental farm are shown in Figure 1. The average values of the daily minimum, medium, and maximum temperature were 11.8 °C, 17.7 °C, and 23.3 °C, respectively. The highest temperature was recorded on May 24, reaching a peak value of 31.8 °C, while lowest temperature was 7.5 °C and was recorded on May 21. On average, the daily minimum, medium, and maximum RH values were 50.6%, 73.1%, and 91.29%, respectively. Overall, the daily RH values were comparable between the different days, except for a considerable decrease of daily RH values that was recorded from 20 May to 25 May (Figure 1).

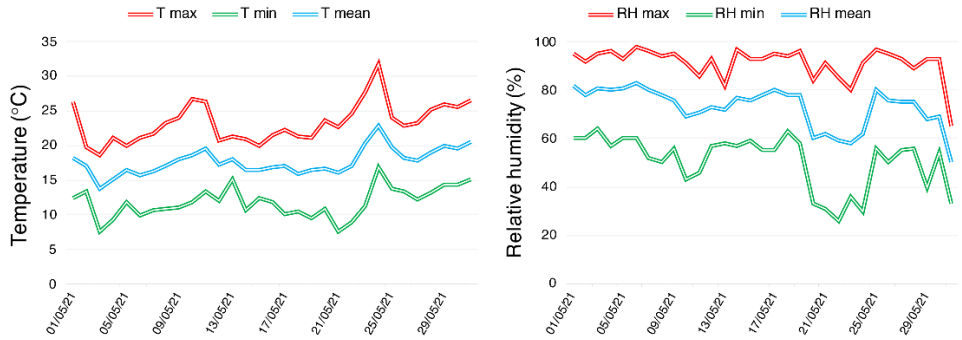


Figure 1. Daily trend of maximum (red line), minimum (green line) and mean (blue line) temperature and humidity measured in May 2021 at "Improsta" Regional Experimental Farm, in Eboli (SA) (40 ° 33 '29 "N; 14 ° 58' 28" E, at 15 m.a.s.l.).

5.3.2 Flowering time

Figure 2 shows the duration of flowering time of the different olive cultivars considered in this study. On average, the flowering time among cultivars was 7 days. Moreover, the shortest and the longest duration of flowering were recorded in 'Pisciottana' (3 days) and 'Femminiella' (10 days), respectively (Figure 2).

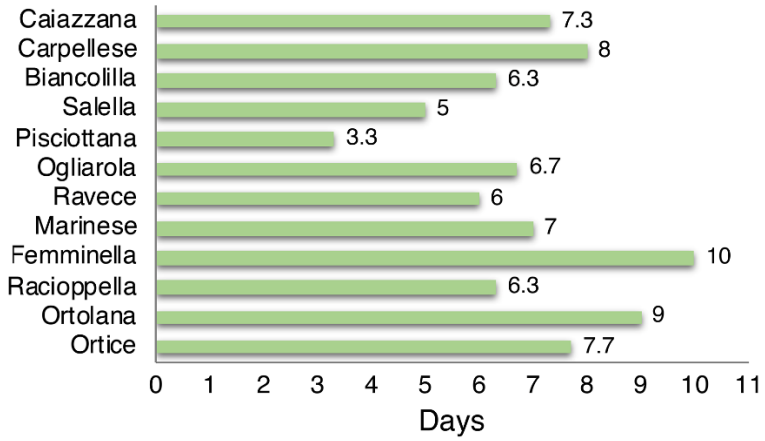


Figure 2. Duration of flowering in 12 olive cultivars from Campania region in Italy.

5.3.3 Pollen viability

According to the ANOVA, all factors tested in this study (i.e., cultivar, temperature, and humidity) had a significant effect on pollen viability over time (Table 2). The viability tests performed on pollen at T_0 showed significant differences between the 12 cultivars (Figure

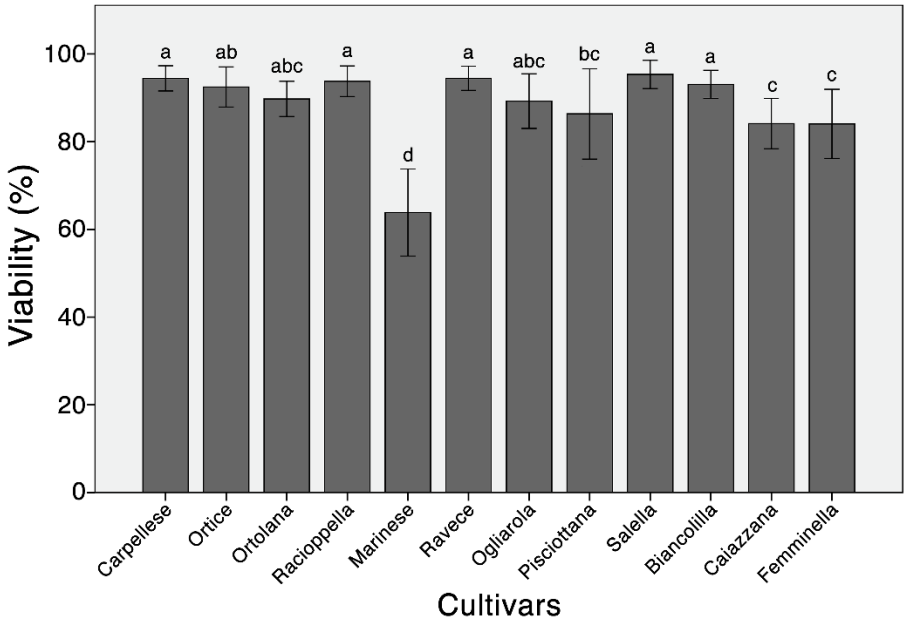
3). However, despite these differences, pollen viability of all cultivars at T_0 ranged between 84% and 95%, except for 'Marinese' in which pollen viability was 64%.

Table 2. Analysis of variance for the effects of cultivar, relative humidity (RH), temperature, or their interaction on pollen viability of *Olea europaea*.

Factor	Sum of Squares	Sig.
Cultivar	165,808.8	***
Time	548,079.9	***
RH	157,134.8	***
Temperature	28,848.1	***
Temperature × RH	27,059.9	***
RH × Cultivar	8670.5	NS
Temperature × Cultivar	13,972.0	NS
Temperature × RH × Cultivar	14,478.7	NS

NS, or *** indicate nonsignificant or significant at $P < 0.001$, respectively.

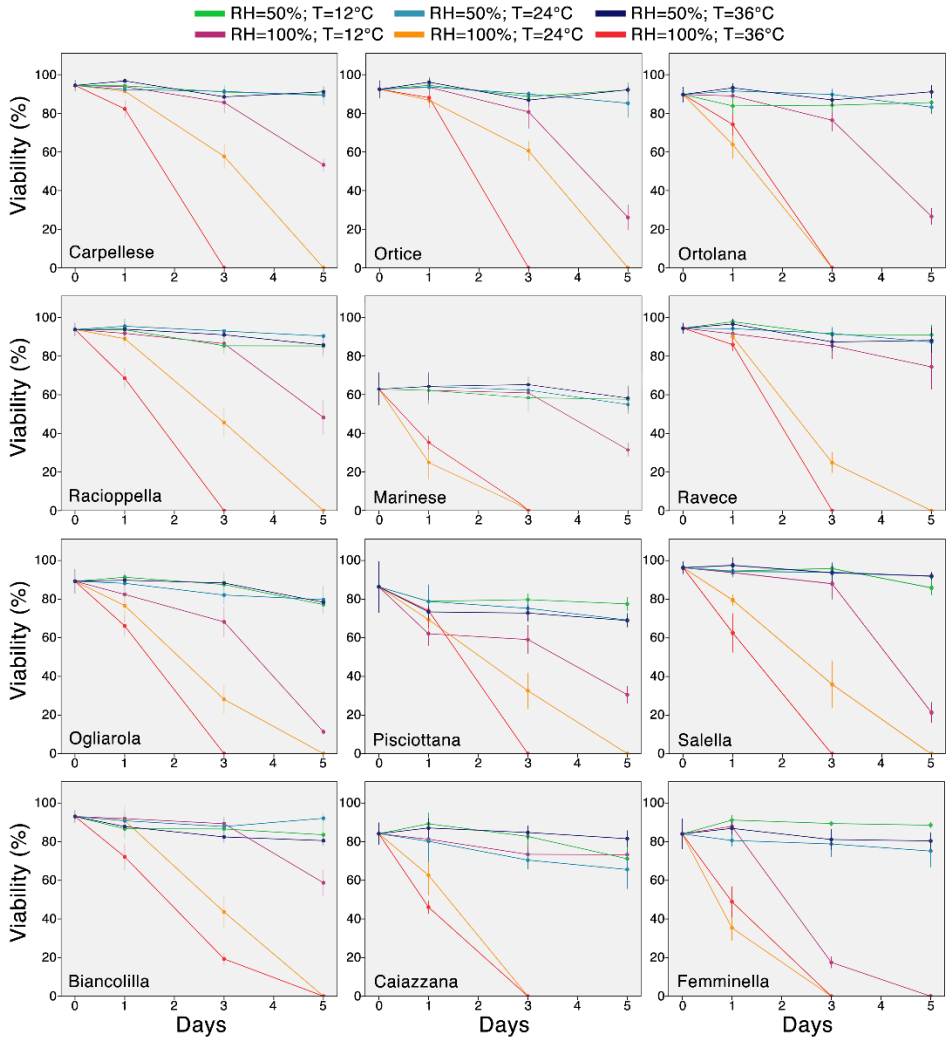
For each cultivar, time-course response of pollen viability was affected by the different combinations of temperature and RH over 5 days incubation. As concern treatments with 50% RH, pollen viability showed no significant decrease over time compared to pollen at T_0 in all cultivars. Notably, pollen preserved a high viability (~80%) both at high (24 – 36 °C) and low (12 °C) incubation temperature over time (Figure 4). A lower pollen viability was found only in 'Marinese' but it was comparable to pollen viability at T_0. Differently from treatments with 50% RH, pollen subjected to 100% RH showed a significant decrease over time in all cultivars with differences due to the incubation temperature. Overall, results showed a drastic loss of viability when pollen was subjected to a combined effect of high humidity (100%) and high temperature (36 °C). Indeed, pollen grains incubated at 100% RH and 36 °C completely lost their viability after 3-days incubation in almost all cultivars. Pollen viability was preserved for more than 3-days incubation at 100% RH and 36 °C only in 'Biancollilla', although with low values (~20%) (Figure 4).



3184

3185 **Figure 3.** Pollen viability of 12 cultivars of *Olea europaea* L. at sampling time (T_0). Letters indicate

3186 significant differences between cultivars ($P < 0.05$). Each data represents the mean ± SE (n = 6).



3187

Figure 4. Viability of pollen from 12 olive cultivars incubated at six different combinations of temperature and humidity for 5 days (T_1-T_5) from sampling (T_0). Each line shows the mean ± SE (n = 6).

A drastic loss of pollen viability also occurred when pollen was subjected to 100% RH and 24 °C, showing considerable variability over time depending on the cultivar. Compared to pollen at 100% RH and 36 °C, pollen at 24 °C showed higher viability over time and preserved its viability longer than under 36 °C. Indeed, pollen under 100% RH and 24 °C remained viable up to 5-days incubation in most of the cultivars except for 'Marinese', 'Caiazzana' and 'Ortolana', in which pollen grains become unviable at 3-days incubation (Figure 4). Differently from 24 °C and 36 °C, the combination of 100% RH and 12 °C showed a more gradual loss of pollen viability over time in most of the cultivars. More specifically, pollen viability remained high over 3-days incubation and never decreased below 60%, except for 'Femminella'. Interestingly, pollen incubated at 100% RH and 12° C showed no significative difference compared to treatments with 50% RH up to 3-days incubation (Figure 4).

5.4. Discussion

5.4.1. Climatic Parameters and Flowering

Recent studies indicate that nearly all European regions will be affected by the impact of climate change [27,41,42]. In this scenario, the Campania region in Italy has already experienced an increase in minimum temperatures of approximately 1.4 °C from 2005 to 2017 [43]. This situation, in agreement with research showing a dramatic global warming since the 1980s [13,44], poses concerns regarding the impact that climate change can have also on restricted geographical areas such as that of the Campania region. *O. europaea* is a typical Mediterranean species whose economic production cycle is extremely long, and fruit production relies on pollen efficiency and fertilization success. Therefore, studies on the interaction between flower biology and environmental parameters involved in climate change scenario are relevant. Our results on flowering period (time and duration) are comparable with those reported for several olive cultivars of the Campania region in the year from 2009 to 2010 [45], who showed that the average duration of flowering was 7 days

and occurred during the second half of May. It is known that environmental factors can affect many aspects of inflorescence development, pollination, and fertilization; in particular, high temperatures can influence the timing of phenological phases such as leaf formation and flowering in many species including *O. europaea* [46–48]. Our data showed that, in the last decade, the flowering time and duration of the olive cultivars considered in this study did not change. However, studies on other species highlighted that short periods of high temperature do not affect flowering phenology but can reduce pollen lifespan so drastically that grains are already dead at the time of anther dehiscence [35]. Indeed, it has been shown that the formation of pollen tetrads and bi-nucleate olive pollen is very sensitive to small increments in temperature in May, when the heat demand for flowering is nearly fulfilled [49]. In addition, besides the satisfaction of heat requirements, it is possible that different olive cultivars require the fulfilment of other conditions for starting the flowering process, such as a mean temperature above 15 °C during the week before the anthesis [12,49]. It should also be considered that the suitable temperature range for metabolic process in *O. europaea* is rather narrow, with an optimal temperature interval of approximatively 10 °C (from 20° C to 30°C) [50]. Therefore, when new cultivars are introduced in specific areas, it is critical to consider climatic requirements and flowering time of these cultivars.

5.4.2. Pollen Viability

The response of pollen to environmental factors is critical in *O. europaea*
, considering that self-incompatibility represents a common phenomenon in most of the olive cultivars [51]. Indeed, olive pollen from different cultivars needs to survive along its journey from the stamen to the stigma of different flowers to ensure the formation of seeds and drupes. Considering the great genetic diversity of olive cultivars in the Campania region, pollen viability represents a crucial feature for the selection of cultivars to be used as pollen donors in a climate change scenario. Our results showed that the decrease in pollen viability over time is highly dependent on the exposure to different combinations of temperature and RH. Pollen viability at sampling time exceeded 80% in almost all cultivars tested in this study. Interestingly, pollen viability of these cultivars was higher compared to

most commercial cultivars from Europe, which generally range between 60% and 70% [52–54]. The low genetic erosion due to the limited replacement of typical olive cultivars with new genotypes in Campania region may have conserved relevant reproductive traits such as pollen viability. On the other hand, commercial cultivars have mostly been selected to overcome self-incompatibility issues to increase plant productivity [55], but probably overlooking reproductive traits such as pollen viability during breeding programs. Our results are in agreement with previous studies, showing that high temperatures can reduce pollen viability more than low temperatures [29,54]. It has already been reported on that temperatures above 22 °C can reduce by 50% the initial pollen viability of *O. europaea* in 1–3 days [54]. However, previous studies mostly overlooked the effect of humidity, since RH was not explicated or fixed, and its effect could not be evaluated. Conversely, our study focused on the effect of different combinations of temperature and humidity to disentangle their effects on pollen functionality. Overall, with this approach, we found that RH has a substantial influence on pollen thermotolerance over time. Previous studies have shown that the exposure of olive pollen to high RH during preincubation can significantly decrease pollen germinability in vitro at low temperatures [37]. Contrastingly, our results showed that high humidity severely increase pollen sensitivity to both low and high temperatures. Specifically, the combination of high temperature (36°C) and high RH (100%) significantly reduced pollen viability already after 1 day of exposure, and pollen was completely unviable after 3 days in most of the cultivars. Conversely, pollen exposed to low RH (50%) preserved high viability (~80%) both at higher (24–36 °C) and lower (12°C) incubation temperatures over 5 days. These differences in pollen longevity could be related to specific mechanisms adopted by pollen to survive hostile environmental conditions [56]. Indeed, pollen grains under low humidity environments can enter a state of complete or partial arrest of metabolic processes associated with a high resistance to environmental stresses [56]. This phenomenon might explain the significant differences of pollen thermotolerance to high or low humidity. Water content of pollen grains generally decrease after flower anthesis and anthers dehiscence when pollen is exposed to the environment [57]. Therefore, from anthesis on, the possibility of pollen to enter in a quiescent state of development and

resist to unfavorable temperatures depends on pollen exposure to environmental humidity. Indeed, especially in self-pollinating species, pollen is dispersed in a well-hydrated state and remains metabolically active; this pollen is generally more sensitive to environmental stresses and has reduced viability, since it needs to germinate rapidly upon landing on the stigma of the same flower [58–60]. Contrastingly, in cross-pollinated species such as the *O. europaea*, pollen needs to survive for a relative long time, and therefore, it needs to reduce its water content to maintain a metabolically inactive state during its journey to the stigma of other flowers. In this case, the balance between the content of water, osmolyte compounds, and stabilizing proteins in pollen grains make the cellular content "glassy" and all the metabolic activities slow down [61]. However, this "glassy" state, responsible for increasing pollen longevity, is influenced by both humidity and temperature exposure of pollen grains [62,63]. Indeed, when pollen is exposed to low temperatures, this state of glassy cytoplasm can also be achieved in conditions of high humidity, and this would explain why pollen exposed to 12 °C and 100% RH preserved a high viability up to 5 days in most of the cultivars we tested. As regards temperatures of 24 °C or 36 °C, their negative effects on pollen viability becomes evident over time only when in combination with exposure to high RH. Overall, the differences in thermotolerance found between the different olive cultivars can be linked to the capability of pollen in adopting specific strategies to face heat stress including dehydration, accumulation of osmolytes, and synthesis of protective molecules such as heat-shock proteins (HSPs). Numerous studies have highlighted the key role of HSPs in activating heat-stress responses in reproductive cells of several plant species [64–68]. To date, HSPs have been identified in *O. europaea* but only in vegetative tissues [69]. In the present study, it is likely that the different responses of pollen to temperature and humidity found between cultivars are due to differences in heat-stress response pathways. In this regard, the screening of HSPs gene expression and synthesis of olive cultivars would provide a better understanding of the molecular mechanisms adopted by pollen to cope provide with heat stress. Moreover, such insights could be useful to select suitable olive cultivars as pollen donor to be used in a climate change scenario.

5.5. Conclusions

Despite the increase in temperature in Campania region over the last decade, no significant change in flowering time of *O. europaea* was found compared to previous studies. However, a drastic loss of pollen viability was found under high temperature and humidity conditions. Overall, the decreasing trend of pollen viability under the different combinations of temperature and humidity was comparable between cultivars, except for few cases. Specifically, most of the olive cultivars showed a significant decrease of pollen viability already after 24 h incubation under 36 °C and 100% RH, and a complete loss of viability after 3 days' incubation in the same conditions. Interestingly, pollen exposed to low RH (50%) preserved high viability both at high and low incubation temperatures over 5 days, indicating that pollen thermotolerance is affected by humidity conditions. In a current scenario of climate change, it is critical to evaluate the effect of temperature on reproductive traits to predict the future impact of global warming on crop yield; on the basis of the results obtained, we could therefore state that the cultivars that showed greater tolerance to extreme temperatures and humidity was Biancolilla (RH 100%–T 36°C), while 'Carpellese', 'Ortice', 'Racioppella', 'Ravece', 'Ogliarola', 'Pisciottana', and'Salella' also showed good tolerance in conditions of RH 100% and 24 °C. However, it becomes evident that other environmental factors such as humidity must be considered when evaluating pollen thermotolerance. Moreover, considering the key role of the heat shock proteins in heat-stress responses, further studies must investigate the molecular mechanism adopted by olive pollen to cope with environmental stresses.

5.6 References

1. Giorgi, F. Climate change hot-spots. Geophys. Res. Lett. 2006, 33, 1–4.
2. Giannakopoulos, C.; Le Sager, P.; Bindi, M.; Moriondo, M.; Kostopoulou, E.; Goodess, C.M. Climatic changes and associated impacts in the Mediterranean resulting from a 2 °C global warming. Glob. Planet. Chang. 2009, 68, 209–224.
3. Gualdi, S.; Somot, S.; Li, L.; Artale, V.; Adani, M.; Bellucci, A.; Braun, A.; Calmanti, S.; Carillo, A.; Dell'Aquila, A.; et al. The circe simulations: Regional climate change

projections with realistic representation of the mediterranean sea. Bull. Am. Meteorol. Soc. 2013, 94, 65–81.

4. Dell'Aquila, A.; Calmanti, S.; Ruti, P.; Struglia, M.V.; Pisacane, G.; Carillo, A.; Sannino, G. Effects of seasonal cycle fluctuations in an A1B scenario over the Euro-Mediterranean region. Clim. Res. 2012, 52, 135–157.
5. Arenas-Castro, S.; Gonçalves, J.F.; Moreno, M.; Villar, R. Projected climate changes are expected to decrease the suitability and production of olive varieties in southern Spain. Sci. Total Environ. 2020, 709, 136161.
6. Benlloch-González, M.; Sánchez-Lucas, R.; Benlloch, M.; Ricardo, F.E. An approach to global warming effects on flowering and Benlloch fruit set of olive trees growing under field conditions. Sci. Hortic. 2018, 240, 405–410.
7. Cirillo, A.; Conti, S.; Graziani, G.; El-Nakhel, C.; Rouphael, Y.; Ritieni, A.; Di Vaio, C. Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters. Agronomy 2021, 11, 1884.
8. Galán, C.; García-Mozo, H.; Vázquez, L.; Ruiz, L.; De La Guardia, C.D.; Trigo, M.M. Heat requirement for the onset of the *Olea europaea* L. pollen season in several sites in Andalusia and the effect of the expected future climate change. Int. J. Biometeorol. 2005,
9. 49, 184–188.
10. Tupper, N. Spanish Olive Oil Under Constant Threat from Climate Change. Available online: https://www.oliveoiltimes.com/ world/spanish-olive-oil-under-constant-threat-from-climate-change/29618 (accessed on 20 December 2021).
11. Ozdemir, Y. Effects of Climate Change on Olive Cultivation and Table Olive and Olive Oil Quality. Horticulture 2016, 60, 65–69.
12. De Micco, V.; Scala, M.; Aronne, G. Effects of simulated microgravity on male gametophyte of Prunus, Pyrus, and Brassica species. Protoplasma 2006, 228, 121–126.

13. Osborne, C.P.; Chuine, I.; Viner, D.;Woodward, F.I. Olive phenology as a sensitive indicator of future climatic warming in the Mediterranean. Plant Cell Environ. 2000, 23, 701–710.
14. Moriondo, M.; Stefanini, F.M.; Bindi, M. Reproduction of olive tree habitat suitability for global change impact assessment. Ecol. Modell. 2008, 218, 95–109.
15. Ponti, L.; Gutierrez, A.P.; Ruti, P.M.; Dell'Aquila, A. Fine-scale ecological and economic assessment of climate change on olive in the Mediterranean Basin reveals winners and losers. Proc. Natl. Acad. Sci. USA 2014, 111, 5598–5603.
16. Rubio, R.; Balaguer, L.; Manrique, E.; Pérez, M.E.; Vargas, P. On the historical presence of the wild olive [*Olea europaea* L. var. Sylvestris (Miller): Lehr.(Oleaceae)] in the Eurosiberian region of the Iberian Peninsula. In Anales del Jardín Botánico de Madrid; Consejo Superior de Investigaciones Científicas: Madrid, Spain, 2002; Volume 59, No. 2; pp. 342–344.
17. Olesen, J.E.; Trnka, M.; Kersebaum, K.C.; Skjelvåg, A.O.; Seguin, B.; Peltonen-Sainio, P.; Rossi, F.; Kozyra, J.; Micale, F. Impacts and adaptation of European crop production systems to climate change. Eur. J. Agron. 2011, 34, 96–112.
18. Poljuha, D.; Sladonja, B.; Šeti´c, E.; Miloti´c, A.; Bandelj, D.; Jakše, J.; Javornik, B. DNA fingerprinting of olive varieties in Istria (Croatia) by microsatellite markers. Sci. Hortic. 2008, 115, 223–230.
19. Ayed, R.B.; Grati-Kamoun, N.; Moreau, F.; Rebaï, A. Comparative study of microsatellite profiles of DNA from oil and leaves of two Tunisian olive cultivars. Eur. Food Res. Technol. 2009, 229, 757–762.
20. Rallo, P.; Dorado, G.; Martín, A. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). Theor. Appl. Genet. 2000, 101, 984–989.
21. Marra, F.P.; Caruso, T.; Costa, F.; Di Vaio, C.; Mafrica, R.; Marchese, A. Genetic relationships, structure and parentage simulation among the olive tree (*Olea europaea* L. subsp. europaea) cultivated in Southern Italy revealed by SSR markers. Tree Genet. Genomes 2013, 9, 961–973.

22. Di Vaio, C.; Nocerino, S.; Paduano, A.; Sacchi, R. Characterization and evaluation of olive germplasm in southern Italy. J. Sci. Food Agric. 2013, 93, 2458–2462.
23. Cicatelli, A.; Fortunati, T.; De Feis, I.; Castiglione, S. Oil composition and genetic biodiversity of ancient and new olive (*Olea europea* L.) varieties and accessions of southern Italy. Plant Sci. 2013, 210, 82–92.
24. Martin, G.C. Olive flower and fruit production dynamics. Acta. Hortic. 1990, 286, 141–153.
25. Lavee, S.; Rallo, L.; Rapoport, H.F.; Troncoso, A. The floral biology of the olive: Effect of flower number, type and distribution on fruitset. Sci. Hortic. 1996, 66, 149–158.
26. Fernández-Escobar, R.; Gomez-Valledor, G.; Rallo, L. Influence of pistil extract and temperature on in vitro pollen germination and pollen tube growth of olive cultivars. J. Hortic. Sci. 1983, 58, 219–227.
27. Vuletin Selak, G.; Perica, S.; Goreta Ban, S.; Poljak, M. The effect of temperature and genotype on pollen performance in olive (*Olea europaea* L.). Sci. Hortic. 2013, 156, 38–46.
28. Biondi, E.; Casavecchia, S.; Pesaresi, S. Nitrophilous and ruderal species as indicators of climate change. Case study from the Italian Adriatic coast. Plant Biosyst. 2012, 146, 134–142.
29. Aronne, G.; Buonanno, M.; De Micco, V. Reproducing under a warming climate: Long winter flowering and extended flower longevity in the only Mediterranean and maritime Primula. Plant Biol. 2014, 17, 535–544.
30. Aronne, G.; De Micco, V.; Scala, M. Effects of relative humidity and temperature conditions on pollen fluorochromatic reaction of Rosmarinus officinalis L. (Lamiaceae). Protoplasma 2006, 228, 127–130.
31. Mousa, Y.M.; Gerasopoulos, D.; Metzidakis, I.; Kiritsakis, A. Effect of altitude on fruit and oil quality characteristics of "Mastoides" olives. J. Sci. Food Agric. 1996, 71, 345–350.
32. Kiritsakis, A.; Markakis, P. Olive oil: A review. Adv. Food Res. 1987, 31, 453–482.

33. Ridolfi, M.; Terenziani, S.; Patumi, M.; Fontanazza, G. Characterization of the lipoxygenases in some olive cultivars and determination of their role in volatile compounds formation. J. Agric. Food Chem. 2002, 50, 835–839.
34. Di Vaio, C.; Nocerino, S.; Paduano, A.; Sacchi, R. Influence of some environmental factors on drupe maturation and olive oil composition. J. Sci. Food Agric. 2013, 93, 1134–1139.
35. Ledesma, N.A.; Kawabata, S. Responses of two strawberry cultivars to severe high temperature stress at different flower development stages. Sci. Hortic. 2016, 211, 319–327.
36. Iovane, M.; Aronne, G. High temperatures during microsporogenesis fatally shorten pollen lifespan. Plant Reprod. 2021, in press.
37. Ferri, A.; Giordani, E.; Padula, G.; Bellini, E. In vitro germinability of pollen grains of olive of new genotypes obtained advanced selections. Italus Hortus. 2009, 16, 77–81.
38. Koubouris, G.C.; Metzidakis, I.T.; Vasilakakis, M.D. Impact of temperature on olive (*Olea europaea* L.) pollen performance in relation to relative humidity and genotype. Environ. Exp. Bot. 2009, 67, 209–214.
39. Dafni, A. Pollination Ecology: A Practical Approach; Oxford University Press: Oxford, UK, 1992.
40. 39.Dafni Dafni, A.; Firmage, D. Pollen viability and longevity: Practical, ecological and evolutionary implications. Plant Syst. Evol. 2000, 222, 113–132.
41. Rapoport, H.F.; Rallo, L. Postanthesis Flower and Fruit Abscission in Manzanillo'Olive. Am. Soc. Hortic. Sci. 1991, 116, 720–723.
42. Giorgi, F.; Nielsen, P.; Ozdemir, Y.; Giannakopoulos, C.; Le Sager, P.; Bindi, M.; Moriondo, M.; Kostopoulou, E.; Goodess, C.M. Coastal and estuarine processes. Coast. Estuar. Process. 2009, 33, 1–360.
43. Biesbroek, G.R.; Swart, R.J.; Carter, T.R.; Cowan, C.; Henrichs, T.; Mela, H.; Morecroft, M.D.; Rey, D. Europe adapts to climate change: Comparing National Adaptation Strategies. Glob. Environ. Chang. 2010, 20, 440–450.

44. Mastrocicco, M.; Busico, G.; Colombani, N. Deciphering interannual temperature variations in springs of the Campania region (Italy). Water 2019, 11, 288.
45. Smirnova, A.; Leide, J.; Riederer, M. Deficiency in a Very-Long-Chain Fatty Acid-Ketoacyl-Coenzyme A Synthase of Tomato Impairs Microgametogenesis and Causes Floral Organ Fusion. Plant Physiol. 2013, 161, 196–209.
46. Di Vaio, C. Caratterizzazione morfologica e bio-agronomica delle cultivar campane di olivo. In Il Germoplasma Dell'olivo in Campania; Regione Campania: Napoli, Italy, 2012; pp. 17–26.
47. Fornaciari, M.; Romano, B.; Pieroni, L.; Ciuchi, P. A regression model for the start of the pollen season in *Olea eropaea*. Grana 1998, 37, 110–113.
48. Schwartz, M.D. Advancing to full bloom: Planning phenological research for the 21st century. Int. J. Biometeorol. 1999, 42, 113–118.
49. Orlandi, F.; Bonofiglio, T.; Ruga, L.; Sgromo, C.; Romano, B.; Fornaciari, M. Relationships between vegeta-tive-reproductive phases of plant species and the meteorological variables in a phenological garden of Central Italy. Ital. J. Agrometeorol. Riv. Ital. Agrometeorol. 2010, 15, 35–43.
50. Orlandi, F.; Ruga, L.; Romano, B.; Fornaciari, M. Olive flowering as an indicator of local climatic changes. Theor. Appl. Climatol. 2005, 81, 169–176.
51. Bonofiglio, T.; Orlandi, F.; Sgromo, C.; Romano, B.; Fornaciari, M. Influence of temperature and rainfall on timing of olive (*Olea europaea*) flowering in Southern Italy. N. Z. J. Crop Hortic. Sci. 2008, 36, 59–69.
52. Montemurro, C.; Dambruoso, G.; Bottalico, G.; Sabetta, W. Self-incompatibility assessment of some italian olive genotypes (*Olea europaea* L.) and cross-derived seedling selection by ssr markers on seed endosperms. Front. Plant Sci. 2019, 10, 1–13.
53. Wu, S.B.; Collins, G.; Sedgley, M. Sexual compatibility within and between olive cultivars. J. Hortic. Sci. Biotechnol. 2002, 77, 665–673.
54. Ferrara, G.; Camposeo, S.; Palasciano, M.; Godini, A. Production of total and stainable pollen grains in *Olea europaea* L. Grana 2007, 46, 85–90.

55. Aguilera, F.; Ruiz Valenzuela, L. Time trend in the viability of pollen grains in the Picual olive (*Olea europaea* L.) cultivar. Palynology 2013, 37, 28–34.
56. Marchese, A.; Marra, F.P.; Costa, F.; Quartararo, A.; Fretto, S.; Caruso, T. An investigation of the self- and inter-incompatibility of the olive cultivars "Arbequina" and "Koroneiki" in the Mediterranean climate of Sicily. Aust. J. Crop Sci. 2016, 10, 88–93.
57. Pacini, E.; Dolferus, R. Pollen developmental arrest: Maintaining pollen fertility in a world with a changing climate. Front. Plant Sci. 2019, 10, 1–15.
58. Firon, N.; Nepi, M.; Pacini, E. Water status and associated processes mark critical stages in pollen development and functioning. Ann. Bot. 2012, 109, 1201–1213.
59. Heslop-Harrison, J. An Interpretation of the Hydrodynamics of Pollen. Am. J. Bot. 1979, 66, 737–743.
60. Franchi, G.G.; Nepi, M.; Dafni, A.; Pacini, E. Partially hydrated pollen: Taxonomic distribution, ecological and evolutionary significance. Plant Syst. Evol. 2002, 234, 211–227.
61. Aylor, D.E.; Baltazar, B.M.; Schoper, J.B. Some physical properties of teosinte (Zea mays subsp. parviglumis) pollen. J. Exp. Bot. 2005, 56, 2401–2407.
62. Buitink, J.; Leprince, O. Glass formation in plant anhydrobiotes: Survival in the dry state. Cryobiology 2004, 48, 215–228.
63. Kravets, E.A. Cytomixis and its role in the regulation of plant fertility. Russ. J. Dev. Biol. 2013, 44, 113–128.
64. Hinojosa, L.; Matanguihan, J.B.; Murphy, K.M. Effect of high temperature on pollen morphology, plant growth and seed yield in quinoa (Chenopodium quinoaWilld.). J. Agron. Crop Sci. 2019, 205, 33–45.
65. Kotak, S.; Larkindale, J.; Lee, U.; von Koskull-Döring, P.; Vierling, E.; Scharf, K.D. Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. 2007, 10, 310–316.
66. Larkindale, J.; Vierling, E. Core genome responses involved in acclimation to high temperature. Plant Physiol. 2008, 146, 748–761.

67. Scharf, K.D.; Berberich, T.; Ebersberger, I.; Nover, L. The plant heat stress transcription factor (Hsf) family: Structure, function and evolution. Biochim. Biophys. Acta (BBA)-Gene Regul. Mech. 2012, 1819, 104–119.
68. AL-Quraan, N.A.; Locy, R.D.; Singh, N.K.; Sun, W.; Montagu, M.; Van Verbruggen, N. Small heat shock proteins and stress toler. Plant Physiol. Biochem. 2002, 1577, 1–9.
69. Hong, S.W.; Vierling, E. Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. Proc. Natl. Acad. Sci. USA 2000, 97, 4392–4397.
70. Assab, E.; Rampino, P.; Mita, G.; Perrotta, C. Heat shock response in olive (*Olea europaea* L.) twigs: Identification and analysis of a cDNA coding a class I small heat shock protein. Plant Biosyst. 2011, 145, 419–425.

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High Temperature and Humidity Affect Pollen Viability and Longevity in *Olea europaea* L.

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Abstract: *Olea europaea* L. is a crop typical of the Mediterranean area that has an important role in economy, society and culture of this region. Climate change is expected to have significant impact on this crop, which is typically adapted to certain pedo-climatic characteristics of restricted geographic areas. In this scenario, the aim of this study was to evaluate the time-course response of pollen viability to different combinations of temperature and humidity. The study was performed comparing flowering time and pollen functionality of *O. europaea* from twelve cultivars growing at the same site belonging to the Campania olive collection in Italy. Pollen was incubated at 12 °C, 22 °C, and 36 °C in combination with 50% RH or 100% RH treatments for 5 days. The results highlighted that a drastic loss of pollen viability occurs when pollen is subjected to a combination of high humidity and high temperature, whereas 50% RH had less impact on pollen thermotolerance, because most cultivars preserved a high pollen viability over time. In the ongoing climate change scenario, it is critical to assess the effect of increasing temperatures on sensitive reproductive traits such as pollen viability to predict possible reduction in crop yield. Moreover, the results highlighted that the effect of temperature increase on pollen thermotolerance should be evaluated in combination with other environmental factors such as humidity conditions. The screening of olive cultivars based on pollen thermotolerance is critical in the ongoing climate change scenario, especially considering that the economic value of this species relies on successful fertilization and embryo development, and also that production cycle of *Olea europaea* can be longer than a hundred years.

Keywords: climate change; germplasm; olive; pollen viability; pollen functionality

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1. Introduction

Climate change will severely impact the Mediterranean Basin with an expected rise in temperatures in the range of 2–5 °C [1–3]. Besides substantial warming, it has been estimated that climate change will result in a significant decrease in precipitation in this region [3,4], which might cause serious economic and ecological changes, influencing plant growth, the attack of pests and weeds, and ultimately, crop yield [5].

The olive (*Olea europaea* L.) is one of the most characteristic crops of the Mediterranean Basin, having a remarkable economic, social, and cultural impact. This species is widely spread and well adapted to the environmental conditions of the Mediterranean Basin. However, the predicted increase in ambient temperature due to global warming may affect plant physiology, phenology, and reproductive biology of this crop, ultimately reducing its yield [6,7].

The Mediterranean region is characterized by a changeable climate, especially in spring season, when daily temperatures can vary considerably. Moreover, it has been shown that inter-annual climate variations can affect flowering time and pollen production [8]. Indeed, previous studies have shown that the flowering time of *O. europaea* is highly dependent on yearly spring temperatures, which are rising steadily over time due to global warming [9,10]. In this scenario, the olive phenology may provide useful indications to evaluate the influence of climate change on plant growth for the whole Mediterranean

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Chapter 6. General Conclusion

Chapter 6. General conclusions

Crops are affected by environmental fluctuations that can drastically decrease yields and quality crops. For this reason, many studies aim to develop novel practical, and sustainable systems to reduce the various problems caused by climate changes. Future climatic changes have great importance for the agricultural sector, and the olive tree sector in particular. At present, new agriculture offers a lot of molecules that affect plant responses to abiotic stresses. In this regard, using natural products proved to be a promising tool to improve tolerance to heat stress and drought and it is possible to do analyses about differences among different olive cultivars in the time-course response of pollen viability to a different combination of temperature and humidity treatments.

This PhD thesis was designed to satisfy the demand for a more innovative, more efficient, and secure agriculture suited for future climate challenges. It aimed to improve the resistance of olive trees to abiotic stresses in particular the quality of drupes and oil using of biostimulants and anti-transpirant products, and to do a screening of Campania cultivars most tolerant to extreme conditions of temperature and humidity. In this work, we obtained novel and interesting results that which could be a solution to mitigate the effects of climate change on olive trees by also improving some qualitative and quantitative aspects.

The first part of this research focused on a preliminary test carried out in the greenhouse on young olive plants (**Chapter 2**), and the results highlighted the importance of biostimulants' application to mitigate the effects of abiotic stresses (high temperatures and drought), with different effects shown between the two watering regimes (100% and 50%). Biostimulants' effects were evident in conditions of water stress, and glycine betaine treatment and algae products (micro-algae and seaweed mix) were reported to improve some agronomic and eco-physiological parameters. Particularly interesting results were obtained with kaolin

applications that caused a considerable two-fold increase in the total polyphenols content in the leaves compared to the control, and a significant increase as well in the antioxidant activity. These results are interesting for improving the quality of olive oils characterized with low phenolic and antioxidant components, with the addition of leaves rich in polyphenols that can be used equally for pharmaceutical purposes. These results have been published in the Journal "*Agriculture*" (Graziani et al. 2022).

Another preliminary greenhouse test was conducted to emphasize the importance of using products based on kaolin and pinolene to improve the biometric, physiological and nutraceutical characteristics of young olive trees subjected to high thermal stress. (**Chapter 3**). As tentative results, foliar application of the anti-transpirant pinolene-based product improved the biometric parameters. Plants sprayed with the tested preparation were characterized by a higher relative water content (RWC) in leaves and a higher value of efficiency of photosystem II (PSII); this positive effect of 1-p-menthene on vegetative growth and development may be due to improved plant water status related to lower transpiration. Kaolin application also reduced stomatal conductance and like the V application, recorded higher values of leaf chlorophyll concentrations (SPAD) at the end of the vegetative season. Interesting effects were recorded with the use of kaolin on the nutraceutical compounds of olive leaves; high contents of total polyphenols were recorded compared to control plants and this product showed a higher value of the antioxidant activity. These results have been published in the Journal "*Agronomy*" (Cirillo et al. 2021).

The second part of this study was carried out in full field on plants in production. We evaluated the impact of different biostimulants to reduce climate damage and improve the performance of plants and the quality of the derived product (**Chapter 4**). In this study, the biostimulants increased the production per plant, in particular the *Trichoderma* by about 70%, which positively influenced the carotenoids content and polyphenols biosynthesis in the drupes as well. Biostimulants based on tropical plant extracts and trichoderma reported changes to the flavor (bitter and spicy), due to an increase in the total polyphenol content in the oil by 32.1% and 19.8% respectively. All biostimulants influenced the oil fatty acid content. Our study demonstrated that it is possible to state that biostimulants affect some

qualitative and quantitative aspects of both the oil and the drupes, improving, in some cases, fundamental parameters that determine consumer satisfaction of a good product, as well as their antioxidant capacity and nutraceutical potential. These results have been published in the Journal "*Agriculture*" (Cirillo et al. 2022).

The aim of the last study was a varietal screening of the Campania olive cultivars in extreme ambient conditions. A drastic loss of pollen viability was found under high temperature and humidity conditions (**Chapter 5**). Overall, the decreasing trend of pollen viability under the different combinations of temperature and humidity was comparable between cultivars, except for few cases. Specifically, most of the olive cultivars showed a significant decrease of pollen viability already after 24 h incubation under 36 °C and 100% RH, and a complete loss of viability after 3 days' incubation in the same conditions. Interestingly, pollen exposed to low RH (50%) preserved high viability both at high and low incubation temperatures over 5 days, indicating that pollen thermotolerance is affected by humidity conditions. In a current scenario of climate change, it is critical to evaluate the effect of temperature on reproductive traits to predict the future impact of global warming on crop yield; based on the results obtained, we could therefore state that the cultivars that showed greater tolerance to extreme temperatures and humidity was Biancolilla. However, it becomes evident that other environmental factors such as humidity must be considered when evaluating pollen thermotolerance. These results have been published in the Journal "*Agronomy*" (Iovane et al. 2021).

Altogether, three were the main finding of this PhD thesis: **(1)** evaluation of biostimulant and antitrasspirant products' effects in influencing ecophysiological, biometric and nutraceutical parameters in young olive trees grown in greenhouses; **(2)** evaluation of biostimulants products' effects in improving qualitative-quantitative parameters of drupes and oil, in olive trees in full production; **(3)** varietal screening of Campania olive cultivars that are more resistant to extreme temperatures and humidity.

Based on the results obtained from these studies it is possible to state that the strategies used in the present study have been shown to be effective in improving the productive and qualitative characteristics of olive tree in a scenario of climate change.

6.1 References

1. Graziani, G., Cirillo, A., Giannini, P., Conti, S., El-Nakhel, C., Rouphael, Y., ... & Di Vaio, C. (2022). Biostimulants Improve Plant Growth and Bioactive Compounds of Young Olive Trees under Abiotic Stress Conditions. *Agriculture*, *12*(2), 227.
2. Cirillo, A., Conti, S., Graziani, G., El-Nakhel, C., Rouphael, Y., Ritieni, A., & Di Vaio, C. (2021). Mitigation of High-Temperature Damage by Application of Kaolin and Pinolene on Young Olive Trees (*Olea europaea* L.): A Preliminary Experiment to Assess Biometric, Eco-Physiological and Nutraceutical Parameters. *Agronomy*, *11*(9), 1884.
3. Cirillo, A., De Luca, L., Graziani, G., Cepparulo, M., El-Nakhel, C., Giordano, M., ... & Di Vaio, C. (2022). Biostimulants Application on *Olea europaea* L. in Mediterranean Conditions Increase the Production and Bioactive Compounds of Drupes and Oil. *Agriculture*, *12*(12), 2173.
4. Iovane, M., Cirillo, A., Izzo, L. G., Di Vaio, C., & Aronne, G. (2022). High temperature and humidity affect pollen viability and longevity in *Olea europaea* L. *Agronomy*, *12*(1), 1.